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Role of docohexaenoic acid/Elovl2 axis in glucolipotoxicity induced apoptosis and secretory dysfunction in pancreatic β-cells.

Rôle de l'axe de l'acide docohexaenoïque/Elovl2 dans l'apoptose et le défaut de sécrétion induits par la glucolipotoxicité dans les cellules β pancréatiques.

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"nella vita potranno toglierti tutto,

ma non potranno mai toglierti

il tuo sapere".

ABSTRACT

Type 2 diabetes (T2D) is a disease characterised by a dysfunction of pancreatic β cell survival and function associated with insulin resistance. In the case of T2D associated with obesity, chronic hyperglycaemia potentiates the deleterious effect of saturated free fatty acids on β cell. This phenomenon is defined as gluco-lipotoxicity (GL). Up to now, limited therapeutic options exist to fight against GL and treat diabetes and none to cure or prevent this disease, in part due to the limited knowledge of β-cell biology in health and disease. To face to the lack of knowledge regarding β cell function in diabetes, the European consortium IMIDIA (http://www.imidia.org) had conducted a multi-parameter analysis that led to the identification of a sub-network of islet-expressed genes associated with glucose tolerance and insulin secretory capacity during development of obesity in mice. Among these genes, I decided to further investigate the role of the very long chain fatty acid elongase 2 (ELOVL2). ELOVL2 is an enzyme involved in the synthesis of ω3-poly-unsaturated fatty acids (PUFAs), especially Docosahexaenoic acid (DHA). I have found that GL decreases *Elovl2* expression and DHA levels in β-cells. I showed that DHA and *Elovl2* over-expression rescues glucose-induced insulin secretion and cytosolic Ca^{2+} influx impaired by GL, suggesting that increased endogenous DHA levels resulting from *Elovl2* up-regulation counteracts the insulin secretion defect associated with GL. In a second part, I found that down-regulation of *Elovl2* drastically potentiates apoptosis induced by GL. In contrast DHA and over-expressed *Elovl2* counteract β cell apoptosis induced by GL. Interestingly, I found that ELOVL2/DHA axis inhibits accumulation of ceramide, which normally mediate β cell apoptosis under GL. It appears that ELOVL2/DHA axis did not inhibit enzyme function involved in *de novo* ceramide synthesis. In contrast, the fat oxidation inhibitor, etomoxir, which markedly enhanced GL-induced cell death, completely inhibits the beneficial effect of ELOVL2/DHA axis. These results suggest that ELOVL2/DHA alter fatty acid partitioning, in favour of mitochondrial fatty acid β-oxydation in order to protect β-cells from apoptosis. Collectively, my results show for the first time a role of the ELOVL2/DHA axis in β-cell dysfunction and apoptosis induced by GL. The existence of this axis could lead to develop new therapies that target DHA synthesis to protect β-cells against the deleterious effect of GL. Finally, although I focus experimental validation on *Elovl2*, the comprehensive data set and integrative network model used to identify this candidate gene represents an important novel resource to dissect the molecular aetiology of β-cell failure following metabolic stress.

Résumé

Le diabète de type II (T2D) est une pathologie caractérisée par une hyperglycémie chronique due au dysfonctionnement ainsi qu'à l'apoptose des cellules β pancréatiques, associée à la résistance à l'action de l'insuline. Dans le cas d'un T2D accompagné d'une obésité, l'hyperglycémie chronique potentialise les effets délétères des acides gras saturés sur la cellule β. Ce phénomène est défini comme étant la gluco-lipotoxicité (GL). Aujourd'hui, peu de cibles thérapeutiques existent afin de contrecarrer les effets de la GL et de traiter/prévenir définitivement le diabète, ceci étant dû en partie au manque de connaissances sur la régulation de la cellule β dans des conditions pathologiques. Dans ce but, le consortium Européen IMIDIA (http://www.imidia.org) a réalisé une analyse multiparamétrique permettant l'identification de gènes exprimés dans les îlots de Langerhans qui sont associés à la tolérance au glucose ainsi que la capacité de l'îlot à sécréter l'insuline chez des souris obèses. Parmi ces gènes, je me suis intéressée au rôle de l'élongase 2 (ELOVL2), enzyme impliquée dans la synthèse d'acides gras ω3-poly-insaturés (PUFAs) et en particulier l'acide docosahexaénoïque (DHA). J'ai pu mettre en évidence que la GL diminue l'expression d'Elovl2 et la quantité de DHA dans les cellules β. J'ai pu montrer que le DHA et la surexpression d'Elovl2 restaurent la sécrétion d'insuline induite par le glucose inhibée par la GL, suggérant qu'une augmentation de la quantité endogène de DHA, via la surexpression d'Elovl2 serait capable de contrecarrer le défaut de sécrétion d'insuline associé à la GL. J'ai pu ensuite montrer qu'une sous-expression d'Elovl2 accroît encore plus l'apoptose des cellules β induite par la GL. Ceci étant contrecarré par une surexpression d'Elovl2 et l'addition de DHA. L'axe Elovl2/DHA diminue drastiquement l'accumulation de céramides, responsable de l'apoptose des cellules β induite par la GL. Néanmoins, cet axe ne semble pas affecter la synthèse *de novo*. En revanche, l'étomoxir (inhibiteur de l'oxydation des acides gas) inhibe totalement l'effet protecteur de l'axe Elovl2/DHA. Ceci suggère donc que l'axe Elovl2/DHA altère le devenir des acides gras dans la cellule en favorisant la dégradation des acides gras par la β-oxydation afin de protéger la cellule β de l'apoptose. En conclusion, mes résultats ont permis de mettre en évidence le rôle de l'axe Elovl2/DHA dans le disfonctionnement et l'apoptose induits par la GL. L'existence de cet axe pourrait conduire à développer de nouvelles thérapies qui cibleraient la synthèse de DHA afin de protéger la cellule β contre les effets délétères de la GL. Enfin, bien que je me sois focalisée uniquement sur la validation d'Elovl2, la base de données créée au cours de cette étude pour identifier de nouveaux gènes impliqués dans le T2D représente une nouvelle

ressource importante pour mieux comprendre la défaillance de la cellule β durant un stress métabolique.

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ABBREVIATION:

3-KSR	3-ketospinganine reductase
aCDase	ceramidase
AIF	apoptosis-inducing factor
ALA	α-linolenic acid (C18:3,*n*-3)
AMPc	cyclic adenosine monophosphate
aSMase	acid sphingomyelinase
Bcl-2	B-cell lymphoma 2
Bak	Bcl-2 homologous killer
BAT	brown adipose tissue
Bax	Bcl-2-associated X protein
C1P	ceramide-1-phosphate
CerS	ceramide synthases
Cers4	ceramides synthase 4
CNS	central nervous system
CPT1	carnitine-palmitoyl-transferase-1
DAG	diacyl-glycerol
DGAT2	diacylglycerol acyltransferase 2
DHA	docosahexaenoic acid (22:6,*n*-3)
DhCerDes1/2	dihydroceramide desaturase 1 and 2
DH-Sph	dihydro-sphingosine
DPA	docosapentaenoic acid (22:5,*n*-6)
ELOVL	Elongation very long chain fatty acids
ER	endoplasmic reticulum
EPA	eicosapentaenoic acid (20:5,*n*-3)
FAHFA	fatty acid esters of hydroxyl fatty acids
FAS	fatty acid synthesis
FFAs	Free Fatty acids
GlcCer	glucosylceramide
GL	Glucolipotoxicity
GLP-1	glucagon-like peptide 1
GSIS	glucose stimulated insulin secretion
Glut4	Glucose type 4 transported
GSK3	Glycogen synthase kinase 3
G6P	Glucose-6-phosphate
HCMV	human cytomegalovirus

HDHA	hydroxydocohexaenoic acid
HFD	high-fat diet
HFHS	high-fat high-sucrose
HLA	hydroxyoctadecadienoic acid
Hox	*homeobox* (Hox)-like domain
HSP90	heat shock protein 90
IFG	impaired fasting glucose
IL-6	Interleukin 6
IMIDIA	Innovative Medicin Initiative for Diabetes
$iPLA_2\beta$	Ca^{2+}-independent phospholipase A2
iR	ionizing radiation
IR	insulin resistance
JNK	c-Jun NH_2-terminal kinase
KAR	3-ketoacyl-CoA reductase
KLF11	Kruppel-like factor 11
LA	linoleic acid (18:2,*n*-6)
LC3B-II	microtubule-associated protein 1 light chain 3BII
LC-CoA	long chain acyl-CoA
MAM	mitochondria associated membranes
MCP-1	monocyte chemotactic protein-1
MUFA	monounsaturated fatty acids
OGTT	oral glucose tolerance test
PASK	Per-Arnt-Sim
PC	pyruvate carboxylase
PtC	phosphatidylcholine
PE	phosphatidylethanolamine
PDX-1	pancreatic and duodenal homeobox 1
PGC1α	peroxisome proliferator activator receptor γ coactivator-1α
PKC	Protein kinase C
PKCδKN	kinase-negative PKCδ
PPAR	Peroxisome proliferator-activated receptors
PUFA	polyunsaturated fatty acids
ROS	Reactive oxygen species
S1P	sphingosine-1-phosphate
SAPs	sphingolipid activator proteins
SCAP	SREBP cleavage-activating protein

SCD-1	stearoyl-CoA desaturase 1
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SM	sphingomyelin
SMS	Sphingomyelin synthase
SMase	sphingomyelinase
SphK1/2	sphingosine kinases 1 and 2
SPT	serine palmitoyl-transferase
SRE	sterol responsive element
SREBP-1c	sterol-regulatory element binding protein 1c
STZ	streptozotocin
VSVG-GFP	vescicular stomatitis virus glycoprotein tagged with GFP
T1D	diabetes type 1
T2D	diabetes type 2
tBid	truncated BH3 interacting-domain death agonist
TER	trans-2,3-enoyl-CoA reductase
TG	triglycerides
TNF-α	tumor necrosis factor α
TZDs	thiazolidinediones
VLCFA	very long chain fatty acids
VLDLs	very low-density lipoproteins
UCP2	Uncoupling protein 2
UPR	unfolded protein response
ZDF	Zucker diabetic fatty
ZF	Zucker fatty

I INTRODUCTION

1 DIABETES

The diabetes is a pathology defined by an abnormal increase of basal glycemia. By 2040, the International Diabetes Federation predicted that 1 adult in 10 will developed diabetes (http://www.diabetesatlas.org); this disease is one of the fasted increasing disease worldwide. Two types of diabetes exist: diabetes type 1 (T1D) or diabetes type 2 (T2D).

1.1 TYPE 1 DIABETES

Type 1 diabetes, formerly defined as "insulin-dependent diabetes mellitus" is characterised by a hyperglycemia due to pancreas failure to produce enough insulin. This deficit is caused by an auto-immune destruction of pancreatic β cells (Pickup et al., 1998). The original causes is largely unknown.

1.2 TYPE 2 DIABETES

Type 2 diabetes (T2D) is characterized by both impaired insulin secretion and its action in target tissues as muscle, liver and fat (Defronzo et al., 2009). In addition, a potential reduction of β-cell mass occurs, even if it is still debated. This type of diabetes is the most common, it represents 90% of diabetic cases, and touches about 5% of the over 40-years population worldwide. The appearance of this disease is relatively slow and mostly appears in elderly people. The study of this disease is really complex, due to the multifactorial essence of the pathology: in one hand there are environmental components among which obesity is the most prominent linked factor, in the other hand there is also contribution of genetics' architecture components (Unger 1995; Forouhi and Wareham 2014). Nowadays, disease's aetiology is still undefined and represents a controversial argument, making very difficult the understanding of its installation and the monitoring of its progression. Despite these controversies, it is generally accepted that association of both genetic and environmental components are involved.

Imbalanced regulation of glucose levels in T2D originates from two dysfunctions: resistance to insulin action on several target tissues, and impaired glucose-secretory response of β cells and

the potential decrease of this cell type number (Klöppel et al., 1985, Butler et al., 2003). Resistance to insulin action targets mainly skeletal muscle, adipose tissue and liver. Insulin resistance (IR) on skeletal muscle causes a decrease of insulin-stimulated glucose uptake of circulating glucose utilisation; it has been shown that approximately 80% of total body glucose uptake occur in skeletal muscle (Abdul-Ghani et al., 2010). IR on the adipose tissue leads to a constant release of free fatty acids (FFAs) on the bloodstream and to a decrease of glucose utilisation by this tissue. Regarding the liver, the loss of the insulin action leads to an extended release of glucose in blood and in parallel to an uncontrolled gluconeogenesis (Bugienesi et al., 2005). The state of chronic hyperglycemia and the deleterious effect associated to it has been defined as glucotoxicity (Butler et al., 2003).

Nowadays limited therapeutic options exist to treat diabetes and none to cure or prevent the disease, in part due to the limited knowledge of β cell biology in health and disease.

2 THE NORMAL B-CELL FUNCTION

2.1 INSULIN AND HOMEOSTASIS OF GLUCOSE LEVELS

In mammals, plasma glucose levels are tightly regulated in order to maintain glycemia. The β cells of endocrine pancreas act as a "glycemic detector" and guarantee an immediate and fine secretion of a hormone called insulin, in response to variations in plasma glucose levels. Insulin is a peptide hormone able to control body glucose homeostasis. Thanks to this regulation, glycemia is always maintained around 1g/l (5mM) in human, despite the daily food intakes and the energy expenditure. Insulin action is in opposition to the action of the counter-regulatory hormones, such as glucagon, epinephrine (adrenalin), norepinephrine (noradrenaline), cortisol, and growth hormone. The main insulin secretion stimulus is glucose (Figure 1), which coordinate the activation of insulin gene transcription, insulin mRNA transcript stability, pre-pro-insulin translation and finally secretion of this peptide (Figure 1). Different stimuli are able to modulate positively insulin release as many nutritional factors (as lipids), hormones (glucagon, glucagon like peptide 1 GLP-1), and the parasympathetic nervous system (through acetylcholine release) (Pickup et al., 1998). After a glucose intake stimulus, insulin is released in a biphasic way, with a first phase burst in insulin secretion that lasts about 10 minutes, and a second slow sustained phase of newly formed vesicles, peaking in 2 to 3 hours.

Figure 1

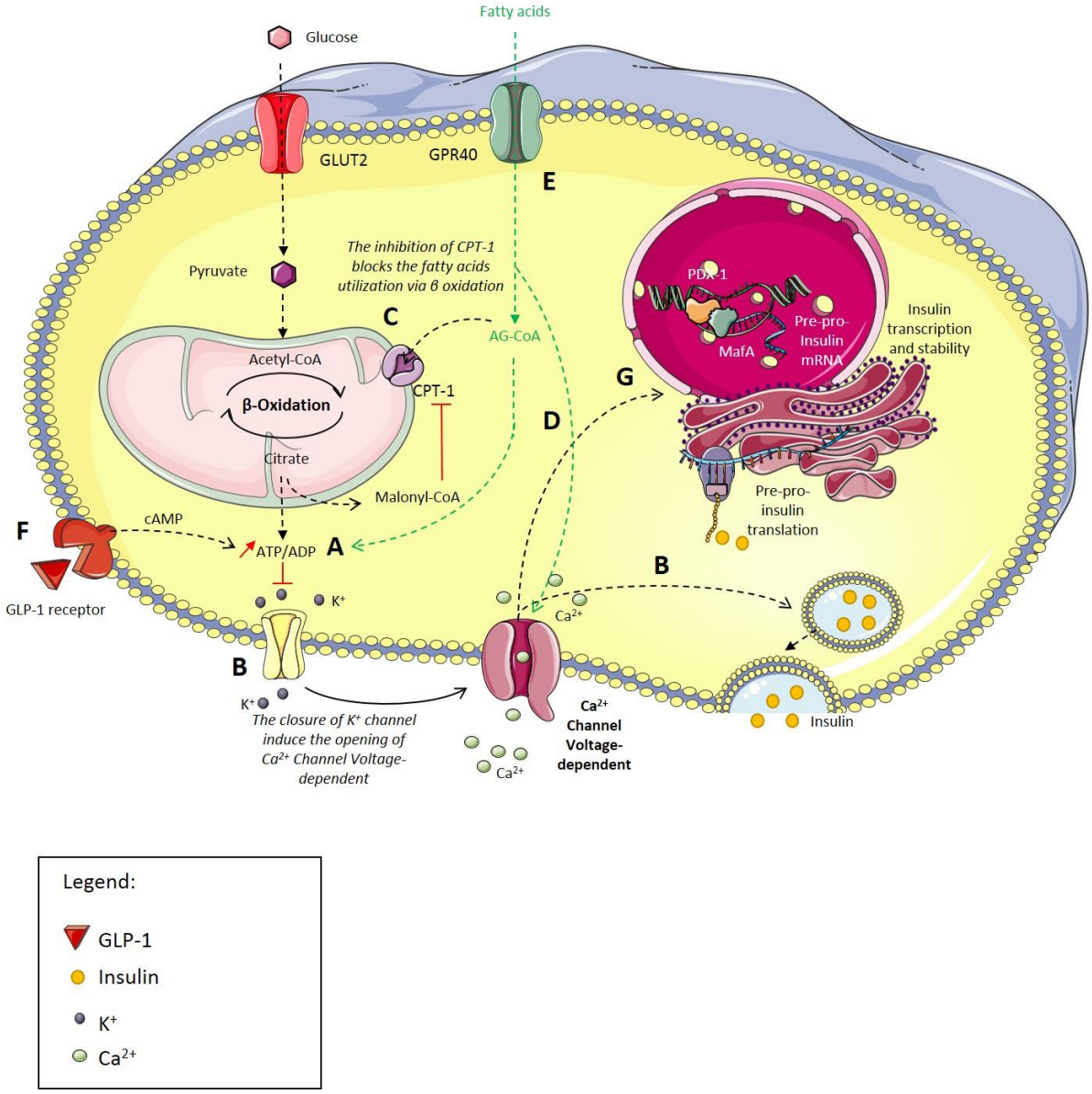

Figure 1. Insulin secretion mechanisms. Glucose metabolism into pancreatic β-cell increases the ATP/ADP ratio (A), the accumulated ATP induces the potassium channel closure, and the consequent membrane depolarization induces activation of a calcium channel, which induces a Ca^{2+} influx responsible for insulin vesicle exocytosis (B). This mechanism can be amplify by other pathways. Glucose metabolism leads to citrate production, which is malonyl-CoA precursor. In pancreatic β-cells, malonyl-CoA accumulation inhibits CPT-1 transporter, which blocks the fatty acids translocation in mitochondria and their utilization by the mitochondrial β-oxidation (C). Fatty acids regulate insulin secretion, via increased Ca^{2+} concentration (D). Fatty acids can also regulates insulin secretion via G-coupled receptor proteins, such as GPR40 (E). Others molecules secreted by others organs, such as GLP-1, potentiate the insulin secretion through cAMP synthesis (F). Glucose coordinates activation of insulin gene transcription by acting on PDX1 and MaFA transcription factors, its mRNA stability and translation (G). CPT-1: carnitine palmitoyl transferase, LC-CoA: long chain acyl-CoA, Glut2: Glucose type 2 transported, GPR40: G-coupled protein receptor 40, GLP-1: glucagon-like peptide 1, cAMP: Cyclic Adenosine Monophosphate, PDX-1: pancreatic and duodenal homeobox 1.

Insulin targets many tissues (Bugianesi et al., 2005); the most important for the regulation of glucose homeostasis, skeletal muscle, liver and adipose tissue. Insulin controls glycogen storage and lipid synthesis. In case of food intake, secreted insulin forces the storage of glucose in liver; activation of the glycogen synthase kinase 3 (GSK3) induces glycogen synthesis, and gluconeogenesis is blocked. In parallel, in adipose tissue, insulin forces adipocyte to store glucose under the form of triglycerides by activating lipogenesis. The insulin receptor belong to the tyrosine kinase receptor family and its activation is known to trigger the AKT/PKB pathway. On muscle, activation of AKT/PKB pathway induces glucose type 4 transporter (GLUT4) translocation to the plasma membrane, in way to increase glucose utilisation. This mechanism is present also in adipose tissue, where lipolysis is blocked, to avoid a competition between free fatty acids (FFAs) with the glucose as an energy source. In this way, after energy intake, the tissues can use the glucose as energy source, which is available in blood. Conversely, during starvation periods, activation of lipolysis in adipocytes increases circulating FFAs that can be used as energy source. The lack of insulin, and consequently of nutrients, reverses all of these responses.

2.2 MECHANISMS OF REGULATION OF INSULIN SECRETION BY GLUCOSE

The intracellular mechanisms able to control the insulin secretion mediated by glucose are complex and are still not completely elucidated (Newgard et al.,, 1995). In β cells, glucose stimulates insulin secretion by generating triggering and amplifying signals. The triggering pathway is well established and involves a series of precise events (Figure 1). Glucose enters via the transporter *Glut2*, which has a high affinity for glucose, but it is easily saturated. In this way, *Glut2* guarantees a rapid transport and an instantaneous equilibration with glycemia variation. Once entered, glucose is phosphorylated by glucokinase in glucose-6-phospate (G6P). The different reactions of oxidative glycolysis of G6P induce ATP accumulation; rises in the ATP-to-ADP ratio which induced closure of ATP-sensitive K^+ (K_{ATP}) channels (Figure 1A). The K^+ ions accumulation at the plasma membrane level induces an increase of the membrane potential. Membrane depolarisation opens voltage-operated Ca^{2+} channels followed by a Ca^{2+} influx which activate the exocytotic machinery and vesicles of insulin granules fuse at the membrane to release insulin (Figure 1B) (Henquin et al., 2000). The amplifying pathway starts to be active once cytoplasmic free Ca^{2+} concentration is elevated. The amplifying pathway

serves to optimize the secretory response not only to glucose but also to other stimuli. In animal model of type 2 diabetes, this pathway seemed to be impaired, and indirect evidence suggests that it is also altered in β-cells of type 2 diabetic patients. Unfortunately, the nature of the amplifying mechanisms is still not defined (Henquin et al., 2000). Also cyclic adenosine monophosphate (AMPc) resulted to play an important role on the insulin secretion amplification in response to glucose (Gromada et al., 1997; Kasai et al., 2002).

3 B CELL FAILURE IN TYPE 2 DIABETES

3.1 ENVIRONMENTAL FACTOR: OBESITY

Among the genetic or environmental factors correlated to T2D, obesity is the most relevant. Indeed, about 85% of T2D patients are overweight; modern lifestyle with abundant nutrient supply and reduced physical activity, typical of developed or developing countries, rises up the incidence of this disease. So much that, some studies support the idea that the lipid metabolism is important as much as (or even more) than glucose metabolism in the development of T2D pathology (McGarry 1992; Shafrir et Raz 2003).

Numerous studies have shown that first occur the IR, and secondly there is the development of hyperglycemia and eventually the T2D (Martin et al., 1992). However, T2D only develops in insulin-resistant subjects with the onset of β-cell dysfunction (Prentki et al., 2002; Poitout et al., 2002, Prentki et al., 2006).

3.2 HYPERLIPIDEMIA AND IR

In case of obesity, the energy intake is higher compared to the energy expenditure; this condition leads to a chronic surplus of lipids. In mammals, the tissue responsible for lipid storage is adipose tissue. Adipocytes with the help of insulin control the amount of circulating FFAs, by stocking the excess of lipids in form of triglycerides (TG) and making them available in case of energy deficiency (Unger et al., 2002). When an excess of FFAs occurs, adipose tissue compensates by hypertrophic (cell size increase) and hyperplastic (cell number increase) growth (Jo et al., 2009). If caloric surplus is chronic, it could happen that adipose tissue fails in

controlling circulating FFAs, and lipids start to be stored in ectopic tissues as muscle, liver and pancreas (Figure 2) (Van Herpen 2007). The sequence of events leading to ectopic accumulation of FFAs has been defined as "overflow hypothesis" (Danforth et al., 2000; Bugianesi et al., 2005); according to this hypothesis, IR is the result of the inability of adipose tissue to increase and store excess of calories. The fat overflow to muscle or liver interferes with insulin signalling, glucose transport/phosphorylation, and glycogen synthesis in muscle and increased hepatic gluconeogenesis (Bugianesi et al., 2005). Accumulation of lipids in liver results from an imbalance among the uptake, synthesis, export, and oxidation of fatty acids.

Figure 2

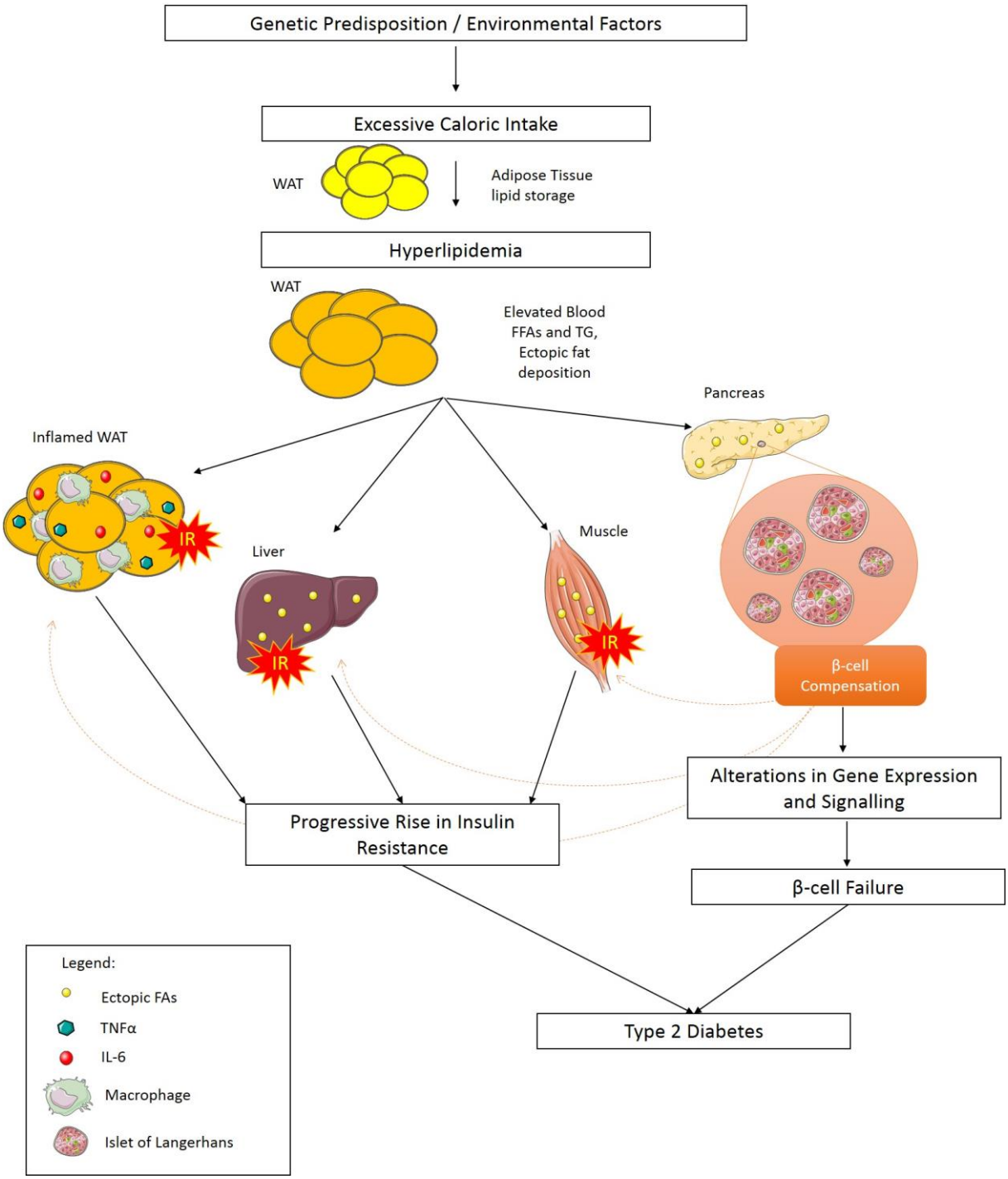

Figure 2. Type 2 Diabetes installation. Model illustrating the influence of calorigenic nutrients and over-nutrition in the aetiology of type 2 diabetes. Individuals subject to environmental factors, such as obesity, or with genetic predisposition, present a dysfunctional balance between food intake and energy expenditure, toward excessive caloric intake. The lipid surplus initially is stored in adipocytes, but when the storage capacity of white adipose tissue (WAT) reach a critical level, lipids start to be ectopically stored in muscle, skeletal cells and pancreas. The hyperlipidemia cause adipose tissue inflammation and the installation of insulin resistance. The pancreatic β-cell initially compensate the increased demand of insulin, by up-regulation of insulin expression and secretion. On other tissue, the insulin demand continue to exacerbate and when a β-cell failure occurs, there is installation of type 2 diabetes. WAT: white adipose tissue, TNF-α: tumor necrosis factor α, IL-6: interleukin 6, IR: Insulin Resistance.

The reason why adipose tissue becomes unable to stock the FFAs surplus is still unknown. For a long time, adipose tissue has been considered as an inert mass of stored energy with insulation and mechanical support properties. Recently, it has emerged as a master regulator of whole body energy homeostasis (Powell et al., 2007); adipose tissue resulted to manage much of the body's response to insulin. Indeed, the elimination of glucose transporter from the surface of mouse adipocytes caused muscle and liver IR installation. Overexpression of GLUT4 in adipocytes surface led to increased insulin-stimulated whole body disposal (Tozzo et al., 1997; Kim et al., 2000). In opposition, *Glut4* gene ablation in mouse adipose surface resulted in IR installation in muscle and liver cells (Abel et al., 2001). Crosstalk between adipocytes and other cells seems to be crucial for IR installation. In case of stress, adipocytes release a multitude of adipocytokines that could communicate with hepatocytes/skeletal muscle cells, and induce an inflammatory state in these tissue (Figure 3). In case of obesity, macrophage infiltration can be clearly observed in expanding adipocytes. The functional capability of these two cell types can overlap and works synergistically, leading to the secretion of inflammatory adipocytokines as TNFα, IL-6, IL-8, macrophage inflammatory protein-1α/β, monocyte chemotactic protein-1 (MCP-1) that has emerged as key regulators of IR (Anghel et Wahli 2007). In plasma of obese individuals, increase of different leucocytes, TNFα, IL-6, MPC-1, the plasminogen activator inhibitor PAI-1, C-reactive protein has been detected (Dandona et al., 2004; Juhan-Vague et al., 2003; Kamei et al., 2006). Adipocytokines act *in loco* modulating adipocyte metabolism: MCP-1, TNFα and IL1β increases adipocyte lipolysis and decrease TG synthesis (Guilherme et al., 2008). While experiments of co-culture of human adipocytes with skeletal muscle cells demonstrate that MPC-1 plays a key role in signalling between the two cell types and impaired insulin signalling and glucose uptake under physiological concentrations (Dietze-Schroeder et al., 2005; Xiang et al., 2007). Exogenously administered TNFα in humans attenuates both insulin signalling and glucose transport in skeletal muscles, a similar effect obtained with FFAs administration (Bouzakri et Zierath, 2007). In both cases, adipocyte dysfunction results from an increase of circulating FFAs, and to an ectopic fat storage (Mittra et al., 2008; van Herpen 2008). In condition of high fat oxidative capacity, concentrations of lipids intermediates such as DAG, ceramide, acyl-CoA, stay low, while situations with high nutrients availability but low fat oxidative capacity lead to increased concentrations of these intermediates (van Herpen 2007). When lipids are stored in non-specialized tissues, they are ineffectively oxidized, giving rise to a phenomenon defined as lipotoxicity. Therefore, insulin signalling pathway is hindered by the fat overload. In muscles, insulin-mediated glucose uptake into myocytes is inhibited by the FFAs, through the interference of the glucose transporter GLUT4 and its' translocation to

the plasma membrane from intracellular vesicles (Kusminski et al., 2009; McGarry 1992; Shulman 2000). On liver, FFAs has been shown to inhibit insulin-mediated suppression of glycogenolysis and gluconeogenesis (Boden et al., 2002). In muscle and liver cells, DAG accumulation can activate specific Protein kinase C (PKC), thereby triggering a serine/threonine kinase cascade, leading to phosphorylation of serine/threonine sites on insulin receptor substrate (IRS-1 and IRS-2) and consequently to the inhibition of insulin signalling (van Herpen 2007, Meshkani et al., 2009) .

Figure 3

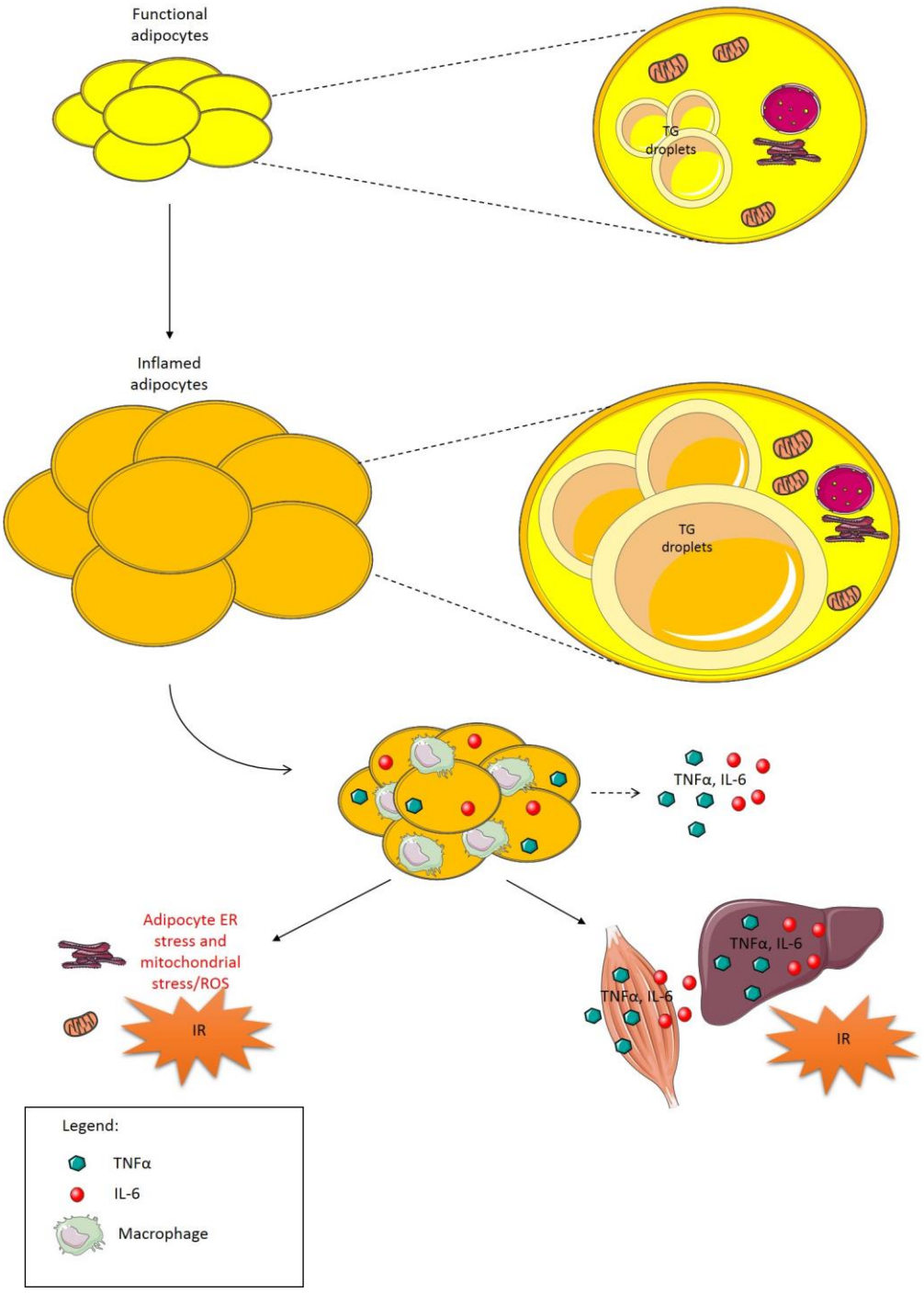

Figure 3. **Adipocyte inflammation mechanism**. Functional adipocytes play a primary role in case of fatty acid excess; they store TG droplets as an energy source to be used as and when required. However, if the stored TG surpass the adipocyte critical mass of expansion, adipocytes activate inflammatory pathways and became source of adipocytokines, such as TNF-α, IL-6 and MCP-1. When inflammation occurs, macrophage start to infiltrate in adipocytes. The perpetual inflammation state induces ER and mitochondrial stress, and ROS production, which culminate with IR. The released adipocytokines and cytokines communicate with other cells, such as hepatocytes and skeletal muscle cells, and induces an inflammatory state, that ultimately leads to IR installation. TG: triglycerides, TNF-α: tumor necrosis factor α, IL-6: interleukin 6, MCP-1: monocyte chemotactic protein-1, ROS: reactive oxygen species, ER: Endoplasmic Reticulum, IR: Insulin Resistance.

3.3 ADIPOSE TISSUE HORMONES AND LIPOREGULATION

Adipose tissue is programmed to protect other tissue in case of fat excess, which have more restricted capacity to store TG under conditions of energy surplus, by secreting hormones. The most relevant hormone is leptin, it is able to determine the partitioning of lipid excess, reducing lipogenesis and enhancing the compensatory FA oxidation capacity of non-adipocytes (Shimabukuro et al., 1997; Wang et al., 1998; Kusminski et al., 2009). Leptin-deficient rodent or rodents with non-functional leptin receptors shown in non-adipose tissues a rapid accumulations of TG, despite a normal food intake (Lee et al., 2001). Another adipocyte-derived hormone, adiponectin, seems to play a crucial role in the protection against lipotoxic insult. Adiponectin complements the leptin anti-steatotic and anti-apoptotic functions, acting as insulin sensitizer, normalizing dyslipidemia and presenting anti-atherogenic actions (Wang et Scherer, 2008). In pancreatic β-cells, adiponectin protects from apoptosis through inhibition of ceramide accumulation and stimulation of S1P production (Holland et al., 2011). Liver and muscle have the ability to dispose of surplus FFAs; liver can export TG as very low-density lipoproteins (VLDLs), while skeletal cells can lowers lipid content by increasing muscle activity to enhance FA oxidation and to reduce the risk of lipotoxicity (Kusminski et al., 2009). However, other tissues such as endocrine pancreas or heart are more vulnerable to a lipid "spill over". Indeed, pancreatic β-cells and the myocardium cells have both limited compensatory FA oxidation capacity as the sole means by which to dispose of excess FFAs (Lee et al., 1994, Shimabukuro et al., 1998, Szczepaniak et al., 2007).

When the liporegulatory system breaks, tissues initially respond adequately to facilitate TG surplus storage. These lipids, once sequestered, are considered as passive and harmless to the cell. However, the saturation of the neutral TG store can exceed the cells' storage capacity and drive hydrolysis back into fatty-acyl CoA. This event surpasses the oxidation capacity of a cell and feeds the unoxidized excess into non-oxidative toxic pathways of FA metabolism, such as the *de novo* ceramide synthesis (Unger 2005). The result of these lipids accumulation is cell dysfunction and apoptosis.

3.4 LIPOAPOPTOSIS

Many *in vivo* and *in vitro* studies demonstrated that FFAs induces cell apoptosis. *In vitro* fatty acid exposure induces apoptosis of hepatocytes (Cazanave et al., 2009; Ricchi et al., 2009), human smooth muscle cells and rat cardiac myocyte (Artwohl et al., 2009, Sparagna et al., 2000), and renal proximal tubular cell line (Katsoulieris 2009; 2010). Different rodents deficient in leptin signalling exist, and they are models of T2D induced by obesity: the ZDF rat (Zucker diabetic fatty) and the *ob/ob* mice present a non-functional leptin receptor, while the *db/db* mice are leptin-deficient. On these models, cardiomyocytes go through apoptosis and cell death, and it is correlated with TG accumulation (Zhou et al., 2000). Moreover, *db/db* mice or C57BL/6 mice fed with HFD presented TG accumulation in liver that lead to hepatocyte apoptosis (Witek et al., 2009). It is important to know that only some lipid species participate to apoptosis induced by lipotoxicity. Saturated FFAs resulted to be much more toxic than mono- or poly- unsaturated fatty acids. *In vitro* studies have been performed by stimulating cells with different fatty acid species; from these studies it appears that only saturated fatty acids such as palmitate (16:0) and stearate (18:0) induce apoptosis (Wei et al., 2006, Pagliassotti et al., 2007). In opposition, mono or polyunsaturated fatty acids (MUFA and PUFA respectively) as oleate (18:1), linoleate (18:2) or palmitoleate (16:1) do not induce apoptosis, but even protect against lipotoxicity induced by saturated FFAs (Wei et al., 2006). The same results have been obtained in cultured human islets (Maedler et al., 2003).

Why do saturated fatty acids cause lipotoxicity and MUFA protect against it? Nolan and Larten addressed this question and proposed a model in which MUFA promote processes of detoxification of saturated FFAs through: 1) enhancing their esterification and incorporation into stable lipid droplets and 2) via enhancement of their consumption by fatty acid β-oxidation (Figure 4) (Nolan et al., 2009). Recently, Hu and colleagues demonstrated a novel mechanism by which the MUFA counteracts palmitate effects. They demonstrated that palmitate induces DESG1, an enzyme responsible for the *de novo* ceramide synthesis, this induction is blocked by MUFA addition to the culture medium, which leads to the recution of ceramide accumulation (Hu et al., 2011).

The mechanisms involved in saturated fatty acids induced apoptosis have been partially determined *in vitro*. Lipotoxicity causes altered mitochondrial functions with cytochrome C release due to reduction in cardiolipin levels (Hardy at al 2003) and the consequent caspase

Figure 4

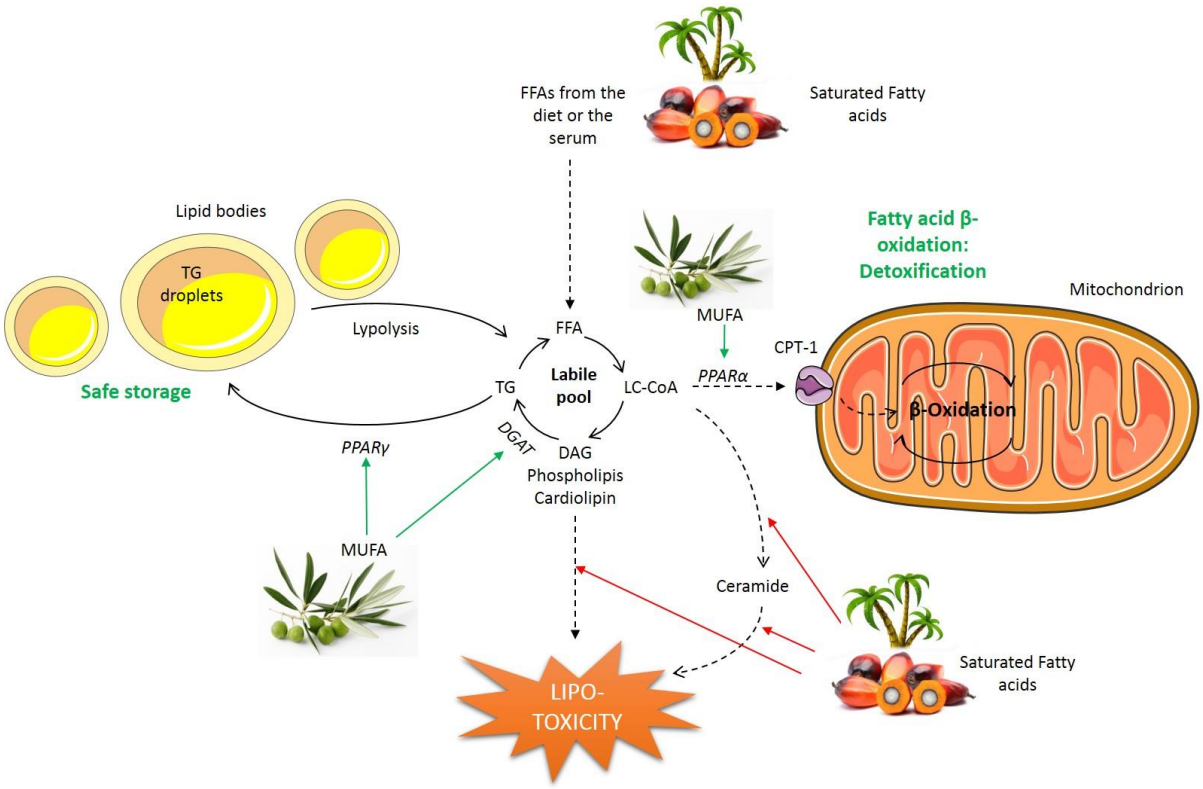

Figure 4. Fatty acid partitioning. Model of fatty acid partitioning during lipotoxicity. Saturated fatty acids and mono unsaturated fatty acids (MUFA) have a different metabolism in cells. Saturated FAs are less well incorpored into triglycerides (TG) compared to MUFA, which are DGAT preferential substrate for TG formation. Saturated FAs are preferentially used for ceramide synthesis, and lead to greater accumulation of DAG and a pattern of phospholipids (PL) with reduced cardiolipin (CL) production. Saturated FAs partitioning is associated with greater lipotoxicity. MUFA are well incorporated into TG and lipid droplets that is a safe mean of lipid storage. In this way, FAs are removed from the functionally active labile pool of lipids. MUFA rather than saturated FAs also activate the nuclear transcription factor PPARα and PPARγ, which respectively promote lipid detoxification via promotion of fatty acid β-oxidation and safe lipid storage. Importantly, MUFA promote the safer partitioning of saturated FAs into TG and fatty acid oxidation pathways. MUFA: Mono unsaturated fatty acids, PPAR: Peroxisome proliferator-activated receptors, TG: triglycerides, DGAT: the diacylglycerol acyltransferase, LC-CoA: long chain Acyl CoA, DAG: diacyl-glycerol. Inspired from Nolan et Larter 2009.

activation (Turpin et al., 2006), endoplasmic reticulum stress (Cazanave et al., 2009, Cunha et al., 2009, Akazawa et al., 2010), and or/oxidative stress as ROS production (Figure 6) (Inoguchi et al., 2000, Saitoh et al., 2008, Yu et al., 2001 Julien thesis).

3.5 B-CELLS COMPENSATION IN RESPONSE TO OBESITY

Different studies have demonstrated that IR installation precedes the development of hyperglycemia (Martin et al., 1992). Longitudinal studies of subject that develop T2D shown an increase in insulin levels in normoglycemic and pre-diabetic phase, which keep glycemia near normal levels despite the installation of IR. This happen because, in response to chronic fuel surfeit and obesity-associated IR, pancreatic β-cells can react and compensate by insulin hypersecretion in order to maintain glycemia (Prentki et al., 2006). As showed in Figure 5, overweight subjects need more insulin and islets respond by increasing their mass, enhancing insulin biosynthesis, and increasing the responsiveness of nutrient-secretion coupling. This compensation mechanism can fail in individuals with susceptible islets, due to genetic and acquired factors, then hyperglycemia can increase and T2D can be installed (Figure 5) (Prentki et al., 2006).

The mechanisms involved on the β cell compensation are not fully understood, but it is clear that both expansion of β cell mass and enhanced β cell function are important (Steil et al., 2001; Jetton et al., 2005; Liu et al., 2002). The stimulating factors implicated on the compensatory islet's response include nutrients, in particular glucose and FFAs, insulin, growth factor signalling, increase levels of incretin, such as glucagon-like peptide 1 (GLP-1), and increase of parasympathetic nervous system activity.

In islets, glucose metabolism play a key role coupling glucose sensing to insulin release (Prentki, 1996; Meglasson et Matschinsky, 1986). *In vivo* the most used model is the non-diabetic Zucker fatty rat (ZF); these rats are insulin-resistant but normoglycemic and, for this reason, they are an excellent model for the study of β cell compensation mechanism. Studies on ZF rats showed an up-regulation of glucose metabolism; the total glucose utilization in islets is 1.5 to 2 fold increased compared to lean rats. This was due to the increased flux through pyruvate carboxylase (PC) and the malate-pyruvate and citrate-pyruvate shuttles and increased glucokinase activity (Liu et al., 2002). The inhibition of these pathways by phenylacetic acid, which inhibits PC, markedly impaired glucose stimulated insulin secretion (GSIS) (Liu et al., 2002).

FFAs supplementation is essential for normal β-cell function, and it is well established that it induces an amplified GSIS (Prentki et al., 2006). The mechanisms by which FFAs do this are not completely defined, but it is clear that at least two pathways are involved. 1) FFAs are

ligands of the G protein coupled receptor FFAR1/GPR40 (Figure 1E), which activates intracellular signalling, including Ca^{2+} influx increase; 2) the malonyl-CoA/long chain acyl-CoA (LC-CoA) pathway, malonyl CoA inhibits FFAs oxidation such that LC-CoA accumulate in the cytosol, favouring the production of lipid signalling molecules (Liu et al., 2002; Roduit et al., 2004, Prentki et al., 1996) .

Regarding the incretin hormones, GLP-1 is likely to be involved in enhancing the β-cell function in compensatory conditions (Figure 1F) (van Citters et al., 2002). Interestingly, GLP-1 and FFAs had synergistic actions in their augmentation of GSIS (Nolan et al., 2006).

Figure 5

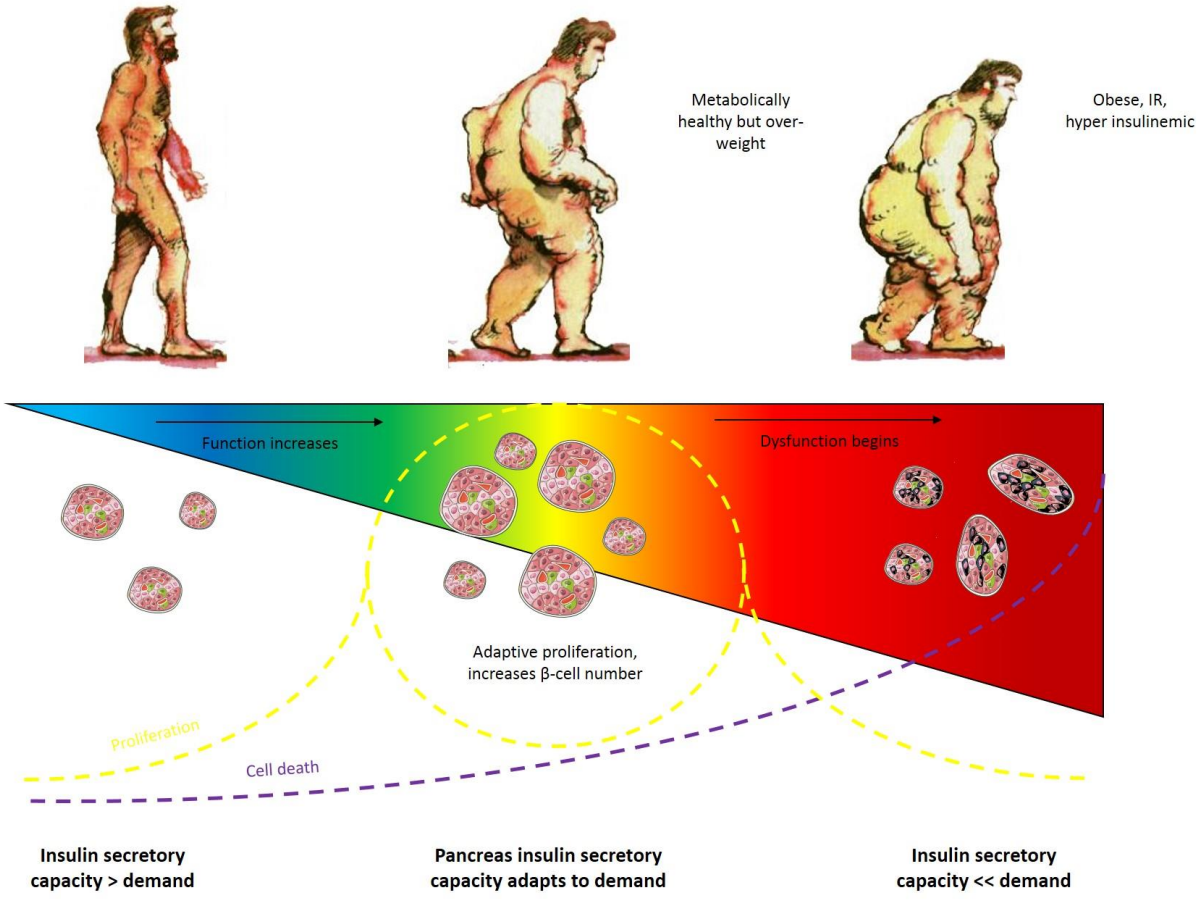

Figure 5. β-cell compensation and β-cell failure. In type 2 diabetes associated with obesity, the whole body is subject to constant lipid surplus. The increased body mass demands increased quantity of insulin, especially when tissues became insulin resistant. Islets of Langerhans adapt their secretory ability by increasing their number (hyperplastic) and their volume (hypertrophic). This compensation mechanism lasts for a limited time-window, depending on environmental factors of genetic predisposition. Over time, islets of Langerhans compensation for the insulin resistance fails, resulting in a progressive decline in β-cell function and apoptosis. Consequentially, subjects progress from normal glucose tolerance, to impaired glucose tolerance and finally to established type 2 diabetes. IR: Insulin Resistance.

Pancreatic islets are richly innervated with parasympathetic and sympathetic neurons (Ahrén et al., 2000). In the last years, more and more studies indicate that the brain plays a principal role on the T2D installation. There are evidences that the brain and increased parasympathetic nervous system activity are involved in β-cell compensatory growth and function (Lamy et al., 2014).

3.6 HYPERGLYCEMIA

The step that precede installation of diabetes is the appearance of chronic hyperglycemia. On human, normal glycemia values are around 5mM of glucose, in a T2D patient this value can climb to 7 mM of glucose in average (Pickup et Crook, 1998). Different tissues such as muscles, kidneys, blood vessels and eyes could have altered function capacity in response to the severity and exposure time to chronic hyperglycemia. On these tissues, the glucose intracellular concentration exceeds the normal quantity and induces excessive activation of metabolic and signalling pathways. For example, high glucose concentration constitutively actives the PKC pathway causing permeabilization of vascular endothelium cells (Wolf et al., 1991), and leading to retinopathy, nephropathy, and vascularopathy (Brownlee, 1992). At the level of skeletal muscle, hyperglycemia interferes with insulin-signalling pathway, and participate to the IR installation (Rossetti et al., 1987). Hyper-activation of the glucose signalling pathways inhibits mechanisms controlled by insulin receptor: for example, activation of IRS-1 and AKT/PKB (by phosphorylation) are inactivated through phosphatase proteins. Hyper-activation of the glucose metabolism can also lead to ROS accumulation and induce apoptosis (Mishra et al., 2005). Hyperglycemic side effects on tissues or cells is a phenomenon has been defined as glucotoxicity (Unger et Grundy, 1985).

3.7 FACTORS THAT LEAD TO B-CELL FAILURE

The factors that determine susceptibility to β-cell failure and T2D installation are complex and not completely defined. At the molecular levels, the most relevant are β-cell “steatosis” and lipotoxicity, defective insulin promoter, glucotoxicity or defective fatty acid β-oxidation (Prentki et al., 2006). Regarding the genetic component, the determination of key playing factors is even more complex. The susceptibility of islets to dysfunction and apoptosis is very dependent on species and genetic background. For examples, gerbil *psammomys obesus* and

human isolated islets, cultured and exposed to elevated glucose, are highly susceptible to dysfunction and apoptosis compared with rodent isolated islets (Kaiser at al 2005; Federici et al., 2001). Among mice strains, studies have shown that female C57BL/6j mice increase their body weight and their blood glucose already during the first week of HFD compared to littermates fed on regular chow diet, and became with time progressively glucose intolerant and insulin resistance (Winzell et Ahrén, 2004). Under HFD, DBA/2 mice congenitally display a strong insulin secretion in response to glucose while 129S2 mice have higher fasting plasma glucose levels (Andrikopoulos et al., 2005). A/J mice compared to C57BL/6j mice are obese-resistant without changes on food intake (Surwit et al., 1995). Recently, it has been shown that C57Bl/6j mice had a defective insulin secretory response to glucose compared to C57Bl/6n mice, a difference likely due to a single mutation in *nicotinamide nucleotide transhydrogenase (Nnt)* gene, a mitochondrial enzyme involved in NADPH production, that could lead to susceptibility to develop glucose intolerance and β-cell dysfunction (Fergusson et al., 2014). Human studies of polymorphisms revealed correlations between T2D and *PPARγ* (Deeb et al., 1998), *PPARγ coactivator 1-α (PGC1α)* (Ek et al., 2002), transcriptor factor *Kruppel-like factor 11 (KLF11)* (Neve et al., 2005), *calpain 10 (CAP10)* (Weedon et al., 2003), *transcriptor factor 7-like 2 (TCF7L2)* (Grant et al., 2006), and *KCNJ11 (Kir6.2)* (Gloyn et al., 2003). The limitation of these polymorphisms is that, for the majority of them, it is uncommon for a significantly increased T2D risk to be shown in more than one or few population cohorts, consistent with enormous heterogeneity in heritability for this condition (Parikh et Groop 2004). An exception are the transcription factors *KCNJ11* and *TCF7L2*; *TCF7L2* polymorphisms are common in Icelandic, Danish and US cohorts, *KCNJ11* polymorphisms are relatively common as indicated by population-based studies and meta-analysis (Gloyn et al., 2003). Finally, epidemiological evidences show an importance of the intrauterine environmental factors in T2D installation. These evidences points to a role for low birth weight or intrauterine growth retardation, concluding that T2D and hypertension have a common origin in sub-optimal development in utero (Barker et al., 1993).

Regarding the deleterious effects of FFAs overload, they seemed to be not deleterious in β-cell until hyperglycemia is not present (Poitout el al 2008). Indeed, in normal state, FFAs are essential β-cell fuels, and prolonged FFAs exposure of pancreatic β-cells increases basal insulin release (McGarry et Dobbins 1999), and permits β-cells adaptation to insulin resistance, as exemplified by the ZF rat studies (Nolan et al., 2006). β-cell studies (both *in vivo* and *in vitro*) using "reasonable" (no toxic) concentration of FFAs and in absence of hyperglycemia, have

been developed to investigate the adaptive process of the β-cell to FFAs and coined the term "lipoadaptation" rather than "lipotoxicity".

3.8 B-CELL FAILURE: MECHANISMS

Since now, it has been presented the chronic glucose and the FFAs effects separately, named respectively as glucotoxicity and lipotoxicity. In β-cells, toxic effects of hyperglycemia and FFAs are exacerbate when these two factors are both present; this condition has been defined as "glucolipotoxicity" (Prentki et al., 2002).

To understand the mechanisms of glucolipotoxicity, a central question is if the lipotoxic effects are due to increased FFA oxidation and a resulting decrease in glucose oxidation or to generation of cytosolic signals via esterification of FFAs. In literature, different authors favour the view that one or several intermediate metabolites generated by FFAs esterification pathway mediate the deleterious effects of chronically elevated FFAs (Poitout et al., 2002). To support this hypothesis there is the evidence that prolonged exposure to FFAs is associated with profound alterations in lipid metabolism and minimal changes in glucose metabolism (Segall et al., 1999). According to this model, in glucolipotoxic conditions cytosolic citrate accumulates, it is the precursor of malonyl coA, which in turn inhibits the carnitine-palmitoyl-transferase-1 (CPT1) (Figure 1D), the key enzyme for β-oxidation pathway. Prolonged CPT1 inhibition results in cytosolic accumulation of LC-CoA, which are proposed to mediate the deleterious effects of chronically elevated FFAs (Prentki et al., 1996). LC-CoA accumulation could directly affects β-cell function, or could serve as a precursor for other active molecules such as diacylglycerols or phospholipids. The precise nature of the downstream effectors of lipid accumulation is unknown, although several candidates have been proposed.

3.8.1 Oxidative stress and UCP2

Glucolipotoxicity effects involved negative regulation of several transcription factors, such as MafA and PDX-1, and, at least in part, these effects are mediated by generation of chronic oxidative stress. Reactive oxygen species (ROS) are physiologically produced in cells during nutrient oxidation: they are produced in mitochondria, in the plasma membrane electron-transporting NADPH oxidase complex (Gray et al., 2011), and in peroxisomes during fat

oxidation (Newsholme et al., 2009). To note is that, pancreatic islets contains particularly low levels of antioxidant enzymes compared to other tissues, that suggests in one side a role for ROS as signalling molecules in insulin secretion and in the other side it suggests that β-cells are particularly at risk for oxidative stress (Figure 6A) (Newsholme et al., 2009, Tiedge et al., 1997). There are some evidence implicating ROS in insulin secretion regulation, it appears that ROS can have opposite effects: stimulating secretion when their concentrations are low, and causing β cell dysfunction when they are chronically produced in high amount (Prentki et al., 2013). Uncoupling protein 2 (UCP2) is activated by ROS and *UCP2* gene is induced by chronically elevated FFAs and glucose (Figure 6A). Increased UCP2 helps to safely dissipate elevated mitochondrial membrane potential and promotes fuel detoxification: energy surplus is then oxidized in heat instead to ATP production (Prentki et al., 2006). The uncoupled oxidative phosphorylation caused impaired insulin-secretion capacity; this is the price that β-cell pays for its survival in the presence of fuel surfeit.

3.8.2 G- coupled protein receptor (GPR40)

The G-coupled protein receptor GPR40 is specifically expressed in brain and pancreatic β-cells (Briscoe et al., 2003) and it is activated by long-chain FFAs (Figure 6D) (Itoh et al., 2003). First studies on this receptor demonstrated that GPR40 mediates the long-chain FFAs amplification of glucose-stimulated insulin secretion (Itoh et al., 2003), indicating a role of this receptor on insulin secretion control by FFAs. The *GPR40* KO mice model confirmed this hypothesis since GPR40-deficient β-cells secrete less insulin in response to FFAs (Steneberg et al., 2005). A parallel study demonstrated that isolated islets from *GRP40* KO mice shortly stimulated with FFAs had markedly reduced insulin release potentiation mediated by FFAs (Latour et al., 2007). More recently, Wagner and colleagues have shown that a specific GPR40 agonist, TUG-469, stimulate glucose-induced insulin secretion through GPR40 in human islets (Wagner et al., 2013). At present, mice models overexpressing GPR40 showed contradictory results on glucose homeostasis. Loss of GPR40 protects mice from obesity-induced hyperinsulinemia, hepatic steatosis, and hypertriglyceridemia increased hepatic glucose output, hyperglycemia, and glucose intolerance whereas overexpression of GPR40 in β-cells of mice leads to impaired β-cell function, hypoinsulinemia, and diabetes (Steneberg et al., 2005). In contrast, another study demonstrated that mice overexpressing GPR40 in pancreatic β-cells showed improved oral glucose tolerance, increase insulin secretion and resulted resistant to HFD induced glucose intolerance (Nagasumi et al., 2009). Interestingly, prolonged FFAs incubation of *GPR40* KO

islets were sensitive to FFAs inhibition of insulin secretion as much as wild type islets (Latour et al., 2007) suggesting that GPR40 is not involved in β-cell failure induced by obesity. Proapoptotic effect of chronic exposure of β-cells to palmitate was also independent of GPR40. However, the potent agonist of GPR40, TUG-469, was protective, whereas inhibition of GPR40 promoted apoptosis (Wagner et al.,. 2013). Therefore, complementary studies result to be necessary to elucidate the GPR40 role on obesity and pancreatic β-cell glucolipotoxicity.

Figure 6

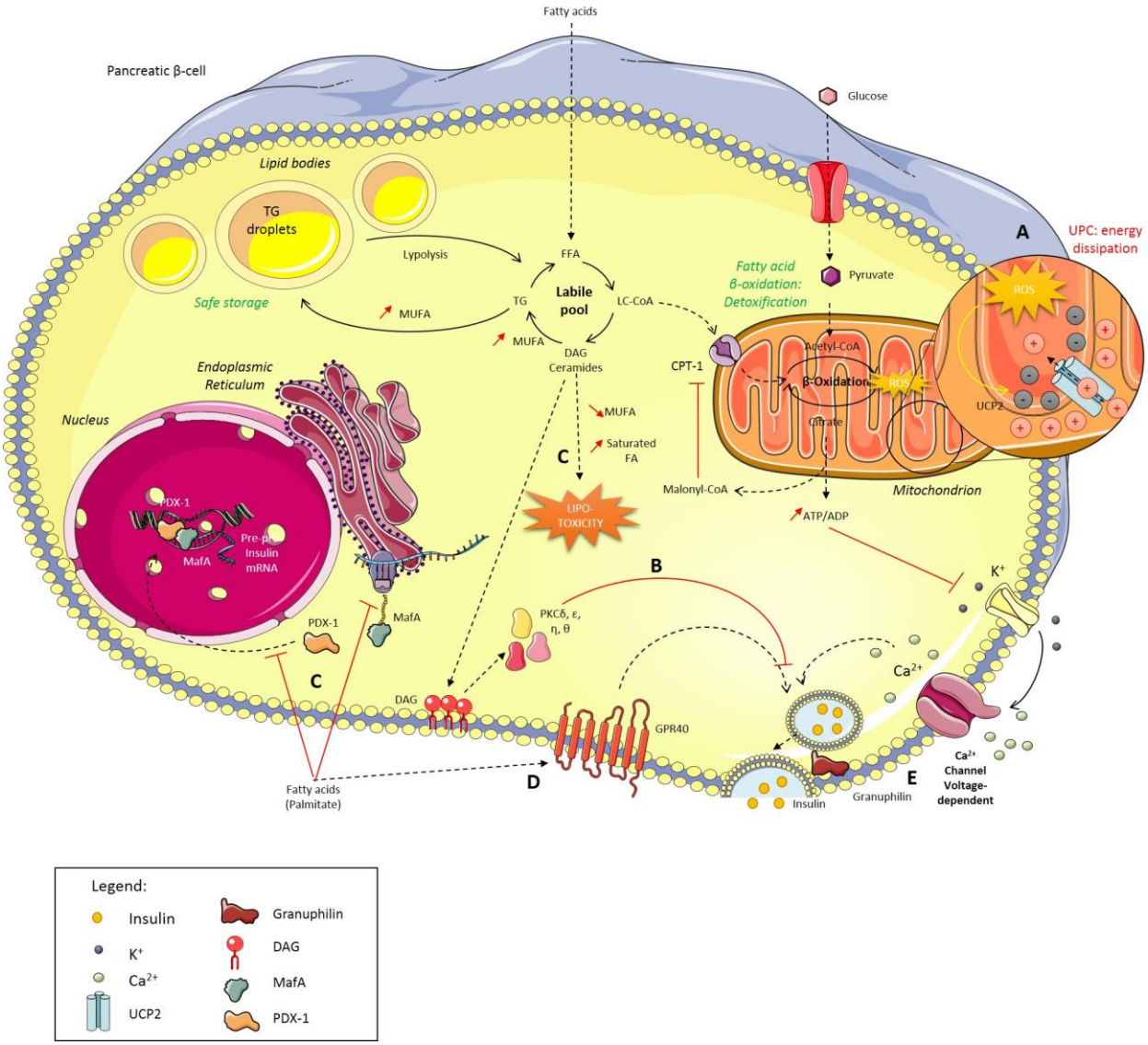

Figure 6. Mechanisms involved on the β-cell failure. The β-cell failure can be caused by different mechanisms. During high palmitate concentration, the detoxification machine can be activated, indeed increased ROS production induces UCP2 activation, which dissipate energy in heat (A). In presence of high glucose concentrations, malonyl-CoA accumulates and inhibits CPT-1, which blocks fatty acid utilization by mitochondrial β-oxidation pathway. The excess of lipids is partitioned in other lipid species, as TG that can be safely stored in TG droplets, or DAG and ceramides. DAG can activate PKC that inhibits insulin secretion (B); ceramides seems to be involved on the inhibition of insulin gene expression and on insulin secretion defects (C). Saturated fatty acids activate GPR40, which induce insulin secretion (D). During glucolipotoxicity, SREBP1-c is activated and it activates granuphilin, which negatively controls insulin granules exocytosis (E). ROS: Reactive oxygen species, UCP2: Uncoupling protein 2, CPT1: carnitine-palmitoyl-transferase-1, DAG: diacyl-glycerol, PKC: Protein kinase C, TG: triglycerides, GPR40: G-coupled protein receptor, FFA: Free fatty acids, LC-CoA: long chain Acyl CoA, MUFA: Mono unsaturated fatty acids, TG: triglycerides.

3.8.3 Protein kinase C (PKC)

Recently, different studies highlighted a glucolipotoxic key role of the protein kinase C (PKC) family. Chronic lipid treatment of muscle, liver and β-cells results in preferential activation of the novel subgroup of PKC isozymes (Figure 6B) (Schmitz-Peiffer et al., 1997; Wrede et al., 2003). The PKC novel group, which include the δ, ε, η, and θ isoforms, requires DAG for activation and does not require Ca^{2+} influx. PKCε deletion augmented insulin secretion and prevented glucose intolerance in HFD-fed mice and PKCε-inhibitory peptide improved insulin availability and glucose tolerance in the diabetic *db/db* mice (Schmitz-Peiffer et al., 2007). *Ex vivo*, functional ablation of PKCε selectively enhanced insulin release in diabetic or lipid-pretreated islets (Schmitz-Peiffer et al., 2007). *In vivo*, overexpression of kinase-negative PKCδ (PKCδKN) in pancreatic β-cells protected mice from HFD-induced glucose intolerant. The islets resulted to have increased size and to be less subject to apoptosis compared to WT animals. *In vitro*, isolated islets and pancreatic β-cells over-expressing PKCδKN were protected from palmitate-induced mitochondrial dysfunction. The mechanism involved was the inhibition of FOXO1 activation and translocation into nucleus (Hennige et al., 2010).

3.8.4 Insulin Exocytosis

Another mechanism involved on FFAs impairment of insulin secretion seems to be at the levels of insulin exocytosis (Figure 6E). The granuphilin plays a role in the docking of insulin secretory granules to plasma membrane. Granuphilin promoter has been demonstrated to be a SREBP-1c target. SREBP-1c is a transcription factor that control the expression of genes involved on FFAs synthesis, β-cell differentiation, and insulin synthesis (MafA expression). Granuphilin and SREBP-1c are activated in several diabetic mouse models and in normal islets treated with palmitate (Kato et al., 2006). Granulophilin over-expression correlates to inhibition of insulin secretion, and, in opposition, knock down or knock out of this protein restores insulin secretion (Kato et al., 2006). To support this study, Olofsson and colleagues demonstrated that long-time islets of Langerhans exposure to glucolipotoxicity inhibits the fusion steps of insulin granule with plasma membrane (Olofsson et al., 2007).

3.8.5 Insulin gene expression

Inhibition of insulin secretion mediated by glucolipotoxicity does not involve only regulation of insulin-signalling pathway and secretory mechanisms but also negative regulation of insulin

gene expression (Figure 6C). Interesting is to note that only saturated fatty acids have been demonstrated to affects insulin gene, and this has been demonstrated to be mediated through ceramide production and accumulation (Kelpe et al., 2003). Kelpe and colleagues have shown that isolated islets exposed to palmitate for long time have decreased insulin mRNA levels, which is not due to changes in mRNA stability, but to direct inhibition of glucose-induced promoter activity (Kelpe et al., 2003). A following study, determined that the transcriptional factors involved are PDX-1 and MafA. Glucolipotoxicity reduces PDX-1 nuclear translocation and blocks the stimulation of MafA mRNA protein expression by glucose (Hangman et al., 2005). Palmitate and ceramide inhibit insulin gene expression through the inhibition of Per-Arnt-Sim (PASK) mRNA and protein levels in MIN-6 cells and cultured islets of Langerhans. PASK over-expression increased insulin gene expression in presence of glucolipotoxicity (Fontés et al., 2009).

3.9 B-CELL APOPTOSIS

β-cell dysfunction in diabetes is not only restricted to impairment of the β-cell secretion and function, but involves also β-cell apoptosis.

3.9.1 β-cell death in human islets

Chronic hyperglycemia has negative effects on β-cell mass and function. A pioneer study, performed on human autopsy pancreata, gave precious information regarding the pathogenesis of islet β-cell failure in T2D. The study was performed on 124 subjects, included T2D patients and those with impaired fasting glucose (IFG). The study highlighted a 40% and 63% loss of islet volume in IFG and T2D subjects compared to the respectively weight-matched controls (Butler et al., 2003). Lean T2D subjects had 41% less β-cell mass compared with lean controls. On this study, it was also investigated if the decrease of islets mass was due to apoptosis or to reduced β-cell proliferation. The frequency of β-cell replication resulted to be very low and no differences among the groups emerged. New islets formation from exocrine ducts (neogenesis) was increased with obesity, but it was comparable between obese, obese T2D, and IFG, while non-diabetic subjects, lean T2D and non-diabetic subject had similar values (Butler et al., 2003). The TUNNEL staining highlights that the frequency of β-cell apoptosis was increased 10-fold in lean and 3-fold in obese cases of T2D compared with their respective non-diabetic control groups. These evidences determined that the decrease of islet β-cell mass was associated

to islet apoptosis. This work supports the idea that the main dysfunction in T2D could be associated to β-cell apoptosis, and that strategies for T2D prevention should be to avoid β-cell apoptosis. Interesting, data of IGF patients suggests that β-cell mass changes is not necessary confined to late-stage T2D (Butlet et al., 2003). Other studies support a role for selective loss of islet β-cells in pathogenesis of T2D (Yoon et al., 2003, Hanley et al., 2010). Culture of human islets of Langerhans culture under high glucose and high palmitate concentration confirmed that glucolipotoxicity induced apoptosis (Federici et al., 2001, Maedler et al., 2001, Maedler et al., 2002, Brun et al., 2015). Interestingly, different animal models for T2D present a β-mass reduction (Bernard-Kargar et Ktorza 2001) correlated with hyperglycemia and glucotoxicity (Jonas et al., 1999, Tanaka et al., 1999).

Ex vivo culture of human islets also showed that only saturated FFAs as palmitate and stearate induced apoptosis. Monounsaturated FFAs as oleate not only did not induce apoptosis, but they also prevented deleterious effects of both palmitic acid and high glucose concentration (El-Assaad et al., 2003, Maedler et al., 2003).

3.9.2 B-cell apoptosis: mechanisms

Nowadays, different mechanisms have been demonstrated to be involved on the apoptosis induced by the glucolipotoxicity (Figure 7). The *in vitro* and *ex vivo* studies point the attention on the fact that only saturated FFAs are involved on β-cell apoptosis. This difference on the pro-apoptotic effects of FFAs is proposed to be due to the greater ability of unsaturated FFAs to form intracellular TG (Figure 4); MUFA rescue palmitate-induced apoptosis by channeling palmitate into TG pools and away from pathways leading to apoptosis (Cnop et al., 2001, Listenberger et al., 2003). Moreover, if triglycerides synthesis is impaired, oleate induces lipotoxicity. A work performed on a selected sub-population of glucolipotoxic resistant β-cells, demonstrated that on these cell's groups the stearoyl-CoA desaturase 1 (SCD-1) was increased. The SCD-1 enzyme is involved on the conversion of palmitate in palmitoleate and steraate in oleate, it can modify the oleate-to-palmitate ratio and consequently it allows the modification of the neutral lipid storage pool generation (Busch et al., 2005; Shimabukuro et al., 1998). These findings indicate that the capacity of a cell to desaturate fatty acids protects from glucolipotoxicity.

Figure 7

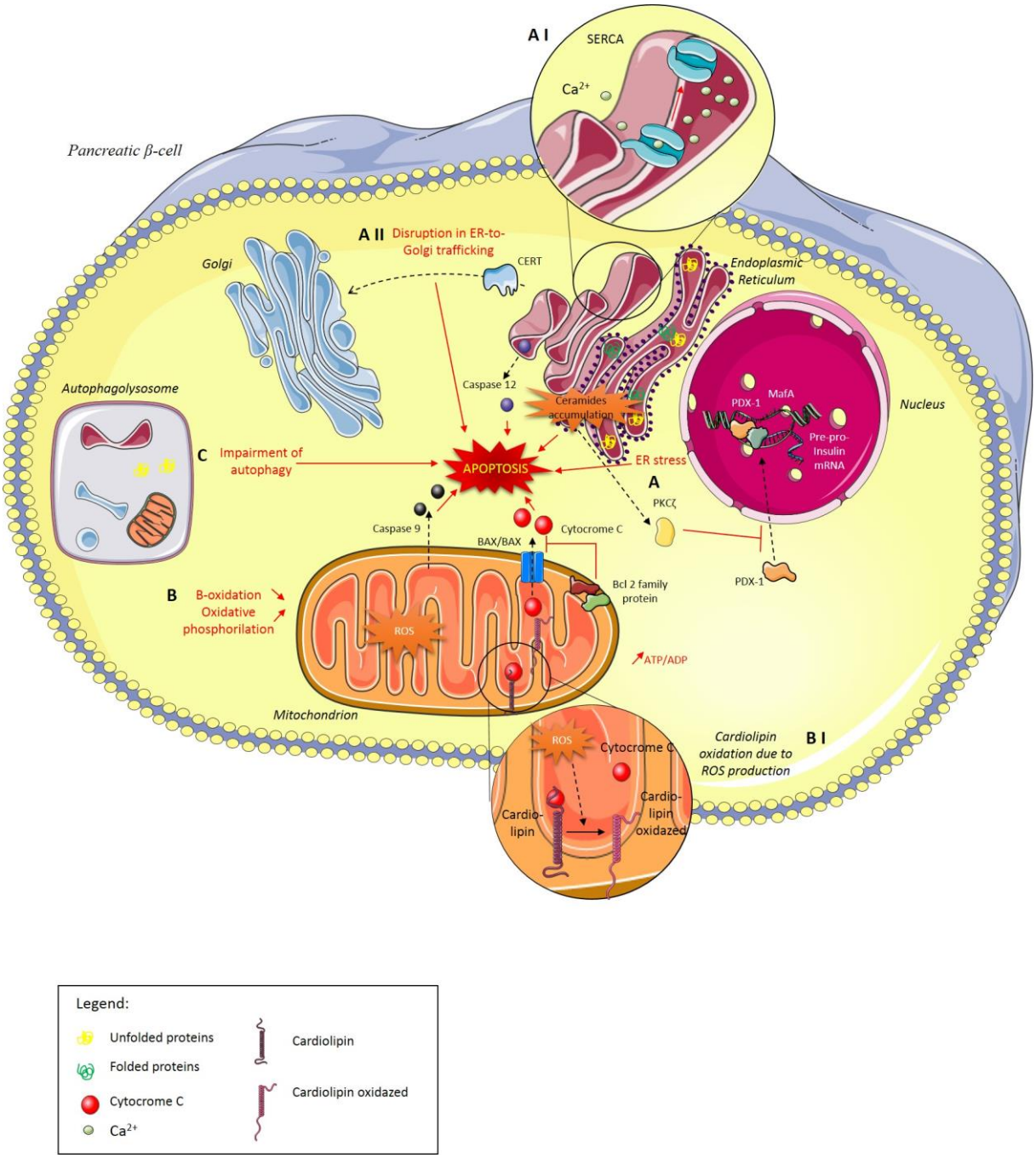

Figure 7. Mechanisms involved on the β-cell apoptosis. The β-cell apoptosis can be caused by different pathways. (A) ER stress is activated during glucolipotoxicity (GL). It can be due to many factors, such as unfolded protein accumulation or impaired ER-to-Golgi protein trafficking. During GL, ceramide accumulation occurs, and it can induce caspase-12 release or PKCζ activation that inhibits PDX-1 nuclear translocation and finally insulin gene transcription. Not only the protein trafficking is impaired, but also lipid trafficking through CERT protein (A II). SERCA pomp can be inhibited by different factors, including high palmitate concentrations, and this inhibition perturbs Ca^{2+} concentrations and mediates ER stress (A I). (B) Another important organelle affected during glucolipotoxicity is the mitochondrion. GL induces the inhibition of β-oxidation and the induction of oxidative phosphorylation. Increased ROS induces caspase 9 release and cardiolipin oxidation, which causes cytochrome C release (B I). During GL, it has also been evidenced impaired autophagy (C). SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase, ROS: Reactive oxygen species, GL: glucolipotoxicity, ER: endoplasmic reticulum, PKC: protein kinase C. PDX-1: pancreatic and duodenal homeobox 1.

3.9.2.1 ER stress

More recently, endoplasmic reticulum (ER) stress has been shown to play a central role on FFAs mediated β-cell apoptosis (Figure 7A). The first evidences came from *in vitro* studies: β-cells chronically exposed to elevated FFAs increased ER stress markers, and caused alteration of ER morphology (Laybutt et al., 2007). Further studies confirmed that ER stress markers were elevated in pancreatic islets of T2D patients (Marchetti el al 2007, Huang et al., 2007). ER stress is activated by an accumulation of unfolded proteins that can be caused by 1) protein overload or 2) alteration of ER milieu that compromise folding efficiency (Biden et al., 2014). In both cases, the unfolded protein response (UPR) is triggered in an attempt to resolve ER stress. β-cells required a highly efficient UPR since the pro-insulin accounts for more than 50% of total protein synthesis (Biden et al., 2014), especially in case of enhanced insulin demand, such as it occurs during obesity in compensation for IR. Mild UPR activation contributes to acute stimulation of pro-insulin biosynthesis by glucose. In human, several rare mutations that cause misfolding of pro-insulin result in ER stress and β-cell failure. Regarding protein misfolding, perturbators of the ER milieu, such as nitric oxide, cytokines, and glucotoxicity, appear to act as ER stressors by diminishing lumenal Ca^{2+} content (Figure 7A I) (Hara et al., 2014). A recent study, demonstrate that both elevated palmitate concentration and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor are more effective at lowering Ca^{2+} compared to cytokines or glucotoxicity (Hara et al., 2014). Intriguing, overexpression of mutant secretory proteins also depleted ER Ca^{2+} normal concentrations, suggesting that this phenomenon occurs as a consequence, as well as a trigger, of ER stress (Hara et al., 2014). Diminished ER Ca^{2+} is necessary to induce the apoptosis in response to cytokines, glucotoxicity, and nitric oxide donors, since SERCA2b overexpression partially rescues the effects of these ER stress agents (Hara et al., 2014; Oyadomari et al., 2001; Cardozo et al., 2005). However, no such causation has been demonstrated for lipotoxic ER stress; ER Ca^{2+} depletion in β cells is better established for other ER stressors than for lipotoxicity. Nevertheless, it is necessary to keep in mind that ER Ca^{2+} is only an indirect measure of the actual state of protein folding. To specifically investigate the protein misfolding, an ER-localizator reported has been created: the vescicular stomatitis virus glycoprotein tagged with GFP (VSVG-GFP), and palmitate did not shown to induce protein misfolding, whereas SERCA inhibitor did (Preston et al., 2009). ER stress induced by protein over-load could arise from enhanced protein biosynthesis or impaired exit protein from the ER. To investigate the second mechanism, β-cells were transfected with the temperature-sensitive VSVG-GFP reporter; accumulation of this protein on the ER allows to

determine the impaired ER to Golgi proteins trafficking. Several groups conclusively showed that lipotoxicity disrupts ER-to-Golgi protein trafficking (Figure 7A II)(Preston et al., 2009, Petremand et al., 2012, Véret et al., 2013). Impaired ER-to-Golgi protein trafficking is an early general defect that leads to protein overload-induced ER stress, resulting in impaired pro-insulin maturation, loss of insulin content and finally to apoptosis. Lipotoxic ER stress slightly differs from the classic UPR, especially through the activation mode and the independent modulation of down streamer pathways. Several mechanism have been proposed to underlie protein trafficking or folding that is compromised by FFAs. Fu and colleagues found that increased phosphatidyl-choline:phosphatidyl-ethanolamine (PC:PE) ratio results in inhibition of SERCA activity and ER stress (Fu et al., 2011). In addition, a general decrease in ER membrane phospholipid unsaturation might impair the morphology of this organelle and the budding of secretory vesicles (Volmer et al., 2013). Lipid raft alteration that accompany chronic saturated FFAs exposure may provide a unique explanation for concomitant protein overload and ER stress (Janikiewicz et al., 2015). Prolonged ER stress might result in apoptosis; indeed ER can sense and transmit apoptotic signals in β-cells in response to lipotoxicity. ER stress can trigger several broad mechanisms that lead to apoptosis, including the transcriptional induction of C/EBP homologous protein ((CHOP)/GADD153), activation of c-Jun NH_2-terminal kinase (JNK), and activation of caspase-12 (Figure 7) (Janikiewicz et al., 2015).

3.9.2.2 Mitochondrial dysfunction

In normal glucose conditions, FFAs catabolism via mitochondrial β-oxidation serves as a significant source of ATP in pancreatic β-cells. In T2D patients, an impaired secretory response to glucose had been shown to be correlated with considerable modifications of mitochondrial function and morphology (Figure 7B) (Janikiewicz et al., 2015). As already discussed, mitochondria are the main source of ROS, and obesity-associated hyperglycemia induces lipotoxicity by augmenting the ROS production in mitochondria (Barlow et affourtit 2013). A persistent imbalance between excessive ROS formation and restricted antioxidant defences leads to oxidative stress and injury, which can eventually damage mitochondria and cause β-cell death (Karunakaran et Park 2013).

Nowadays, little is known about the mechanisms that trigger mitochondria-dependent apoptosis in β-cells. Mitochondria respond to pro-apoptotic stimuli promoting the release of intramembranous proteins, such as cytochrome c and apoptosis-inducing factor (AIF) into the cytoplasm, which consequently induce the apoptosomes formation. Initiator caspase-9 becomes

activated, and it activates executioner capsases-3, -6, and -7. During the first phases of apoptosis, stimulation of ROS production on the inner mitochondrial membrane stimulates cardiolipin oxidation. Oxidation of cardiolipin changes the interaction strength with cytochrome c, which it is subsequently released into the cytoplasm (Figure 7B I). The particular susceptibility of cardiolipin to oxidation results from its enrichment with polyunsaturated FA residues, in particular linoleate and arachidonic acid (Hishikawa et al., 2014). Notably, cardiolipin is also a target of the proapoptotic B-cell lymphoma 2 (Bcl-2) family protein member truncated BH3 interacting-domain death agonist (tBid), which facilitate pore formation in the outer mitochondrial membrane by two other proapoptotic members, Bcl-2-associated X protein (Bax) and Bcl-2 homologous killer (Bak) (Paradies et al., 2014).

3.9.2.3 Ceramide

Lipid metabolism seems to play a key role on FFAs-induced apoptosis in β-cells: while the transformation of FFAs surplus in neutrals lipids seemed to be beneficial, transformation of FFAs in other lipids such as ceramide mediated the negative effect of palmitate (Lupi et al., 2002). Increase of circulating FFAs that occur in obesity drives an accumulation of FA-CoA, which can be esterified into complex lipids, such as ceramide or DAG. In pancreatic β-cells, ceramide are one of the most important mediators of FA-derived β-cell failure and apoptosis. Even if the precise mechanism is still not clear, toxic effects of ceramide have been reported to occur mainly through the promotion of the *de novo* ceramide synthesis pathway (Lupi et al., 2002; Véret et al., 2011). In isolated rat islets, ceramide accumulation diminishes proinsulin mRNA levels; interestingly, inhibitors of ceramide synthesis such as Myriocin or Fumonisin B1 block the deleterious effects of palmitate on β-cell viability (Kelpe et al., 2003). Ceramide also activate protein kinase C ζ, which inhibits the transcriptional activity of PDX-1 (Galadari et al., 2013). Even if the effects of the *de novo*-ceramide synthesis on FFAs induced β-cell dysfunction are well established, the involvement of sphingomyelin signalling pathway is still on debate. In INS-1 cell line, Ca^{2+}-independent phospholipase A2 ($iPLA_2\beta$) has shown to promote the generation of ceramide via the hydrolysis of sphingomyelin, whereas the administration of a neutral sphingomyelinase (SMase) inhibitor reversed the mentioned effects (Lei et al., 2010). Moreover, $iPLA_2\beta$-derived ceramide generation was linked to the ER stress-associated mitochondrial dysfunction and activation of the intrinsic apoptotic pathway (Lei et al., 2010). Interestingly, over-expression of glucosylceramide synthase in association to chronic palmitate resulted to reciprocally modulate free cholesterol in the ER. In this study, the loss of

sphingomyelin content in the ER resulted to be a key event for the β-cell lipotoxicity, which leads to perturbation of ER-to-Golgi protein trafficking and the initiation of ER stress (Boslem et al., 2013). Another recent study demonstrated the importance of both the *de novo* and sphingomyelinase pathways as mediator of the glucolipotoxicity effects on pancreatic β-cells (Manukyan et al., 2015). Therefore, additional experiments need to be performed to clarify the role of sphingomyelinase in β cell gluco-lipotoxicity.

Ceramide could also act as crosstalk link between ER and mitochondria. Ceramide-induced alterations in mitochondria membrane integrity has been proposed to induce apoptosis in β-cells (Véret et al., 2014). Our laboratory demonstrated that caspases 3/7 were activated in INS-1 cells during palmitate mediated β-cell death, and overexpression of ceramide synthase 4 (Cers4) potentiated the palmitate- and stereate-induced accumulation of ceramide and enhanced apoptosis through the production of specific ceramide species, such the ones with C18:0, C22:0, and C24:1 chain (Véret et al., 2013). These ceramide could act as lipid second messengers which mediates a cross-talk between ER and mitochondria. Interestingly, Chipuk and colleagues demonstrated that sphingolipid metabolism coordinates BAK/BAX activation; they found that the activation of SMase led to the induction and translocation of the pro-apoptotic protein Bax in mitochondria, with simultaneous cytochrome c leakage into cytosol (Chipuk et al., 2012). Interesting, sphingolipid alterations due to glucolipotoxicity resulted to be not a consequence of ER stress, since this alterations were neither induced by pharmacological ER stressors and neither overcome by chemical chaperone. Sphingolipids metabolism seems to be implicated on defective ER-to-Golgi protein trafficking and to enhance ER stress and apoptosis under glucolipotoxicity (Boslem et al., 2011).

3.9.2.4 Defective autophagy

Macroautophagy (normally referred as autophagy) is a cell machinery that allows the elimination of pathogens, dysfunctional organelles, and protein aggregates. It is activated in case of presence of deleterious elements or in case of nutrient deficiency. In normal nutrient conditions, low levels of constitutive autophagy is sustained for cellular quality control purposes; while in case of stress stimuli, autophagy serves as survival mechanism (Figure 7C) (Janikiewicz et al., 2015). Only recently appeared the first studies that connect autophagy pathway and intracellular lipids in pancreatic β cells; these studies suggests that autophagy might plays a role in the pathogenesis of T2D. *In vivo*, a substantial number of autophagosomes

has been reported in ZDF rats (Li et al., 2006), *db/db* mice and C57BL/6 mice fed with HFD (Ebato et al., 2008). Electron microscopy analysis of T2D pancreatic islets confirmed the presence of abnormally overloaded autophagosomes, indicating signs of altered autophagy (Masini et al., 2009). A mice model for autophagy, the *Atg7* KO mice, when fed with HFD displayed impaired glucose tolerance, pronounced failure in the compensatory increase in β-cell mass and an increase in the accumulation of caspase 3-positive apoptotic cells (Ebato et al., 2008). *In vitro* studies supported additional evidences: INS-1 cells exposed to palmitate exhibited at different time points the induction of authophagosomes, confirmed by increases in microtubule-associated protein 1 light chain 3B II (LC3B-II) levels (Komiya et al., 2010). Interestingly, activation of autophagy seemed to play a protective role against palmitate-induced death of INS-1 cells (Choi et al., 2009). However, recent studies have suggested that glucolipotoxicity could inhibit autophagy rather than stimulate it (Las et al.,. 2011). Indeed, prolonged exposure of INS1 cells to glucolipotoxicity showed accumulation of autophagosomes resulting from an inhibition of autophagic turnover and impairment in lysosomal acidification (Las et al., 2011). Nevertheless, a recent study by Biden and colleagues provided the first *in vivo* demonstrations that obesity increases autophagic flux in pancreatic β-cells (Chu et al., 2015). These observations support the hypothesis that compromised autophagy renders β-cells more susceptible to glucolipotoxicity and can predispose individuals to T2D.

Autophagy defects have several implications for other organelles. As already discussed, under states of excess of nutrient and of insulin resistance, the increased need of insulin synthesis and secretion create a bulk of armful protein aggregates and organelles that need to be eliminated through the autophagic machinery. Maintenance of the quality of the ER and mitochondria in insulin-secreting-β-cells is critical for their survival. In autophagy-deficient β-cells, the presence of functionally defective, malformed mitochondria and cisternal distension of the ER appeared to contribute to the reduction of the insulin secretory capacity (Ebato et al., 2008). Importantly, it was shown that *in vivo* activation of autophagic flux in β-cells play a central role to protect against induction of ER stress (Chu et al., 2015).

4 CERAMIDE

As already discussed, lipotoxic condition is often associated with a potential increase of triglyceride levels. Triglycerides are good lipotoxic biomarkers, but probably their exaggerated accumulation is harmful or even protective (Cnop et al., 2001; Kelpe et al., 2002). Therefore, for a long time, the intermediate diacylglycerol (DAG) has been proposed to be a putative lipotoxic agent, as mediator of insulin resistance (Shulman et al., 2014). However, among the myriad of lipids that accrue under gluco-lipotoxic conditions, it has been determined that sphingolipids, especially ceramide and its metabolites, have the most deleterious effect (Chaurasia et Summers 2015). The sphingolipid family is then the mainly implicated in the deleterious effects of the gluco-lipotoxicity on pancreatic β-cell and more generally in the T2D pathology.

Sphingolipids for a long time have been considered as inert components of cellular membranes. On the last years, an exponential number of works began to show that these membrane lipids can also act as active molecules playing a role on the regulation of different cellular processes, such as proliferation, differentiation and apoptosis. Unger and colleague defined ceramide as "the most important of the deleterious routes" underlying the lipotoxic events (Chaurasia et Summers 2015; Unger et al., 2002).

4.1 SPHINGOLIPIDS STRUCTURE AND TOPOLOGY

Sphingolipids are present on all eukaryotic and some prokaryotic membranes, in a portion between 10 to 20% of all membrane lipids. Their distribution on plasma membrane is functional: they shown a preference for partitioning into ordered domains. Sphingolipids domains have functional purposes, and the interaction with cholesterol modulate the sphingolipid raft fluidity in way to create platforms where signalling transducing complexes could organize (Ramstedt et Slotte 2006). Vertically, sphingolipids and glycerospholipids are unequally distributed between the two plasma membrane leaflets. The more complex sphingolipids, such as sphingomyelin (SM) and glycosphingolipids, are mostly confined on the external membrane layer. The maintenance of proper lipid asymmetry is essential for the mechanical stability of the membrane. Moreover, local or global changes of the lipid asymmetry

are important signal for cell cycle progression, apoptosis and platelet coagulation (Ikeda et al., 2006).

4.2 SPHINGOLIPIDS METABOLISM

Ceramide are the precursors for all complex sphingolipids and can be generated in mammals through the *de novo* biosynthesis and sphingomyelinase activity (Figure 8). Recently, a new pathway of ceramide formation has been defined; the salvage pathway allows the re-formation of ceramide from complex sphingolipids (Chaurasia et al., 2015).

The *de novo* synthesis starts on the cytoplasmic face of the ER by the condensation of L-serine and palmitoyl-CoA to form 3-ketosphinganine. This reaction is catalysed by serine palmitoyl-transferase (SPT) complex. 3-ketosphinganine is directly reduced in dihydro-sphingosine (DH-Sph) by the 3-ketosphinganine reductase (3-KSR). DH-Sph is the substrate of ceramide synthases (CerS); CerS add fatty acyl-CoAs of different chain length to the DH-Sph backbone to produce dihydro-ceramide (Chaurasia et al., 2015). Finally, dihydro-ceramide are transformed into ceramide by a desaturase, the dihydroceramide desaturase 1 and 2 (DhCerDes1/2), which inserts 4-5 trans double bond (Figure 8) (Wegner et al., 2016). Depending on the acyl-CoA used by CerS, ceramide are classified on different species, which differs by the degree of saturation of the acyl chains, and by the chain length that in mammals could varying between 14 to 32 carbons (Merril et al., 2005). After *de novo* generation, dihydroceramide and ceramide are transported from the ER to the Golgi apparatus via: 1- the ceramide transport protein CERT (Hanada et al., 2003) or 2- by a vesicular process (Funato et Riezman 2001), where they are metabolized into complex sphingolipids, such as sphingomyelin and dihydro-spingomyelin. Complex sphingolipids can be then transported from the Golgi apparatus to the plasma membrane via vesicles, and here ceramide can condensate with a carbohydrate moiety to form glycosphingolipides (Wegner et al., 2016). The *de novo* generated ceramide can also be degraded into sphingosine in ER by neutral/alkaline ceramidase. This reaction could occur at the plasma membrane levels and on the ER membrane (Hannun et al., 2008).

The catabolic pathway delivers 50% to 90% of cellular ceramide content. In plasma membrane, sphingomyelinases, ceramidases or glycosidases in combination with *sphingolipid activator proteins* (SAPs) mediate the hydrolytic release of sphingomyelin and glycosphingolipids. These lipids are further degraded in the late endosome or lysosome into ceramide by acid

sphingomyelinase (aSMase). Ceramide could be degraded into sphingosine by ceramidases (aCDase). Sphingosine can leave the endosome or lysosome and be modified by the sphingosine kinases 1 and 2 (SphK1/2) to form sphingosine-1-phosphate (S1P), which is a pro-survival lipid, or can be delivered to the ER to be recycled with CerS to produce newly ceramide species (Figure 8) (Wegner et al., 2016).

Figure 8

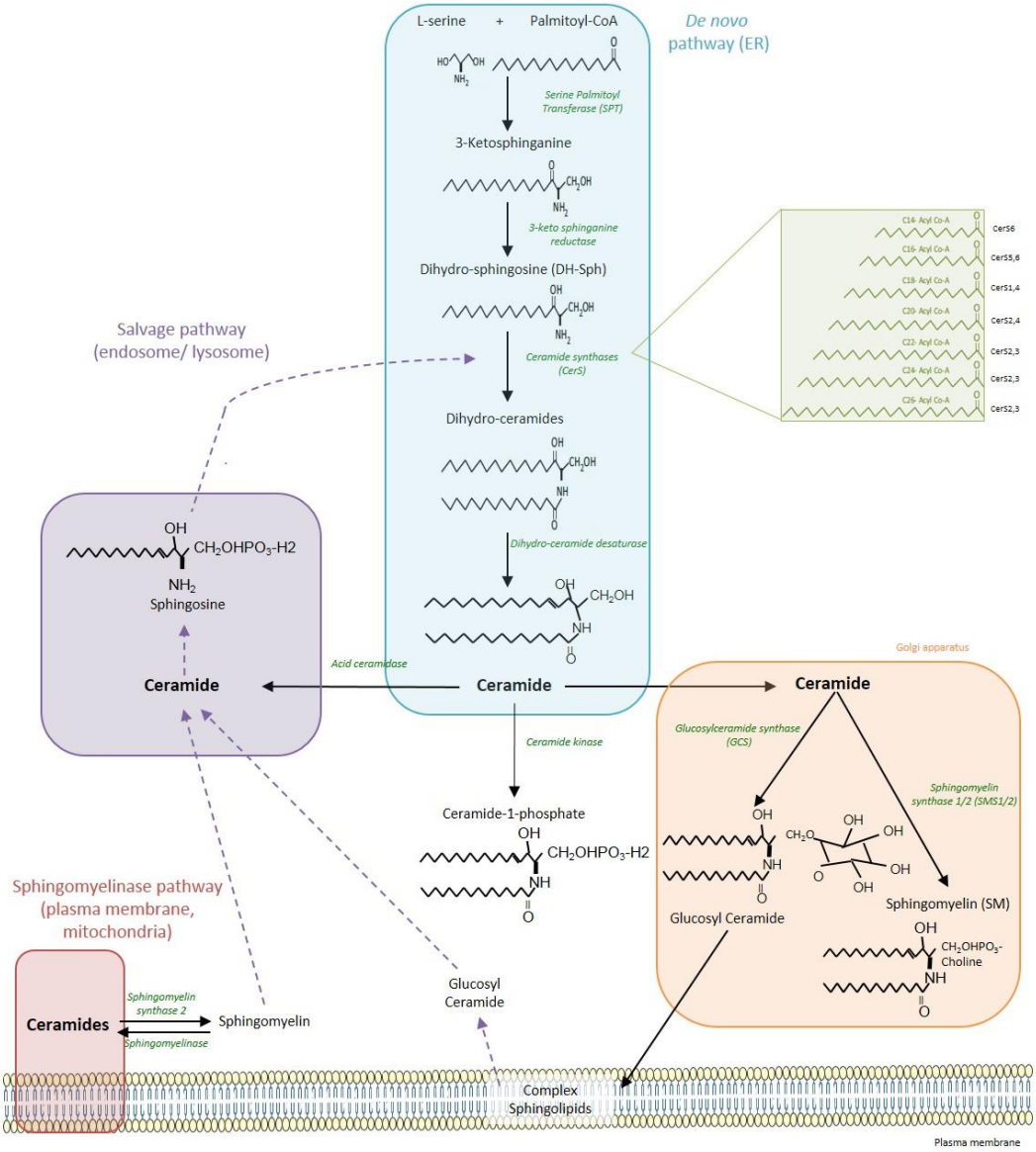

Figure 8. Metabolism of ceramide. The *de novo* ceramide synthesis starts at the surface of the endoplasmic reticulum (ER) by the condensation of palmitoyl-CoA and serine through serine palmitoyl transferase (SPT). 3-ketosphinganine is reduced by 3-ketosphinganine reductase (3-KSR). The ceramide synthesis (CerS) presents specific preferences for the acyl-CoA chain used on the condensation step with dihydro-sphingosine. Glucosylceramide (GluCer) and sphingomyelin (SM) are synthesised at the Golgi, where the newly formed ceramide are transported. From the plasma membrane SM and GluCer can be recycled or degraded via salvage pathways. The sphingosine, obtained by the salvage pathway, can be used by CerS and produce new ceramide. CerS: ceramide synthasis.

4.3 SPHINGOLIPIDS AND CELL SIGNALLING

Some sphingolipids are bioactive molecules, they can act as signalling molecules and activate protein kinase C or they can induce apoptosis. As example, the phosphorylated form of sphingosine or ceramide, sphingosine-1-phosphate (S1P) and ceramide-1-phosphate (C1P), respectively are known signalling molecules with a role on different biological functions. It is interesting the balance of these two signals; indeed, in mammals, Sph and Cer have been demonstrated to induce cell apoptosis, while S1P is involved on cell survival and proliferation (Pettus et al., 2002). Into the cells, it has been proposed that a sphingolipid biostat exists and determine cell fate; it is determined by the balance between ceramide and S1P levels. If the balance shifts in favour of ceramide, cell apoptosis is induced whereas if the balance shifts in favour of S1P, cells activate proliferation and survival pathways (Spiegel et al., 2003).

4.4 ENZYMES INVOLVED IN THE DE NOVO CERAMIDE BIOSYNTHESIS

4.4.1 Serine palmitoyl-transferase (SPT)

The first enzyme involved in the *de novo* synthesis of ceramide is SPT. It is localized on the external membrane of the ER, and it is the rate limiting step enzyme of the *de novo* biosynthesis pathway. SPT is a heterodimeric complex, composed by three subunits, SPLTC1, 2, and 3. The catalytic site is present on SPLTC2, while SPLTC1 and 3 are regulatory subunits (Hanada et al., 2003; Honermann et al., 2006). In particular, SPLTC3 subunits over expression correlated with SPT activity. Other two proteins have been demonstrated to enhance SPT activity: ssSPTa and ssSPTb. In human, it exists 4 distinct human SPT isozymes able to control SPT activity (Han et al., 2009). Despite SPT is a housekeeping gene, its activity is regulated transcriptionally and post-transcriptionally, and results to be up-regulated in certain types of stress, suggesting a putative role on the induction of the apoptosis (Hanada et al., 2003).

4.4.2 3-Ketosphinganine reductase

The 3-ketosphinganine produced by SPT is a short-lived intermediate that is rapidly converted into sphinganine by 3-ketosphinganine reductase. Very little is known regarding the regulation of this enzyme.

4.4.3 Ceramide synthases (CerS)

The third step is performed by ceramide synthases. In mammals, six CerS isoforms have been identified, which present specific preference for specific fatty acyl-CoA chain length (Chaurasia et al., 2015). Much of the diversity in the sphingolipids pool results from this reaction. These proteins mainly localize in the ER, even if last year studies evidences the possible localization of these enzymes in mitochondria, mitochondria associated membranes (MAM) or in the nucleus and perinuclear structures (reviewed in Wagner et al., 2016; Mesicek et al., 2010). CerS isoforms differ in substrate specificity and tissue distribution, and dysregulation of these proteins have been associated to different pathophysiological processes. CerS seem to be regulated at different levels, at the epigenetic, transcriptional, post-transcriptional and post-translational levels (Wegner et al., 2016), even if clear regulatory mechanisms remain to be elucidated.

The protein topology is still predictive, but it is assumed that CerS possess at least six transmembrane domains. CerS enzymes own a *translocation associated membrane protein* (Tram)- *longevity assurance gene* (Lag)- *ceroid lipofuscinosis neuronal 8* (CLN8) (TLC) domain, which possess a lag1p-motif (Jiang et al., 1998; Winter et al., 2002). Except for CerS1, all CerS isoforms present an N-terminal *homeobox* (Hox)-like domain, which can interact with the DNA, but until now there are not evidences that indicate that CerS can act as transcription factor and the function of their Hox-like domain remains unknown up to date (Wegner et al., 2016).

All CerS protein have the same affinity for their sphingolipid substrate: DH-Sph. They differ from each other on the different affinity for the acyl-CoA used for the reaction to produce dihydroceramide (Figure 8). In mammals, DH-Sph can be N-acylate by different fatty acid with chains length comprises between 14 to 28 carbons. These fatty acids can be saturated or mono-unsaturated. An exception are the testis and skin, where it is possible to find ceramide species with very long (26 to 32 carbons) and poly-unsaturated chains (Rabionet et al., 2008). Nowadays, there is still not a consensus on the CerS acyl-CoA specificity. The most part of the studies are performed on yeast, and this is a limiting factor since this organism did not produce the same fatty acid than mammals, so there is the possibility that results obtained could be incorrect. Anyway, it is generally accepted that CerS1, CerS5 and CerS6 are involved on the synthesis of ceramide with chain length comprises between 14 and 18 carbons (Riebeling et al., 2003; Mizutani et al., 2005). The other ceramide synthases, CerS2, CerS3 and CerS4, are

involved on the formation of ceramide with 18 carbons or more, and in particular, CerS3 produces the very long chain ceramide, until 32 carbons (Rabionet et al., 2008). Generation of CerS mutated mice showed that the lack of these enzymes is lethal or have serious impact on the whole organism (Wegner et al., 2016). CerS2-mutated mice showed that the lack of C22-C24 ceramide production, was compensated by increased levels of C16-ceramide, and consequently the total amount of ceramide remained unchanged (Pewzner-Jung et al., 2010). CerS2-mutated mice also presented age dependent degeneration of grey and white matter of cerebellum (Imgrund et al., 2009). CerS4 deficiency has been investigated in skin. CerS4 knock out mice presented altered lipid composition in sebum, which resulted to be more solidified; the dilated and obstructed pilary canals finally caused hair loss (Ebel et al., 2014). Interesting is the data from CerS6-mutated mice, these mice were protected from diet-induced insulin resistance and steatohepatitis (Turpin et al., 2014). Recently, CerS2 KO presented compensatory increased in CerS6 and C16 ceramide, which predisposed mice to steatohepatitis (Pewzner-Jung et al., 2010; Raichur et al., 2014). In future, the creation of conditional CerS knock-out mice will better elucidate these enzymes' role. However, from the existing study it emerges that the deregulation of one CerS can be counterbalanced by the over-expression of the other isoforms (Pewzner-Jung et al., 2010). The study of these enzymes is even more complexified by the fact that they could act as dimers. Indeed, one study demonstrated that co-expression of a catalytically inactive CerS5 inhibited CerS5 activity, while CerS2 activity were enhanced by co-expression on CerS5 (Laviad et al., 2012). All these features make the investigation of CerS's role complex.

4.4.4 Dihydroceramide desaturase

The dihydro-ceramide desaturases, *Degs1* and *2*, insert a double bond that determines many of the unique biophysical properties of ceramide. *Degs1* is ubiquitously express in most tissue, while *Degs2* is express mostly in skin and gut. On the *de novo* ceramide synthesis process, the dihydro-ceramide are transient intermediate. For a long time, dihydro-ceramide have been considered inert, and only recent studies revealed that these lipids have distinct and non-overlapping biological function with ceramide (Chaurasia et Summers 2015). Indeed, they resulted to be involved in different pathway, such as the induction of the autophagosome formation, to mediate the hypoxia response, the control of cell cycle (Siddique et al., 2015). For example, Kraveda and colleagues tested the effect of DEGS1 knock down in human neuroblastoma cells. The effect were an accumulation of dihydro-ceramide and the inhibition

of the cell growth (Kraveda et al., 2007). Interesting, in metabolic disorders, dihydro-ceramide resulted to be a good biomarker (Siddique et al., 2015). Obese patients and obese mouse models showed DEGS1 decreased expression. *In vitro*, DEGS1 loss-of-function prevented pre-adipocyte adipogenesis and decrease lipid accumulation (Barbarroja et al., 2015). *In vivo*, pharmacological inhibition of DEGS1 impaired adipocyte differentiation (Barbarroja et al., 2015). The balance dihydro-ceramide/ceramide results to be an important signal for the cells, which can leads to changes in cell fate.

4.5 CERAMIDE AND PROTEIN INTERACTION

Different *in vitro* and *in vivo* experiments showed interaction between ceramide and proteins. *In vitro*, Chalfant and colleagues showed that ceramide could activate the serine-threonine phosphatases PP1 and PP2A (Chalfant et al., 2004). Some other studies confirmed that ceramide are involved on the activation of these phosphatases, which dephosphorylate target proteins, such as *retinoblastoma protein* (Dbaibo et al., 1995), specific PKC (Lee et al., 1996), AKT/PKB (Zhou et al., 1998). It has also been demonstrated that ceramide can activate PKCζ, which is involved on inhibition of the AKT/PKB pathway (Bourbon et al., 2002; Bellini et al.,. 2015).

4.6 CERAMIDE AND TUMOURS

In the pathology of cancer, ceramide have a particular role, and the findings on this field are important for the understanding of these lipids' role and function. Indeed, many tumours correlated with a decrease in ceramide content. As example, in human head and neck squamous cell carcinoma Cer18:0 levels decreased and the decrease amplitude was correlated with higher incidence of lympho-vascular invasion and pathologic nodal metastasis (Karahatay et al., 2007). In different *in vitro* studies, addition of ceramide precursors, such as palmitate or sphingosine, or the short-chain ceramide analogue (C6-ceramide) induced apoptosis of cancerous cells (Hardy et al., 2003). The pharmacological inhibition of *de novo* ceramide pathway made cancer cells more resistant to pro-apoptotic molecules. The first evidences on this field lead to conclude that ceramide induce death, growth inhibition and senescence in cancer cells. However, last year studies demonstrated that this simple picture is no longer true. Endogenous produced ceramide have different functions, which are context-dependent, regulated by subcellular-membrane localization and by the presence or absence of direct targets.

Mesecek and colleagues demonstrated in HeLa cells that the ionizing radiation (iR) induced *de novo* synthesis of ceramide, through specific activation of CerS2, 5 and 6 isoforms (Mesicek et al., 2010). Investigation demonstrated that CerS are involved on the synthesis of opposing anti- and pro-apoptotic ceramide. They demonstrated that these three CerS isoforms might coexist as a complex, and their combined activation could determine a balance between pro- and anti-apoptotic signals. CerS2 seemed to play a protective role: its over-expression partially protected cells from the iR apoptosis, while its knock-down demonstrated that CerS2 was involved in the synthesis of C24-ceramide, which is considered as a pro-survival ceramide. CerS5 was implicated in C16-ceramide biosynthesis and mediated iR-induced apoptosis (Mesicek et al., 2010). Similarly, studies performed on head and neck cancer cells demonstrated opposite role of CerS1 and CerS6, which were involved on the production of C18- and C16-ceramide, respectively. In one hand, CerS1 overexpression or C18-ceramide treatment suppressed tumor growth, while CerS6/C16-ceramide promoted cancer cell proliferation (Karahatay et al., 2007; Senkal et al., 2011). Mechanistically, generation of C16-ceramide regulated ER stress, through regulation of ER-Golgi membrane network (Senkal et al., 2011). Inhibition of *de novo* C16-ceramide production in cancer cells modifies Ca^{2+} influx in ER, altering the Golgi/ER membrane integrity and inducing apoptosis (Senkal et al., 2011).

Another evidence that highlight the role of ceramide in cancer comes from experiments performed on the protein that can be activated by ceramide, or are involved in ceramide biosynthesis pathway. The ceramide transporter CERT, is involved on the transport of ceramide from the ER to the trans-Golgi for sphingomyelin synthesis. Downregulation of CERT sensitizes cancer cells to chemotherapy agents (Saddoughi et Ogretmen 2013). It is hypothesized that an increased accumulation of ceramide leads to ER stress and cell death (Swanton et al., 2007). Another well-known downstream target of ceramide is PP2A. In cancer, PP2A acts as tumor suppressor. The mechanism by which ceramide directly activates PP2A is still elusive; however, a recent study demonstrate that ceramide can directly bind to SET/I2PP2A, which is a biological inhibitor of PP2A and an oncoprotein (Mukhopadhyay et al., 2009). Specific ceramide preferentially bind I2PP2A (C6- and C18-ceramide), and the result of this interaction is PP2A re-activation. As evidenced in various cancer tissue and cells, I2PP2A acts as oncogene if its expression is exacerbate (Mukhopadhyay et al., 2009). Interestingly, transient overexpression of I2PP2A constitutively inhibits PP2A activity and binds ceramide as a biological sponge, leading to the protection against ceramide-mediated *c*-

Myc degradation and resistance to ceramide-mediated anti-proliferative effect (Mukhopadhyay et al., 2009).

The connection between ER and mitochondria is also important in the effect of ceramide. Ceramide induce apoptosis and degradation of anti-apoptotic protein such as XIAP by a proteosomal process (Kroesen et al., 2003) or can inhibit BCL-2 (Ruvolo et al., 1999), through phosphatase proteins PP2A, and PP1. Ceramide also stimulate pro-apoptotic pathway by activating various effectors such as BCL-Xs and caspase 9 (Chalfant et al., 2002), or BAX (Siskind et al., 2010). Ceramide could also induce BCL-2 dephosphorylation through ER stress induction (Sauane et al., 2010). Siskind and colleagues showed a link between *de novo* ceramide production in ER and the apoptotic mitochondria pathway (Siskind et al., 2010). They demonstrated that BAK, which is part of BCL-2 pro-apoptotic protein family, activated long-chain ceramide generation. They hypothesize that BAK induced CerS activation at post-transcriptional level. In this way, specific ceramide species were produced (C16-, C18- and C20-ceramide) and apoptosis was induced (Siskind et al., 2010). This study points the attention on the link between ceramide and BCL-2 family protein, and suggest that ceramide can modulate the balance between anti-apoptotic proteins (XIAP, BCL-2) and pro-apoptotic proteins (BCL-Xs, BAX, caspase 9) toward the pro-apoptotic side. It is also interesting to see the polyvalent role of ceramide during apoptotic cascades: they can act both upstream and downstream of these cascades.

As already discussed, subcellular localization of ceramide during apoptosis is still not completely elucidated. Some studies indicate that ceramide could be present on mitochondria, but the origin of these ceramide remain undefined. Since sphingomyelinase acid are localized on the inter-membrane mitochondria space, it has been proposed that ceramide produced on this organelle can play a role in mitochondia apoptosis, through an increased permeabilization of the outer mitochondria membrane (Wegner et al., 2016). Siskind and colleagues showed that ceramide are able to form a stable channel in mitochondria outer membrane leading to the exit of proteins that induced apoptosis, such as cytochrome C. Ceramide cause channel disassembly, and it is likely due to direct interaction with pro-apoptotic proteins (as BCL-Xs) (Siskind et al., 2010). Since ER communicate with mitochondria via mitochondria-associated membranes (MAM) that is closed juxtaposition of these organelles, it is possible that the ceramide-induced apoptosis is performed in MAM (Wegner et al., 2016).

Even if apoptotic signalling pathway induced by ceramide start to be well-known and defined, little is known regarding the effect of each ceramide species and about CerS in apoptosis

induction. Over-expression or knock down of CerS highlighted that CerS1 and CerS5 isoforms sensitizes the tumoral tissues to the apoptotic chemo-therapeutic agents (Senkal et al., 2007; Min et al., 2007). Regarding CerS2, CerS3, Cers4 and Cers6, their role during apoptosis induction remains unclear. CerS6 isoform is involved on C16-ceramide production, and the inhibition of this enzyme induces cell death via the ER stress induction in some cancer cell lines (Koybasi et al., 2004; Mesicek et al., 2010). In some tumors, increased C16-ceramide could have a proliferative role (Koybasi et al., 2004; Senkal et al., 2009).

The information that we possess regarding ceramide and cancer are fragmentary and still uncomplete. However, from the evidences presented in this section, we can see that ceramide can integrate many signals and play a role on the balance between apoptosis and cell proliferation.

4.7 CERAMIDE AND INFLAMMATION

As discussed above, obesity is often associated with a chronic low-level of inflammation in adipose tissue in due to excessive fuel load. Excessive nutrient availability and an inflamed environment seem to co-operate and induce sphingolipid production. In human, inflammatory agents, such as TNF-α or IL-6, strongly correlated with plasms levels of ceramide (de Mello et al., 2009). Different studies, demonstrated a cross-talk between cytokines and ceramide. In hepatocytes, neutral SMase has been demonstrated to increase the ability of IL1β to phosphorylate JNK, probably through modulation of the phosphorylation pattern of IL1β receptor associated kinase (Karakashian et al., 2004). Another study performed on endothelial cells, demonstrate that TNF-α activate neutral SMase, and the cooperative actions of these two factor leads to endothelial nitric oxide synthase activation (De Palma et al., 2005). A successive work confirmed that TNF-α-mediated SMase activation, and demonstrated that PKC mediated TNF-induced translocation of neutral SMase from Golgi apparatus to plasma membrane (Clarke et al., 2008). These experiments highlight an important cross talk between inflammation and ceramide, which can be involved in IR installation induced by lipotoxicity (de Mello et al., 2009).

4.8 SPHINGOLIPIDS AND T2D/OBESITY

During obesity and metabolic disease, different studies profiled sphingolipid metabolites, but results are still confusing. Different groups determined a sphingolipid accumulation in plasma and muscle of insulin resistant patients; the correlation was restricted to specific ceramide species, and it was independent of obesity, DAGs and FFAs (Dubé et al., 2011). Other studies found a strong correlation between plasma ceramide, IR, and T2D (Haus et al., 2009). However, a consensual correlation between ceramide and IR has still not been reached, because other groups reported that IR installation in animals could be independent of ceramide accumulation (Galbo et al., 2013).

For a better understanding of these type of studies, it is important to keep in consideration that ceramide are biosynthetic intermediates that do not remain into a steady-state concentration; it is important to investigate the metabolic fluxes in which they are involved. Moreover, it is still not elucidated the subcellular localization of these crucial pool of sphingolipids, and neither their temporal fluctuations.

SPT enzymes are the rate-limiting step for *de novo* ceramide biosynthesis. In yeast, their Km for L-serine and palmitoyl-CoA are close to the physiological concentrations, and this makes these enzymes sensitive to little fluctuations of substrate concentrations (Pinto et al., 1992). Thanks to these two characteristics of STP enzymes, in case of fuel surplus, free FAs can be used for an aberrant ceramide production. Ceramide accumulation can directly induce some cellular dysfunctions and/or integrate others lipotoxic pathways.

In ZDF rat, increased TG and ceramide levels in islets of Langerhans was correlated with cell death (Shimabukuro et al., 1998). In other tissues, such as liver, skeletal muscle, adipose tissue, an increase of ceramide content was detected when animals were fed with HFD (Chocian et al., 2010). Human studies evidenced a higher accumulation of ceramide in skeletal muscle of IR subjects compared to insulin-sensitive subjects (Coen et al., 2010). Interestingly, DAG concentration was unchanged. Intramyocellular triglycerides accumulated into type I myocytes and not in type II myocytes. Type I myocytes are glycolytic fibres, while type II are oxidative. Since glycolytic fibres are poor in mitochondria number compared to the oxidative ones, it is possible that type I myocytes are not able to eliminate FFAs surplus into heat or energy and ended to use them through other metabolic pathways, such as the *de novo* ceramide biosynthesis pathway (Coen et al., 2010). The same effect was observed on cardiomyocytes, where the

accumulation of exogenous FFAs is correlated with ceramide production and apoptosis (Park et al., 2008). Ceramide content increases in skeletal muscle, liver and adipose tissue in T2D. These studies strongly suggest a role of ceramide in diabetes etiology and the gluco-lipotoxic phenomenon. Ceramide accumulation in these tissues contributes to the installation of an insulin-resistance state. As proof of a role of ceramide in IR installation, the effects of the inhibition of *de novo* ceramide biosynthesis were tested. Obese rodents improved their glucose homeostasis when they were treated with myriocin, a potent inhibitor of SPT (Holland et al., 2007). The same amelioration was also evidenced on genetically obese animals, the rat ZDF. During lipotoxic cardiomyopathy, myriocin also counterbalanced the lipotoxic effects, by improving systolic function and prolonging the survival rate (Park et al., 2008). Another study confirmed that a diet enriched with saturated fatty acids induced IR via ceramide accumulation in skeletal muscle of mice, and treatment with myriocin reversed the glucose intolerance and insulin resistance state. The rescue phenotype was due to an enhanced insulin-stimulated AKT and glycogen synthase kinase 3β phosphorylation (Ussher at el 2010).

4.9 CERAMIDE PATHWAYS INVOLVED IN INSULIN RESISTANCE

In skeletal muscle, Stratford and colleagues investigated how ceramide inhibit insulin signalling pathway, they found that ceramide have a double action on AKT/PKB signalling pathway: AKT dephosphorylation due to the activation of protein phosphatase PP2A and blocking membrane translocation of AKT/PKB (Stratford et al., 2004). The second mechanism is mediated through PKCζ activation. Interestingly, it has been demonstrated that ceramide's action depends on plasma membrane (PM) structural organization and especially on caveolin-enriched domain (CEM) abundance. Indeed, in pre-adipocytes, which are loaded of caveolin-enriched domains, ceramide are concentrate at the levels of these domains. In these conditions, ceramide preferentially activated PKCζ. At the contrary, in fibroblasts, which have low caveolin-enriched domains, ceramide-activated PP2A pathway dominated to inhibit AKT/PKB (Blouin et al., 2010).

In adipose tissue, ceramide also inhibit activation of the AKT/PKB pathway and Glut4 translocation in response to insulin (Summers et al., 1998). Origin of ceramide on adipose tissue is still unclear and there's still controversies about their role in cytokine secretion and cytokine action adipose cells (Chaurasia et al., 2015). TNFα is known to play an important role in IR installation, in lipolysis activation on WAT and in inhibition of the differentiation of the

mesenchymal cell into adipocytes. As already discussed, TNFα activates sphingomyelinase and induces ceramide production (De palma et al., 2006). In brown adipose tissue, TNFα-stimulated ceramide production is involved in impaired insulin induction of Glut4 mRNA expression (Fernandez-Veledo et al., 2006). In WAT, TNFα-induced ceramide accumulation mediates inhibition of insulin receptor activation (Grigsby et al., 2001). *In vivo*, hyperinsulinemia and elevated TNFα levels associated with obesity may be involved in up-regulation of expression of ceramide biosynthesis enzymes observed in various obese models (De Mello et al., 2009). In obese mice model, the interplay between ceramide and TNFα has also been investigated. Ceramide levels in plasma was increased in *ob/ob* mice compared to WT mice. Cultured adipocytes showed that ceramide and sphingosine induced cytokines gene expression, included TNFα (Samad et al., 2006). However, another study, demonstrated that in cultured adipocytes, inhibition of the *de novo* ceramide biosynthesis had no effect on TNFα expression (Yang et al. 2009).

In liver, there is still some controversies about the role of ceramide in the installation of IR. While in adipose tissue and skeletal muscle ceramide are clearly involved in IR installation, ceramide accumulation in liver seems to be not sufficient to induce IR installation. Different authors investigated what is happening when lipid balance is altered in liver, and if this lipid unbalance could lead to IR installation. Monetti and colleagues generated a mice over-expressing in liver the diacylglycerol acyltransferase 2 (DGAT2), an enzyme involved in the final step of TG synthesis. Liver-DGAT2 mice developed hepatic steatosis, and the lipid balance was modified: there were an increased amount of TG, DAG, ceramide and unsaturated FAs in liver. However, these mice did not presented abnormalities in plasma glucose and insulin levels, glucose and insulin tolerance, and liver responded properly during hyperinsulinemia (Monetti et al., 2007). Another study, investigated the importance of liver secretion of triglyceride-rich VLDLs. Mice with defective ability to secrete VLDL from the liver, resulted in an increase of DAG and ceramide in this tissue. Despite this VLDL secretion defect, TG content of the other tissues were unaffected in these mice. Glucose tolerance, insulin response and hepatic glucose production were also unaffected (Minehira et al., 2008). These findings indicate that installation of whole body IR seems not to be controlled by a lipid unbalance in the liver, at least when triglycerides metabolism is modified. In these works, liver ceramide were increased and did not influenced IR installation. However, ceramide can act as signalling molecules and in these works plasma ceramide levels were not quantified. Another study investigated the effects of blocking *de novo* ceramide pathways in models where obesity and

IR were already onset. They demonstrated that ceramide inhibition in *ob/ob* mice and DIO mice reduced weight gain, enhanced metabolism and energy expenditure, decrease hepatic steatosis and improved glucose homeostasis (Yang et al., 2009). Similar results were obtained in mice were IR was induced by glucocorticoid or lard oil infusion and treated with myriocin (Holland et al., 2007). Among sphingolipid metabolites, some studies focused on the role of glycosphingolipids, and connected these lipids to insulin responsiveness. While ceramide act on AKT/PKB pathway, glycosphingolipids can directly inhibit the phosphorylation of insulin receptor and IRS-1. Indeed, these lipids can induce IRS receptor exclusion from specific membrane domains where it is phosphorylated. Interestingly, inhibition of glucosylceramide synthase improved glucose tolerance and insulin response in obese-induced mice and animal models of IR (Langeveld et al., 2009). From KO mice studies, ceramide synthesis enzymes resulted to play different roles on the diet-induced IR installation. The impaired very-long-chain ceramide synthesis, investigated on CerS2 haploinsufficient mice, conferred to mice higher susceptibility to diet induced IR and steatohepatitis (Raichur et al., 2014). CerS2 in liver resulted to play a crucial role in hepatic fatty acid uptake, indeed, CerS2 null mice presented reduced hepatic triacylglycerol levels, independently from the diet (Park et al., 2014). In opposition, CerS6-mutated mice were protected from IR, and CerS6 overexpression correlates with insulin resistance (Turpin et al., 2014).

4.10 CERAMIDE AND PANCREATIC B-CELLS

One of the pioneer study on pancreatic β-cells gluco-lipotoxic dysfunction made in ZDF rats, has correlated β-cells mass decrease and the diminution of the insulin secretion to an increase of ceramide content and to increased SPT1 mRNA levels in islets of Langerhans (Shimabukuro et al., 1998; Shimabukuro et al., 1998). During gluco-lipotoxicitic conditions, different studies performed on islets of Langerhans demonstrated the induction of the *de novo* ceramide biosynthesis, which is involved in the inhibitory effects on insulin gene expression (Poitout et al., 2008). The *de novo* ceramide biosynthesis occurs when excess of palmitate start to accumulate in cells as palmitoyl-CoA and it is no longer metabolized by the β-oxidation pathway. In islets of Langerhans and the pancreatic β MIN-6 cell line, inhibition of insulin gene expression performed by ceramide is mediated by ERK1/2 (Fontes et al., 2009). ERK can be inactivated by PP2A and, in INS-1 cell line, ceramide accumulation inhibits ERK cascades through PP2A phosphatase activation (Guo et al., 2010). Another mechanism involved on the ceramide inhibition of insulin gene transcription involved protein kinase C. PKCζ is activated

by ceramide and it is able to phosphorylate and inactivate PDX, a transcription factor that regulates insulin gene expression (Galadari et al., 2013).

The other gluco-lipotoxicitic effect on pancreatic β-cells is the induction of apoptosis, where ceramide seemed to play an important role. In culture, it has been demonstrated that ceramide analogue (C2-ceramide) blocks cell proliferation and induces apoptosis of pancreatic β cell lines and isolated islets of Langerhans (Veluthakal et al., 2005). At the contrary, inhibition of *de novo* ceramide biosynthesis blocks palmitate induced apoptosis of pancreatic β-cells (Maedler et al.,., 2003 ; Shimabukuro et al.,., 1998). Treatment of ZDF rats with the β-cyclo-serine, a SPT inhibitor, partially reduces islets apoptosis (Shimabukuro et al., 1998). It is interesting to note that mono-unsaturated fatty acids did not increase the ceramide pool, and this could be the reason why these fatty acids did not induce β-cell apoptosis. In INS-1 β cells, it has been evidenced that C2-ceramide reduce mitochondrial membrane potential and induces cytochrome C release into the cytoplasm, demonstrating that ceramide induced-apoptosis is through the mitochondrial pathway (Veluthakal et al., 2005). It is well established that ER stress can trigger the β-cell apoptosis. It has been demonstrated that, during the ER stress, Ca^{2+}-independent phospholipase A_2 (iPLA$_2$β) expression is induced by SREBP-1 activation. During ER stress, ceramide accumulated, and this accumulation seems to occur by an iPLA$_2$β-dependent induction of neutral SMase (lei et al., 2010). Then, the axes iPLA$_2$β/ceramide activate the β-cell apoptosis through the mitochondria pathway. Importantly, ER-stress induced apoptosis via the iPLA$_2$β/ceramide axis was also observed in human islets (Lei et al., 2012). Despite all these findings, in MIN-6 cells, one study showed that palmitate induces ER stress without the induction of the *de novo* ceramide synthesis (Thorn et al., 2010). However, other recent studies have reported that in MIN-6 cells palmitate induced *de novo* ceramide synthesis (Boslem et al., 2013; Wehinger et al., 2015; Manukyan et al., 2015). Interesting, Boslem and colleague work linked palmitate-induced ceramide production to ER stress and apoptosis in pancreatic β-cells. They demonstrated the ceramide accumulation was not a consequence of ER stress and was not overcame decreasing ER stress (Boslem et al., 2011).

Works performed on our lab, demonstrated that gluco-lipotoxicity in pancreatic β-cells (INS-1) induces ceramide accumulation, and that regulation of the *de novo* ceramide biosynthesis pathway modified their susceptibility to gluco-lipotoxic apoptosis. Interestingly, ceramide accumulation was restricted to specific ceramide species accumulation (C18, C22 and C24:1) due to CerS4 up-regulation. CerS4 overexpression in β cells amplified ceramide production and gluco-lipotoxic-induced apoptosis, whereas CerS4 knock-down partially reduced apoptosis

(Véret et al., 2011). Recently, another study found that palmitate (C14, C16, C20:1, C24) induces a specific ceramide production in MIN-6 cells. This production involved both the *de novo* and the sphingomyelinase pathway (Manukyan et al., 2015). Differences in ceramide species production are likely due to different culture conditions (1% FBS versus 15% FBS) and to intrinsic differences of the pancreatic β cell lines. Induction of ceramide accumulation could cause alteration of ER lipid raft, which was causally linked to ER stress. Palmitate perturbed sphingomyelin and free cholesterol content in ER lipid rafts, leading to disruption of ER lipid rafts, perturbation of protein trafficking and initiation of ER stress (Boslem et al., 2013). Another mechanism involved in palmitate-induced apoptosis in β-cells implied caveolin-1. Caveolin are proteins associated with lipid rafts and are involved in many cellular processes, such as vesicle trafficking, signal transduction and cell fate. In particular, they can modulate cell death: in response to oxidative stress, caveolin is phosphorylated and can control mitochondrial permeabilization, caspase activation and inhibition of pro-survival signals (Wehinger et al., 2015). A recent study connected glucolipotoxicity and caveolin, the loss of mitochondria transmembrane potential, caused by ceramide accumulation, induced ROS formation which promoted caveolin-1 phosphorylation and consequently the apoptosis. This study demonstrated that caveolin-1 phosphorylation promoted palmitate-induced ROS-dependent apoptosis in pancreatic β-cells (Wehinger et al., 2015).

4.11 CERAMIDE, OBESITY AND TYPE 2 DIABETES COMPLICATIONS

Ceramide effects on insulin signalling pathways and apoptosis are implied in different T2D complications. Ceramide production in response to palmitate or HFD, induced apoptosis of different cell types, such as cardiomyocyte, macrophages, glomerular cell and retinal cells (Hickson-Bick et al., 2000; Prieur et al., 2010; Boini et al., 2010; Cacicedo et al., 2005). Ceramide accumulation in plasma can induce V-LDL aggregation and lead to atherosclerosis (Schissel et al., 1996). It has also been demonstrated that in macrophages and vascular endothelial cells, ceramide forward the lipotoxic pro-inflammatory effects (Prieur et al., 2010; Xiao-Yun et al., 2009). As proof, the treatment of a mouse model of atherosclerosis with myriocin reduced apparition of symptoms (Hojjati et al., 2005).

5 IMIDIA

Nowadays limited therapeutic options exist to treat diabetes and none to cure or prevent this disease, in part due to the limited knowledge of β-cell biology in health and disease. To face to the lack of knowledge regarding the β-cell function in diabetes, a consortium combining most part of the European diabetes experts has been created. This consortium, IMIDIA (http://www.imidia.org), aimed to improve the β-cells function and to determine new biomarkers for the treatment and the monitoring of this disease. In order to better understand the installation of the diabetes correlated to obesity, six different mouse strains (C57BL/6j, DBA2/J, 129S2, AKR, A/J and BALB/cJ) has been fed with chow diet or HFD (high sucrose) for 90 days. Mice from these six strains were phenotyped for glucose homeostasis, insulin resistance and islet morphometric analyses at 2, 10, 30 or 90 days of HFD versus regular chow diet. These parameters have been complemented by deep sequencing of mouse islet RNA under the same conditions and at the same time-points. After an initial evaluation and comparison of diet-induced diabetes in the different strains, the phenotype measurements has been integrated with islet gene expression data in a network-based analysis. This multi-parameter analysis led to the identification of a sub-network of islet-expressed genes associated with glucose tolerance and insulin secretory capacity (Figure 9). The results of the extensive phenotypic and islet gene expression data collected during this study has been analysed and organized into a database. The network analysis highlighted new candidate genes that could play a key role in regulating glucose stimulated insulin secretion in mouse islets. Among these genes, we decided to further investigate the role of the *very long chain fatty acid elongase 2 (Elovl2)* (Figure 9).

Figure 9

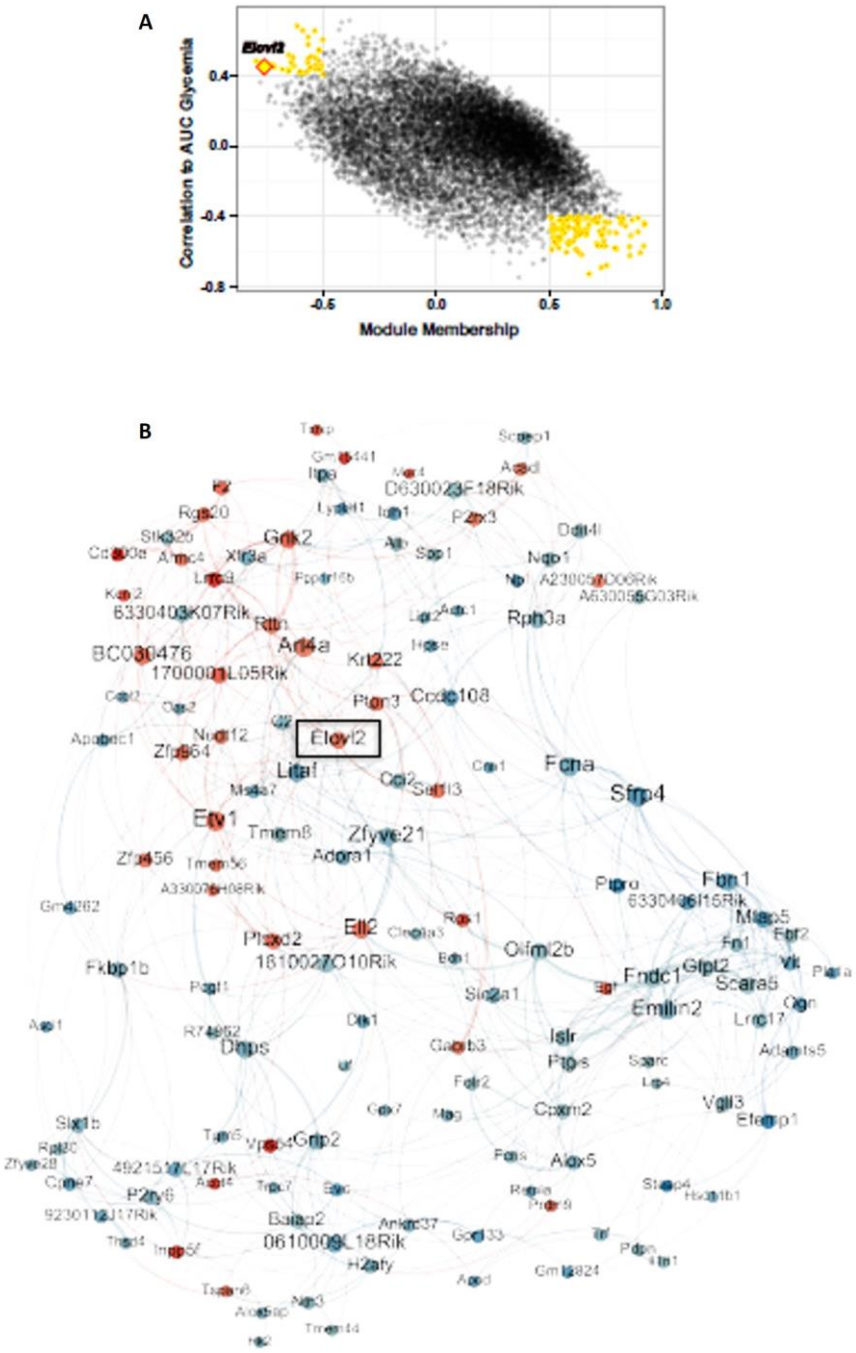

Figure 9. Elovl2 correlation to glycemia and connection with other genes. Elovl2 gene expression correlate with AUC glycemia in IMIDIA's study (A). Elovl2 gene is included in a module, modules group genes that are associated for similarity of expression profile. Here is reported Elovl2 connections with the others module genes (B). AUC: Area Under the Curve. Adapted from Bellini et al 2016..

6 FATTY ACID SYNTHESIS AND ELONGASES

In mammals, fatty acids synthesis is performed by two different enzyme systems: 1) the *de novo* synthesis where palmitic acid is formed from low-molecular-weight precursors; 2) enzymatic chain elongation during which fatty acids already present are converted in more complex fatty acids (Figure 10).

6.1 DE NOVO SYNTHESIS

In mammals, *de novo* fatty acid synthesis (FAS) takes place in the cytoplasm. It involved fatty acids up to 16 carbons (palmitic acid). The fatty acid machinery involves a homodimeric and multifunctional complex of 250kDa, this complex hosts seven different enzymatic activities in two catalytic centres. The reaction starts by the elongation of a primer, as acetyl or propionyl, with two-carbon units donated from malonyl-CoA and the reduction using NADPH. The repetition of this reaction seven times in a cyclic manner enables fatty acid synthesis to finally produce the saturated C16 fatty acid, the palmitic acid (Figure 10) (Jakobsson et al., 2006; Wakil et al., 1983).

6.2 FATTY ACID ELONGATION

Nugteren in 1965 performed the pioneer studies regarding fatty acid elongation. Through isolation of ER from liver rat, he defined that fatty acid elongation takes place in the microsomal re-constituted fraction, which is clearly distinguished from the *de novo* synthesis that takes place in the cytoplasm (Figure 10) (Nugteren et al., 1965). A significant portion of the fatty acids produced by FAS and fatty acids derived from the diet are further elongated into long chain fatty acid with 18- up to 26-carbons, defined as very long chain fatty acids (VLCFA) (Figure 10). The main location of VLCFA synthesis is ER, and the enzymatic steps involved on this process are principally the same as described for FAS. The difference compared to FAS synthesis stays of the fact that the four principal successive steps of VLCFA elongation are performed by individual proteins, which may be physically associated (Nugteren et al., 1965; Kohlwein et al., 2001). Three of these enzymes are located to the cytoplasmic face of the ER membrane, while the enzyme performing the third step is suggested to be embedded in the membrane (Jakobsson et al., 2006). The first step of the reaction starts with the condensation

of an acyl-CoA (n) molecule and malonyl-CoA, generating the β-ketoacyl-CoA. The following reaction is then the reduction of the β-ketoacyl-CoA to β-hydroxyacyl-CoA, through a NADPH-dependent mechanism. In the third step, β-hydroxyacyl-CoA is subsequently dehydrated resulting in enoyl-CoA (C4 +2 = C6), which finally it is reduced by enoyl-reductase completing in this way the elongation cycle and generating an extended acyl-chain (n+2) (Figure 10).

Figure 10

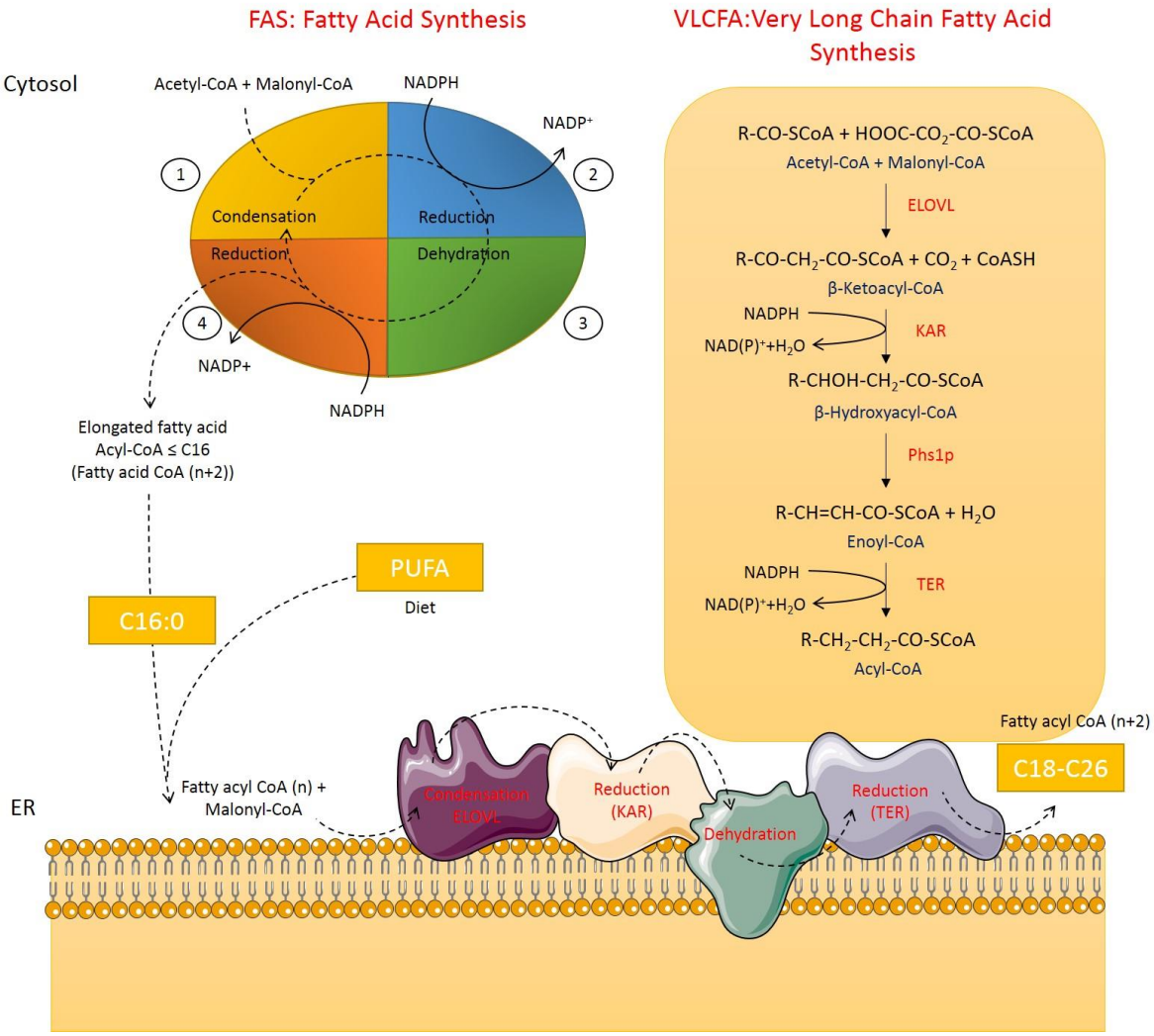

Figure 10. Fatty acid synthesis and fatty acid elongation. Fatty acid synthesis (FAS) multimeric complex synthetizes de novo the fatty acids and elongate fatty acid chain until C16 carbons producing palmitate. Fatty acids with more than C16 chain length are produced in the Endoplasmic Reticulum by the VLCFA (Very long chain fatty acid) elongase. Elongases are the rate limiting step and perform the first reaction by the condensation of an acyl-CoA molecule with malonyl-CoA. The product, β-ketoacyl-CoA, is further reduced by β-ketoacyl-CoA reductase (KAR). The third reaction is a dehydration, performed by Phs1p, to form enoyl-CoA. Finally enoyl-CoA is reduced by trans-2,3-enoyl-CoA reductase (TER) to give the final extended acyl-chain FA.

Following the discovery of the VLCFA synthesis, several attempts have been made to purify the enzymes involved on the ER elongation process, but the hydrophobic properties of these proteins brought to unsuccessful outcomes. Bernert and Sprecher were the first to be able to solubilized and partially purify one of the protein involved on this process, the β-hydroxyacyl-CoA dehydrase. The enzyme did not show condensation and acyl-CoA reductase activity, indicating that the chain elongation system is constituted by discrete enzymes, in contrast to the cytosolic FAS multi-domains machine (Bernert et Sprecher 1979). Nugteren, in his study also postulated that the rate-limiting step was the first reaction, and other studies, through indirect evidences, support this finding (Bernert et Sprecher 1977; Moon et al., 2001). These evidences support the idea that the introduction of a condensing enzyme is sufficient for specific VLCFA synthesis.

6.3 THE ELONGASES

Extensive biochemical studies have been performed on the VLCFA elongation machinery, and they highlighted the existence of several distinct elongation pathways in mammals (Sprecher et al., 1974). When finally the genomic sequences for these enzymes became available, the study of the separate enzymes and their respective properties became possible through cloning experiments, even if their purification remains unsuccessful (Kihara et al., 2012). The enzymes that perform the condensation step are called ELOVL (Elongation very long chain fatty acids). In mammals it exist seven ELOVLs. Based on the presence of similar motifs, such as KXXEXXDT, HXXHH, HXXMYXYY, TXXQXXQ, these proteins are grouped ad a gene/protein family: the *Elongase* gene and ELONGASE protein family respectively (Jakobsson et al., 2006). Comparative studies evidences that these enzymes are evolutionarily conserved among the species: mouse and yeast have approximately 30% of sequence similarity in each mouse-mouse or mouse-yeast pair (Jakobsson et al., 2006). Among the ELONGASE family members, high sequence similarity is seen between each other, this has been seen for nematode, yeast, mouse and human (Leonard et al., 2004).

Regarding the other elongation steps, in 2003 Moon and co-workers identified and characterized the two mammalian enzymes that catalyse the reduction of 3-ketoacyl-CoA and trans-2,3-enoyl-CoA; these enzymes have been called 3-ketoacyl-CoA reductase (KAR) and trans-2,3-enoyl-CoA reductase (TER) respectively (Moon and Horton 2003). Regarding the dehydration step, few years later Phs1p has been determined to be the VLCFA dehydratase,

revealing in these way the last missing component of the elongation cycle (Denic and Weissman 2007) (Figure 10).

6.3.1 The ELOVL proteins

The membrane topology of the ELONGASE family is still predictive and the sequence suggest the existence of five to six transmembrane-spanning, due to the presence of several hydrophobic amino acids. The mouse and human ELOVL proteins all contains lysine or arginine residues at the carboxyl terminus, which are proposed to function as ER retrieval signals (Jakobsson et al., 2006; Leonard et al., 2004). The yeast VLCFA synthesis elongases and reductases co-localize to areas in the yeast cell that correspond to the ER, or perhaps early Golgi (Kohlwein et al., 2001). Immuno-fluorescent data showed that human ELOVL4 is located in close vicinity to the ER, and the ectopic expression of the murine ELOVL3 fused to GFP in mammals cells also indicates that the protein localises to the ER (Grayson et Molday 2005; Jakobsson et al., 2006).

Some ELOVL contains a potential N-glycosylation motif. ELOVL3 has been shown to be glycosylated in position 6-9 of the N-terminal domain, which is efficiently modified with a high mannose oligosaccharide both *in vivo* and *in vitro* (Monne et al., 1999; Tvrdik et al., 1999). The N-terminal tail with the first trans-membrane region enables the correct insertion of the protein to the ER membrane (Tvrdik et al., 1999). The glycosylation near the N-terminal has also been found in ELOVL4, even if this modification seems not to be essential for the protein expression and localization (Grayson et Molday 2005). ELOVL1 also contains a potential N-glycosylation motif, but the fact that this motif is buried into a putative membrane-spanning region lets to think that ELOVL1 is most likely to be not glycosylated. Regarding ELOVL2, no putative glycosylation sites have been found on this protein. The functional effects of divergent glycosylation pattern among the ELONGASE family components has still not been investigated.

Co-immunoprecipitation experiments shown that ELOVL4 forms multisubunit complexes; this suggest that the enzyme works as homodimer (Grayson et Molday 2005). It is not clear if this mechanism involve other ELONGASE.

The condensing domain of known condensing enzyme of the FAS complex present a strictly conserved cysteine-containing catalytic triad. The sequence of the ELONGASES lack this homolog sequence to the conserved catalytic domain of the FAS complex. At the same time, all the ELONGASE proteins contain an absolutely conserved signature sequence motif located

within either the juxta-cytosolic transmembrane helix regions or within the cytosolic loop. In yeast, the region has been analysed and it has been hypothesized that these motifs might be arranged into a catalytic ring, forming the entrance of an intramembrane substrate-binding pocket. The active site faces the cytosol, and the length of the fatty acid chain is determined by a lysine more or less close to the luminal surface. This lysine residue is located on the sixth transmembrane helix and the distance between the lysine and the active site determines the length of the fatty acid produced (Denic et Weissmen, 2007).

The ELONGASE present a conserved histidine motif also present in desaturase enzymes. This address the question if the ELONGASE are reductase or condensing enzymes. Different experiments confirmed that ELOVL proteins are the condensing enzyme and the rate-limiting step of the VLCFA synthesis. As example, one study performed *in vitro* on HEK-293 cells, showed that cells transfected with an expression plasmid containing *Elovl6* open reading frame, had six-fold higher elongation activity compare to cells transfected with an empty vector. Moreover, in absence of NADPH, the end product was the 3-ketostearoyl-CoA when the cells were fed palmitoyl-CoA, confirming again the condensation reaction (Moon et al., 2001).

In yeast, PUFA are normally not synthesized, and heterologous co-expression of the ELOVL-related enzyme PEA1 (from *Caenorhabditis elegans*) with the fatty acid desaturases required for the synthesis of C20 polyunsaturated fatty acid resulted in accumulation of arachidonic acid from linoleic acid and eicosapentaenoic acid from alpha-linolenic acid (Beaudoin et al., 2000).

Another evidence that support the ELOVL primary role on VLCFA synthesis is the fact that ELOVL proteins present divergent tissue expression pattern and substrate-specific activity, while the reductases are expressed in all human and murine tissues tested so far and show no fatty acid specificity (Jakobsson et al., 2006).

Nowadays, the ELONGASE family is characterized by seven mammalian homologues (Figure 11), and it comprises both enzymes that are both ubiquitously expressed and also more tissue specific enzymes. They are termed ELOVL1 to ELOVL7 and each one present substrate specificity and controls the first rate-limiting step of the VLCFA elongation (Jakobsson et al., 2006, Nagunuma et al., 2011, Kihara et al., 2012). Even if the sequence gene is known, the high protein hydrophobicity makes difficult the purification and the characterization of these proteins. As consequence, the bigger part of the data regarding mammalian ELOVL enzymes derived from gene expression analysis and from studies of Knock Out mutant mice.

Figure 11

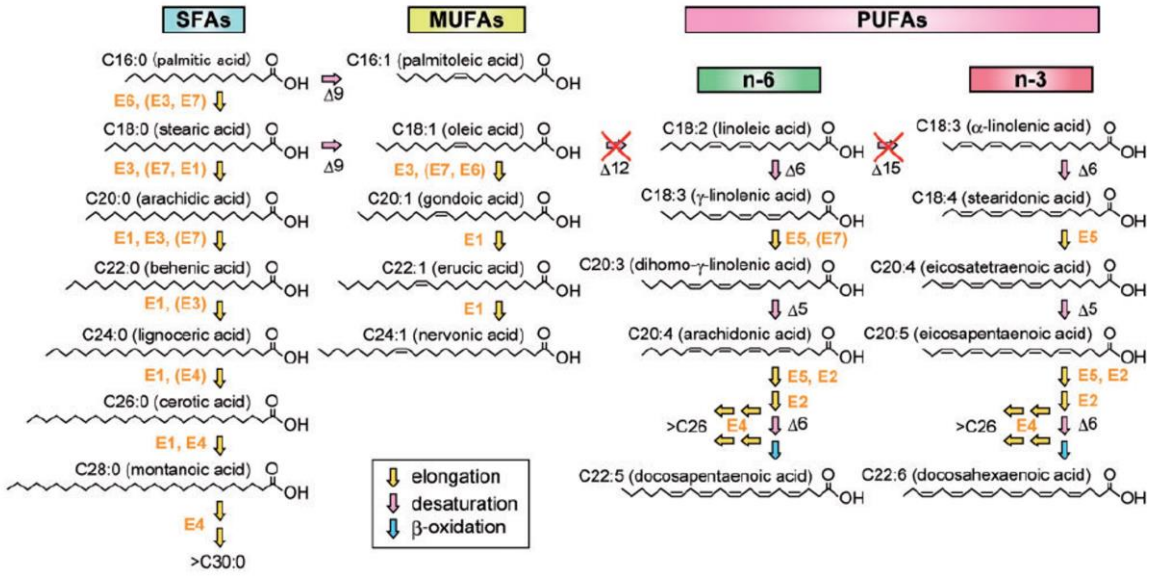

Figure 11. Fatty acid elongation in mammals. Here represented the FA elongation pathway. Elongases involved in each pathway are illustrated. In humans, the Δ12 and Δ15 desaturase do not exist, and the *ω*-6 and ω-3 poly-unsaturated fatty acids cannot be synthetized *de novo*. E*x* and Δ*x* represent Elovl*x* enzyme and Δ*x* desaturase respectively, where *x* denotes the enzyme number. Elovl indicate in parenthesis means that they have weak functions in the indicated reactions. Figure adapted from Kihara et al 2012.

6.3.1.1 Elovl1

The *Elovl1* gene has been discovery through sequence homology to *Elovl3*. Yeast complementation studies implied *Elovl1* function in the synthesis of saturated fatty acids up to 26 carbons in length and in the formation of membrane lipids such as sphingolipids (Tvrdik et al., 2000). Saturated VLCFA are proposed to be important for the tight packaging of membrane lipids in raft structures, and might have a function as organisers in intracellular transport and signalling events by creating micro-environments (Jakobsson 2006). In yeast, it has been shown that changes in acyl-chain length of membrane lipid constituent affects membrane-residing enzymes activities and also membrane fusion/budding events (Destarges et al., 1993, Durrens et al., 1995). These studies suggest that in mammals *Elovl1* could be important in membrane-related functions related to sphingolipids metabolism.

The expression pattern of *Elovl1* is ubiquitous, suggesting a housekeeping gene role, necessary for the maintenance of membrane integrity. The tissues where the expression levels of this gene are particularly elevated are the highly myelinated parts of the central nervous system (CNS),

such as the corpus callosum and the spinal cord (Jakobsson et al., 2006). Two mouse genetic models deficient in nerve myelination, *Quarking (qk)* and *Jimpy (jp)* present a mutation in the myelin-associated glycoprotein (*Mag*) and the proteolipid protein (*Plp*) respective genes. Both strains show conspicuously decreased elongation activity and VLCFA levels in the brain, which correlate well with the reduced *Elovl1* mRNA expression (Tvrdik et al., 2000). Noteworthy is the fact that *Elovl1* mRNA level seems to correlate with the severity of the elongation defect, indeed the more severe *jimpy* phenotype present lower *Elovl1* mRNA levels compared to the *quarking* mice, which has a less severe phenotype (Jakobsson et al., 2006).

6.3.1.2 Elovl2

As *Elovl1*, *Elovl2* gene has also been identified through *Elovl3* sequence homology studies. In contrast to *Elovl1*, *Elovl2* shows a tissue specific gene expression pattern, tissues with the higher mRNA levels include testis and liver, while mild-gene expression is found in brain, kidney and white adipose tissue (Tvrdik et al., 2000). The first studies performed in yeast and HEK cells demonstrated that over-expression of both human and murine *Elovl2* resulted on elongation of arachidonic acid (20:4,*n*-6), eicosapentaenoic acid (20:5,*n*-3) (EPA), docosatetraenoic acid (22:4,*n*-6) and docosapentaenoic acid (22:5,*n*-3) and no activity has been detected for saturated/monounsaturated substrates (Leonard et al., 2002, Moon et al., 2001). Nevertheless, a minor functional divergence between murine and human protein has been found: mouse ELOVL2 is able to elongate γ-linolenic acid (C18:3,*n*-3) to some degree, while the human form is not. Wang and colleagues determined the fatty acids elongase products of rat primary hepatocytes infected with *Elovl2* adenovirus. After infection, the cells were treated with ^{14}C-labeled 16:0, 20:4,*n*-6, or 20:5,*n*-3 supplemented medium for 24 hours. Ad-*Elovl2* infected cells showed 2-fold increased 20:4,*n*-6 elongation into 22:4,*n*-6, and 37-fold increase 20:5,*n*-3 elongation into 24:5,*n*-3. ELOVL2 showed no C16 substrate-specific elongation (Wang et al., 2008).

Generation of the *Elovl2* knock out (KO) mice demonstrated that this elongase is indispensable for normal sperm formation and fertility in male mice (Zadravec et al., 2011). The mice lacking *Elovl2* presented a complete arrest of spermatogenesis, with seminiferous tubules presenting only spermatogonia and primary spermatocytes without further germinal cells. The heterozygous *Elovl2* male mice also presented abnormal sperm formation: i.e. impaired formation and function of haploid spermatids. At the levels of spermatocytes, it was found mostly a reduced levels of C28:5 and C30:5,*n*-6 PUFAs, whereas in the serum there were

distorted levels of C20 and C22 PUFAs from both the *n*-3 and *n*-6 series. Surprisingly, dietary supplementation of docosahexaenoic acid (22:6,*n*-3) (DHA) did not restored the male fertility in *Elovl2*($^{+}$/$^{-}$) mice (Zadravec et al., 2011). A second work on *Elovl2* KO mice has been focused on liver and on the hepatic lipid composition. The *Elovl2* KO mice displayed an important decrease in 22:6,*n*-3 (DHA) levels, and docosapentaenoic acid 22:5,*n*-6 (DPA), with a consequent accumulation of 22:5,*n*-3 and 22:4,*n*-6 in both liver and serum. This work demonstrated for the first time that *in vivo* ELOVL2 primarily control the elongation process of PUFAs with 22 carbons to produce 24-carbon precursors for DHA and DPA,*n*-6 formation. Metabolically, mice lacking *Elovl2* are resistant to diet-induced weight gain and hepatic steatosis. The main affected genes on *Elovl2* KO mice was the lipogenic transcriptional regulator sterol-regulatory element binding protein 1c (SREBP-1c) and its downstream target genes. The changes in fatty acid metabolism were reversed by dietary supplementation with DHA. Therefore, these data suggest that hepatic DHA synthesis mediated by ELOVL2 controlled *de novo* lipogenesis, lipid storage and fat mass expansion in a SREBP-1c dependent manner (Pauter et al., 2014).

Different studies demonstrate the key role of ELOVL2 in the ω3 DHA synthesis (Zadravek et al., 2001; Pauter et al., 2014; Wang et al., 2008). Elongases substrate specificity present often some overlap, and ELOVL5 is the enzyme with more substrate overlap with ELOVL2. A recent paper investigated the selectivity of ELOVL2 toward DPA,*n*-3 which is the penultimate precursor of DHA. It emerged that the cysteine at the position 217 in Elovl2 replaced by a tryptophan at the equivalent position in ELOVL5 seems to be the responsible for their differing abilities to elongate DPA,*n*-3 (Gregory et al., 2013).

At present, few studies have focused on ELOVL2 regulation and mechanisms, and no work is focused on glucolipotoxicity and pancreatic β-cells. In diabetes context, *Elovl2* gene expression seemed to be modulated in retina (Tikhonenko et al., 2010). An important number of studies also associated DNA methylation status of *Elovl2* with ageing, and proposed this protein as human age prediction marker (Garagnani et al., 2012, Zbiec-Piekarska et al., 2015, Kananen et al., 2016).

6.3.1.3 Elovl3

Elovl3 has been identified thanks to its impressive increase in gene expression in mouse brown adipose tissue (BAT) upon animal cold stimulation (Tvirdik et al., 1997). In addition to cold exposure, *Elovl3* gene expression has also been found to be stimulated by injection of

norepirephrine and by feeding the animals with HFD, indicating that this gene is under sympathetic control in brown adipose tissue. *Elovl3* gene expression has been detected during perinatal development and immediately after birth, at the moment when brown adipose tissue is recruited, suggesting a correlation with the recruitment of brown fat (Tvrdik et al., 1997). Complementation studies in yeast showed that *Elovl3* has a putative role in elongation of saturated and monounsaturated fatty acid up to 24 carbons length (Tvrdik et al., 2000). In addition to be expressed on brown adipose tissue, *Elovl3* is also expressed in the hair follicles of the skin and in liver, where its expression is under a strong circadian control (Tvrdik et al., 1997, Brolinson et al., 2008). The *Elovl3*-KO mice presents a particular skin phenotype with tousled fur, the sebaceous glands show general hyperplasia and the fur has impaired water repulsion and increased trans-epidermal water loss. The hair lipid contents is unbalanced, there is an exaggerate accumulation of eicosenoic acid (20:1) and a reduced amount of 22-26 fatty acid species (Westerberg et al., 2004). A following study, investigate *Elovl3* role during cold-stress. The impaired skin barrier, typical of these mice, increased heat loss and the mice showed impaired ability to hyper-recruit the brown adipose tissue. As compensation effect, the muscle shivered in order to maintain body temperature. In brown tissue, during the initial cold stress, *Elovl3*-KO mice showed a transient decrease in the capacity to elongate saturated fatty acids into very long chain fatty acid. The consequences are a diminished ability to accumulate fat and reduced metabolic capacity within the brown fat cells on *Elovl3*-deficient mice (Westerberg et al., 2006). These evidences support the theory that ELOVL3 plays an important role on maintenance of the lipid homeostasis for triglyceride and lipid droplet formation. More recently, it has been shown that vitamin D regulates fatty acid composition through ELOVL3 in white adipose tissue. Upon vitamin D interaction with its nuclear receptor, it inhibiting ELOVL3 expression receptor by occupying a negative-response element in the promoter proximal region of *Elovl3* gene (Ji et al., 2016). In a contest of obesity, ablation of *Elovl3* resulted in reduced hepatic lipogenic gene expression and triglyceride content, with consequently reduced *de novo* fatty acid synthesis and uptake. Interesting, *Elovl3*-deficient mice were not able to compensate the effects of an increased food expenditure by increasing food intake, despite the reduced serum levels of leptin, and the increased expression of orexigenic peptides in the hypothalamus. These results suggest that the C20-22 saturated and monounsaturated VLCFA produced by ELOVL3 are indispensable for correct fatty acid uptake and storage in adipose tissue, and appropriate synthesis of liver triglycerides (Zadravec et al., 2010).

6.3.1.4 Elovl4

Elovl4 has been particularly studied in human since different mutations of this protein have been found to cause tissue-specific maculopathy and/or neuro-ichthyotic disorders. The first discovery has been its association to the eye diseases Stargardt-like dystrophy and in autosomal dominant macular dystrophy. The Stargardt-like macular dystrophy patients suffer from an eye disorder that leads to loss of vision, and this disease has been found to be due to three independent mutations in the last exon (IV) of the *Elovl4* gene (Edwards et al., 2001, Zhang et al., 2001, Agbaga et al., 2008). In rodents, *Elovl4* is significantly expressed in adult retina, skin, testis, and lens (Mandel et al., 2004). Through a gain-of-function approach, it was evidenced that ELOVL4 is required for the synthesis of C28 and C30 saturated fatty acids and of C28-C38 very long chain polyunsaturated fatty acids (Agbaga et al., 2008). The creation of a total *Elovl4*-mutant mouse showed a primary role of this gene in skin formation: homozygous mice displayed scaly, wrinkled skin, and severely compromised epidermal permeability barrier function, and died few hours after the birth (Vasireddy et al., 2007). Lipid analysis of the *Elovl4*-mutated homozygous mice epidermis showed a global decrease in VLCFA with 28 or more carbon chain in both ceramide/glucosylceramide and the free fatty-acid fraction. In particular, these mice skin lacked the epidermal-unique omega-O-acylceramide, which are the key hydrophobic components of the extracellular membranes (Vasireddy et al., 2007). Despite skin abnormalities and early death, no obvious abnormalities appeared in internal organs. The mice carrying *Elovl4*-mutation in heterozygous state presented progressive photoreceptor degeneration (Vasireddy et al., 2007).

6.3.1.5 Elovl5

Elovl5 gene expression profile indicates high expression levels in testis, adrenal glands and liver, while mild-expression resulted on all other tested tissues. ELOVL5 is involved on the elongation of different polyunsaturated long-chain fatty acid, in particular, it elongates C18-C20 carbons-length fatty acids, and it seems to have not the ability to elongate PUFA substrates beyond C22 carbons (Leonard et al., 2000). Adenoviral *Elovl5* delivery in C57BL/6 livers increased hepatic and plasma levels of dihomo-gamma-linolenic acid (20:3,*n*-6) and suppressed hepatic arachidonic acid (20:4,*n*-6) and docosahexaenoic acid (22:6,*n*-3) content (Wang et al., 2008). Generation of a KO mouse for ELOVL5 showed that the elongation of gamma-linolenic (18:3,*n*-6) to dihomo-gamma-linolenic acid (20:3,*n*-6) and stearidonic (18:4,*n*-3) to omega3-

arachidonic acid (20:4,*n*-3) needs *Elovl5*, partially confirming what was previously found. *Elovl5* deletion induce an accumulation of C18 substrates and a consequent decrease of the downstream products as arachidonic acid and DHA. Metabolically, lack of endogenously synthetized PUFAs leads to the activation of SREBP-1c and its target genes: the fatty acid synthesis is constantly induced and it culminates in the development of hepatic steatosis in KO mice (Moon et al., 2009).

6.3.1.6 Elovl6

While FAS is producing palmitic acid (16:0), it appear that the majority of fatty acid have a length of C16 to C18 carbon atoms in mammalian cells. The enzyme responsible for the C16 fatty acid elongation is ELOVL6. ELOVL6 has a pivotal role in the elongation of saturated and monounsaturated long chain fatty acid, it can elongate palmitic and palmitoleic acid (16:1,*n*-7) in stearic acid (18:0) and oleic acid (18:1,*n*-9), respectively (Matsuzaka et al., 2002, Moon et al., 2001). The expression pattern of this enzyme showed that it is ubiquitously expressed, especially in tissue with high lipid content such as brown adipose tissue, white adipose tissue, liver and brain (Moon et al., 2001). The first study performed on an *Elovl6* KO mice showed that deletion of this *Elongase* protects from hyperinsulinemia, hyperglycemia and hyperliptinemia, even if these mice become obese and develop hepatosteatosis when fed with HFD. The results showed the importance of ELOVL6 on hepatic fatty acid composition, and the fact that changes of the hepatic fatty acid composition could improve the insulin resistance and hyperglycemia, independently from the energy balance and stress (Matsuzaka et al., 2007). However, these results have been recently challenged by Moon and co-workers. They demonstrated that ELOVL6 mutated mice fed with HFD did not shown altered development of obesity, fatty liver, hyperglycemia, or hyperinsulinemia. They suggested that the accumulation of palmitoleic (16:1,*n*-7) and vaccenic (18:1,*n*-7), observed in *Elovl6* ($^{-/-}$) mice could largely replace the roles of oleic acid (18:1,*n*-9) *in vivo*, and that deletion of ELOVL6 did not protected mice from the development of hepatic steatosis or insulin resistance (Moon et al., 2014). More recently, it has been show that absence of ELOVL6 decreases hepatic inflammation, oxidative stress and liver injury induced by an enriched-cholesterol diet (Kuba et al., 2015). However, ELOVL6 deletion in this study promotes gallstone formation. A following study investigated *Elovl6* role in pancreatic β-cells. The idea of the article was to show that regulation of monounsaturated fatty acid synthesis through ELONGASE might alter saturated FAs susceptibility of β-cells such as FAs-induced ER stress and apoptosis. The Knock down of

Elovl6 diminished the palmitate elongation in stearate, and consequently increased palmitoleate production. The result were an attenuated pamiltate-induced ER stress and apoptosis. In opposition, *Elovl6* over-expression increased palmitate elongation to stearate and palmitate-induced ER stress and apoptosis resulted to be higher (Green et Olson 2011). In human, genetic studies of a population from southern Spain connected genetic ELOVL6 variations with insulin sensitivity (Morcillo et al., 2011). A study focused on β-cells functions indicated that ELOVL6 could be involved in insulin secretory capacity. Islets from *Elovl6* KO mice fed with high-fat high-sucrose (HFHS) showed decrease islet compensatory hypertrophy and improved GSIS compared to the WT mice (Tang et al., 2014). Altogether, these studies showed the importance of ELOVL6 in C16 fatty acid elongation, and indicated a possible role of this enzyme in diabetes installation and IR. Further evidences are necessary to clearly elucidate ELOVL6 role in metabolic diseases.

6.3.1.7 Elovl7

Elovl7 is the last elongase that have been discovered (Tamura et al., 2009). Genome-wide gene expression analysis of clinical prostate cancer cells indicated that ELOVL7 is over-expressed in these cells. Tamuka and colleagues performed the first functional study of this enzyme. *In vitro* fatty acid elongation assay and fatty acid composition analysis indicated that ELOVL7 is preferentially involved in fatty acid elongation of saturated very-long-chain fatty acids (20:0) (Figure 11). *In vivo*, HFD promoted the growth of prostate cancer tumours over-expressing *Elovl7*. Therefore, ELOVL7 and the VLCFA synthetized by this enzyme could be key molecules to elucidate the association between fat dietary intake and prostate carcinogenesis (Tamura et al., 2009).

The Knock down of *Elovl7* attenuated the prostate cancer cell growth and modified the very-long-chain fatty acids, the phospholipids and the neutral lipids, such as cholesterol ester (Tamuka et al., 2009). Last year, a study demonstrated that ELOVL7 is involved on human cytomegalovirus (HCMV) replication, since saturated very-long-chain fatty acids are required for the production of infectious virion progeny. HCMV infection promoted *Elovl7* mRNA expression by more than 150-fold, through mTOR and SREBP-1. *Elovl7* knock-down impaired HCMV-induced fatty acid elongation and consequently HCMV particle release and infectivity per particle. In opposition, *Elovl7* over-expression enhanced HCMV replication (Purdy et al., 2015).

6.3.2 Elongase expression

Most of the knowledge that we actually have concerning *Elovl* mRNA expression comes from gene expression studies. Very little is known regarding the control of *Elovl* mRNA or protein expression and the control of their enzymatic activity. Thanks to predictive programs, several putative modification sites have been identified on ELOVL protein, such as glycosylation, but their role is still undefined. Regarding the post-transcriptional control of any elongase, no much data exit. A recent study, performed in yeast, demonstrated that Elo2, which correspond to mammal ELOVL5, is regulated by phosphorylation. Elo2 phosphorylation required GSK3 and it is induced upon inhibition of TORC1 (Zimmermann et al., 2013). Genetic modification of the phosphorylation site led to the modification of the ceramide spectrum and to constitutive activation of autophagy (Zimmermann et al., 2013).

The limited knowledge that we have regarding the transcriptional control of ELOVL genes indicates that often their expression is associated to regulators of fatty acid metabolism.

6.3.2.1 Sterol regulatory element-binding proteins

SREBPs enzymes regulate the expression of different genes involve in *de novo* fatty acid synthesis, in fatty acid uptake proteins, and genes involved in cholesterol and phospholipid metabolism (Brown et Goldstein 1997). SREBP proteins are tightly regulated at transcriptional and post-transcriptional levels. After translation, the precursor proteins localized at the ER membrane level and formed a complex with the SREBP cleavage-activating protein (SCAP). SREBPs activation is regulated by intracellular fatty acid and sterol levels; its activation occurs through a proteolytic cleavage that allows SREBP translocation into the nucleus (Brown et Goldstein 1997). Three different SREBP isoforms have been identified: SREBP-1a and SREBP-1c are isoforms derived from alternative splicing of the same gene, while SREBP-2 is derived from a different gene (Shimomura et al., 1997). SREBP-1c is involved in fatty acid metabolism, SREBP-2 is involved on cholesterol synthesis, while SREBP-1a mainly regulates fatty acid synthesis genes. SREBP-1c has been found to be rapidly induced by insulin in primary cultures of hepatocytes. Indeed, in liver, SREBP-1c emerged to be a major mediator of insulin action, contributing to the coordinate regulation of carbohydrate and lipid metabolism (Dentin et al., 2004). Most lipogenic genes, such as ACC and FAS, present on their promotor region a sterol responsive element (SRE), where SREBP can bind. Regarding elongase enzymes, in liver of transgenic mouse that over-express SREBPs, it has been found that *Elovl6* expression is up-

regulated, and it resulted to be a SREBP direct target (Moon et al., 2001). This data was confirmed by another study where the gene expression pattern of transgenic mice lacking SREBP isoforms and over-expressing nSREBP-1c or nSREBP-2 has been investigated. It emerged that nSREBP-2 directly controlled the expression of genes involved on fatty acid synthesis in liver. In particular, nSREBP-2 activated the monounsaturated fatty acid synthesis pathway and, in a less extent, the polyunsaturated fatty acid pathway. The monounsaturated pathway included *Elovl6* gene up-regulation, and the polyunsaturated pathway *Elovl*6 and *Elovl*2 up-regulation (Horton et al., 2003). In a feedback manner, SREBP enzymes seems to be controlled by the elongases. Both mice mutated for *Elovl*5 and *Elovl*2 have up-regulated *Srebp-1c* gene expression as well as its downstream genes (Moon et al., 2008; Pauter et al., 2014). Indeed, it is well established that arachidonic acid and DHA could inhibit SREBP-1c activation. The lack of production of these lipids cause SREBP-1c nuclear translocation and activation of *de novo* fatty acid synthesis. Surprising, *Elovl*5-KO mice emerged to develop hepatic steatosis, a phenotype that is reversed through DHA diet supplementation (Moon et al., 2008). Conversely, *Elovl*2-KO mice fed with HFD did not develop hepatic steatosis (Pauter et al., 2014). The different effects of SREBP regulation by ELONGASE and the hepatic steatosis induction need to be further investigated.

6.3.2.2 Peroxisome proliferator-activated receptor

Peroxisome proliferator-activated receptors (PPAR) are ligand-activated transcription factors that regulate genes involved in cell differentiation and metabolic processes. In particular, they play a role in lipid and glucose homeostasis, so far that they are defined as lipid and insulin sensors. These receptors are expressed in many tissues, including adipose tissue, liver, and muscle. Interesting, their expression profile and distribution differs among the different tissues, which leads ultimately to different and specific-tissue outcomes. The PPAR family comprises three isoforms: PPARα, PPARβ/δ and PPARγ. These three isoforms have different tissue distribution, ligand specificity and physiological role. All of them participate to energy balance (reviewed in Grygiel-Gorniak et al., 2014). PPARα is expressed mainly in metabolically active tissues, such as liver, hearth, skeletal muscle, intestinal mucosa and brown adipose tissue. This receptor is activated by low lipid levels and it is implicated in fatty acid metabolism. PPARγ is mainly expressed in white and brown adipose cells and plays a key role in the regulation of adipogenesis, energy balance, and lipid biosynthesis. This receptor resulted to be involved also in lipoprotein metabolism and insulin sensitivity. PPARβ/δ is the less studied isoform, it is

expressed ubiquitously, even if the higher expression profile has been found in tissues involved on lipid metabolism. In skeletal muscle and cardiac muscles, it participates in fatty acid oxidation, it regulates blood cholesterol concentration and glucose levels (Grygiel-Gorniak et al., 2014). The ligand binding cavity of PPAR receptors is particular: it is bigger compared to most nuclear receptors, and it can bind a variety of natural lipophilic acids, such as essential fatty acids. Of high interest, is the fact that DHA and EPA are well known PPAR agonists (Grygiel-Gorniak et al., 2014). Among the natural ligands of PPARs there are not only the essential fatty acids, but also the eicosanoids, such as leukotriene B_4 or prostaglandin PGJ_2 (Krey et al., 1997). Both essential fatty acids and eicosanoids are required at relatively high concentrations (approximately 100µM) for PPAR activation (Plutzky et al., 2000).

Jakobsson and colleagues investigated the mechanism involved in ELOVL3 over-expression in BAT upon cold-stress stimulation. *In vitro*, primary brown adipocytes cultured with a mixture of norepinephrine, dexamethasone and Wy-14643, a PPARα agonist, were induced to a high oxidative state. Under these conditions, *Elovl3* expression was strongly induced. To determine the role of PPARα on *Elovl3* induction, *PPARα*-KO mice were put under cold stimulation, and *Elovl3* mRNA was no longer inducted (Jakobsson et al., 2005). In contrast, PPARα agonist did not affect *Elovl1* gene expression, which is also involved on the synthesis of saturated VLCFA. Successively, it has been shown that in liver, differently from BAT, *Elovl3* mRNA induction is not controlled by PPARα (Brolinson et al., 2011). Rat fed with PPARα agonist presented increased hepatic elongase activity and *Elovl1*, *Elovl5*, *Elovl6* gene expression (Wang et al., 2005). Later on, it has been shown that PPARα is required for hepatic induction of ELOVL5, ELOVL6 and SCD1 in mice (Wang et al., 2006). Rat primary hepatocytes over-expressing ELOVL2 and ELOVL5, but not ELOVL6, attenuated PPARα regulated genes involved on cholesterol metabolism (Wang et al., 2008).

7 ω-3 POLY-UNSATURATED FATTY ACID

ω3 poly-unsaturated fatty acids (PUFAs) have been reported to have beneficial effects on obesity-associated metabolic disorders. The most important ω3 PUFAs are eicosapentaenoic acid (20:5*n*-3) (EPA) and docosahexaenoic acid (22:6,*n*-3) (DHA). These fatty acids cannot be synthetized from internal produced fatty acids, but are synthesized from precursors provide by the diet. They can be synthetized by using α-linolenic acid (ALA, C18:3,*n*-3) or linoleic acid (LA, 18:2,*n*-6) in the organism. However, the conversion rate seems to be apparently modest, and direct intake assures more the optimal consumption (Martínez-Fernández et al., 2015). These fatty acids are naturally present on fish oil.

Figure 12

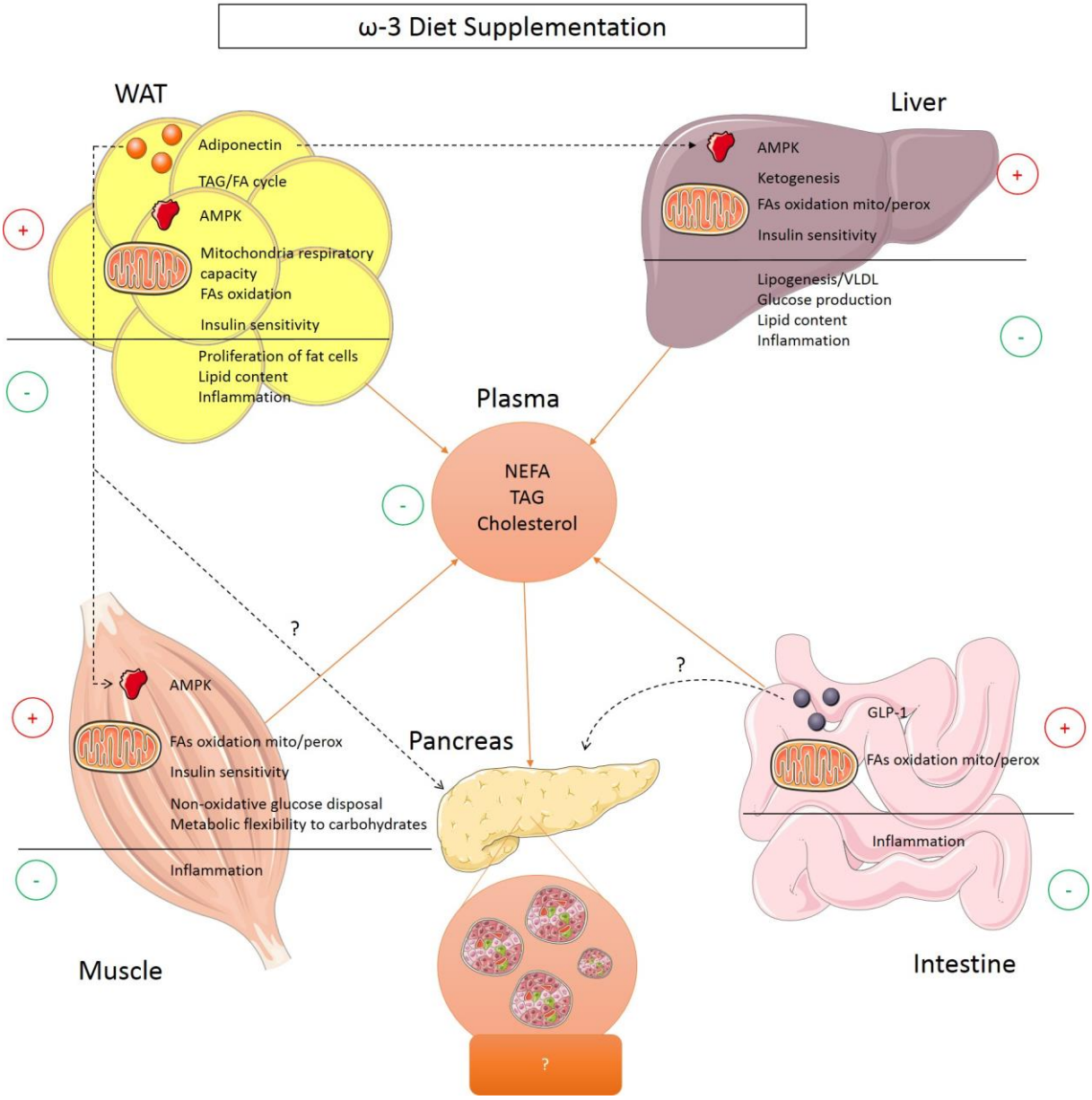

Figure 12. ω-3 PUFAs diet supplementation effects. ω-3 PUFAs supplementation causes different effects on tissues. The common effects found in different tissues, such as liver, white adipose tissues (WAT) and muscle, are increased insulin sensitivity, FAs oxidation and decrease inflammation. In WAT, they specifically resulted to induce adiponectin secretion, which cause AMPK activation pathway also in other tissues, and decreased fat cell proliferation and lipid content. In liver, ω-3 PUFAs decreased glucose production, lipogenesis and lipid content and in muscle ω-3 PUFAs increased non-oxidative glucose disposal and metabolic flexibility to carbohydrates. Regarding the direct effects of ω-3 PUFAs supplementation in pancreatic β-cells, they are almost unexplored.

7.1 ω-3 PUFAS AND ADIPOSE TISSUE

ω-3-diet supplementation has been demonstrated by different studies to decrease body weight gain and fat mass. This data leads to a massive investigation of the PUFAs effects on adipose tissue (Figure 12) (reviewed in Martínez-Fernández et al., 2015).

ω3 PUFAs in WAT control the expression of different genes. The most important target is the *peroxisome proliferator-activated receptor (PPAR)* family. As already described, transcriptional factors of this family are strongly activated by DHA and EPA. PPARγ activation in adipose tissue is known to stimulate expression of genes involved in differentiation of fat cells. The other two PPAR isoforms can be also activated by EPA and DHA in adipose tissue; PPARα and PPARδ stimulates FAs oxidation in mitochondria and peroxisome (Luquet et al., 2005; Flachs et al., 2005), controlling fatty acid uptake, β-oxidation and energy uncoupling. In mice fed with HFD supplemented with PUFAs, abdominal adipose tissue presented increased mitochondria biogenesis and increased expression of *carnitine-palmitoyl-trasferase 1 (Cpt-1)* mRNA. *Ex vivo*, tissue fragments or isolated adipocytes presented increased mitochondria β-oxidation upon ω-3 supplementation (Flachs et al., 2005). Some studies investigated the effects of ω3 PUFAs supplementation in HFD-fed mice in adipose tissue mass and neogenesis. It was evidenced that EPA and DHA, but not ALA, could reduce accumulation of body fat by limiting hypertrophy and hyperplasia of fat cells (Ruzickova et al., 2004).

Increased β-oxidation and inhibition of fat cell proliferation and differentiation may explain the anti-obesity effects of DHA and EPA. As reviewed by Martínez-Fernández and colleagues, many studies evidenced a decrease in body weight gain and adipose mass following HFD supplemented with ω3 PUFAs, even if some studies did not evidenced any change on body weight compared to the littermates fed with HFD-only (Flachs et al., 2005; Ruzickova et al., 2004; Martínez-Fernández et al., 2015).

Another important effect of ω3 PUFAs in adipose tissue is the induction of adiponectin secretion. Different studies showed that EPA/DHA supplementation, independently from the diet, can increased plasma adiponectin levels (Flachs et al., 2006). A possible mechanism is proposed by Iwaki and colleagues: they found a functional PPAR-responsive element in human adiponectin promoter. PPARγ, in collaboration with liver receptor homolog-1, cooperates on the transcriptional activation of adiponectin gene (Iwaki et al., 2003).

In animals fed with HFD, the glucose transporter GLUT4 and glucose transport are inhibited and correlated with IR installation. Different studies demonstrated an increase of GLUT4 expression (Gonzalez-Periz et al., 2009) and a better GLUT4 translocation in response to insulin in mice fed with HFD diet supplemented with EPA/DHA (Taouis et al., 2002; Kusminski et al., 2009). In some works, it has been highlighted an amelioration of AKT phosphorylation with ω-3 supplemented diet (reviewed in Martínez-Fernández et al., 2015). The effects of PUFAs on GLUT4 and on AKT-insulin signalling pathways could explain the reason why in some studies ω3 PUFAs-supplemented-diet resulted to have insulin sensitizing properties.

Finally, ω3 PUFAs are well known anti-inflammatory lipids. Lipotoxicity strongly mediates its negative effects through inflammation and macrophage infiltration in adipose tissue (Anghel et Wahli 2007). Both in obese rodents and human, ω3 PUFAs resulted to ameliorate adipose tissue inflammation. There are several mechanisms proposed to be involved in the anti-inflammatory action of PUFAs in WAT. They involve the reduced production of pro-inflammatory adipocytokines and increased release of anti-inflammatory adipocytokines from adipose tissue, and reduced macrophages infiltration. Different studies reported that DHA or EPA HFD-supplementation decrease Mcp-1, IL-6 and Tnf-α mRNA expression in WAT and in parallel increased adiponecting gene expression (Perez-Echarri et al., 2008). DHA and EPA can also modulate the utilization of arachidonic acid, and competitively inhibit the production of *n*-6 PUFAs-derived pro-inflammatory lipid mediators (reviewed in Martínez-Fernández et al., 2015).

DHA and EPA control inflammation induction also by resolvins and protectins. Indeed, ω3 PUFAs can be used for the synthesis of anti-inflammatory ω3 PUFAs-derived lipid mediators called protectins and resolvins (Martínez-Fernández et al., 2015). Eicosanoid and N-acylethanolamines/Endocannabinoid, such as EPEA and DHEA, are also lipids derived from EPA and DHA, which possess anti-inflammatory effects (Balvers et al., 2012). Recently, a novel class of fatty acid esters of hydroxyl fatty acids (FAHFA) with anti-inflammatory properties has been discovered (Kuda et al., 2016). These lipokines derived from DHA and ALA have been found in serum and WAT after ω3 PUFAs supplementation. DHA esterification to 9- and 13-hydroxyoctadecadienoic acid (HLA) or 14-hydroxydocohexaenoic acid (HDHA) DHAHLA, termed 9-DHAHLA, 13-DHAHLA, and 14-DHAHDHA, were synthetized by adipocytes and had anti-inflammatory and pro-resolving properties (Kuda et al., 2016).

7.2 ω-3 PUFAS AND LIVER

ω-3 have multiple effects on the liver. They activate PPARα which increase FAs oxidation in both peroxisome and in a less extent in mitochondria. FAs oxidation in liver prevents steatosis in case of starvation/fasting (Grygiel-Gorniak 2014). Increase of FAs concentration activates PPARα causing the stimulation of PPARα-regulated genes and activation of the oxydation machinery. This activation results in increased energy burning and reduced fat storage. Ineffective PPARα sensing or decreased fatty acid oxidation results in hepatic steatosis and steatohepatitis, in particular during overnight or prolonged fasting (Rao et Reddy 2004). In PPARα deficient mice, it was observed a severe hepatic steatosis development, which was prevented and even reversed by administration of PPARα agonist. PPARα agonist (WY-14,643) administration was able to up-regulate mRNA for liver fatty acid binding protein and peroxisomal β-oxidation enzymes (Ip et al., 2003).

As already described previously, ω3 PUFAs are natural inhibitor of SREBP-1 protein. Therefore, another mechanism involved in the ω-3 effects in liver, is a decrease of *Srebp-1* mRNA expression. The decrease of this gene leads to the inhibition of lipogenic gene expression and consequently to lower formation of FAs, TAG, and VLDL (Flachs et al., 2014).

A third pathway, which seems to be involved in the effect of ω3 PUFAs in liver, is activation of AMPK. In liver, it has been show that PUFAs supplementation needs AMPKα2 to preserve hepatic insulin sensitivity. This effect was correlated with the induction of adiponectin secretion by adipose tissue and the reduction of diacylglycerol content in liver (Jelenik et al., 2010).

As a final pathway, ω3 PUFAs could forms anti-inflammatory derived lipid mediators, named ω3 PUFAs-derived resolvins and protectins (Gonzáles-Périz et al., 2009). These active lipid mediators protect hepatocytes from oxidative stress and DNA damage during necroinflammatory liver injury (Gonzáles-Périz et al., 2009).

The activation of all of these pathways induces a decrease of liver TAG, VLDL-TAG secretion and an increase of insulin sensitivity in hepatocytes (Figure 12) (Flachs et al., 2014).

7.3 ω-3 PUFAS AND SKELETAL MUSCLE

In skeletal muscle, ω3 PUFAs prevent IR installation in mice fed with HFD, and they were correlated with a decrease of muscle TAG content (Storlien et al., 1991). As occurred in liver and adipose tissue, DHA and EPA also have hypolipidemic effects due to a metabolism switch in muscle; ω3 PUFAs decreased lipogenesis and increase lipid oxidation (Flachs et al., 2014). Adiponectin secreted in response to DHA and EPA may stimulate AMPK in muscle, and stimulates FAs β-oxidation (Yamakauchi et al., 2002).

Another mechanism involved in muscle protection from IR installation by PUFAs is PPAR-γ activation. Thiazolidinediones (TZDs) are insulin-sensitizing drug and are potent agonist of PPAR isoforms. Compared to adipose tissue, PPAR-γ is not highly expressed in muscle. However, generation of mice depleted of PPAR-γ in muscle develop IR, and TZDs treatment was ineffective to improve the insulin sensitivity (Hevener et al., 2003). In obese animals, PPARδ activation regulates FAs burning capacity in skeletal muscles. It was also implicated in the metabolic response in case of endurance exercise by controlling the number of oxidative myofibers (Luquet et al., 2005).

ω3 PUFAs can also accumulate in phospholipids of cell membranes and modulate oxylipin metabolism (Storlien et al., 1991). Interestingly is the fact that DHA content in muscle membrane phospholipids increased following exercise in human. Higher levels of long-chain saturated fatty acids are mainly find in phospholipids disposed in lipid rafts compared to non-raft region (London et Brown 2000). Lipid rafts are characterised by lower fluidity and to this reason they facilitate membrane signalling with harboured molecules such as receptors, adaptors, kinases, and lipids required for signal initiation and transduction (London et Brown 2000). Interesting, in pancreatic β-cells, lipid rafts have been found to be associated with the cellular glucose transport activity of glucose transporter 2 (GLUT2). Increased localization of GLUT2 in lipid rafts resulted to lower glucose transport levels, due to high GLUT2-binding stomatin content in the rafts of pancreatic β-cells (Ohtsubo et al., 2013). It is well documented that ω3 PUFAs incorporation disrupts lipid raft function in several cell types (Chan et al., 2014). Methyl-β-cyclodextrin, a specific cholesterol binding agent, disrupts lipid rafts and enhances GLUT2-mediated glucose transport by GLUT2 releasing in nonraft membrane regions (Ohtsubo et al., 2013). Methyl-β-cyclodextrin has been found to promote also closure of potassium channels and enhances insulin granule exocytosis (Xia et al., 2004). These evidences

suggests that ω3 PUFAs disrupt properties and functions of lipid rafts in pancreatic β-cells and may have similar effects of methyl-β-cyclodextrin on GLUT2 localization and ion channel activity, which would result in improved insulin secretion.

To summarize, ω3 PUFAs have principally two effects in muscle: they prevent IR and they preserve metabolic flexibility to carbohydrates. The result is a better glycogen synthesis at the basal and insulin stimulated condition, which allow a better tissue function and preservation under lipotoxicity (Figure 12).

7.4 ω-3 PUFAS AND B-CELLS

Very few studies investigated the effects of ω3 PUFAs in pancreatic β-cells. *In vivo*, only one study explored islet function after ω3 PUFAs dietary supplementation. This work, performed on female C57BL/6j mice, showed improved insulin responsiveness to glucose of isolated islets from mice fed with a chow diet supplemented with ω3 PUFAs, due to increased glucose oxidation. However, isolated islets from mice fed with HFD supplemented with ω3 PUFAs did not increased insulin secretion, and ω3 PUFAs did not protected β-cell function from lipotoxicity (Winzell et al., 2006). However, this study did not evidenced the classical and expected changes in body weight, glucose homeostasis and insulin sensitivity in response to ω3 PUFAs supplementation (Winzell et al., 2006). Other *in vivo* studies have been performed in FAT-1 transgenic mice. *Fat-1* gene derives from *Caenorhabditis elegans* and mediates the conversion of *n*-6 PUFA to *n*-3 PUFA, this conversion is normally impossible in mammals cells. Islets isolated from these mice secrete more insulin in response to glucose, GLP-1, leucine and glutamine stimulation compared to those from WT mice. Moreover, transgenic islets were resistant to cytokine-induced cell death (Wei et al., 2010). Interestingly, streptozotocin (STZ) administration normally induced β-cell destruction, but and in *Fat-1* transgenic mice this phenotype was attenuated. The mechanism involved seem to be an up-regulation of the basal autophagy and amelioration of the autophagy flux (Hwang et al., 2015).

In vitro, ALA and DHA addition to the culture medium of MIN-6 cells enhances glucose-stimulated insulin secretion, through GRP40 receptor stimulation (Itoh et al., 2003). Interestingly, addition of EPA in culture medium counteracted insulin secretion defect induced by palmitate in both INS-1 cells and cultured mouse islets (Kato et al., 2008). The mechanism involved SREBP1-c inhibition by EPA since the protective effects of EPA were abolished in

SREBP1-null islets. Importantly, EPA administration to KK-Ay mice, a model of obesity and T2D, ameliorated insulin secretion impairment of its islets (Kato et al., 2008).

Exception made for the study by Winzell and colleagues, there are no more investigation on the effect of ω3 PUFAs supplementation on islets function and adaptation to lipotoxicity. In order to understand the effects of ω3 PUFAs supplementation in glucose homeostasis and insulin response, it would be interesting to investigate the effect of these lipids on pancreatic β-cell function.

7.5 ANIMAL EXPERIMENTS

An impressive number of studies investigated the effects of ω3 PUFAs diet supplementation (reviewed in Martínez-Fernández et al., 2015). In many cases, divergent results have been found. Regarding the body weight, some studies showed that ω3 PUFAs supplementation could decrease body weight and fat mass and in some cases a significant reduction in some fat deposit have been evidenced, while other works did not report any change in body weight or fat mass (Martínez-Fernández et al., 2015).

ω3 PUFAs supplementation in a wild range of studies emerged to have effects on the lipid metabolism, lowering the triglyceride content (Martínez-Fernández et al., 2015).

Regarding the insulin sensitivity, the majority of studies demonstrated an improvement in fasting glycemia and insulinemia, and some of them also a better glucose tolerance and insulin response. However, a study performed on *fa/fa* Zucker rats did not evidenced any amelioration due to an ω3 PUFAs diet supplementation (Figueras et al., 2011). These controversial outcomes could be due to the different animal models of diabetes used, to the type and formulation of ω3 PUFAs, or to the dosage and the duration of the treatment.

An interesting model is the *Fat-1* transgenic mice, even if there are conflictual outcomes from this model. These mice express the FAT-1 gene from *Caenorhabditis elegans*, which have the peculiarity to be able to convert *n*-6 to *n*-3 PUFAs *in vivo*. This model allows to study the effects of endogenous production of ω3 PUFAs. When fed with HFD, these mice emerged to be protected against an increase of weight gain; while under isocaloric diet or low-fat diet, they do not show any body weight differences compared to wild type mice (Belchior et al., 2015). In conflicts with these outcomes, White and colleagues did not observed differences in body weight and adiposity in *Fat*-1 mice compared to wild type mice fed with HFD (White et al.,

2010). It is difficult to understand the reason why the two work's outcome is in opposition, since they used the same transgenic mice, and they both fed the mice with HFD for 8 weeks. Further experiments are necessary to elucidate *Fat*-1 mice susceptibility to HFD. A following study of the same group evidenced a difference on adipose cells size from epididymal tissue: *Fat*-1 mice adipocytes were mostly mid-sized compared to the large of very large-sized wild type adipocytes (White et al., 2015). Regarding glucose homeostasis markers, slightly differences have been found, but HFD-fed *fat*-1 mice have low fasting glucose and improvements in insulin sensitivity in some studies (Belchior et al., 2015), while in others it was not detected any significant changes in insulin sensitivity (Ji et al., 2009). Despite these discrepancies on the effect of ω3 PUFAs on adipose tissues among the different studies, in most of them, endogenous ω3 PUFAs increment was shown to be beneficial in glucose tolerance (White et al., 2010, Belchior et al., 2015, White et al., 2015, Ji et al., 2009). Another group investigated the effects of endogenously produced ω3 PUFAs in the streptozotocin (STZ)-induced diabetic mice. Their work was particularly focused on the pancreatic response. Interestingly, STZ-induced *Fat*-1 mice resulted to be protected from the development of hyperglycemia, β-cell destruction and reduced insulin content compared to STZ-induced wild type mice (Bellenger et al., 2011).

Even if sometimes the results are contradictory, most of works, leaded in mice, documented beneficial effects of *in vivo* ω3 PUFAs supplementation in insulin sensitivity and glucose metabolism under IR condition linked to obesity.

7.6 HUMAN EVIDENCES

ω-3 supplementation effects had also been investigated in human subjects with obesity and metabolic syndrome features. The results were mostly in accordance with animal outcomes: indeed, most of the clinical trials in humans strongly supported a beneficial effects on hypertriglyceridemia of ω3 PUFAs supplementations, even if the results are heterogeneous depending the health status of individuals.

7.6.1 Healthy individuals

Among the different human trials outcome, it is well established that ω3 PUFAs have beneficial effects in blood lipids, in particular TAG. In most of the studies, the fish oil intake is negatively correlated with the risk to develop T2D. Interestingly, some studies looked at the circulating

ω3 PUFAs, rather than dietary intake ω3 PUFAs, and individuals with higher ω3 PUFAs plasma levels presented a lower risk to develop T2D (Djoussé et al., 2011). Regarding glucose metabolism, some studies reported no changes or either impairment in glycemia after ω3 PUFAs administration and lower or no change of plasma insulin in response to ω3 PUFAs (review in Flachs et al., 2014).

In healthy individuals, ω3 PUFAs supplementation seems to induce changes in glucose metabolism. Indeed, ω3 PUFAs might interfere with insulin secretion, it was observed decreased circulating insulin levels and a consequent rise in blood glucose. ω3 PUFAs induced a switch in substrate metabolism; it was observed an increase of whole-body fat oxidation and a decrease in carbohydrate oxidation. The non-oxidative glucose metabolism is documented to increase insulin sensitivity of skeletal muscle and indicates high metabolic flexibility to carbohydrates. We can conclude that, in healthy individuals, the ω3 PUFAs acute administration gives controversial results regarding the effects on insulin sensitivity and glycemic control and other studies are necessary to investigate these different effects and their cause. However, the beneficial effects of ω3 PUFAs on blood lipids, in particular TAG, are clear and the majority of studies confirm that, in the long term, ω3 PUFAs have a beneficial effect.

7.6.2 Subjects with metabolic syndrome

ω3 PUFAs intake by obese people provided none or only marginal effects on body weight, but its association with physical activity and/or reduced calorie intake had an additive-weight-reducing effects. Interestingly, in several human genetic association studies, the ω3 PUFAs contents has been negatively correlated with WAT, plasma lipids and obesity (reviewed in Flachs et al., 2014). MacLean and colleagues performed a meta-analysis study in individuals with metabolic syndrome, their analysis showed that ω3 PUFAs significantly lowered plasma TAG compared to control, even if ω3 PUFAs did not affected total cholesterol levels, HDL-cholesterol, LDL-C, and fasting glycemia (MacLean et al., 2004).

Regarding the glucose homeostasis, several trials performed in obese children, or in young overweight or obese individuals, demonstrated that ω3 PUFAs supplementation ameliorate impaired glucose tolerance and insulin resistance, independently from changes in body weight

(Ramel et al., 2008, Dangardt et al., 2012). In adults patients similar studies have been performed and showed different outcomes.

7.6.3 Patients with Type 2 Diabetes

The first experiments with ω3 PUFAs supplementation led on animals, greatly encouraged the ω3 PUFAs supplementation studies on T2D patients. However, the first trial results were not encouraging, and showed either no or detrimental effects on glucose homeostasis (reviewed in Flachs et al., 2014). The subsequent studies evidenced no change in glycemic control after ω3 PUFAs supplementation, while supported hypotriglyceridemic evidences of ω3 PUFAs. Interestingly, a recent randomized clinical trial revealed a positive effect of purified EPA-based supplementation on glucose homeostasis of overweight T2D patients (Sarbolouki et al., 2013).

As already discussed, the deterioration of insulin sensitivity in obese condition could be due to different mechanisms such as lipotoxicity or glucolipotoxicity, and it is linked to an impaired metabolic flexibility. In animals, different studies document that ω3 PUFAs increased lipid catabolism, and this mechanism could counteract the lipotoxic damage on insulin signalling. The most part of human trials are focused on the ω3 PUFAs effects on glucose homeostasis and insulin resistance. A very restricted number of these studies explored the effects of ω3 PUFAs on metabolic flexibility on T2D patients. One study performed by Mostad and colleagues evidenced by indirect calorimetry that ω3 PUFAs supplementation initially increase glucose utilization during the first week of treatment, and successively, at the 9^{th} week, decrease glucose utilization by stimulating lipid utilization (Mostad et al., 2006). Actually, the data available regarding the ω3 PUFAs effects on the metabolic flexibility are insufficient to determine a clear outcome. However, these data suggest that ω3 PUFAs probably induce time-dependent changes in the capacity to oxidize carbohydrate and lipid fuels. This could explain the divergent results obtained on glucose homeostasis and insulin sensitivity, which are mostly negative in a short-time window and started to be neutral or beneficial in a long term studies.

II OBJECTIVES

During glucolipotoxicity, fatty acid partitioning has been shown to be modified. Excess of fatty acids, mimicked *in vitro* by the addition of palmitate, activate different lipotoxic pathways that affect glucose metabolism and insulin signalling (Prentki et al 2008). The β-cells handle with difficulties the lipid surplus; palmitate and high glucose concentration inhibit mitochondrial β-oxidation and lead the cell to use lipids through other metabolic ways (Poitout et al 2002). Different works demonstrated that high doses of palmitate led to the production and accumulation of ceramide in pancreatic β-cells (Véret et al 2011; Boslem et al 2011; Manukyan et al 2015). Ceramide accumulation under glucolipotoxicity resulted to be extremely toxic for β-cells. Indeed, these lipids have been involved in insulin gene expression inhibition, ER stress, and finally β-cell apoptosis induction (Lupi et al 2002; Lei et al 2010; Boslem et al 2011).

On this picture, we decided to investigate the role of ELOVL2/DHA axis in glucolipotoxicity. Very little is known in literature regarding the ω3 PUFAs metabolism in pancreatic β-cells. DHA and EPA resulted to have a beneficial role in different cell types, controlling different pathways, such as autophagy, insulin signalling pathways, *de novo* fatty acids synthesis (Martinez-Fernandez et al 2015; Flachs et al 2014). They influence different genes such as *Srebp*, *PPAR*, *AMPK*, and induce the expression of others genes as *CPT-1* and *GLUT4* (Luquet et al 2005; Flachs et al 2005; Gonzalez-Periz et al 2009; Flachs et al 2014). Metabolically, they ameliorate insulin sensitivity, diminish fat mass, decrease adipose tissue inflammation and protect from diabetes installation (Martinez-Fernandez et al 2015; Flachs et al 2014). In β-cells, only one study investigated the *in vivo* effect of DHA supplementation in β-cell functioning (Winzell et al 2006). While the endogenous ω3 PUFAs production has been investigated only in *Fat-1* over-expressing mice (Wei et al 2010). The *in vitro* studies demonstrated that ω3 PUFAs could increased insulin secretion via GPR40 receptor, and counterbalance palmitate toxic effects on insulin secretion, via SREBP-1c inhibition (Kato et al 2008).

In this context, the objectives of my thesis work have been: 1- to investigate the role of ELOVL2/DHA axis in β-cell insulin secretion in normal and glucolipotoxic conditions; 2- to investigate the role of ELOVL2/DHA axis in β-cells palmitate-induced apoptosis during glucolipotoxicity; 3- to determine the *in vivo* effects of DHA supplementation and the effects of ELOVL2 lack on glucose homeostasis by focusing on islet of Langerhans functions.

1 ARTICLE 1

Type 2 diabetes associated to obesity is one of the most common disease of developed and developing countries. The glucolipotoxicity is the better concept to describe what happen when the disease is installed and it is going to progress. During glucolipotoxicity we have a toxic effect due to fat (lipotoxicity) and glycemia which is no anymore properly regulated (glucotoxicity). In diabetes, a lot of attention is given to the tissues that control the circulating glucose and fatty acid levels. When lipids overload became too important, adipose tissue fails to stock fat, and the exceeding fatty acids start to be ectopically stored. Tissues as liver and skeletal muscle work as spongy and stored as much as possible the exceeding fat. However, more or less all body tissues ectopically store fat, included the pancreas, and they are subjected to lipotoxicity too. Lipotoxicity can be handled by the body for a determinate time window, depending on environmental factors and on genetic predisposition. Lipotoxicity can lead to the insulin resistance installation. When it happens, the tissues start to be subject to not only fat overload but also glucose overload, and glucotoxicity pathways became active. The phenomenon of glucolipotoxicity starts to act on tissues.

During all the different phases of the disease, the equilibrium is maintained if the pancreatic β-cells continue to respond properly to glycemic changes. The pancreatic β-cells have the ability to increase their insulin secretion ability by hypertrophy and hyperplasia. During the first phases of obesity and insulin resistance, β-cells increase in number and in volume, and respond properly to the insulin resistance. In diabetes aetiology, dysfunction of adipose tissue, liver and skeletal muscle play an important role, but β-cells failure is the determining factor that leads to the disease outbreak.

Despite this, pancreatic β-cells are not deeply studied and their function in normal or in disease conditions is not completely analysed. To compensate the lack of this knowledge, the European consortium IMIDIA (Innovative Medicine Initiative for Diabetes) has been created. The consortium aims were to improve the β-cells function and determine new diagnostic biomarkers for the monitoring and treatment of Diabetes. In order to achieve this goal, RNAseq analysis were performed in isolated islets of mice fed with chow versus HFD. To take in account of the genetic background component, analysis have been performed in 6 different mice strains (C57BL/6j, DBA2/J, 129S2, AKR, A/J and BALB/cJ). The RNAseq profiles have been

complemented with physiological tests, such as OGTT and ITT. From the integration of the physiological test and the gene expression changes, a list of candidate genes important for the β-cells function has been created. From this list, we choose *Elovl2* and we decided to investigate this protein function in β-cells and diabetes linked to obesity.

The part that I take care has been to test if *Elovl2* is a potent regulator of β-cell function in glucolipotoxicity. The objective was to determine if *Elovl2* is regulated during glucolipotoxicity and if this enzyme is protecting β-cells from metabolic stresses, or if it is implied on the side effects of glucolipotoxicity. A better knowledge of this protein's role in glucolipotoxicity could reveals a new gene that indicate T2D installation, and the inhibition or activation of this protein could be used to counterbalance the glucolipotoxicity side effects and protect pancreatic β-cells.

Molecular phenotyping of multiple mouse strains under metabolic challenge uncovers *Elovl2* as a novel regulator of glucose-induced insulin secretion

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Summary

Defective insulin secretion by pancreatic β cells underlies the development of type 2 diabetes (T2D). High fat diet-fed mice are commonly used to study diabetes progression, but studies are usually limited to a single strain, such as C57Bl/6J. Here, we use a systems biology approach to integrate large phenotypic and islet transcriptomic data sets from six commonly used non diabetic strains fed a high fat or regular chow diet to identify genes associated with glucose intolerance and insulin secretion. One of these genes is *Elovl2*, encoding very long chain fatty acid elongase 2. ELOVL2 is responsible for the synthesis of the polyunsaturated fatty acid, docosahexaenoic acid (DHA). We show that DHA rescues glucose-induced insulin secretion and cytosolic Ca^{2+} influx impaired by glucolipotoxicity, and that *Elovl2* over-expression is able to restore the insulin secretion defect under these conditions. We propose that increased endogenous DHA levels resulting from *Elovl2* up-regulation counteracts the insulin secretion defect associated with glucolipotoxicity. Although we focus our experimental validation on *Elovl2*, the comprehensive data set and integrative network model we used to identify this candidate gene represents an important novel resource to dissect the molecular aetiology of β cell failure following metabolic stress.

Introduction

A complete or relative decrease in insulin secretion by pancreatic β-cells underlies the development of, respectively, type 1 and type 2 diabetes. These diseases impose a huge burden to welfare systems, both in Europe and in other developed and developing countries, affecting ~8% of the adult population and consuming US$160 b per annum (International Diabetes Federation, 2015). In particular, a sedentary lifestyle and a high-fat high-sucrose diet consumption play a central role in the setting of insulin resistance and type 2 diabetes, which develops when insulin secretion by the β-cell becomes insufficient to overcome insulin resistance (Unger 1995, Forouhi and Wareham 2014). Due, in large part, to our limited knowledge of β-cell biology in health and disease, there are so far limited therapeutic options to treat diabetes and none to cure or to prevent the disease.

Many current studies have evaluated the role of specific mechanisms such as the action of reactive oxygen or nitrogen species, ER stress, sphingolipid metabolism, or autophagy in β-cell demise (Poitout and Robertson 2002, Unger, Clark et al. 2010, Bellini, Campana et al. 2015). Although these mechanisms are normal physiological responses to changes in environmental conditions, they may ultimately lead to cell deregulation and death, in particular when the intensity of the original stimuli exceeds a given threshold and is long-lasting. Importantly however, besides the intensity of the triggering signal, the particular genetic architecture of the cells also dictates their sensitivity to these signals. Thus, C57Bl/6J mice display a defective insulin secretory response to glucose compared to C57Bl/6N mice (Fergusson, Ethier et al. 2014), a difference due to a single mutation in the *Nnt* gene (Toye, Lippiat et al. 2005) that alters the susceptibility to develop glucose intolerance and β-cell dysfunction (Fergusson, Ethier et al. 2014). Therefore, investigating, in a controlled way, how specific signals lead to different β-cell fates depending on the genetic background, is a critical goal of β-cell research.

Approaches to examining the β-cell response to palmitate have previously been deployed in clonal β-cells and human islets using microarray and RNAseq analyses (Xiao, Gregersen et al. 2001, Busch, Cordery et al. 2002, Malmgren, Spegel et al. 2013, Cnop, Abdulkarim et al. 2014). One of the limitations of these earlier studies is the use of *in vitro* models of lipotoxicity, which induce more rapid and harmful effects than those that may occur *in vivo*. A few similar studies have been performed in mice under high fat (HF) diet (Winzell and Ahren 2004, Peyot,

Pepin et al. 2010, Roat, Rao et al. 2014). Indeed, mice fed on a high fat diet increase their body weight and show increased blood glucose and insulin levels during the first weeks of exposure to the modified diet compared to littermates fed on regular chow. The HFHS diet-fed mice become severely glucose intolerant and insulin resistance progressively worsens with time (Winzell and Ahren 2004). However, the development and severity of the diabetic phenotype depends on several factors, including the choice of genetic background. In addition the composition of the diets administered, the housing conditions and the experimental setup for mouse phenotyping and downstream analysis all have an impact on the disease severity. The C57Bl/6J line is established as the strain of choice for the HFHS diet model of diabetes as the mice are reported to develop a more severe diabetic phenotype in response to the diet compared to other strains (Surwit, Feinglos et al. 1995, Winzell and Ahren 2004). This has led to the notion of resistant and susceptible strains, where the genetic differences in metabolic response are more important for the development of obesity and diabetes than the increased calorific intake itself (Surwit, Feinglos et al. 1995, Poussin, Ibberson et al. 2011). Although several past studies have compared the glycemic and insulinogenic response of different strains on a high fat or a regular diet (Funkat, Massa et al. 2004, Goren, Kulkarni et al. 2004, Andrikopoulos, Massa et al. 2005, Alexander, Chang et al. 2006, Gallou-Kabani, Vige et al. 2007, Fearnside, Dumas et al. 2008), a systematic evaluation combining phenotypic with islet genomic data is lacking. This kind of analysis could help to identify new targets associated with β-cell dysfunction induced by metabolic stress in an unbiased manner.

Here, we analysed the effect of a high fat-high sucrose (HFHS) diet on six commonly used laboratory mouse strains in parallel using carefully controlled housing and experimental conditions. These strains were chosen as they are amongst the most commonly used and show marked differences in susceptibility to diet-induced obesity and β-failure (Kooptiwut, Zraika et al. 2002, Andrikopoulos, Massa et al. 2005, Alexander, Chang et al. 2006, Gallou-Kabani, Vige et al. 2007, Berglund, Li et al. 2008, Warden and Fisler 2008). Mice from these six strains were phenotyped in depth for glucose homeostasis, insulin resistance and islet morphometric analyses at 2, 10, 30 or 90 days of HFHS versus regular chow (RC) diet. This was complemented by deep sequencing of mouse islet RNA under the same conditions and at the same time-points. After initial evaluation and comparison of diet-induced diabetes in the different strains, we integrated the phenotype measurements with islet gene expression data in a network-based analysis. This multi-parameter analysis led to the identification of a sub-network of islet-expressed genes associated with glucose tolerance and insulin secretory capacity. Finally, we provide evidence that elongase 2 (*Elovl2*), the enzyme involved in very long chain fatty acid synthesis, highlighted by our network analysis, is a key player in regulating glucose-stimulated insulin secretion in mouse islets in the context of β-cell dysfunction.

Results

Different mouse strains show distinct susceptibilities to developing a HFHS induced metabolic phenotype

Mice from six genetically different mouse strains (C57Bl/6J, DBA/2J, 129S2/SvPas, AKR/J, A/J and BALB/cJ) were fed a HFHS or RC diet for 2, 10, 30 or 90 days, after which time various phenotypic and molecular measurements were performed. Many parameters, such as glucose tolerance, insulin secretion, mouse weight and glycemia were recorded (see Methods for details). At each time-point pancreatic islets were isolated and mRNA extracted for deep sequencing (RNA-Seq). The experiment was designed so that there were at least six biological replicates for each strain/time-point/diet combination.

Over the three-month period the mouse strains showed different responses to HFHS diet. DBA/2J mice were characterized by severe weight gain in HFHS-diet fed animals compared to controls (Figure 1A) and this was accompanied by a gradual decrease in glucose tolerance (Figure 1B). In contrast, AKR/J mice showed a transient decrease in glucose tolerance up to day 30 which then started to normalize at day 90. This was accompanied by a gradual increase in body weight in HFHS-diet fed mice compared to controls. This situation was similar for 129S2/SvPas and A/J mice, which showed a gradual increase in body weight accompanied by a decrease in glucose tolerance. C57Bl/6J mice showed later onset of glucose intolerance starting at day 30, and associated with a moderate weight gain, whereas BALB/cJ mice showed massively decreased glucose tolerance at early time-points, but showed no difference in body weight gain between HFHS diet and chow-fed mice. Insulinemia, both basal and glucose-stimulated, was dramatically increased in DBA/2J mice on HFHS diet (Figure 1C, D), and this was accompanied by a small increase in β-cell to α cell ratio although this was not statistically significant (Suppl. Figure 1D). No clear differences in β to α cell ratio were observed for the other strains on HFHS versus RC diet, except for AKR/J mice where a small but significant increase was observed in HFHS-diet fed mice at day 90.

Interestingly, DBA/2J was the only strain displaying a clear diabetic phenotype from the outset with significantly higher fasting glycemia at all time-points (Suppl. Figure 1A). These data indicate that DBA/2J and BALB/cJ show the most severe glucose intolerance (with a marked difference in insulin secretion), and as C57BL6 these three strains appear to show a progressive worsening of disease with time. In contrast, AKR/J, 129S2/SvPas and A/J mice

show more transient glucose intolerance, which starts to improve at three months, indicating that the latter strains may be better able to adapt to metabolic stress.

The mouse strains showed a differential adaptation to HFHS-diet in terms of insulin secretory response to oral glucose. In order to measure the degree of compensatory response to HFHS diet, we calculated an insulinogenic index (AUC insulin/AUC glycemia during oral glucose tolerance test) for each time-point and rank ordered the strains according to the difference in insulinogenic index between HFHS-diet fed and RC-diet averaged over all time-points (Table 1). Overall, DBA/2J mice show the largest insulin adaptation response to HFHS-diet, followed by AKR/J and C57Bl/6J, with 129S2/SvPas, BALB/cJ and A/J showing the lowest adaptation.

Strain	Insulinogenic Index Difference (HFHS-RC)
DBA/2J	0.0163
AKR/J	0.0033
C57Bl/6J	0.0030
A/J	-0.0014
BALB/cJ	-0.0021
129S2/SvPas	-0.0038

Table 1. Insulinogenic index difference between HFHS and RC-diet fed strains. The index is calculated as the mean difference over all four time-points

Islet transcript levels are strongly influenced by strain

Sample similarity calculated from the islet transcriptomics data (Wang, Mezlini et al. 2014) shows a clear separation of the mouse samples into six clusters of similar samples (Figure 2A). These sample clusters closely correspond to the different strains used in the study, rather than the diet, thus indicating that the transcriptional profiles of islets are more closely related to genetic legacy than to environmental factors.

Several islet pathways are modified at the transcriptional level in response to HFHS-induced metabolic stress

In order to identify specific metabolic and signaling pathways that were up- or down-regulated by HFHS-diet in islets from different mouse strains, we performed gene set enrichment analysis (GSEA) (Subramanian, Tamayo et al. 2005) comparing gene lists ranked by fold change (HFHS vs RC) against Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Figure 2B shows a heat map of 10 of the most significantly enriched pathways at day 2, representing initial adaptation to HFHS diet (for all time-points see Suppl. Figure 2). The strains are ordered along the x-axis according to insulinogenic index (Table 1). DNA replication, oxidative phosphorylation, biosynthesis and proteasome pathways are up-regulated in the most insulinogenic strains, DBA/2J and AKR/J. Gene expression alterations in C57Bl/6J are less marked but oxidative phosphorylation and proteasome pathways are up-regulated in addition to fatty acid metabolism. The least insulinogenic strains, 129S2/SvPas, BALB/cJ and A/J show no evidence for modulation of these pathways. Interestingly, extracellular matrix (ECM) genes are down-regulated in AKR/J, DBA/2J and to a lesser extent in A/J, suggesting that modulation of ECM could be linked to beta cell adaptation (or maladaption) to metabolic stress.

Global network analysis highlights key pathways and processes affected in the different mouse strains

The results described above revealed that different mouse strains exhibit distinct patterns of islet gene expression regardless of diet. By comparing HFHS with RC fed mice we detected pathways specifically activated or inhibited under metabolic stress, but we were unable to detect pathways or mechanisms that are modified over time and which could influence normal islet function in RC fed animals. Indeed, we observed changes in many phenotypic parameters (eg. glucose tolerance, insulinemia, glycemia, body weight) over time (Figure 1 and Suppl. Figure 1), suggesting that, even on a regular diet, changes in islet gene expression correlate with measures of islet function. In order to capture the diversity of both islet transcriptomics and phenotypic data collected in a single analysis, we therefore constructed a transcriptional network using samples from both RC and HFHS fed mice. We then integrated the RNA-Seq and phenotypic data together with annotations of known pathways and gene ontology categories into a global network. The analysis and integration steps are described in detail in Methods. To perform the integration, we first analysed the RNA-Seq data to identify gene co-expression modules using Weighted Correlation Gene Network Analysis (WGCNA). Such modules are based on the concept that genes sharing more neighbours in a correlation network are more likely to be functionally related and could be under the same transcriptional control (Zhang and Horvath 2005). Each module was then correlated with the phenotypic traits

to identify those related to particular traits. We identified 'hub' genes in the modules and included these in our final network: these are genes that are highly correlated to other genes nodes within a module and where expression changes may either directly or indirectly influence expression of a large number of other genes in the network. Modules were also tested for enrichment against known pathways and gene ontology categories. The gene expression modules, 'hub' genes, enriched pathways, Gene Ontology (GO) categories and phenotypic traits were integrated together into a single network. An annotated representation of the network is shown in Figure 3. In the figure, each line represents a connection between two nodes, and connections of different types are shown as different colors. Nodes that cluster together have a higher number of shared connections and may therefore represent functionally similar groups of nodes. Manual exploration of the network revealed that several of these node clusters represented distinct sets of pathways, biological processes or related phenotypic traits. Annotations for these node clusters are shown in the figure. We identified distinct node clusters related to focal adhesion, immune response, actin cytoskeleton, MAPK signaling, lipid metabolism, carbohydrate metabolism, oxidative phosphorylation, DNA replication, cell cycle, insulin secretion, glycemia/glucose tolerance and β/α cell mass. As might be expected, β/α cell mass nodes appear close to glucose tolerance and focal adhesion related nodes, indicating that they are quite strongly related in the network. This network captures all of the genomics and physiological results from our mouse experiment into a single model. As such it represents a unique resource that can be used to investigate biological processes and underlying genes that are most affected over time in the mouse strains fed on both HFHS and RC diet. The network, which can be explored within an appropriate software tool such as Cytoscape (Shannon, Markiel et al. 2003), is available as supplementary data.

A sub-network of islet genes is correlated to glucose tolerance

According to the main goal of our study, we focused on identification of genes correlated to glucose tolerance. We interrogated the global network above and identified a gene co-expression module that was significantly correlated to both oral glucose tolerance and insulin secretion. The correlation of this and other modules to the phenotypic traits is shown in Suppl. Figure 2. The AUC glycemia trait measured is an indicator of glucose intolerance: the higher the AUC is, the worse the glucose intolerance. We then searched for genes correlated to both the module and oral glucose tolerance; such genes may represent genes of key influence that are both highly connected within the module, and may also affect the trait. A scatter plot of the correlations of all genes to the module and to oral glucose tolerance is shown in Figure 4A. In the figure, yellow points indicate genes that show the highest correlations to both the trait and

the module. These genes were then used to create a sub-network of genes related to glucose tolerance. Similar approaches have previously been used to generate gene-based networks using reference mouse strains (Mori, Liu et al. 2010, Davis, van Nas et al. 2012, Langfelder, Castellani et al. 2012, Tu, Keller et al. 2012, Chaudhuri, Khoo et al. 2015). A representation of the sub-network is shown in Figure 4B). Interestingly, *Sfrp4* is identified as a key gene in this network (indicated by a dotted box in Figure 4B). This gene was previously identified as a key marker for diabetes in humans and was negatively correlated to glucose tolerance (Mahdi, Hanzelmann et al. 2012). *Sfrp4* is a secreted protein that acts as a modulator of *Wnt* signaling. Indeed, we find that this sub-network is significantly enriched for secreted and extracellular matrix proteins (GO:0031012~extracellular matrix, FDR=3.54e-4; Table S1) indicating that extracellular matrix organization could play an important role in determining the glucose responsiveness of mouse islets.

Elovl2 expression positively correlates to glucose intolerance and insulin secretion

We identified another gene, coding for elongase 2 (Elovl2), the enzyme catalyzing very long-chain fatty acid synthesis, which was both highly connected within the sub-network and showed good correlation to AUC-glycemia. *Elovl2* (indicated by a solid box in Figure 4B) belongs to the fatty acid elongase enzyme family, producing docahexaenoic acid (DHA), a polyunsaturated fatty acid that is an important component of brain, testis and retina and displays beneficial effect in different cell systems (Guillou, Zadravec et al. 2010). *Elovl2* has not been studied in pancreatic islets of Langerhans, although its ability to produce DHA (Pauter, Olsson et al. 2014), might have beneficial effects on β cell functions.

Scatter plots of *Elovl2* gene expression against AUC-glycemia and insulinemia are shown in Figure 5. Correlations of *Sfrp4* with the same traits is shown for comparison. *Elovl2* expression is positively correlated to both AUC glycemia (glucose intolerance) and AUC insulinemia (Figure 5A, C) in both HFHS and RC fed mice (orange and grey regression lines respectively). These data suggest that *Elovl2* may have a regulatory role in β cell function, with declining *Elovl2* expression contributing to impaired insulin secretion.

DHA restores glucose-induced insulin secretion inhibited by gluco-lipotoxicity

In order to gain further insight into the potential role of *Elovl2* in the defective glucose-induced insulin secretion induced by gluco-lipotoxicity, we treated the β-cell line MIN6, as well as mouse

islets, with 0.4 mM BSA_conjugated-palmitate in the presence of various concentrations of glucose. Palmitate, one of the most abundant free fatty acids (FFA) in plasma, has been shown to have detrimental effects on β cell function, including impairment of glucose-induced insulin release (Sako and Grill 1990, Zhou and Grill 1994), β-cell connectivity and responses to incretins (Gremlich, Bonny et al. 1997, Hodson, Mitchell et al. 2013, Kang, Deng et al. 2013), and insulin gene expression (McGarry and Dobbins 1999, Ritz-Laser, Meda et al. 1999, Kelpe, Moore et al. 2003). The chronic adverse effects of FFA on β-cell function are potentiated by the presence of hyperglycaemia, a phenomenon that has been termed "gluco-lipotoxicity" (Weir, Laybutt et al. 2001, Prentki, Joly et al. 2002). We found that either 0.4 mM of palmitate or 25 mM glucose increase *Elovl2* mRNA levels but not protein expression (Figure 6A and C). A similar effect was observed in C57BL6 mice islets (Figure 6B and D). In contrast, the concomitant presence of palmitate almost completely abolished high glucose-induced *Elovl2* mRNA expression in both MIN6 cells (Fig. 6A) and mouse islets (Fig. 6B). Palmitate, at high glucose levels, decreased Elovl2 protein quantity in MIN6 cells and mouse islets (Fig. 6C and D). Interestingly, the decrease of *Elovl2* protein was associated with decreased levels of DHA (Table 2).

MIN6 cell treatments	DHA levels (µg/M cells)
Glucose 5mM	0.35 ± 0.07
Glucose 5mM +Pal 0.4mM	0.26 ± 0.05
Glucose 25mM	0.30 ± 0.06
Glucose 25mM + Pal 0.4mM	0.25 ± 0.05*,%

Table 2. DHA levels in MIN6 cells treated for 24 hours with different concentration of glucose in presence or not of palmitae (0.4µM). Data are means + SEM of 6 different experiments. *P<0.05 glucose 25mM vs glucose 25mM+Pal 0.4mM, %P<0.05 glucose 5mM vs glucose 25mM+Pal 0.4mM; two-tailed student t-test.

To determine whether palmitate inhibition of ELOVL2 expression mediates the effects of the FFA on to reduce insulin secretion, we tested the effect of DHA on MIN6 cells co-treated with palmitate in the presence of various concentrations of glucose (Figure 7). As previously observed, pretreatment of MIN6 cells with palmitate in the presence of 5 or 25 mM glucose for 24h decreased glucose-induced insulin secretion (Figure 7A). Interestingly, addition of 100 µM DHA partially restored the defective insulin secretion provoked by gluco-lipotoxicity in MIN6 cells (Figure 7A). Palmitate treatment also led to a lowering of the insulin content of MIN6 cells

(Table 3A). In isolate islets, palmitate treatment had a slight tendency to decrease the insulin content (Table 3B). Under these conditions, addition of DHA induced a slight restoration of insulin content in MIN6 cells (Table 3A) and had no effects in isolates islets (Table 3B). Finally, when we normalized glucose-induced insulin secretion to insulin content, we observed that DHA completely restored stimulated insulin secretion (Figure 7B). A similar effect of DHA on insulin secretion was found in C57Bl/6J mouse islets under gluco-lipotoxic conditions (Figure 7C and D).

A

Insulin content (ng/100k cells)	Glucose 5mM	Glucose 5mM +Pal 0.4mM	Glucose 25mM	Glucose 25mM +Pal 0.4mM
BSA 0.125%	12.00 ± 0.55	2.27 ± 0.32	10.44 ± 1.21	1.85 ± 0.12
DHA 100µM	10.41 ± 0.75	3.16 ± 0.42	10.02 ± 1.35	3.19 ± 0.33**

B

Insulin content (µg insulin/islet)	Glucose 5mM	Glucose 5mM +Pal 0.4mM	Glucose 25mM	Glucose 25mM +Pal 0.4mM
BSA 0.125%	75.8 ± 18	56.8 ± 7.6	74.2 ± 16.8	47.2 ± 10
DHA 100µM	62.6 ± 24.3	69.2 ± 19.5	60.1 ± 22.3	40.8 ± 8.7

Table 3. Insulin content in MIN6 cells (A) and C57BL/6 islets (B) under gluco-lipotoxic conditions, with or without DHA 100µM. **P<0.01 compared to the same condition without DHA.

DHA restores glucose-induced cytosolic Ca^{2+} fluxes inhibited by gluco-lipotoxicity

Since glucose-induced insulin secretion is driven by increases in intracellular free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in β cells (Rutter 2004, Gilon, Chae et al. 2014) we determined whether DHA could restore intracellular Ca^{2+} dynamics under gluco-lipotoxic conditions. MIN6 cells were exposed to palmitate in the presence of 25 mM glucose with or without 100 µM DHA for 24h. They were then loaded with trappable fluorescent Ca^{2+} probe, Fura-2, and perifused sequentially with low

(2 mM) and elevated (20 mM) glucose concentrations. Elevated glucose elicited similar $[Ca^{2+}]_{cyt}$ oscillations in control and DHA-treated MIN6 cells (AUC, arbitrary units, controls vs DHA: 4.95 ± 0.43 vs 5.38 ± 0.37, *n*=3 separate experiments, NS (Figure 7E and F). In contrast, cells treated with palmitate displayed lowered glucose-induced $[Ca^{2+}]_{cyt}$ oscillations compared to control conditions (controls vs palmitate: 4.95 ± 0.43 vs 3.60 ± 0.36, *n*=3, p=0.07, Figure 7E and F). To assess whether DHA could provide a protective action towards palmitate exposure, MIN6 cells were treated simultaneously with DHA and palmitate. In the presence of DHA, the inhibitory effect of palmitate on glucose-induced $[Ca^{2+}]_{cyt}$ oscillations was abrogated (palmitate vs DHA-palmitate: 3.60 ± 0.36 vs 5.41 ± 0.52, n=3, $p<0.05$) and $[Ca^{2+}]_{cyt}$ levels were restored (DHA-palmitate vs controls: 5.38 ± 0.37 vs 4.95 ± 0.43, *n*=3, NS, Figure 7E and F). MIN6 cells perifused with 20 mM KCl, palmitate- and DHA-treated cells displayed similar Ca^{2+} dynamics as control MIN6 cells (Figure 7E and F), indicative of an action of DHA to correct defective metabolic signalling by glucose. Taken together these results are consistent with a protective role for DHA in palmitate-induced intracellular Ca^{2+} disruption in response to glucose stimulation.

Role of *Elovl2* in the restoration of the insulin-secretion defect induced by gluco-lipotoxicity

In the addition to the effect of DHA, we determined the effect of *Elovl2* over-expression in β cells. For this purpose, we infected MIN6 cells with an adenovirus encoding human *Elovl2* isoform. Over-expression of *Elovl2* was associated with an increase of DHA levels, and gluco-lipotoxicity treatment did not affected the DHA level (Suppl. Figure 3). We then tested the effect of *Elovl2* over-expression on insulin secretion in MIN6 treated with palmitate in the presence of various concentrations of glucose (Figure 8A). *Elovl2* over-expression by itself is able to modulate glucose-induced insulin secretion, but this effect it is lost when we normalized glucose-induced insulin secretion to insulin content (Figure 8C). More importantly, over-expressed *Elovl2* partially restored the insulin secretion decrease due to gluco-lipotoxicity in MIN-6 cells (Figure 8A). When we normalized glucose-induced insulin secretion to insulin content, we observed that *Elovl2* over-expression completely restored stimulated insulin secretion (Figure 8C). However, in absolute values, the *Elovl2* over-expression glucose-stimulated insulin secretion under glucolipotoxicity was lower compared to the levels obtained by DHA addition (Ad-*Elovl2* vs DHA: 56.12 ± 1.9 vs 86.76 ± 6.8). We hypothesized that the cells could have limited DHA precursor. For this reason, we performed the same experiment supplementing the cells with EPA, which is DHA precursor. Under gluco-lipotoxicity condition and EPA supplementation, *Elovl2* over-expression partially restored the insulin secretion

decrease due to gluco-lipotoxicity in MIN-6 cells to comparable levels than DHA (Figure 8B) (Ad-*Elovl2* vs DHA: 102.62 ± 5.0 vs 86.76 ± 6.8). When we normalized glucose-induced insulin secretion to insulin content, we observed that *Elovl2* over-expression completely restored stimulated insulin secretion (Figure 8D), and increased the stimulated insulin secretion by itself in the non-palmitate conditions (Figure 8D). *Elovl2* over-expression did not restored the palmitate-lowered insulin content (Table 4A), neither with EPA supplementation (Table 4B).

A

Insulin content (µg/100k cells)	Glucose 5mM	Glucose 5mM +Pal 0.4mM	Glucose 25mM	Glucose 25mM +Pal 0.4mM
Ad-GFP	12.08 ± 1.43	3.06 ± 0.38	13.04 ± 1.01	2.49 ± 0.23
Ad-Elovl2	11.17 ± 1.93	2.68 ± 0.40	12.36 ± 1.46	1.85 ± 0.24

B

Insulin content (µg/100k cells) +EPA 50µM	Glucose 5mM	Glucose 5mM +Pal 0.4mM	Glucose 25mM	Glucose 25mM +Pal 0.4mM
Ad-GFP	14.00 ± 0.88	8.61 ± 0.94	18.35 ± 2.82	5.47 ± 0.23
Ad-Elovl2	11.86 ± 1.39	6.02 ± 0.57	12.88 ± 0.67	5.07 ± 0.41

Table 4. Insulin content in MIN6 cells transduced with Ad-GFP or Ad-Elovl2 under gluco-lipotoxic conditions, with or without EPA 50µM.

Altogether, these results suggest that DHA produced by *Elovl2* may play a key role in protecting β cells from the effects of lipotoxicity and maintaining and adapted insulin secretion rate.

Discussion

This study integrates transcriptional and phenotypic data obtained from multiple mouse strains to provide a global view of the changes occurring in mouse islets in response to challenge with a HFHS diet. Such dietary insults, and the consequent obesity, are thought to drive, at least in part, the increased incidence of type 2 diabetes in genetically susceptible individuals and eliciting insulin resistance and β-cell failure (Kahn, Zraika et al. 2009). However, the genetic basis of the considerable inter-individual variability in the responses to metabolic challenges is still poorly understood (Yaghootkar, Scott et al. 2014). Using an array of genetically-distinct mouse models we were able here to identify modules of islet genes which were co-induced and that were associated with the observed phenotypic traits. These genes may thus reasonably be assumed to reflect – or to contribute to - the adaption or misadaption to the metabolic stresses leading to both insulin resistance and to β cell failure. One such module, which correlated to both glucose tolerance and insulinemia, contained at its center a gene, *Elovl2*, whose association with islet function was not previously known. We investigated the role of this gene further and show that *Elovl2*/DHA axis regulates insulin secretion defect induced by pancreatic β cell (gluco)-lipotoxicity.

Phenotypically we observe that mice from the different strains show a distinct response to HFHS diet. This is in accordance with a previous study from Gauguier and colleagues (Fearnside, Dumas et al. 2008) showing strain-specific patterns of adaptation to HFHS diet (Funkat, Massa et al. 2004, Alexander, Chang et al. 2006, Gallou-Kabani, Vige et al. 2007, Berglund, Li et al. 2008, Fearnside, Dumas et al. 2008, Warden and Fisler 2008). Intriguingly, we found that in BALB/cJ and C57Bl/6J strains a clear relationship between adiposity and glucose tolerance since the mice developed glucose intolerance without becoming obese. Indeed, BALB/cJ mice on HFHS diet did not develop obesity or increased body weight compared to chow-fed controls although they displayed a dramatic intolerance to glucose as early as day 10. However, the percentage of fat mass (measured with an EchoMRI) is strongly increased in HFHS-fed BALB/cJ mice after 90 days of diet (1.6 fold, data not shown) suggesting that this is likely due to early ectopic fat storage in insulin target tissues. In comparison DBA/2J mice, which present early signs of obesity compared to mice fed with regular chow, have a 3.6 fold increase in the percentage of fat mass at three months, largely reflecting storage in classical adipose tissue depots (subcutaneous, inguinal etc.) These data underline the important phenotypic differences between the mouse strains and suggest that they may be at least in part due to differential underlying response of the β-cells.

Supporting this view, our data show that islet transcriptional profiles cluster together by strain rather than by diet, and that each strain has a distinct diabetic phenotype indicating that strain-specific/strain-dependent changes occurring over time in both RC and HFHS diet fed mice may be important to link transcriptomics signatures to islet function. For this reason, in order to capture the extent of diversity measured, we performed a data-driven analysis including transcriptomics and phenotypic data from all sample data (HFHS and RC) collected. The approach is based on the assumption that each mouse represents an individual in a population with a particular phenotype, and that the phenotype is determined by both genetic (here the strain), and environmental factors (here the diet and age of the mice). The result of these approaches is a global network linking together transcriptomics, phenotypic and pathway information and represents a unique view of the mouse islet transcriptome. We present the network here to illustrate the diversity of information that we obtained, and that, although we chose to follow-up on a single gene, *Elovl2*, the network contains a large amount of as yet unexplored data, and can be used as a resource for future studies on mouse islets.

We identified a sub-network of genes correlated to glucose tolerance and insulinemia, and found that *Elovl2* gene expression was amongst the most strongly associated with both glucose intolerance and insulin secretion within the sub-network. *Elovl2* encodes the enzyme that synthetizes DHA (C22:6), an omega-3 series very long chain poly-unsaturated fatty-acid (PUFA) (Guillou, Zadravec et al. 2010). In mammals, the synthesis of DHA requires a dietary supply of its essential precursor, α-linolenic acid (C18:4) (Burr and Burr 1973). Biosynthesis of DHA involves the formation of 24-carbon intermediates, which are subsequently desaturated prior to chain-shortening through partial β−oxidation (Voss, Reinhart et al. 1991, Sprecher 2000). *Elovl2* and *Elovl4* have been shown to elongate PUFA with C26-C34 long chain (Leonard, Bobik et al. 2000, Wang, Torres-Gonzalez et al. 2008). However, a recent study has shown that, *in vivo*, *Elovl2* is specifically responsible for the synthesis of DHA (Pauter, Olsson et al. 2014). Expression of *Elovl2* has been found in various tissues such as liver, testis, uterus, placenta, mammary gland, retina and certain areas of the brain, all of which are tissues that are documented as having significant levels of DHA (Tvrdik, Westerberg et al. 2000, Ohno, Suto et al. 2010). DHA is also known to play a critical role in brain development and neuroprotective effects (Gharami, Das et al. 2015).

Concerning metabolic diseases such as obesity (insulin resistance) and T2D, several studies have shown that saturated fatty acids decrease insulin secretion and worsen insulin sensitivity,

whereas PUFAs such as DHA preserve or improve insulin secretion and sensitivity (Sato, Kawano et al. 2010, Liu, Xue et al. 2013). However, most of these studies are based on exogenous PUFAs originating from fish oil (Bhaswant, Poudyal et al. 2015) and the role of endogenously synthesized PUFAs as opposed to PUFAs taken up through the diet, is relatively unknown. In mice, a study from Jacobsson's group has shown a role of *Elovl2* in sperm maturation (Zadravec, Tvrdik et al. 2011). More recently, the same group demonstrated that *Elovl2* positively influenced *de novo* lipogenesis by modulating hepatic levels of key lipogenic genes (Pauter, Olsson et al. 2014). However, *Elovl2*$^{-/-}$ mice were resistant to hepatic steatosis and diet-induced weight gain, implying that hepatic DHA synthesis via *Elovl2* could also regulate lipid storage and fat mass expansion. To date, the expression and role of *Elovl2* have not been examined in pancreatic islets or β cells. Our study reveals that *Elovl2* is expressed in the pancreatic β cell line MIN6, in rodent islets, and a survey of recently-published transcriptomic data (Bramswig, Everett et al. 2013, Blodgett, Nowosielska et al. 2015) also reveals detectable, although low, levels of *ELOVL2* mRNA human adult β cells. Interestingly, *Elovl2* expression was also up-regulated ~2-fold in mouse islets in response to deletion of the tumour promoter Liver Kinase B1 (LKB1) (Kone, Pullen et al. 2014), a condition in which glucose-stimulated insulin secretion is also enhanced (Swisa, Granot et al. 2015).

We demonstrate here that high glucose levels, or palmitate at low glucose, induced *Elovl2* mRNA expression *in vitro* whilst paradoxically reducing ELOVL2 immunoreactivity. These data suggest a dual regulatory mechanism of *Elovl2* (mRNA versus protein) by high glucose and palmitate. Whilst a detailed study of the time courses of these changes was not performed, we speculate that up-regulation of ELOVL2 mRNA may drive an early increase in *Elovl2* protein expression, and thus DHA synthesis, which is subsequently reversed by *Elovl2* degradation. *In vitro* and *in vivo*, exogenous DHA has been shown to amplify (directly or not) insulin secretion (Bhaswant, Poudyal et al. 2015). Therefore, up-regulation of endogenous ELOVL2 and consequently *Elovl2* may lead to the local production of DHA, activating glucose-induced insulin secretion. Nonetheless, we found that palmitate blocks both the increase in ELOVL2 mRNA elicited by glucose in MIN6 cells and islets, and that this action of palmitate was associated with a decrease of *Elovl2* protein and also of DHA levels. To our knowledge, this is the first demonstration that *Elovl2* expression and activity are regulated in islets in response to high glucose and high palmitate.

Previous studies have shown that high palmitate represses the induction of genes elicited by glucose, such as the Per-Arnt-Sim kinase (Fontes, Semache et al. 2009) thus contributing to

the lipotoxic effects of this saturated fatty acid. Since our data suggest a strong relationship between ELOVL2 expression and insulin secretion we decided to explore the role of *Elovl2* regulation by palmitate in glucose-induced insulin secretion. As previously described, (gluco)-lipotoxicity drastically inhibited glucose-induced insulin secretion in MIN6 cells and in mouse islets. As a first approach, we found that a chronic treatment with low concentration of DHA restored glucose-induced insulin secretion but was not able to fully counteract the decrease of insulin content. Furthermore, adenovirus-mediated overexpression of human *Elovl2* restored glucose-induced insulin secretion under these conditions. The mechanisms underlying glucose-stimulated insulin secretion are intrinsically linked to Ca^{2+} influx (Rutter 2004, Gilon, Chae et al. 2014) and (gluco)-lipotoxicity is known to alter Ca^{2+} influx into β cells (Hodson, Mitchell et al. 2013). Indeed, at high glucose levels, palmitate blunted glucose-induced Ca^{2+} increases in MIN6 cells. Interestingly, DHA completely normalized the glucose response (while having no effect on the effects of KCl). Together, these results suggest that endogenous *Elovl2*/DHA axis could play a critical role to limit deleterious effect of saturated fatty acids such as palmitate on insulin secretion.

This study reports a data-driven analysis integrating islet transcriptomics with multiple phenotypic measurements across mouse strains and identifies *Elovl2* at the center of a sub-network of genes related to glucose tolerance and insulinemia. *Elovl2* expression in islets is positively correlated with the appearance of glucose intolerance and to insulin secretion. Our *in vitro* results suggest that increased *Elovl2* expression in the pancreatic islets might represent a means through which insulin secretion is increased in response to insulin resistance in obesity and pre-diabetes. Conversely, examined *in vitro,* gluco-lipotoxicity itself inhibits the expression of *Elovl2*, a process which may contribute to decompensation, and relatively impaired insulin secretion, during the progression of prediabetes to the full blown disease. Thus, in the diabetic state, *Elovl2*-calalysed DHA production is no longer sufficient to counteract the deleterious effects of high levels or free fatty acids and glucose. Previous studies have shown that PUFAs including DHA may play a protective role against the development of metabolic diseases mainly by regulating insulin sensitivity (Bjursell, Xu et al. 2014, Pinel, Rigaudiere et al. 2016). Our study reveals for the first time that DHA could also mediate its effect by acting directly on pancreatic β cells. Moreover, these findings uniquely identify endogenous production of DHA by *Elovl2* as a novel regulator of the pancreatic β cell response to gluco-lipotoxicity. Whether or not similar mechanisms are operative in human islets remains to be examined.

Materials and Methods

The experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40).

Animal housing and diet. Male mice from six different strains: C57Bl/6J, DBA/2J, A/J, AKR/J, 129S2/SvPas and BALB/cJ were housed on a 12-hour light/dark cycle and were fed a standard rodent chow (SAFE A04) or high-fat high-sucrose (HFHS) diet (SAFE 235F, with 46% fat expressed in Kcal/kg), ad libitum. They we imposed to HFHS diet at 8 weeks of age.

Oral glucose tolerance test (OGTT)

Mice were food deprived for 5 hours prior to an oral administration of 2g/Kg of 30% glucose. Blood was sampled from the tail vein at 0, 15, 30, 60, 90 and 120 min in order to assay glucose concentration with a glucometer (A. Menarini Diagnostics, France). At 0, 15 and 90 min blood was taken to measure plasma insulin with Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., #90080).

Insulin tolerance test (ITT)

Mice were food deprived for 5 hours prior to intra-peritoneal administration of insulin (Novorapid®) at 0.5UI/Kg. Blood was sampled from the tail vein at 0, 15, 30, 45, 60, 90 and 120 min in order to assay glucose concentration with a glucometer (A. Menarini Diagnostics, France).

Pancreas α- and β-cell mass

Isolated pancreas were fixed in 10% buffered formalin and embedded in paraffin wax within 24 h of removal. Head-to-tail sections (4 µm lengthwise) were cut and incubated overnight at 37°C on superfrost slides. Slides were submerged sequentially in xylene followed by decreasing concentrations of industrial methylated spirits for removal of paraffin wax. Slides were blocked in 5% (vol./vol.) goat serum in Tris buffered saline with 0.05% (vol./vol.) Tween (TBST) for 20 min at room temperature then incubated sequentially with primary (1 : 200 mouse anti-glucagon ab10988, Abcam, Cambridge, UK) and secondary glucagon antibodies (1 : 200 rabbit anti-mouse IgG antibody : TRITC conjugate; T-2402, Sigma), for 60 minutes

followed by a further block in 5% goat serum in TBST prior to sequential incubation in primary (1 : 50 Guinea pig anti-insulin, Dako A0564) and secondary insulin antibodies (1 : 400 goat anti guinea pig AlexaFluor 488, A11073, Invitrogen). Hoeschst (2 µM) was included in the final incubation as a fluorescent nuclear stain and sections were mounted using FluorSave mounting Reagent (Merck 345789). Six longitudinal sections, at 40 micron intervals were imaged per pancreas, with the SpGreen and SpOrange filters respectively on a Zeiss Axio Imager M1 (Carl Zeiss, Jena, Germany) operated through the Metafer software (MetaSystems, Waltham, MA, USA). A custom designed Metafer software classifier enabled mapping of all pancreatic tissue. Definiens image analysis software (Munich, Germany) quantified all fluorescent images.

Islet preparation

Mice were killed by cervical dislocation. The collagenase solution was injected through bile duct, and the injected pancreas were incubated at 37°C for collagenase digestion. The digestion reaction was stopped by the addition of cold HBSS buffer supplemented with BSA. The digested pancreas were washed different times. A four layers density gradient were performed in order to isolate the islets from the exocrine tissue. Islets were handpicked under a stereomicroscope.

Cell culture.

Mouse Insulinoma MIN-6 cells were grown in DMEM high glucose medium buffered with 15% fetal bovine serum (FBS), 50µM 2-β-mercaptoethanol and 100 U/ml penicillin/streptomycin. Prior to each experiment, cells were plated for 48 h in 96-well plates (100x103 cells/well) for insulin secretion test; in 6-well plates (1x106cells/well) for RNA and protein; in 50mm plates (5x106 cells/plate) for analysis of sphingolipids and fatty acid species by LC/MS/MS. Palmitate was administered to the cells as a conjugate with fatty acid-free BSA. Briefly, dried aliquots of palmitate in ethanol were dissolved in PBS containing 5% BSA to obtain a 4 mM stock solution. The molar ratio of FFA to BSA was 5:1. The FFA stock solutions were diluted in DEME supplemented with 1% FBS (described hereafter as incubation medium) to obtain a 0.4 mM final concentration at a fixed concentration of 0.5% BSA.

In vitro insulin secretion measurements:

MIN6 cells were seeded in 96-well plates, after 48 hours they were treated for 24 hours with fatty acids (palmitate, DHA and EPA). Cells were then pre-incubated in DMEM w/o glucose for 30 minutes (deprivation phase), in KREBS containing 0.1% fatty-acid free BSA and 2 mM glucose for 30 min (stabilization phase). Insulin secretion was measured following a 30 min incubation in KREBS containing 0.1% fatty-acid free defatted BSA with 2mM glucose or 20mM

glucose. The cells were lysated with protein extraction buffer and used for protein and insulin content quantification. The insulin concentration in the medium was determined with Ultra Sensitive Mouse Insulin ELISA Kit (EUROBIO). Each condition was tested in triplicate.

Islets were cultured in 2 ml of RPMI 1640 containing 10 mM glucose and supplemented with penicillin-streptomycin (500 U/ml), 2 mM L-glutamine, 10µM Hepes, 1mM Na Pyruvate, 50µM 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum. Islets were maintained free floating at 37 °C in a humidified atmosphere of 95%O2–5%CO2 for 24h. Islets has been stimulated ex vivo for 48 h in culture medium supplemented with fatty acids (palmitate, DHA) in the presence of various glucose concentrations. Then, islets (batch of 5 islets in triplicate) in a 96-filtered-well plate were pre-incubated in KRBH—0.05% BSA with 2.8 mM glucose for 30 minutes, followed by 60 min incubation in KRBH—0.05% BSA with 2.8 or 16.7 mM glucose to measure glucose-induced insulin secretion. The insulin concentration in the medium was quantified by ELISA (EUROBIO). Each condition was tested in triplicate. Groups of 5 islets in triplicate were lisated with protein extraction buffer and used for the ELISA insulin content quantification.

Cytosolic Ca^{2+} imaging.

Imaging of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in MIN6 cells was performed using the trappable intracellular fluorescent Ca^{2+} dye Fura-2TM-AM (Invitrogen) as previously described (Ravier and Rutter 2010, Marmugi, Parnis et al. 2016). Imaging data were analyzed with ImageJ software using an in-house macro and the fluorescence emission ratios were derived after subtracting background.

Western (immuno-) Blotting.

Equal amounts of proteins were separated by 10% SDS-PAGE and then transblotted to nitrocellulose membrane. Blots were probed with either a polyclonal anti-Elovl2 antibody, stripped, and re-probed with a polyclonal anti-β actin antibody. Immunoreactive bands were visualized by enhanced chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Quantitative PCR.

Total RNA was isolated from tissues or cells using the RNeasy mini kit (Qiagen). Total RNA (4 µg) from each sample was reverse transcribed with 40 U of M-MLV Reverse Transcriptase (Invitrogen) using random hexamer primers. The primers used for PCR were derived from mice sequences and designed using OLIGO6. Real-time quantitative PCR amplification reactions were carried out in a LightCycler 1.5 detection system (Roche) using the LightCycler FastStart DNA Master plus SYBR Green I kit (Roche). Reverse transcribed RNA (10 ng) was used as the template for each reaction. All reactions were run in duplicate with no template control. The PCR conditions were: 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. mRNA transcript levels of 4 housekeeping genes (rpL19, Tbp, cyclophilin a, 18S) were assayed. Since similar results were obtained with the 4 housekeeping genes, only Tbp was retained for normalization of other transcripts.

Statistical analysis of qPCR data. Data are expressed as means ± S.E.M. Significance was assessed by the Student's t test, unpaired and two-tailed with suitable correction for multiple tests. P values less than 0.05 were considered as significant.

RNA seq analysis:

At least 150 islets were were selected and transferred to a 1.5mL DNA/RNA free tube. After centrifugation (1000 rpm, 10s) and disposal of the supernatant, islets werewere lysed in 350µL RLT Buffer (Qiagen, France). Samples were kept at -80°C before RNA extraction and RNA seq. The Sequencing library preparation was performed using 200 ng of total RNA input with the TrueSeq RNA Sample Prep Kit v2-Set B (RS-122-2002, Illumina Inc, San Diego, CA) producing a 275bp Fragment including adapters in average size. In the final step before sequencing, 8 individual libraries were normalized and pooled together using the adapter indices supplied by the manufacturer. Pooled libraries have then been clustered on the cBot Instrument form Illumina using the TruSeq SR Cluster Kit v3 - cBot - HS(GD-401-3001, Illumina Inc, San Diego, CA) sequencing was then performed as 50 bp, single reads and 7 bases index read on an Illumina HiSeq2000 instrument using the TruSeq SBS Kit HS- v3 (50-cycle) (FC-401-3002, Illumina Inc, San Diego, CA) .

Processing of RNA-Seq reads. 50nt single-end reads from 341 mouse islet samples (mean ~40Mio reads/sample) were mapped to mm9 reference genome using Tophat software version

1.3.3 (Trapnell, Roberts et al. 2012) with option -–segment-length=20. File format conversions and statistics for aligned reads were performed using *Samtools (Version 0.1.16)*. Read counts for genes were generated using *htseq-count (Version 0.5.3p3)* with UCSC mm9 refFlat genes in GTF format as a reference. Percent mapped reads were calculated and 3 samples were removed with low percentage (<45%) of mapped reads. The remaining 338 samples were used for subsequent analysis.

Differential expression analysis of RNA-Seq data. Differentially expressed genes were detected using the Limma package in R. The raw count data were first filtered for an average of 5 reads in all the samples, normalized to library size, and then transformed to log2-cpm (counts per million reads) using the *voom* function in R. Empirical Bayes moderated t statistics and corresponding p-values were then computed for the HFHS vs RC comparisons at each time-point for each of the six mouse strains using the Limma package in R. P values were adjusted for multiple comparisons using the Benjamini Hochberg procedure (Benjamini and Hochberg 1995). Genes with an adjusted p-value <= 0.05 were considered as differentially expressed.

Gene co-expression network analysis. Weighted gene correlation network analysis (WGCNA) (Langfelder and Horvath 2008) was performed on islet RNA-Seq data in R (Gentleman, Carey et al. 2004). RNA-Seq data were first normalized using the trimmed mean (TMM) method in *edgeR* (Robinson, McCarthy et al. 2010) and filtered to remove the 25% least co-variant genes. Co-expression networks were constructed by calculating adjacency matrices for each data set using a soft-thresholding power of 7 and Spearman correlation using pairwise complete observations. A topological overlap matrix (TOM) was then calculated from each adjacency matrix, converted to distances, and clustered by hierarchical clustering using average linkage clustering. Modules were identified by dynamic tree cut with a cut height of 0.995 with a minimum module size=20, using the hybrid method. Module *eigengenes* (the first principal component of the module) were calculated and similar modules were merged together using a module *eigengene* distance of 0.15 as the threshold. Module *eigengenes* were correlated to mouse phenotypic traits using Spearman correlation. In the same way, any gene, inside or outside a module, can be correlated to a module *eigengene*, and we call this the correlation of the gene to the module.

Pathway analysis of differential expression. *Gene set enrichment analysis (GSEA)* was performed on lists of genes ranked according to decreasing fold-change (HFHS vs RC) against the KEGG pathway subset of *MSigDB Version 3* canonical pathway (c2 cp) gene sets using 10000 permutations for empirical p-value calculation (Subramanian, Tamayo et al. 2005). P values were corrected for multiple comparisons using the Benjamini Hochberg method (Benjamini and Hochberg 1995).

Construction of global network. A network was created with genes, gene co-expression modules, phenotypic traits and pathways/GO categories represented as nodes and the relationships between them represented as edges. The edges were assigned between network nodes as follows. Gene co-expression modules generated from 338 sample RNA-Seq data across 48 conditions were tested for enrichment against MSigDB V3 canonical pathways (c2, cp) and biological process GO categories (c5, bp) using the hypergeometric density distribution function *dhyper* in R (ref). An edge of type 'Module enriched in pathway' or 'Module enriched in GOBP' was created between a module node and a pathway/GO category node if the enrichment p-value (unadjusted) <= 0.001. The unadjusted p-value was used here instead of the FDR because the p-value was being used as a relative score rather than to test statistical significance. An edge of type 'Gene belongs to pathway' or 'Gene belongs to GOBP' was created for each gene in the intersection between the module and the pathway/GO category. An edge of type 'Gene belongs to module' was created between a gene and a co-expression module if the absolute correlation to the module's eigengene >= 0.7 (such genes are referred to as 'hub' genes). An edge of type 'Module correlated (-) to trait' or 'Module correlated (+) to trait' was created between a module node and a trait node for Spearman correlations with p-value <= 0.05. An edge of type 'Gene correlated (-) to trait' or 'Gene correlated (+) to trait' was created if the absolute Spearman correlation, spearman's |r| >= 0.6. A lower stringency cut-off was required for module-trait correlations than for gene-trait correlations because the gene-trait correlations were generally much stronger than module-trait correlations. Finally, for visualization purposes the network was pruned to remove all nodes of degree <=1.

Figure legends

Figure 1. **Impact of HFD and age on metabolic parameters**. Boxplots showing differences between HFHS (yellow) and RC (green) diet in the 6 mouse strains over time for (A) Body weight (g), (B) AUC glycemia measured during the glucose tolerance test, (C) Basal

insulinemia (ng/ml) measured at the start of the glucose tolerance test, and (D) Stimulated Insulinemia (ng/ml) measured at 90 minutes following glucose administration. The bottom and top of the boxes represent the first and third quartiles, with the horizontal line representing the median. The upper whiskers represent the third quartile plus 1.5x IQR (interquartile range); The lower whiskers represent the first quartile minus 1.5x IQR. Outlier points beyond this range are indicated above or below the whiskers. Statistical significance between HFHS and RC at each time-point was measured using the two-sided Student's t-test and p-values were corrected for multiple comparisons using the Benjamini Hochberg FDR method (Benjamini and Hochberg 1995).(Benjamini and Hochberg 1995). Statistically significant comparisons following FDR correction (FDR <= 0.05) are indicated by a double asterisk. Marginally significant comparisons (raw p-value <= 0.05) are indicated by a single asterisk.

Figure 2. **Transcriptomic analysis across mouse strains and diets**. (A) Sample similarity heatmap generated from gene expression data. Sample similarities were calculated by first calculating Euclidean distances between mean gene expression profiles for each sample group, followed by similarity calculation on the distance matrix using the *affinityMatrix* function of *SNFtool* in R (Wang, Mezlini et al. 2014). Darker color indicates higher affinity between samples. The strains corresponding to the sample clustering are indicated on the right of the heatmap. (B) Heatmap of 10 selected pathways enriched in HFHS vs RC for the 6 mouse strains at day 2. Colors correspond to degree of enrichment: red= positive enrichment; blue = negative enrichment. Enrichment scores were calculated using GSEA against MSigDB V3 canonical pathways (see Methods for details). Only enrichment scores >=3 with a corresponding FDR <= 0.3 are represented as colors in the heatmap.

Figure 3. **Network representation integrating gene modules, pathways and phenotypic traits**. See Methods for details of the network generation. Colors represent different relationship types and are included for illustrative purposes only. The figure is overlaid with manual annotations referring to particular pathways or traits that are approximately represented at that position in the network. Network visualisation was produced using *Gephi 0.8.2.* Network files are available as supplementary material.

Figure 4. **A gene co-expression module correlated to insulin secretion and oral glucose tolerance**. (A) Scatter plot of AUC glucose correlation against module membership (correlation to module) for all genes. Genes with the best correlations to both the module (Spearman's |r|

>=0.5) and to AUC glycemia (Spearman's |r| >=0.4) are highlighted by red points outlined in yellow. These genes were used to generate the network in (B). *Elovl2* is indicated by a yellow diamond outlined in red. (B) Network generated from selected module genes. Node size is proportional to degree and node color indicates correlation to AUC glycemia (blue: negative correlation; red: positive correlation). Edges (connections) between nodes indicate correlation between genes (blue: negative; red: positive). *Elovl2* (solid box) and *Sfrp4* (dotted box) are indicated in the network. Full details of network generation are described in Methods.

Figure 5. **Scatter plots showing correlations between *Elovl2* and *Sfrp4* islet gene expression with AUC glycemia and insulinemia**. (A) *Elovl2* is positively correlated with AUC glycemia: Pearson's *r* = 0.39, p-value= 8.82e-05 (all samples); *r* = 0.48, p-value= 0.018 (HFHS samples); *r* = 0.45, p-value= 0.028 (RC samples). (B) Sfrp4 is negatively correlated with AUC glycemia: Pearson's *r* = -0.52, p-value= 6.16e-08 (all samples); *r* = -0.51, p-value= 0.01 (HFHS samples*);* *r* = 0.51, p-value= 0.01 (RC samples). (C) *Elovl2* is weakly positively correlated with AUC insulinemia: Pearson's *r* = 0.26, p-value= 0.01 (all samples); *r* = 0.18, p-value= 0.4 (HFHS samples); *r* = 0.37, p-value= 0.07 (RC samples). (D) *Sfrp4* is negatively correlated with AUC insulinemia: Pearson's *r* = -0.6, p-value= 1.057e-10 (all samples); *r* = -0.56, p-value= 0.004 (HFHS samples); *r* = -0.62, p-value= 0.001 (RC samples).

Figure 6: **Effects of prolonged exposure to glucose and palmitate on *Elovl2* mRNA and protein level in pancreatic β cells and mouse islets**. MIN-6 (A) and C57BL6 isolated islets (B) were cultured for 24h at 5mM or 25mM glucose in absence (control) or presence of 0,4mM Palmitate. Elovl2 mRNA levels was determined by fluorescence-based RT-qPCR as described in Materials and Methods. Results (means ± SE) of three independent experiments are expressed as the ratio between the signal of Elovl2 and the signal of the housekeeping gene rpL19 (ribosomal protein L19). MIN-6 and C57BL/6J isolated islets were cultured as already described (C, D), after 24 hours or 48 hours respectively, protein lysate were harvested and Elovl2 protein levels were checked by immunoblotting. Results (means ± SEM) of three independent experiments are expressed as the ratio between the signal of Elovl2 and the signal of the β-Actin. *P<0.05, **P<0.01, ***P<0.001.

Figure 7 : **DHA reverses the deleterious effects of prolonged exposure of MIN6 cells and mouse islets to elevated glucose and palmitate concentrations and reverses palmitate-induced defects in glucose-stimulated Ca^{2+} influx in MIN6 cells.** MIN6 cells or C57BL6

mouse islets were cultured for 24 or 48 hours respectively at 5 mM or 25mM glucose in the absence (control) or presence of 0.4mM palmitate (Pal) and DHA (100µM). After 24 or 48 hours, an insulin secretion test was performed as described under Materials and Methods, in order to determine the basal (2mM or 2.8mM glucose) or stimulated (20mM or 16.7mM glucose) insulin secretion. Cells or islets, were lysed at the end of the experiment to allow quantification of protein and insulin content. Secreted insulin and total insulin content were determined by ELISA assay. Results (means ± SEM) of four independent experiments with MIN6 cells are expressed as ng secreted insulin/protein (A), ng secreted insulin/insulin content (B). MIN6 cell insulin content is expressed as ng secreted insulin for 10^5 cells (starting number, see Materials and Methods) (Table 3A). Results (means ± SE) of four independent experiments with islets are expressed as ng secreted insulin/islet/min (C), ng secreted insulin/insulin content (D). Islet insulin content is expressed as ng secreted insulin/islet (Table 3B. Cytosolic free Ca^{2+} levels ($[Ca^{2+}]_{cyt}$) were measured in MIN6 cells exposed to high glucose (30mM)-BSA in the presence or absence of BSA-conjugated palmitate (400 µM) or BSA-conjugated DHA (100 µM) for 24h. Cells were loaded with Fura-2TM-AM (Invitrogen) in Krebs-Ringer bicarbonate HEPES (KRBH) buffer and then perfused with KRBH supplemented with 2 mM glucose (G2), 20 mM glucose (G20) or 20 mM KCl (KCl) as indicated. Representative $[Ca^{2+}]_{cyt}$ traces were obtained from three independent experiments. (B) Area under the curve (AUC) for the $[Ca^{2+}]_{cyt}$ rises induced by 20 mM glucose (top panel) or 20 mM KCl (bottom panel). Values are means ± SEM. **P<0.01, ***P<0.001; two-tailed student t-test.

Figure 8. **Elovl2 over-expression reverses palmitate-induced defects in glucose-stimulated insulin secretion**. MIN6 cells were trasduced with Ad-GFP (control) or Ad-Elovl2 prior to be cultured for 24 hours at 5 mM or 25mM glucose in the absence (control) or presence of 0.4mM palmitate (Pal) and EPA (50µM). After 24, an insulin secretion test was performed as described under Materials and Methods, in order to determine the basal (2mM glucose) or stimulated (20mM glucose) insulin secretion. Cells, were lysed at the end of the experiment to allow quantification of protein and insulin content. Secreted insulin and total insulin content were determined by ELISA assay. Results (means ± SEM) of four independent experiments with MIN6 cells are expressed as ng secreted insulin/protein (A), ng secreted insulin/insulin content (C). MIN6 cell insulin content is expressed as ng secreted insulin for 10^5 cells (starting number, see Materials and Methods) (Table 4A). The same experiment were performed adding to the stimulation medium 50µM EPA. Results (means ± SEM) of four independent experiments with MIN6 cells are expressed as ng secreted insulin/protein (B), ng secreted insulin/insulin content (D). MIN6 cell insulin content is expressed as ng secreted insulin for 10^5

cells (starting number, see Materials and Methods) (Table 4B). Values are means ± SEM. *p<0.05, ***P<0.001; two-tailed student t-test.

Supplementary Figure legends

Suppl. Figure 1. **Evolution of glycemia and islet cell mass across mouse strains and diets**. Boxplots showing differences between HFHS (yellow) and RC (green) diet in the 6 mouse strains over time for (A) Fasting glycemia (mg/dL), (B) Beta cell area (% of total islet area), (C) Alpha cell area (% of total islet area), and (D) Beta cell : Alpha cell ratio. The bottom and top of the boxes represent the first and third quartiles, with the horizontal line representing the median. The upper whiskers represent the third quartile plus 1.5x IQR (interquartile range); the lower whiskers represent the first quartile minus 1.5x IQR. Outlier points beyond this range are indicated above or below the whiskers. Statistical significance between HFHS and RC at each time-point was measured using the two-sided Student's t-test and p-values were corrected for multiple comparisons using the Benjamini Hochberg FDR method (Benjamini and Hochberg 1995). Statistically significant comparisons following FDR correction (FDR <= 0.05) are indicated by a double asterisk. Marginally significant comparisons (raw p-value <= 0.05) are indicated by a single asterisk.

Suppl. Figure 2. **Heat map showing correlations between module *eigengenes* and mouse phenotypic traits: darker colors indicate higher spearman correlation (red: positive correlation; blue: negative correlation).** The red box indicates the correlations corresponding to the selected module. In the figure a darker color indicates stronger correlation between a module and a trait (correlations for the selected module are indicated by a red box). The correlations to glucose tolerance and insulin secretion are shown by the darker blue squares, indicating a negative correlation between the module and these 2 traits.

Suppl. Figure 3. **DHA levels in MIN6 cells infected with Ad-GFP and Ad-Elovl2.** (A) DHA levels quantified at basal conditions (Glucose 25mM) and at glucolipotoxic condition (Glucose 25mM+Pal 0.4mM). Results (means ± SEM) of three independent experiments are expressed as ng/10^6 cells. *P<0.05, **P<0.01; two-tailed student t-test.

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Abbreviations.

$[Ca^{2+}]_{cyt}$, intracellular free Ca^{2+} concentration; DHA, docosahexaenoic acid; FFA, free fatty acids**;**

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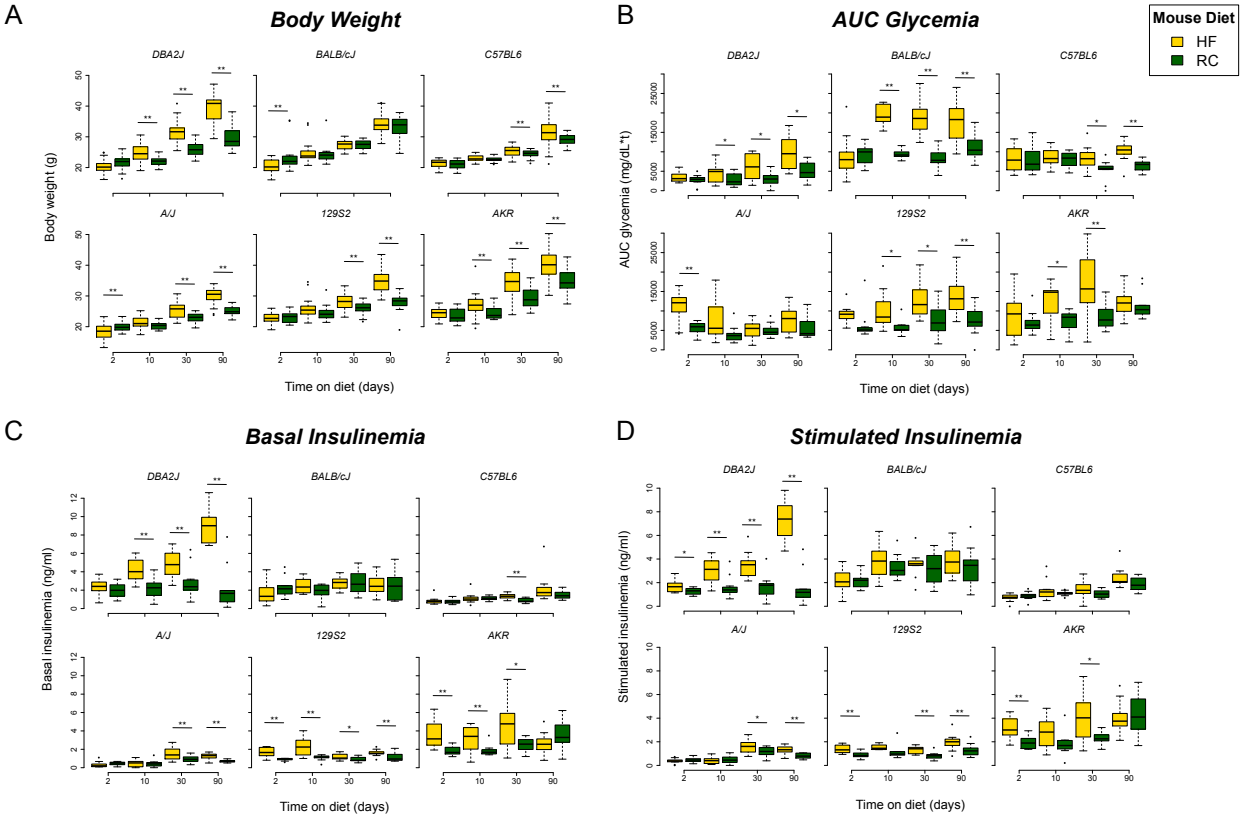
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Time on diet (days)
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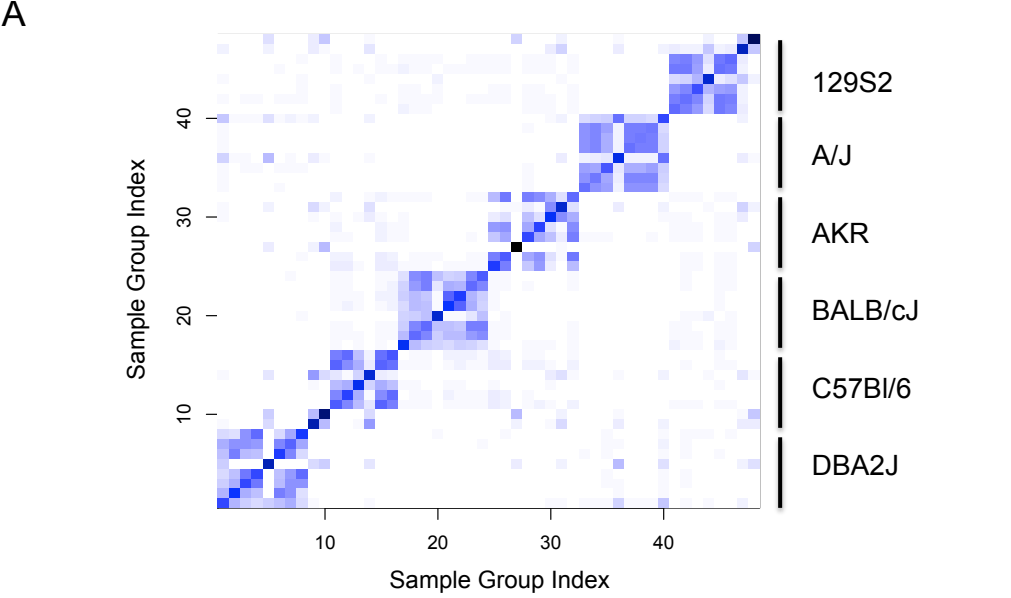
Figure 1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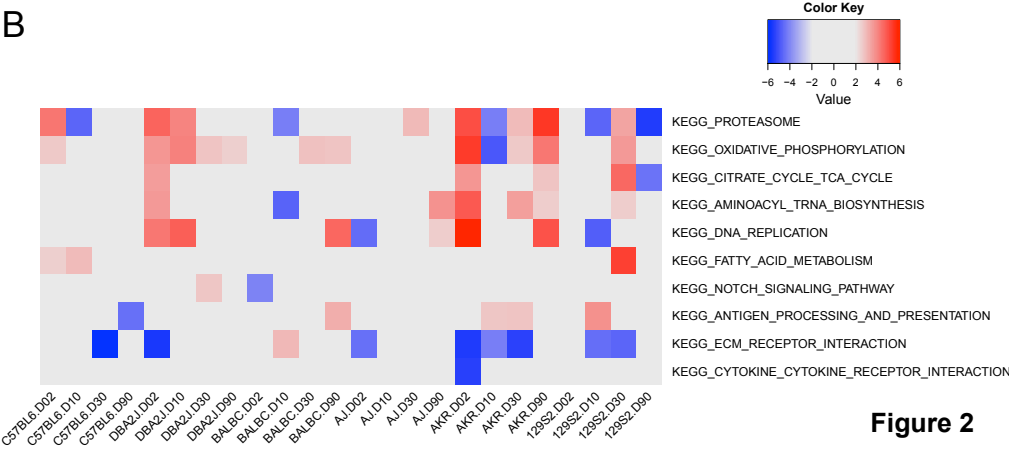
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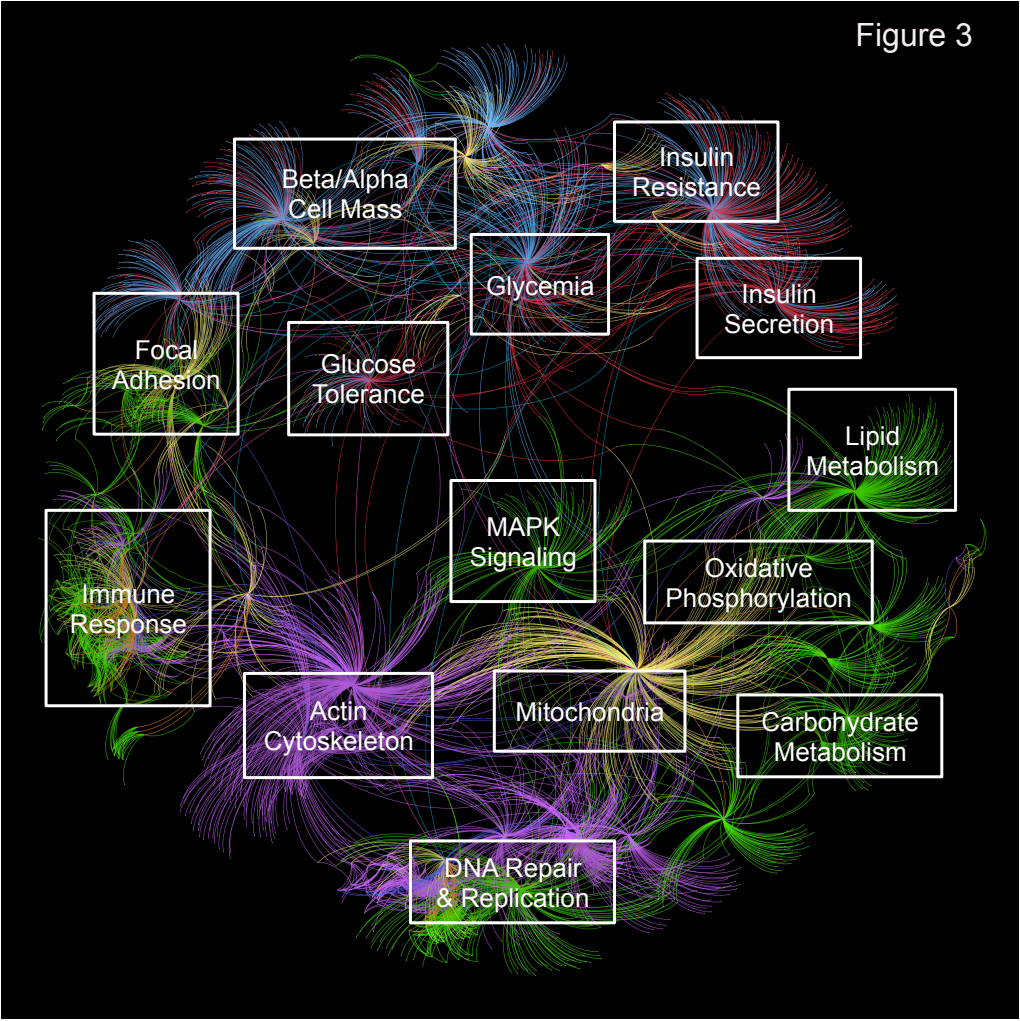
Figure 2

Figure 3



Gene belongs to pathway
Gene belongs to GOBP
Gene belongs to module
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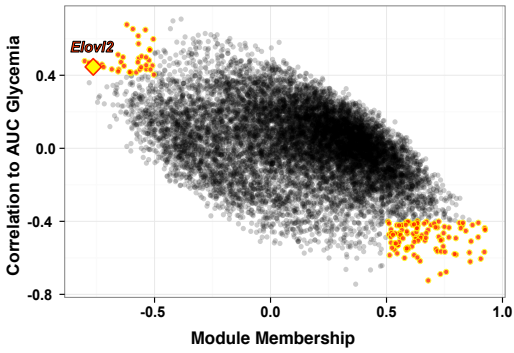
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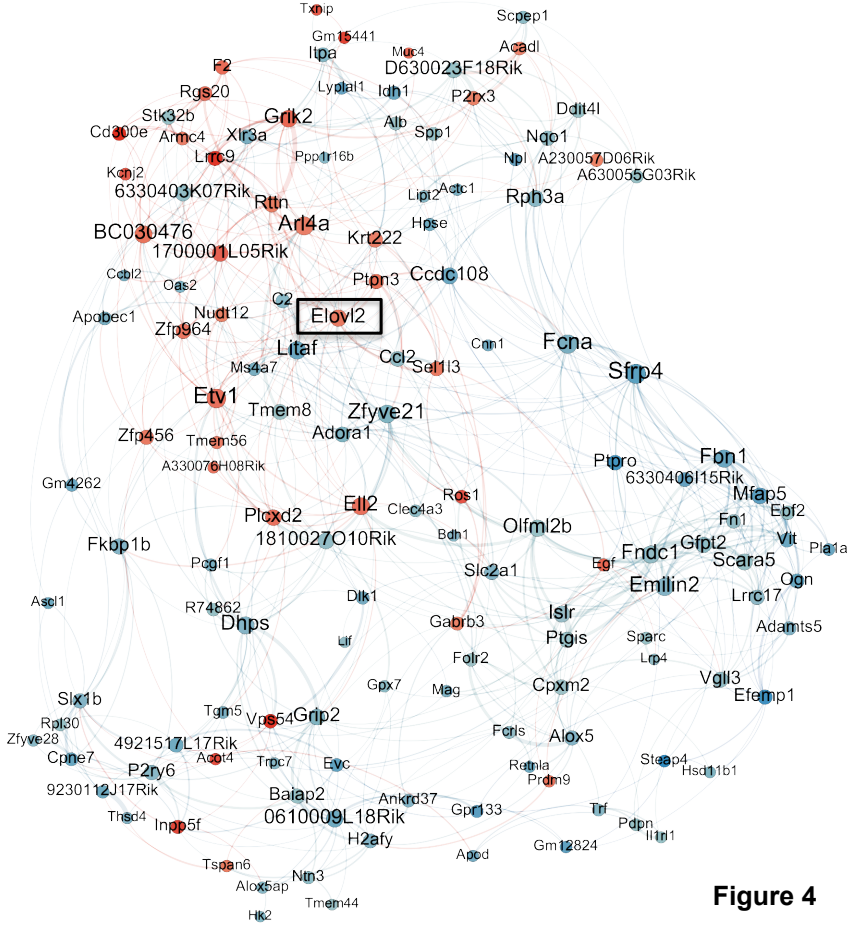
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Idh1
P2rx3
Stk32b
Grik2
Alb
Ddit4l
Cd300e
Armc4
Xlr3a
Spp1
Nqo1
Lrrc9
Ppp1r16b
Npl
A230057D06Rik
Kcnj2
A630055G03Rik
6330403K07Rik
Rttn
Lipt2
Actc1
Rph3a
BC030476
Arl4a
Krt222
Hpse
1700001L05Rik
Ccbl2
Oas2
Ptpn3
Ccdc108
C2
Apobec1
Nudt12
Elovl2
Zfp964
Litaf
Cnn1
Fcna
Ccl2
Sell3
Sfrp4
Ms4a7
Etv1
Tmem8
Zfyve21
Zfp456
Tmem56
Adora1
A330076H08Rik
Ptpro
Fbn1
6330406I15Rik
Gm4262
Mfap5
Ros1
Ell2
Clec4a3
Ebf2
Plcxd2
Fn1
Olfml2b
Fkbp1b
1810027O10Rik
Bdh1
Gfpt2
Vit
Fndc1
Pla1a
Pcgf1
Egf
Scara5
Slc2a1
Ogn
Emilin2
Ascl1
Dlk1
Lrrc17
R74862
Dhps
Islr
Gabrb3
Adamts5
Ptgis
Sparc
Lif
Folr2
Lrp4
Vgll3
Gpx7
Mag
Cpxm2
Efemp1
Slx1b
Tgm5
Vps54
Grip2
Rpl30
Fcrls
Alox5
Zfyve28
4921517L17Rik
Cpne7
Aqp4
Trpc7
Evc
Ratnla
Steap4
P2ry6
Prdm9
Hsd11b1
9230112J17Rik
Baiap2
Ankrd37
Thsd4
Inpp5f
0610009L18Rik
Gpr133
Trf
Pdpn
H2afy
Il1rl1
Gm12824
Aspd
Tspan6
Ntn3
Alox5ap
Tmem44
Hk2

Figure 4

Figure 5

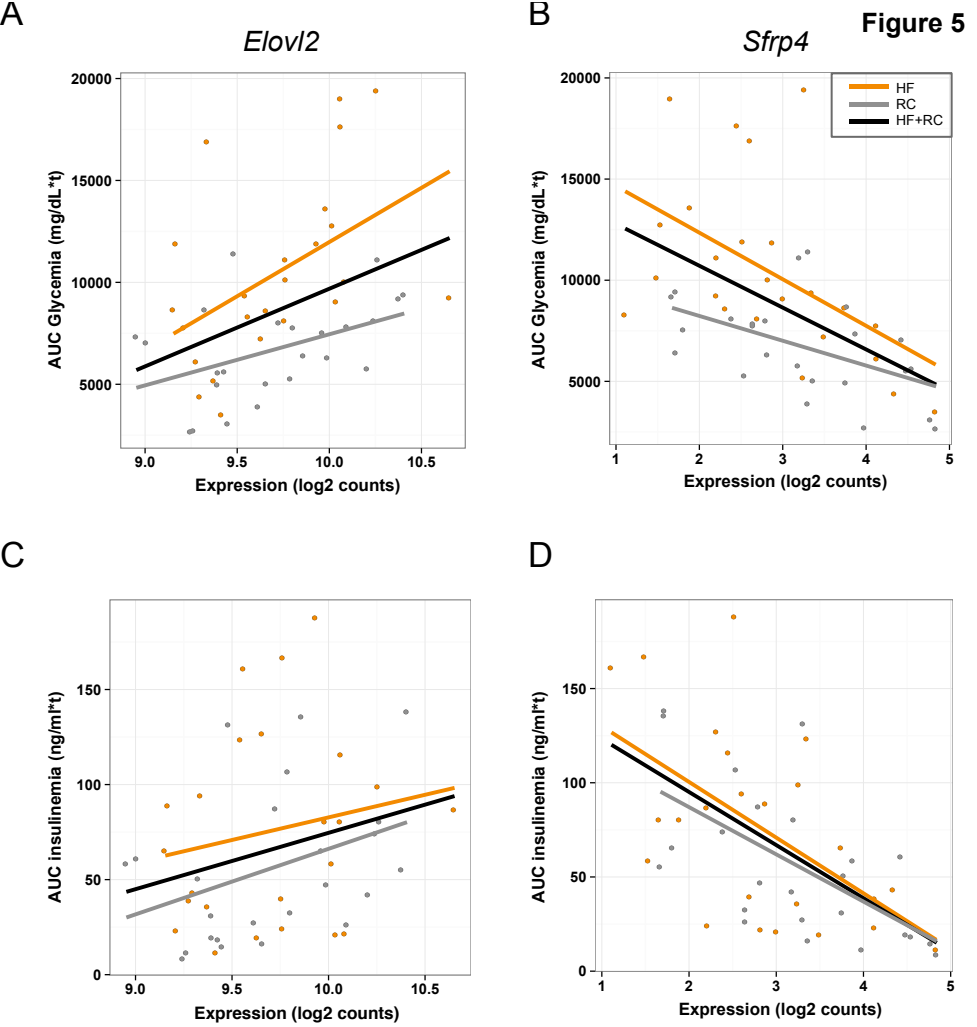
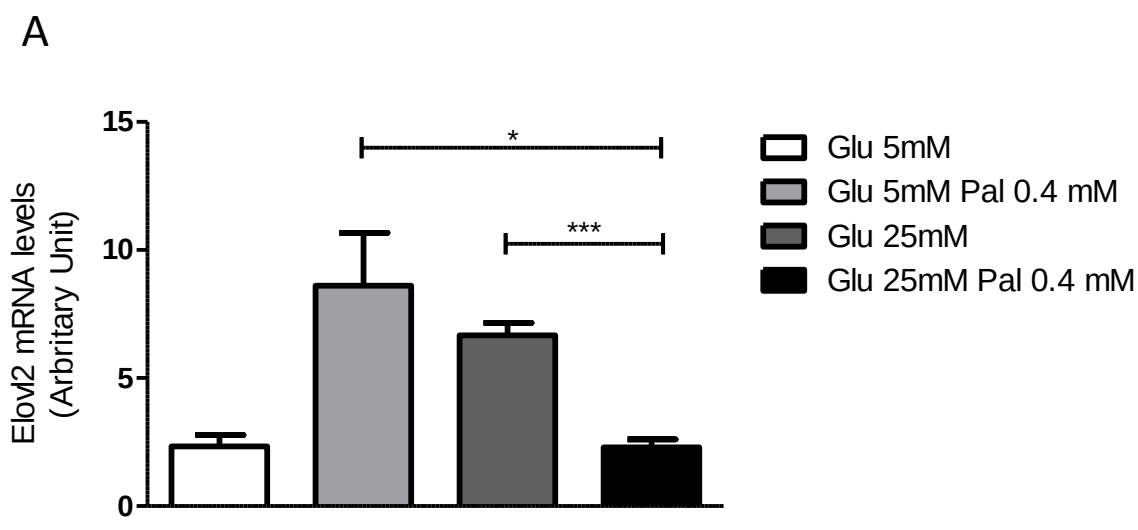
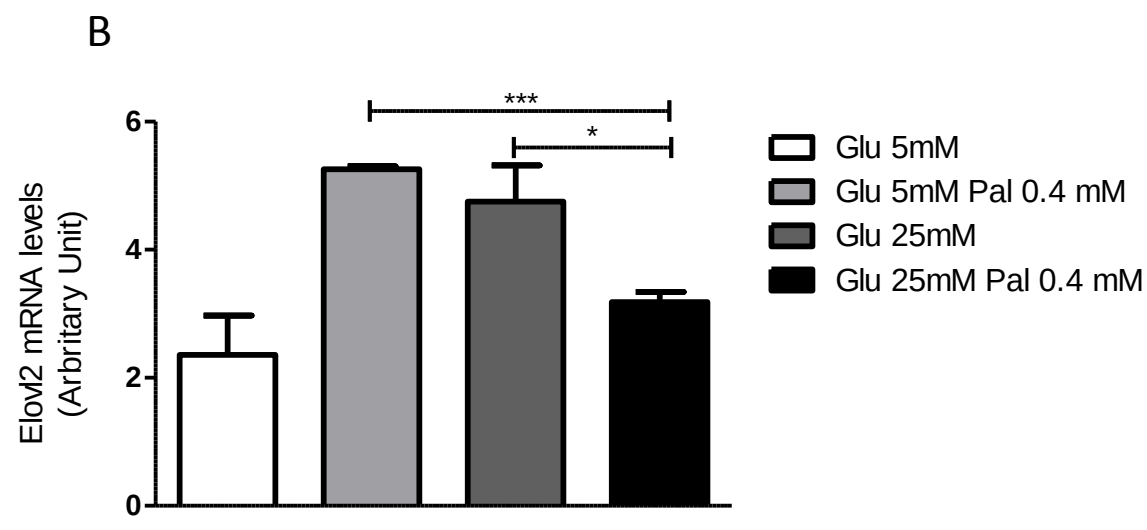
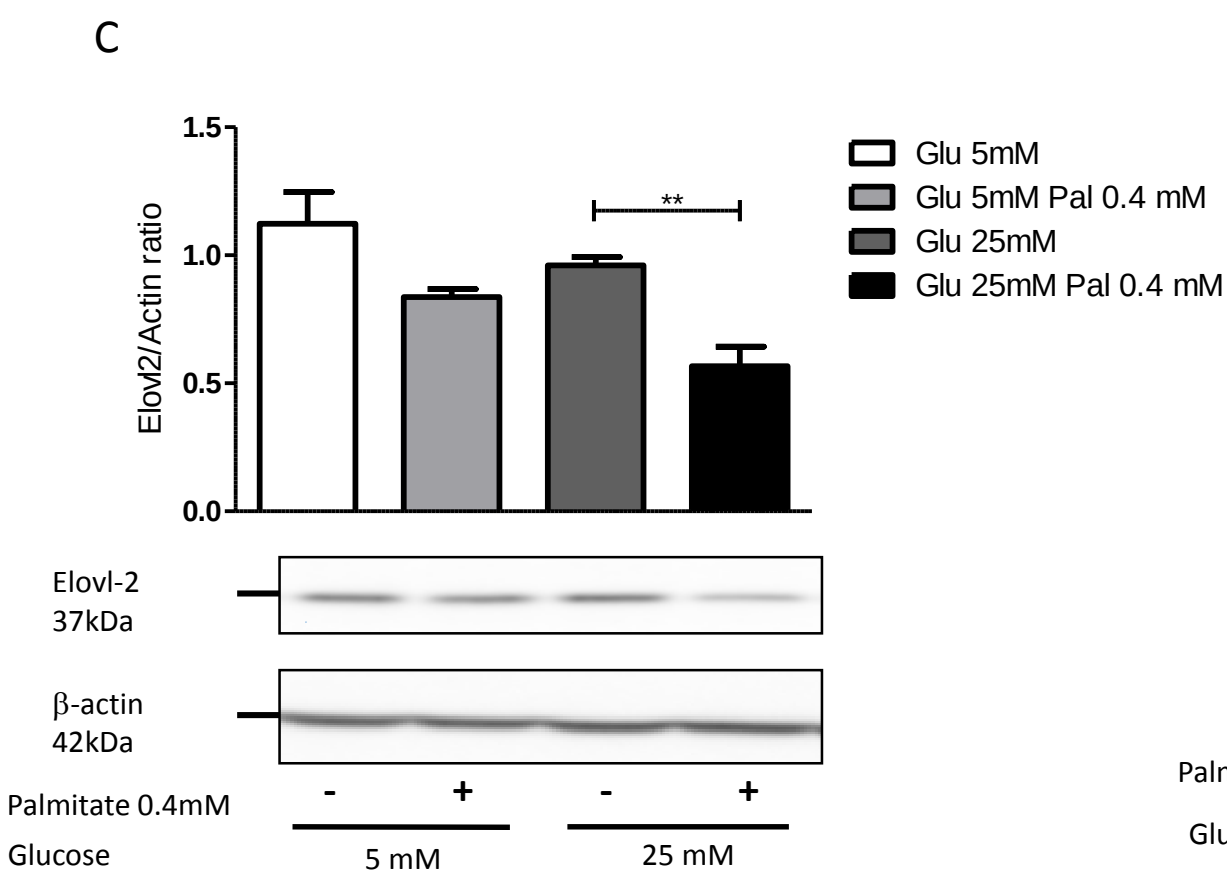

A
Elovl2
AUC Glycemia (mg/dL*t)
20000
15000
10000
5000
9.0
9.5
10.0
10.5
Expression (log2 counts)
B
Sfrp4
HF
RC
HF+RC
AUC Glycemia (mg/dL*t)
20000
15000
10000
5000
1
2
3
4
5
Expression (log2 counts)
C
AUC insulinemia (ng/ml*t)
150
100
50
0
9.0
9.5
10.0
10.5
Expression (log2 counts)
D
AUC insulinemia (ng/ml*t)
150
100
50
0
1
2
3
4
5
Expression (log2 counts)

Figure 6


A
Elovl2 mRNA levels
(Arbritary Unit)
15
10
5
0
*
***
Glu 5mM
Glu 5mM Pal 0.4 mM
Glu 25mM
Glu 25mM Pal 0.4 mM


B
Elovl2 mRNA levels
(Arbritary Unit)
6
4
2
0
***
*
Glu 5mM
Glu 5mM Pal 0.4 mM
Glu 25mM
Glu 25mM Pal 0.4 mM


C
Elovl2/Actin ratio
1.5
1.0
0.5
0.0
**
Glu 5mM
Glu 5mM Pal 0.4 mM
Glu 25mM
Glu 25mM Pal 0.4 mM
Elovl-2
37kDa
β-actin
42kDa
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM

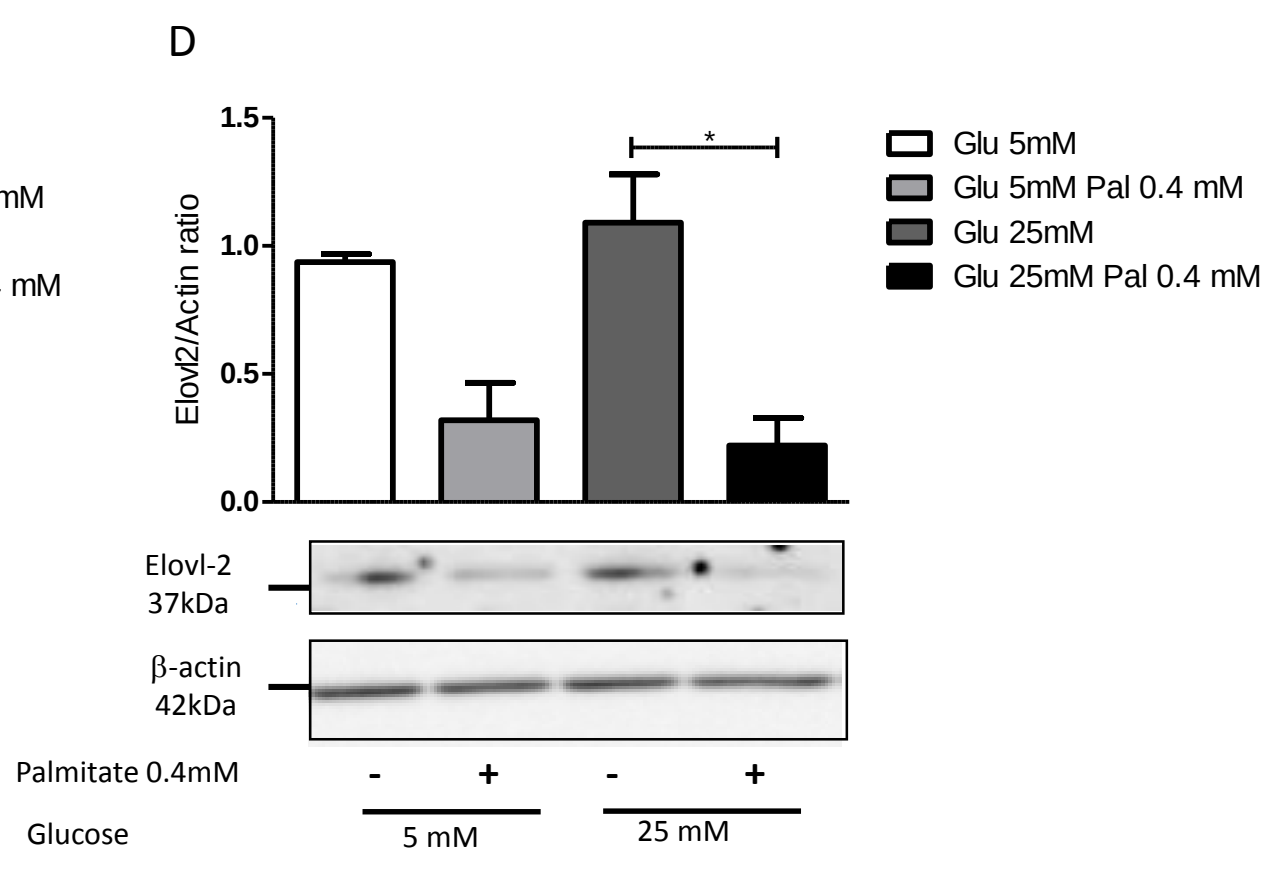
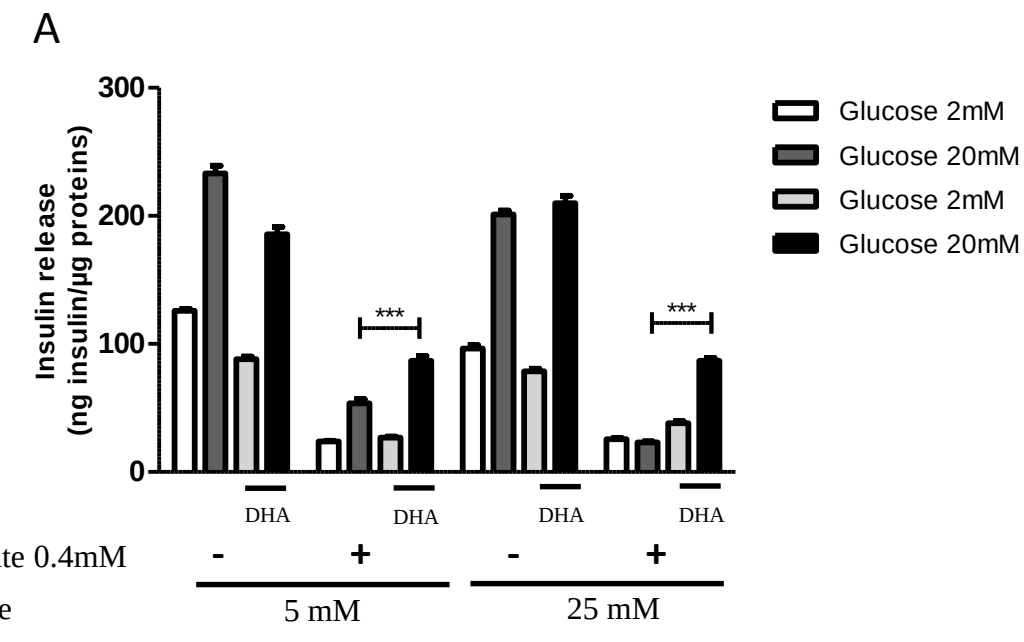
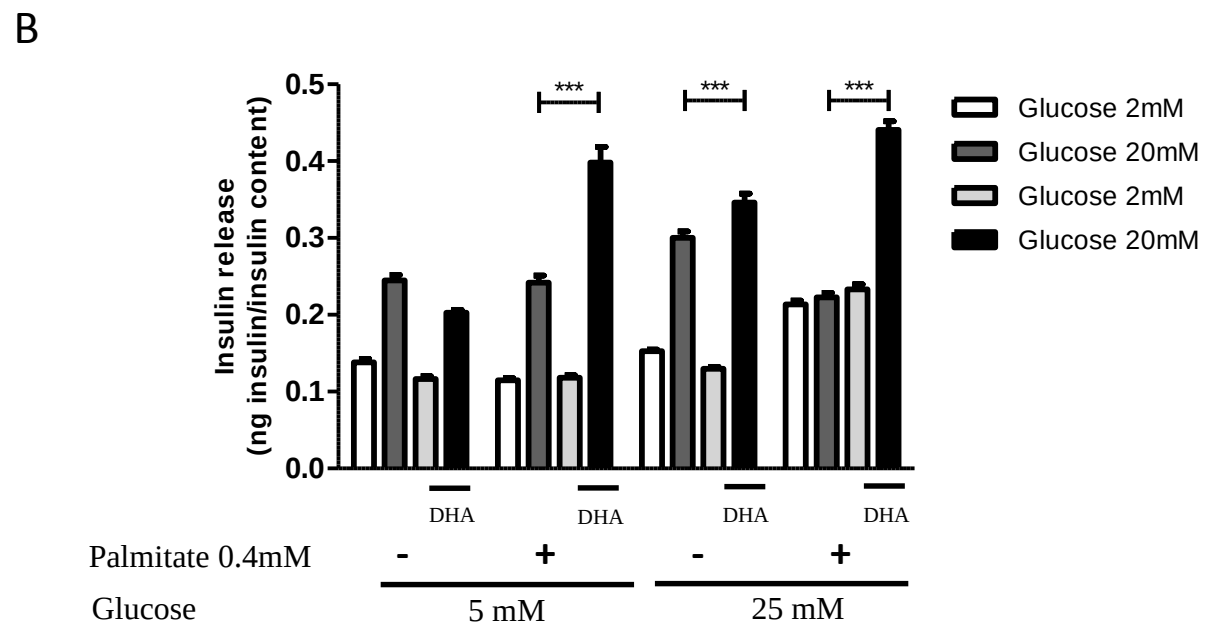
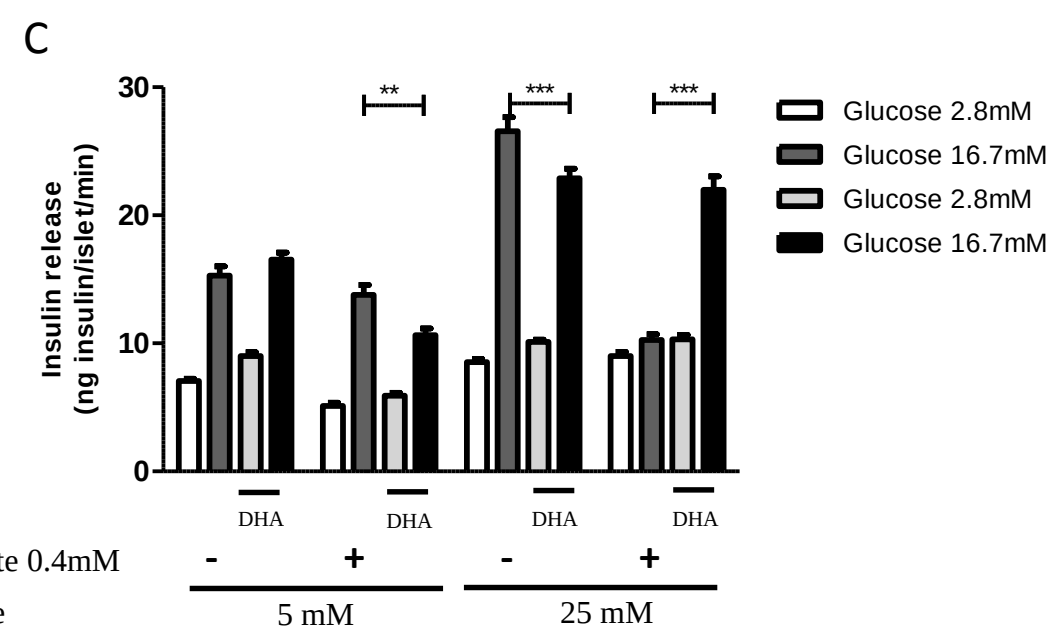
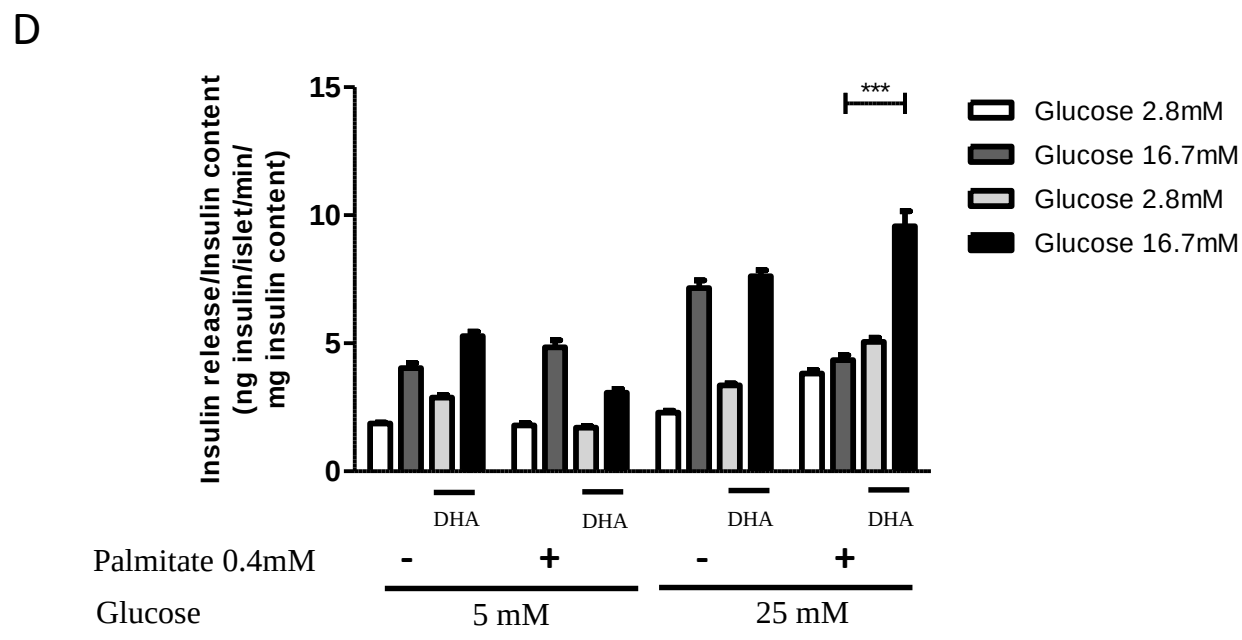

D
Elovl2/Actin ratio
1.5
1.0
0.5
0.0
*
Glu 5mM
Glu 5mM Pal 0.4 mM
Glu 25mM
Glu 25mM Pal 0.4 mM
Elovl-2
37kDa
β-actin
42kDa
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM

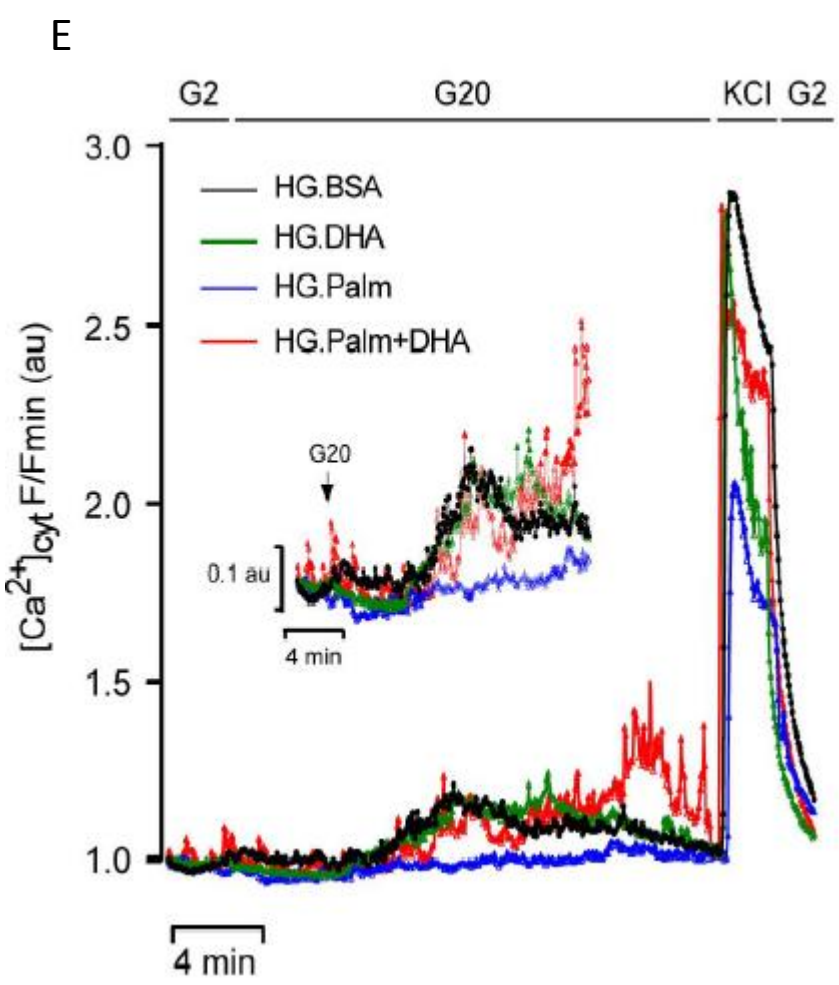
Figure 7


A
Insulin release
(ng insulin/µg proteins)
300
200
100
0
***
***
DHA
DHA
DHA
DHA
Glucose 2mM
Glucose 20mM
Glucose 2mM
Glucose 20mM
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM


B
Insulin release
(ng insulin/insulin content)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
***
DHA
DHA
DHA
DHA
Glucose 2mM
Glucose 20mM
Glucose 2mM
Glucose 20mM
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM


C
Insulin release
(ng insulin/islet/min)
30
20
10
0
**
***
***
DHA
DHA
DHA
DHA
Glucose 2.8mM
Glucose 16.7mM
Glucose 2.8mM
Glucose 16.7mM
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM


D
Insulin release/Insulin content
(ng insulin/islet/min/
mg insulin content)
15
10
5
0
***
DHA
DHA
DHA
DHA
Glucose 2.8mM
Glucose 16.7mM
Glucose 2.8mM
Glucose 16.7mM
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM


E
G2
G20
KCl
G2
3.0
2.5
2.0
1.5
1.0
[Ca2+]cyt F/Fmin (au)
HG.BSA
HG.DHA
HG.Palm
HG.Palm+DHA
G20
0.1 au
4 min
4 min

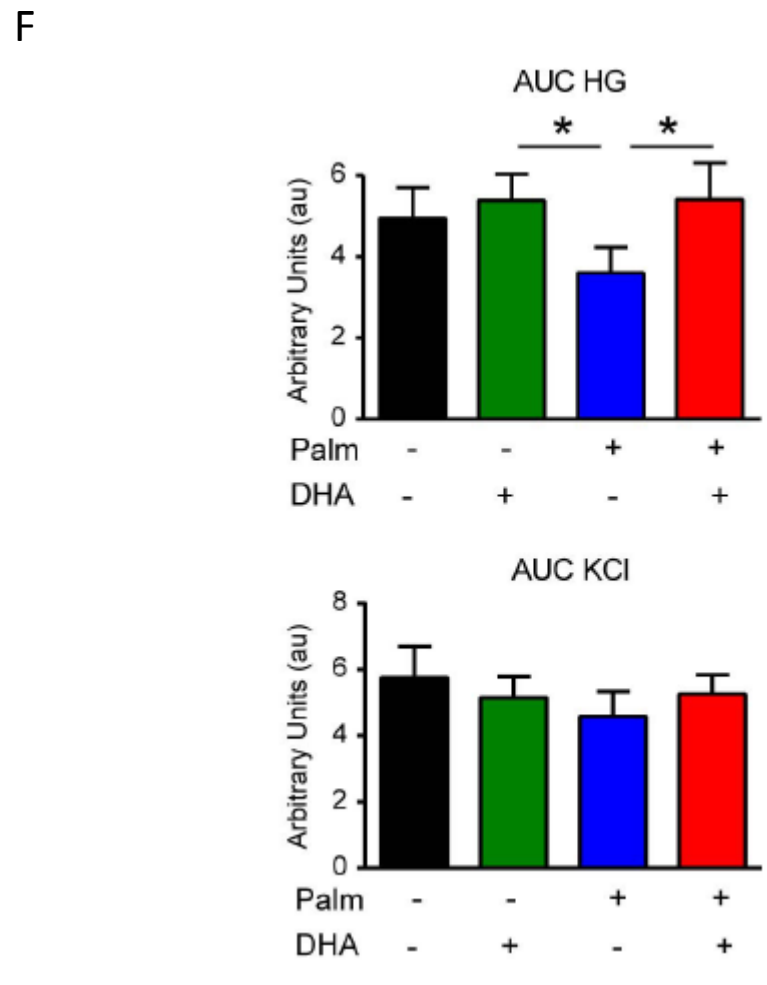
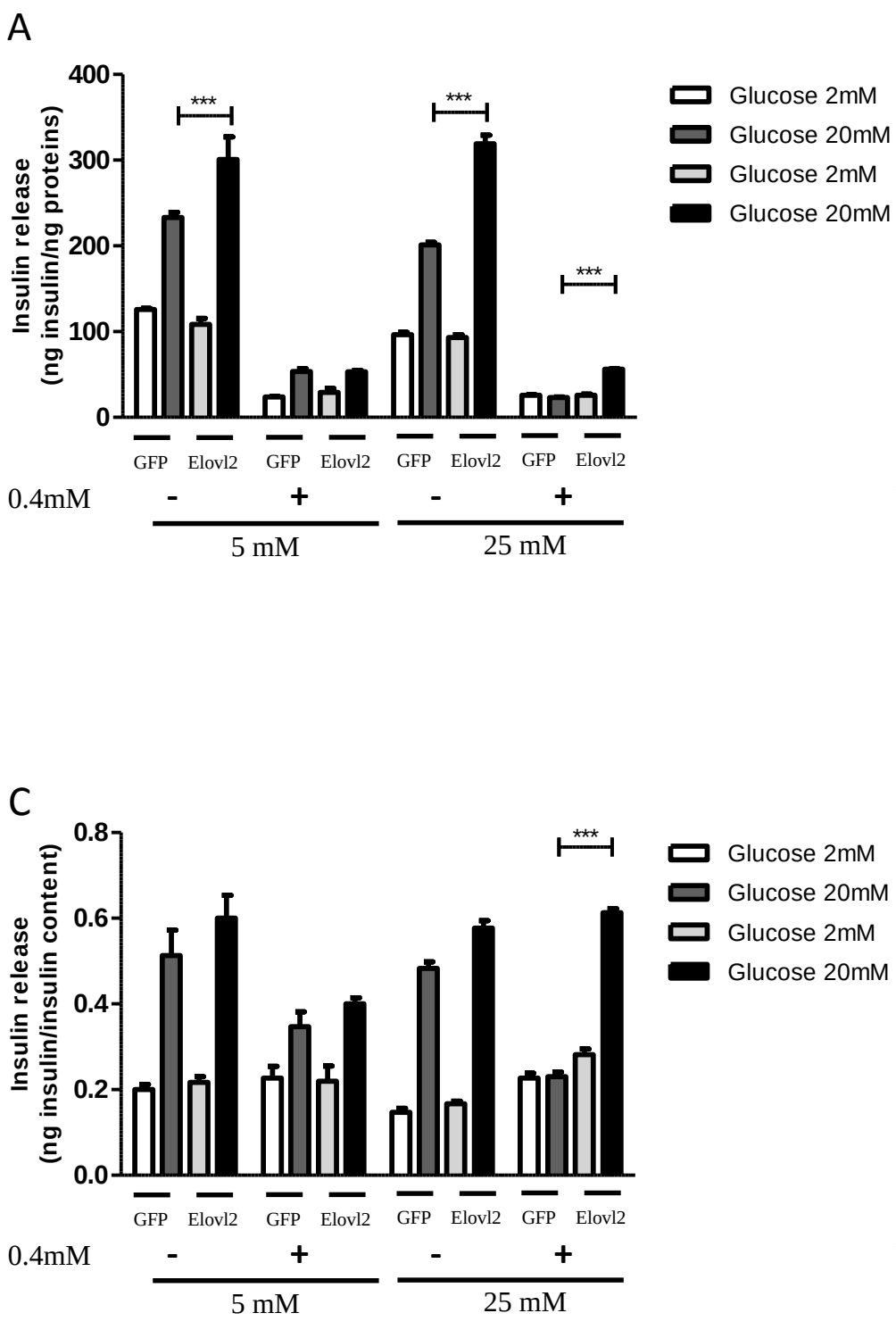
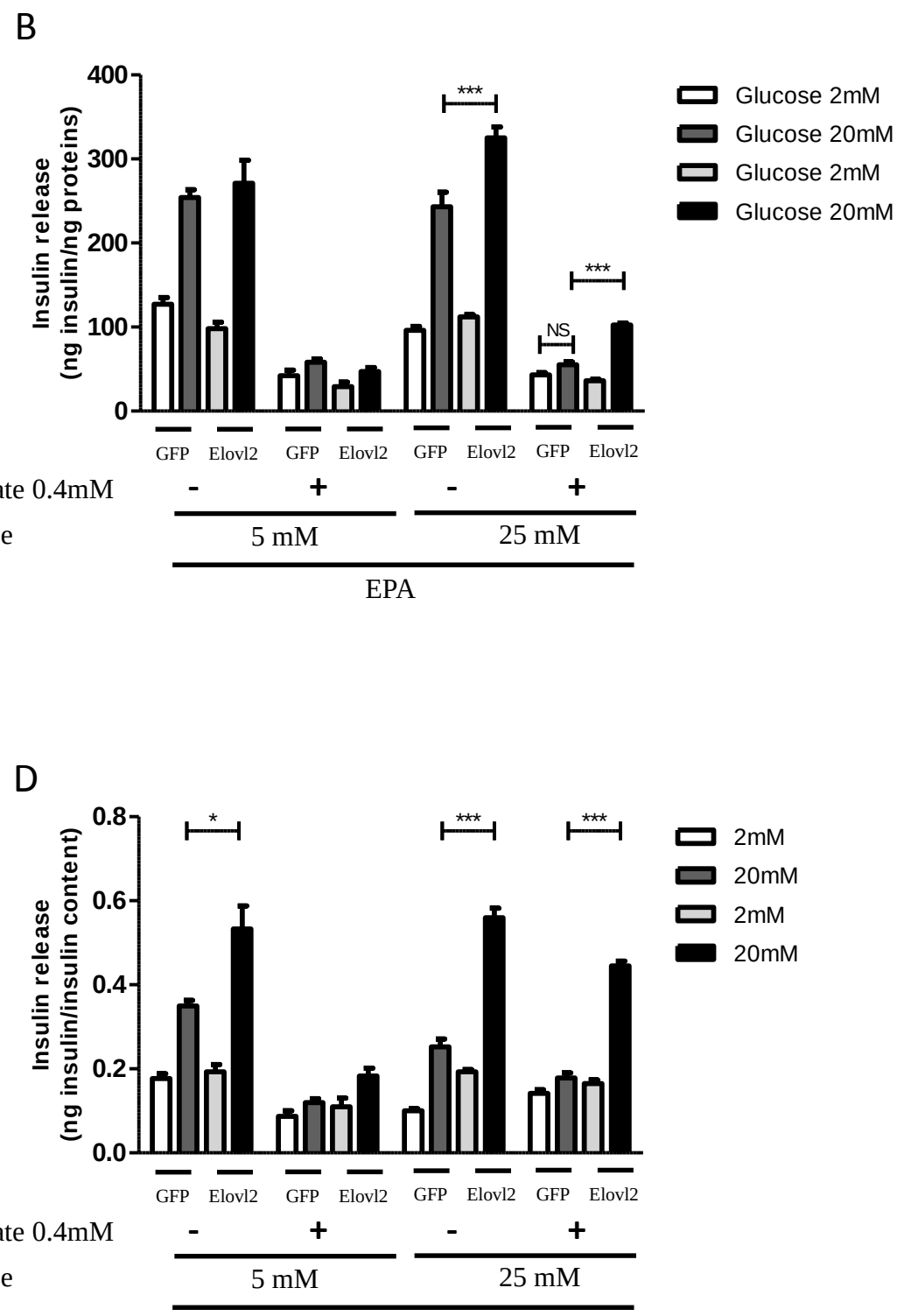

F
AUC HG
*
*
Arbitrary Units (au)
6
4
2
0
Palm
-
-
+
+
DHA
-
+
-
+
AUC KCl
Arbitrary Units (au)
8
6
4
2
0
Palm
-
-
+
+
DHA
-
+
-
+

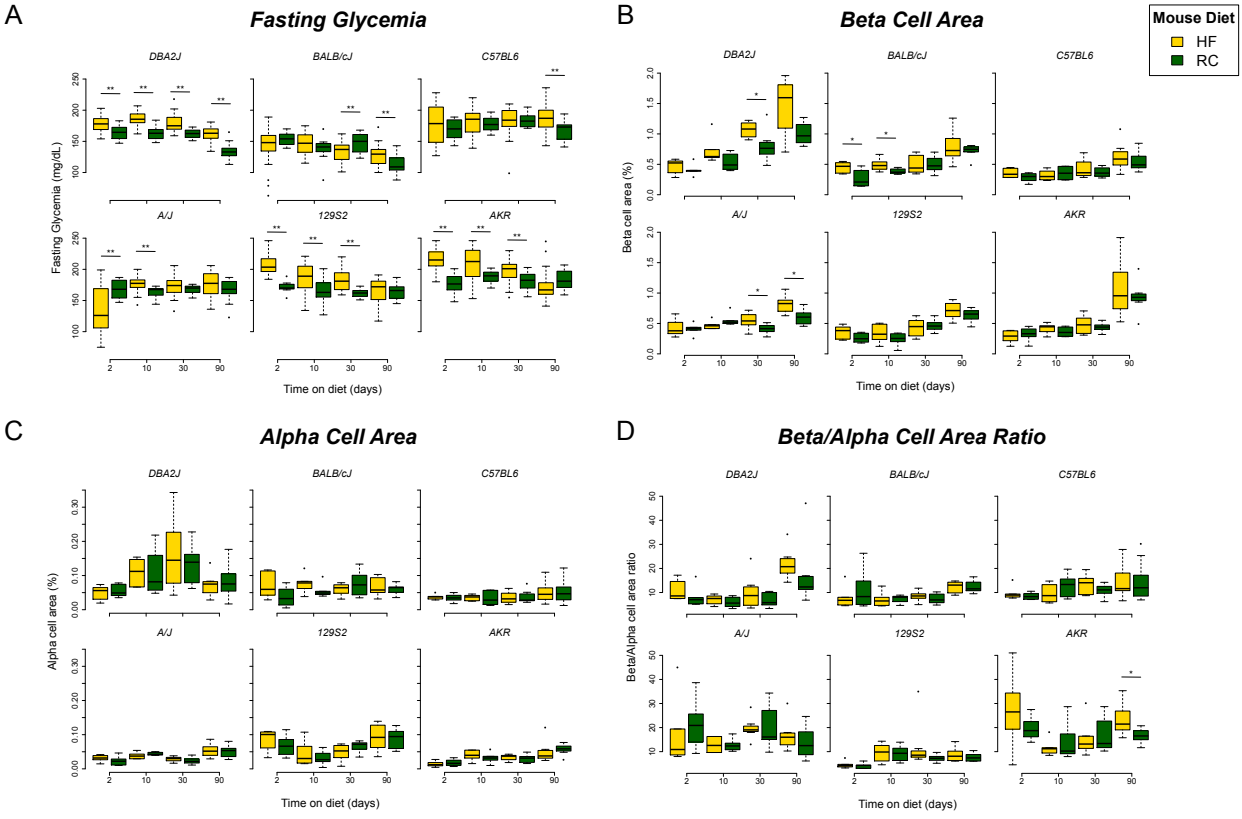
Figure 8


A
Insulin release
(ng insulin/ng proteins)
400
300
200
100
0
***
***
***
Glucose 2mM
Glucose 20mM
Glucose 2mM
Glucose 20mM
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM

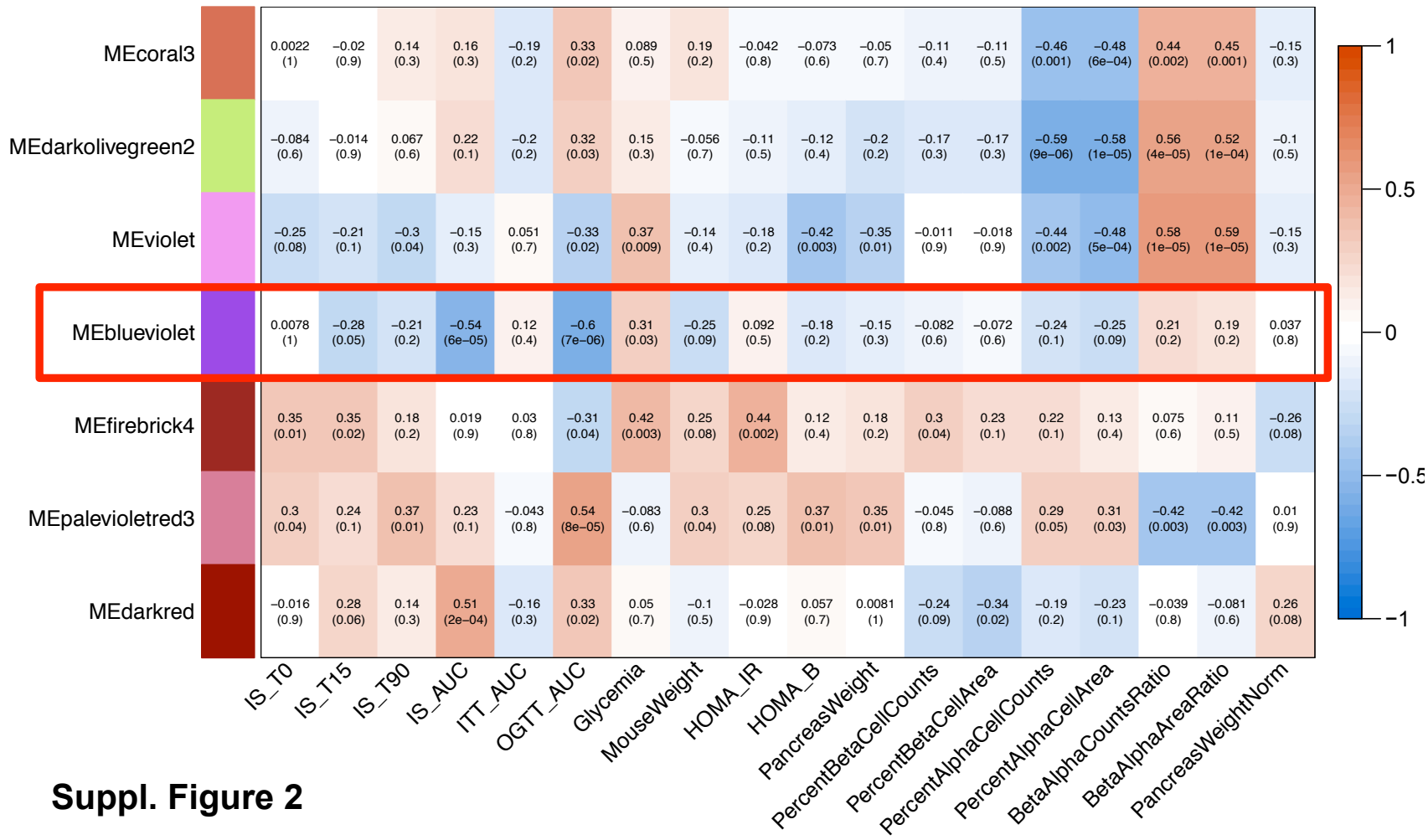

B
Insulin release
(ng insulin/ng proteins)
400
300
200
100
0
***
NS
***
Glucose 2mM
Glucose 20mM
Glucose 2mM
Glucose 20mM
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM
EPA

C
Insulin release
(ng insulin/insulin content)
0.8
0.6
0.4
0.2
0.0
***
Glucose 2mM
Glucose 20mM
Glucose 2mM
Glucose 20mM
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM

D
Insulin release
(ng insulin/insulin content)
0.8
0.6
0.4
0.2
0.0
*
***
***
2mM
20mM
2mM
20mM
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
GFP
Elovl2
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
25 mM
EPA



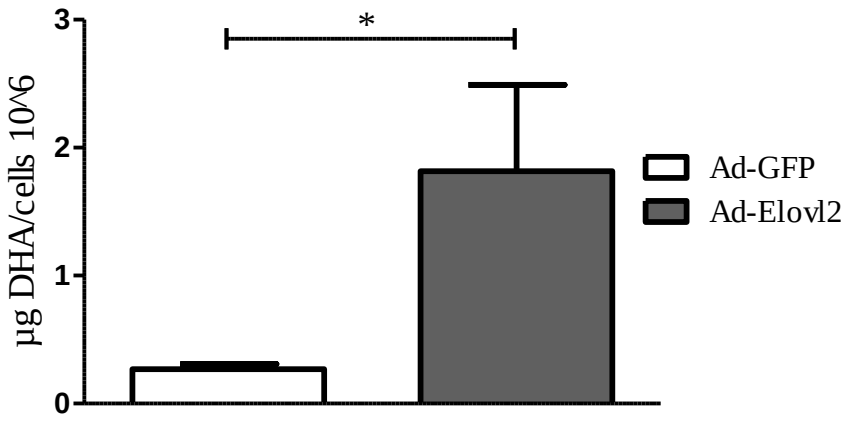
Suppl. Figure 1



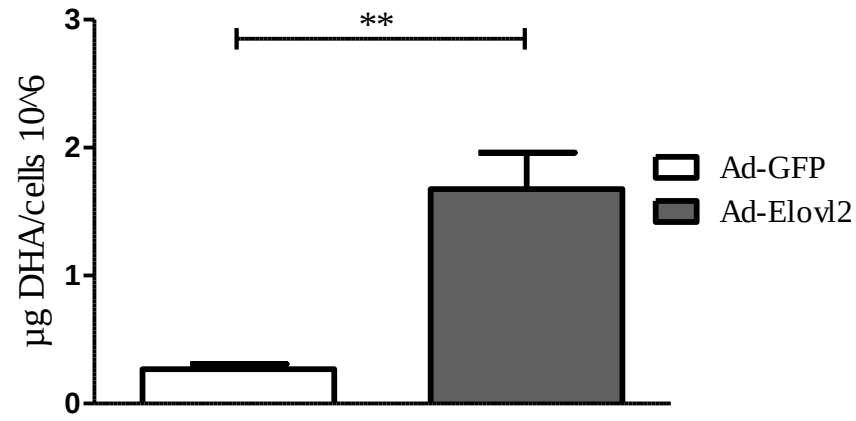
Suppl. Figure 2

Suppl. Figure 3

A



B



1 ARTICLE 2

During glucolipotoxicity, pancreatic β-cells are affected by two main dysfunction: 1- impaired glucose-stimulated insulin secretion and 2- cell death. On the second article, we focused on pancreatic β-cell apoptosis induced by glucolipotoxicity. High glucose and palmitate concentration can cause β-cell apoptosis via different pathways, such as ER stress, mitochondria dysfunction and defective authophagy (Janikiewicz et al 2015). Among the different lipid species produced by palmitate, ceramide have been identified as the main mediator of lipotoxic stress (Bellini et al 2015). Ceramide resulted to mediate ER stress (Boslem et al 2011) and β-cell apoptosis (Véret et al 2011). Inhibitors of *de novo* ceramide pathway, such as myriocin or fumonisin B1, partially block the palmitate-deleterious effects on β-cell viability (Kelpe et al 2003).

Regarding the fatty acid elongases, ELOVL, few works are present in literature that connect these proteins to metabolic syndrome (Jakobsson et al 2006). Both *Elovl5*- and *Elovl6*-knock out mice developed hepatic steatosis (Moon et al 2009; Matsuzaka et al 2007), but, the lack of Elovl6, protected mice from diet induced hyperinsulinemia, hyperglycemia and hyperleptinemia (Matsuzaka et al 2007). Regarding *Elovl2*, total knock out mice were resistant to diet induced weight gain and hepatic steatosis compared to wild type mice (Pauter et al 2014). Unfortunately, regarding the glucose homeostasis nothing is known.

ELOVL2 is known to be the enzyme responsible for the endogenous DHA synthesis (Zadravec et al 2011; Pauter et al 2014). ω-3 fatty acids, when supplemented via diet, are known to have beneficial role in plasma TAG, to control fat mass and to ameliorate glucose homeostasis (Martinez-Fernandez et al 2015). Regarding their direct effects on pancreatic β-cells, limitate number studies exist in literature. *In vivo*, only one study investigated HFD/ω-3 supplemented diet effects on β-cells, highlighting no effects (Winzell et al 2006). *In vitro*, a study determined that DHA addition was improving the glucose-stimulated insulin secretion (Itoh et al 2003). To date, DHA effects under glucolipotoxicity have never been investigated in pancreatic β-cells.

From these bases, we decided to investigate *Elovl2* role in glucolipotoxicity of pancreatic β-cells. In particular, we wanted to explore: 1- ELOVL2 role in pancreatic β-cells, in order to determine if this protein is mediating the glucolipotoxic effects or protecting from them; 2- the mechanisms involved.

Protective role of docosahexaenoic Acid/Elovl2 axis in gluco-lipotoxicity-induced apoptosis of pancreatic β-cells

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Short title: Gluco-lipotoxicity and Elovl2/ docosahexaenoic Acid axis in pancreatic β cells

Corresponding author :

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Abstract

Dietary ω3-polyunsaturated fatty acids (PUFAs), and especially DHA, have been shown to influence glucose homeostasis in normal and pathological conditions. Nevertheless, the role of endogenously synthesized ω3-PUFA compared to ω3-PUFAs taken up from the diet in this context is relatively unknown. This study was undertaken to determine whether endogenously synthesized DHA by the very long-chain elongase 2 (Elovl2), could regulate β cell apoptosis induced by palmitate with high glucose levels. We found that palmitate with high glucose decrease Elovl2 expression in INS-1 β cells. Down-regulation of Elovl2 by specific siRNA drastically potentiated β cell apoptosis induced by gluco-lipotoxicity. In contrast, over-expressed Elovl2 and exogenous DHA partially inhibits INS-1 β cell death induced by this treatment. Inhibition of INS-1 apoptosis by DHA was independent of GPR120 receptor but was associated with a decreased formation of pro-apoptotic ceramides induced by gluco-lipotoxicity. Elovl2/DHA axis partially restored the flow of ceramide from the ER to Golgi by blocking the decrease of ceramide transporter CERT. However, this pathway seemed to be insufficient to promote β cell survival in response to DHA. Finally, the fat oxidation inhibitor etomoxir markedly enhanced gluco-lipotoxicity induced β cell apoptosis and totally prevent the protective effect of Elovl2/DHA axis. In conclusion, our results suggest that the Elovl2/DHA axis plays a protective role against gluco-lipotoxicity-induced apoptotic β cell death by shifting saturated fatty acid partitioning to fat oxidation.

Keywords: Apoptosis; Mitochondrial β-oxydation ; Ceramide; DHA; Elovl2; Gluco-lipotoxicity ; Pancreatic β cells ; type 2 diabetes.

Introduction

Pancreatic β-cell dysfunction plays a central role in the pathogenesis of type 2 diabetes. Indeed, during the development of insulin resistance associated to obesity, islets of Langerhans have been shown to increase their β cell mass and the production of insulin[1]. Glycemia will be maintained until pancreatic β-cells become unable to produce sufficient quantities of insulin, at which point type 2 diabetes will begin. In animal models of diabetes, the failure of β-cell mass adaptation has been link to β-cell apoptosis[2–4]. Interestingly, in vitro studies have shown that saturated free fatty acids (FFAs) may be directly responsible for the defective adaptation of β-cell turnover[5–7]. Importantly, the chronic adverse effects of saturated FFA on β-cell function and viability are potentiated by the presence of hyperglycaemia, a phenomenon that has been termed "gluco-lipotoxicity"[8].

The molecular mechanisms underlying the pathogenesis of gluco-lipotoxicity in pancreatic β-cells are not completely understood. The sphingolipid metabolites, ceramides, have been suggested to be important mediators of saturated FFA-induced β cell dysfunction and apoptosis[9,10]. In contrast, monounsaturated fatty acids (MUFAs) such as palmitoleate and oleate exhibited opposite effects[6,11]. The latter fatty acids did not affect apoptosis but counteracted the toxic effects of gluco-lipotoxicity with improved parameters of β-cell function. Interestingly, palmitate and another saturated fatty acid, stearate (C18:0), but not of palmitoleic acid (C16:1), have been shown to increase de novo synthesis of ceramide[12]. Another class of fatty acids, namely ω-3 polyunsatured fatty acids (PUFAs) such as docosahexaenoic (DHA; C22:6 n-3), has been shown to also modulate lipotoxicity either through enhancing insulin signalling in skeletal muscle or liver[13]. Indeed, dietary ω-3 PUFAs, and especially DHA, have been shown to influence hepatic liver metabolism and prevent the development of liver steatosis[14–17]. At present, the role of endogenously synthesized ω-3 PUFAs compared with ω-

3 PUFAs taken up from the diet on (gluco)-lipotoxicity is relatively unknown, especially at the levels of pancreatic β cells.

In mammals, biosynthesis of very long-chain ω-3 PUFAs, such as DHA, requires a dietary supply of the essential precursor of the ω-3 series, α-linolenic acid[18]. The biosynthesis of ω-3 PUFAs occurs via sequential desaturation and elongation steps by the Δ5- and Δ6-fatty acid desaturases (FADSs) 1 and 2, and the fatty acid elongation by two very long-chain fatty acids elongases, Elovl5 and 2[19]. Elovl5 has been shown to specifically elongates γ-linolenic acid (C18:3 n-6) and stearidonic acid (C18:4, n-3)[20]. More recently, Jacobsson and colleagues by using Elovl2-ablated mice demonstrated that the major in vivo substrates of Elovl2 are C22:5 n-3 and C22:4 n-6, supporting the idea that this elongase completes the final elongation step in the synthesis of DHA and DPA (C22:5 n-6)[21]. Synthesis of DHA, requires a 24-carbon intermediates, which are subsequently desaturated prior to chain shortening through partial β-oxidation[22,23].

Interestingly, using a system biology approach to integrate large phenotypic and islet transcriptomic data sets from mice under regular chow versus high fat diet, we identify Elovl2 as a potent islet genes associated with glucose intolerance and insulin secretion (Bellini et al. submitted). Relatively few studies have evaluated the direct effects of DHA on pancreatic β cells whereas the proper role of Elovl2 in pancreatic β cells has not been explored up to now. Therefore in the present study, we examined whether palmitate in the presence of low and high glucose concentrations (i.e. gluco-lipotoxic conditions) alters Elovl2 expression and could contribute to β cell dysfunction and apoptosis. Our data reveal that palmitate decreases Elovl2 expression and DHA levels in β cells. Down-regulation of Elovl2 predispose β cell to apoptosis induced by glucolipotoxicity. In contrast, exogenous DHA or restoring Elovl2 expression counteract the negative effects of gluco-lipotoxicity as well as improving β cell function.

Altogether, our data suggest that the Elovl2/DHA axis might play an important role in the survival of endocrine β cells under gluco-lipotoxic conditions.

Materials and Methods

Materials

Tissue culture media and HBSS were from Life Technology. [γ^{32}P]ATP was purchased from Perkin Elmer. Palmitate, DHA, EPA, fatty acid-free BSA, AICAR, Fumonisin B1, anti β-Actin antibody and collagenase type V were from Sigma Aldrich. Diacylglycerol kinase and Etomoxir were from Calbiochem. Apo-ONE homogenous caspase-3/7 assay was from Promega. All solvents were from Merck Eurolab or Fisher Scientific. C2-ceramide were from Avanti polar lipids. AH7614, GSK 137647, GW9508 were purchased from Tocris Biotechnology. D609, PPMP were from Enzo. Anti-HA, anti-Elovl2, anti-PARP, anti-CERT and anti-β-actin antibodies were from Covance, InterChim, Cell Signalling and Abcam, respectively. siRNA against Elovl2 and control siRNA, lipofectamine LTX and RNAiMAX were purchased from Invitrogen. Metabolex 36, AZ670, AZ423 were kindly provided from AstraZeneca. Ad-Elovl2 were purchased from SignaGen Laboratories.

Cell culture conditions

Rat Insulinoma INS-1 cells were grown in RPMI 1640 medium buffered with 10mM HEPES containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate, 50µM 2-β-mercaptoethanol and 100 U/ml penicillin/streptomycin. Prior to each experiment, cells were plated for 48 h in 96-well plates ($22x10^3$ cells/well) for MTT assay and caspase-3/7 activity; in 6-well plates ($1x10^6$cells/well) for RNA, protein and ceramide levels; in 50mm plates ($5x10^6$ cells/plate) for analysis of sphingolipids and fatty acid species by LC-MS/MS and GC-MS, respectively.

Fatty acids (Palmitate, DHA and EPA) were added to cell medium as a conjugate with fatty acid-free BSA. Briefly, dried aliquots of fatty acid in ethanol were dissolved in PBS containing 5% BSA to obtain a 4mM stock solution. The molar ratio of FFA to BSA was 5:1. The FFA stock solutions were diluted in RPMI supplemented with 1% FBS (described hereafter as incubation medium) to obtain a 0.4mM palmitate final concentration, and 0.1mM or 0.01mM DHA or EPA final concentrations.

Cell transfection

INS-1 cells and MIN-6 cells were transiently transfected with 30 nmol of sequence-specific siRNA against Elovl2 and control siRNA using Lipofectamine RNAiMAX (Invitrogen). Cells and islets were transfected with Ad-Elovl2 provided from SignaGen Laboratories. Tests were performed 48 hours after infection.

Western Blotting

Equal amounts of proteins were separated by 10% SDS-PAGE and then transblotted to nitrocellulose. Blots were probed with either anti-PARP (Cell Signalling), anti-Elovl2 (Interchim), anti-V5 (Invitrogen), anti-CERT (Abcam) antibody, stripped, and re-probed with a polyclonal anti-βactin antibody (Sigma). Immuno-reactive bands were visualized by enhanced chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Measurement of caspase-3/7 activity

Caspase-3/7 activity assays were performed with the Promega Apo-ONE Homogeneous Caspase-3/7 Assay kit as described previously[24]. Briefly, lysis buffer containing fluorogenic Z-DEVD-R110 substrate was added to each well and fluorescence was measured every 6 min over a 210 minutes period using a Fluostar plate reader set at 37°C (excitation at 485 nm and emission at 530 nm). Caspase-3/7-specific activity was expressed as the slope of the kinetic in arbitrary units. Each experimental condition tested was performed in triplicate.

Analysis of the Intracellular Distribution of Fluorescent Ceramides

INS-1 cells were plated in 6 well plate and grown on a glass coverslip and cultured as previously described[25]. At the end of the treatments, cells were loaded with 2.5 µM BODIPY-C5-Cer or NBD-C6-Cer (as 1:1 complex with fatty acid free BSA) in RPMI 1640 at 4 °C for 30 min. After loading, cells

were incubated 30 min at 37 °C in RPMI 1640 containing 5 mM or 30 mM glucose ± 0.4 mM palmitate with or without DHA and fixed with 0.5% glutaraldehyde solution in PBS for 10 min at 4 °C. Samples were immediately observed and analyzed with a fluorescence microscope (Olympus BX-50) equipped with a fast high resolution charge-coupled device camera (Colorview 12) and an image analytical software (Analysis from Soft Imaging System GmbH).

Quantitative PCR

Total RNA was isolated from INS-1 cells using the RNeasy mini kit (Qiagen). Total RNA (4 µg) from each sample was reverse transcribed with 40 U of M-MLV Reverse Transcriptase (Invitrogen) using random hexamer primers. Real-time quantitative PCR amplification reactions were carried out in a LightCycler 1.5 detection system (Roche) using the LightCycler FastStart DNA Master plus SYBR Green I kit (Roche). Reverse transcribed RNA (10 ng) was used as the template for each reaction. All reactions were run in duplicate with no template control. The PCR conditions were: 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. mRNA transcript levels of 4 housekeeping genes (rpL19, Tbp, cyclophilin a, 18S) were assayed. Since similar results were obtained with the 4 housekeeping genes, only rpL19 was retained for normalization of other transcripts.

Lipid extraction and sample preparation for LC/MS/MS

Cellular lipids were extracted by modified Bligh and Dyer procedure[26] with the use of 0.1 N HCl for phase separation. C17-Cer (30 pmol) employed as internal standards, was added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v) and aliquots were taken out to determine total phospholipid content. Samples were concentrated under a stream of nitrogen, redissolved in methanol, transferred into autosampler vials and subjected to consecutive liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Enzymatic measurement of ceramide levels

Ceramide levels in cellular extracts were measured by the diacylglycerol (DAG) kinase enzymatic method as previously described[24]. Briefly, aliquots of the chloroform phases from cellular lipid extracts were resuspended in 7.5% (w/v) octyl-β-D-glucopyranoside/5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6.6). The enzymatic reaction was started by the addition of 20 mM DTT, 0.88 U/ml E. coli DAG kinase, 5 μCi/10 mM [γ-^{32}P]ATP and the reaction buffer (100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM MgCl2, and 2 mM EGTA). After incubation for 1 h at room temperature, lipids were extracted with chloroform/methanol/HCl (100:100:1, v/v) and 1 M KCl. [γ-^{32}P]-ceramide phosphate was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a phosphorimager (Storm, Amersham). Known amounts of bovine ceramide standards were included in each assay. Ceramide levels are expressed as fmol by nmol of phospholipid (PL) levels. Each measurement was done in duplicate.

Measurement of total cellular phospholipids

Total phospholipids present in cellular lipid extracts used for ceramide analysis were quantified as described previously[24] with minor modifications. Briefly, a mixture of 10N H2SO4/70% perchloric acid (3:1, v/v) was added to lipid extracts which were incubated for 30 min at 210°C. After cooling, water and 4.2% ammonium molybdate in 4 N HCl/0.045% malachite green (1:3 v/v) was added. Samples were incubated at 37°C for 30 min, and absorbance was measured at 660 nm.

Analysis of ceramide species by LC-MS/MS

Analysis of ceramide species was performed by LC-MS/MS as described previously (Berdyshev et al 2006). Instrumentation employed was an API4000 triple quadrupole mass spectrometer (Applied Biosystems) interfaced with an automated Agilent 1100 series liquid chromatograph and autosampler (Agilent Technologies). Briefly, sphingolipids were ionized via electrospray ionization (ESI) with detection via multiple reaction monitoring (MRM) in positive ions mode. Ceramide molecular species were resolved using a 3 x 100 mm X-Terra XDB-C8 column (3.5 μm particle size, Waters, Milford,

MA) and a gradient from methanol/water/formic acid (61:39:0.5, v/v) with 5 mM ammonium formate to acetonitrile/chloroform/water/formic acid (90:10:0.5:0.5, v/v) with 5 mM ammonium formate at a flow rate of 0.5 ml/min. MRM transitions monitored for the elution of ceramide molecular species were as follows: m/z 510>264, 14:0-Cer; m/z 538>264, 16:0-Cer; m/z 540>284, 16:0-DHCer; m/z 552>264, 17:0-Cer (internal standard); m/z 564>264, 18:1-Cer; m/z 566>284, 18:1-DHCer; m/z 566>264, 18:0-Cer; m/z 568>284, 18:0-DHCer; m/z 594>264, 20:0-Cer; m/z 596>284, 20:0-DHCer; m/z 624>284, 22:0-DHCer; m/z 650>264, 24:1-Cer; m/z 652>284, 24:1-DHCer; m/z 652>264, 24:0-Cer; m/z 654>284, 24:0-DHCer; m/z 680>264, 26:1-Cer; m/z 682>264, 26:0-Cer; m/z 708>264, 28:1-Cer; m/z 710>264, 28:0-Cer.

Standard curves for each of ceramide molecular species were constructed via the addition of increasing concentrations of the individual analyte to 30 pmol of the structural analogs of the sphingolipid classes used as the internal standards. Linearity and the correlation coefficients of the standard curves were obtained via a linear regression analysis. The standard curves were linear over the range of 0.0 – 300 pmol of each of the sphingolipid analytes with correlation coefficients (R2) >0.98. Parameters of declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were determined for each individual analyte by the infusion of the corresponding standards. Turbo-V ion source was operated at 550°C, GS1=40, GS2=50, and Curtain Gas=20. Correction for ion suppression by the matrix was controlled by creating standard curves in the presence of total lipid extract from human pulmonary artery endothelial cells (4 nmol total lipid phosphorus).

Statistical analysis

Data are expressed as means ± S.E.M. Significance was assessed by the Student's t test unpaired and two-tailed. P values less than 0.05 were considered as significant.

Results

Palmitate with high glucose reduces Elovl2 expression in pancreatic INS-1 β cells

In order to determine the effect of gluco-lipotoxicity on Elovl2 expression, we first we tested the effect of 0.4 mM palmitate, in the presence of various concentrations of glucose in INS-1 cells. Using real-time quantitative PCR, we found that 5 mM glucose levels and palmitate have no effect on Elovl2 mRNA levels until 24h of treatment (Fig.1A). In contrast, 30 mM glucose transiently decrease Elovl2 mRNA levels at 6h with a return to control levels at 24h. Interestingly, we found that palmitate with high glucose induces a time-dependent decrease of Elovl2 mRNA levels (Fig.1A). Indeed, palmitate with high glucose decrease by 41 % Elovl2 mRNA levels as soon as 6 h which persists until 24h (60% of decrease). In agreement, western-blot analysis using a specific antibody against Elovl2 shows that palmitate in the presence of high glucose reduces Elolv2 protein levels in INS-1 cells (Fig.1B). We also previously found that palmitate with high glucose decrease Elovl2 protein levels in islets of Langerhans from mice (Bellini et al 2016 submitted). Altogether, these results suggest that palmitate with high glucose down-down-regulates Elovl2 expression in pancreatic β cells.

Elovl2 expression regulates palmitate with high glucose-induced INS-1 β-cell apoptosis

Palmitate is known to stimulate β cell apoptosis in the presence of high glucose concentrations[5,24,27]. A specific siRNA against Elovl2 drastically reduces (97 %) its mRNA levels in INS-1 cells (Fig.2A). Interestingly, down-regulation of Elovl2 increases by itself caspase-3/7 activity in INS-1 cells (Fig.2B). As previously described, at 5 mM glucose, palmitate slightly increases caspase-3/7 activity in INS-1 cells (Fig. 2B)[5,24]. However,

inhibition of Elovl2 expression increase by 4.3-fold caspase-3/7 activation induced by palmitate in the presence of 5 mM glucose (Fig.2B). More importantly, caspase-3/7 activation by palmitate in the presence of high glucose concentrations was drastically increased (4.7 fold increase compared to siControl) by inhibition of Elovl2 (Fig.2B). Altogether, these results suggest that a decrease of Elovl2 expression/functions and therefore endogenous ω3 PUFAs production plays a central role in the control of β cell apoptosis induced by palmitate with high glucose.

Overexpression of Elovl2 inhibits palmitate with high glucose-induced apoptosis in INS-1 β cells

Stable over-expression of Ad-hElovl2 did not modified expression of endogenous Elovl2 (mRNA levels) but drastically increased mRNA levels of hElovl2 in INS-1 cells (Fig.3A). Over-expression of hElovl2 in INS-1 cells was also confirmed by the expression of V5-hElovl2 protein determined by western-blotting (Fig.3B). As expected, over-expression of Elovl2 increased basal levels of DHA in INS-1 cells by 2-fold (data not shown). In INS-1 cells over-expressing Elovl2, induction of caspase-3/7 activation by palmitate in the presence of 30 mM glucose was inhibited by 33.3% compared to vector-transfected cells (Fig.3C). We also examine the effect of Elvol2 on PARP cleavage, which represents caspase-3 activation. As expected at 5 mM glucose, palmitate is unable to cleave PARP (Fig.3D). In contrast, with 30 mM of glucose, palmitate significantly reduces the amount of PARP. Interestingly, in INS-1 cells over-expressing hElovl2, palmitate with high glucose is unable to induce PARP cleavage (Fig.3D). Since Elovl2 has been shown to be responsible for the synthesis of DHA in vivo[21], we explored if addition of DHA in culture medium also could inhibits apoptosis induced by palmitate. Addition of 10 μM of DHA drastically inhibits caspase-3/7 activation (Fig.3E) but also PARP cleavage (Fig.3F) induced by palmitate with high glucose. DHA has been shown to

mediate some of is effect through its interaction with a G-protein coupled receptor, GPR120[28]. However, potent GPR120 agonists, such as Metabolex, AZ670, AZ423 and GSK137647, are unable to inhibit caspase-3/7 activation induced by palmitate with high glucose (Fig.4A). Moreover, addition of a potent GPR120 antagonist, AH7614, do not counteract the inhibitory effect of DHA on caspase-3/7 activation by palmitate (Fig.4B). DHA can also be converted to docosahexaenoyl ethanolamine (DHEA) which could mediate their beneficial effect such as their anti-inflammatory properties[37–40] by acting on CB1 receptor. DHEA was unable to inhibit caspase-3/7 activation by palmitate (Fig. 4C). Altogether, these results support the idea that endogenous production of PUFAs such as DHA by Elovl2, independently of G protein couple receptor, plays a protective role against apoptosis of INS-1 cells induced by palmitate in the presence of high concentrations of glucose.

Elovl2/DHA axis alters palmitate-induced ceramide production in INS-1 β cells

Palmitate with high glucose concentrations has been shown to induce β cell apoptosis through ceramide accumulation[6,27]. Our results suggest that a receptor-independent intracellular function of DHA protects INS-1 cells from apoptosis induced by palmitate with high glucose concentrations (Fig.4). Therefore, we determine if the Elovl2/DHA axis could regulate intracellular pathways such as ceramide accumulation. In a first step, using the DAG kinase assay, which determined ceramide levels after phosphorylation to ceramide-1-phosphate[29], we found that palmitate at 5 mM glucose, increase ceramide levels in INS-1 cells, a mechanism potentiated by 30 mm glucose (Fig.5A and B). Addition of DHA as low as 10 μM drastically inhibits ceramide accumulation at both 5 and 30 mM glucose (Fig.5A and B). Over-expression of Elovl2 in INS-1 cells also decrease ceramide accumulation induced by palmitate. We previously showed that gluco-lipotoxicity induces β cell apoptosis through a dual mechanism involving the de novo ceramide biosynthesis and the formation of ceramides with specific N-

acyl chain lengths rather than an overall increase of ceramide content[24]. DHA treatment has no effect on ceramide species levels at 5 mM glucose with or without palmitate at the exception of reducing C16-ceramide levels (Fig.5C and D). However, low dose of DHA partially decreases accumulation of the specific ceramide species, namely C18:0, C22:0, C24:0 and C24:1-ceramides, induced by gluco-lipotoxicity (Fig.5E and F). Altogether, these results suggest that the protective effect of Elovl2/DHA axis against palmitate-induced INS-1 death is mediated by counteracting the accumulation of pro-apoptotic ceramides in INS-1 cells.

Elovl2/DHA axis protects INS-1 β cells partially restores ceramide trafficking impaired by palmitate in INS-1 cells

We have previously showed that palmitate stimulates ceramide accumulation in β cells either by increasing de novo synthesis[24] or by inhibiting their transport toward Golgi apparatus[25]. DHA treatment is unable to alter expression of ceramide synthase 4 (data not shown) which is involved in the accumulation of ceramides with specific N-acyl chain lengths in response to palmitate in β cells[24]. Then, we explored the effect of DHA on the ceramide transporter (CERT) expression. As expected, palmitate with high glucose levels reduces the amount of CERT in INS-1 cells (Fig.6A). Addition of DHA in the medium increases CERT levels by itself and counteracts the inhibition of CERT expression induced by palmitate with high glucose (Fig.6A). In agreement with our previous data, we found that palmitate with high glucose strongly reduced BODIPY-C5-Cer fluorescence accumulation in the Golgi apparatus region (Fig. 6B), supporting an impairment of ceramide flow from the ER to the Golgi apparatus in INS-1 cells. Interestingly, DHA treatment partially restores fluorescence accumulation in Golgi (Fig.6B and 6C). Indeed, palmitate with high glucose decreases by 40% BODIPY fluorescence in cells treated with DHA compared to a 80% reduction in untreated cells (Fig.6C). CERT play a central role in the synthesis of sphingolipid such as sphingomyelin (SM) and glucosyl-ceramide

(GluCer) by transferring ceramide from the ER to Golgi apparatush[30]. Potent inhibitors of SM synthase GluCer synthases, D609 and PPMP, respectively, which potentiate caspase-3/7 activation by palmitate with high glucose (Fig.6D). However, both inhibitors are unable to inhibit the protective effect of DHA on β cell survival in glucolipotoxic conditions (Fig.6B). Moreover, DHA is unable to counteract caspase-3/7 activation (Fig.6C) and PARP cleavage (Fig.6D) induced by exogenous ceramide, C2-ceramide. Altogether, these data suggest that Elovl2/DHA axis inhibits ceramide accumulation probably by partially restoring ceramide transport between ER and Golgi apparatus in INS-1 cells. However, it looks like that additional pathways are required for Elovl2/DHA axis to inhibit β cell apoptosis induced by palmitate with high glucose.

Elovl2/DHA axis protects INS-1 β cells by stimulating mitochondrial β-oxidation of palmitate

Coenzyme A esterification of palmitate, the first step of FFA metabolism, is required for the toxic action of fatty acids at elevated glucose[5]. Palmitoyl-CoA is at the time the precursor for ceramide synthesis but also a substrate for the mitochondrial β-oxydation pathways[31]. As previously showed, AICAR, an activator of AMPK which increase β-oxidation of fatty acids[32], drastically inhibit caspase-3/7 activation (Fig.7A) and PARP cleavage (Fig.7B) by palmitate with low and high glucose. This is associated with a decrease of ceramide accumulation. In contrast, etomoxir, an inhibitor of carnitine palmitoyltransferase I (CPT-I) that catalyses the rate-limiting step of the β-oxidation of FFAs, markedly amplified caspase-3/7 activation (Fig.7C) and PARP cleavage (Fig.7D) induced by palmitate with high glucose. Importantly, etomoxir allows to palmitate at 5 mM glucose to induce similar apoptosis caused by the combined presence of high glucose and palmitate (Fig.7C). Interestingly, etomoxir completely inhibit the protective effect of DHA on caspase-3/7 activation (Fig.7C) and PARP cleavage (Fig.7D) induced by palmitate with high glucose. Moreover, in the presence of etomoxir, over-

expression of Elovl2 is unable to inhibit caspase-3/7 activation induced by palmitate at both glucose concentrations. Altogether, these data suggest strongly suggest that the β-oxidation of palmitate plays a central role in the beneficial effect of the Elovl2/DHA axis in INS-1 cells.

Discussion

Saturated FFAs are known to mediated β cell dysfunction and apoptosis which contribute to dysregulation of glucose homeostasis and later on T2D installation[27,33]. Interestingly, there are growing evidences pointing to the beneficial effect of others FFA taken up from the diet, namely ω3 PUFAs, on glucose homeostasis by regulating insulin secretion and sensitivity[13]. Recently, we identify Elovl2, an elongase involved in PUFAs synthesis, as a novel regulator of β cell function under normal and obesity conditions (Bellini et al. 2016 submitted) supporting that endogenous ω-3 PUFAs produced by β cells could also contribute to regulate glucose homeostasis. In the present study, we found that gluco-lipotoxicity down-regulate Elovl2 expression in β cells. This decrease was reflected by a significant reduction of DHA levels in β cells. In agreement with previous studies, we found that gluco-lipotoxicity induced β cell apoptosis, suggesting that concomitant Elovl2 down-regulation participate to these phenomenon. Indeed, down-regulation of Elovl2 by a siRNA strategy, drastically increased β cell apoptosis induced by gluco-lipotoxicity. Moreover, over-expression of Elov2 in β cells partially protect them from apoptosis induced by gluco-lipotoxicity. Previous study have shown that PUFAs such as EPA could counteract the deleterious effect of palmitate on β cell dysfunction[34]. Jacobsson and colleagues have recently provided evidence that liver Elovl2 is responsible for the synthesis of circulating DHA[21]. Therefore, we tested if the beneficial effect of Elovl2 overexpression could be reproduced by addition of DHA in the culture medium. Interestingly, low concentrations of DHA (10 μM) completely inhibited β cell apoptosis induced by gluco-lipotoxicity. Altogether, these results support the idea that an Elovl2/DHA axis is a new regulator of β cell survival in gluco-lipotoxic conditions.

Part of effect of DHA has been shown to be mediated by its interaction with a rhodopsin-like G protein-coupled receptor (GPCR), GPR120[28]. GPR120-mediated modulation on insulin sensitivity, pancreatic insulin secretion, and β-cell mass have suggested that GPR120 could be targeted as an anti-diabetic treatment[35]. Moreover, using a combined data from human islet gene expression, genetics, and function, Taneera et al. have recently shown that GPR120 can protect pancreatic islets from lipotoxicity[36]. Since DHA is acting at relatively low concentrations, we evaluated the role of its receptor in the protective effect of DHA. Nevertheless, several potent agonist were unable to inhibit apoptosis induced by gluco-lipotoxicity and AH7614, an antagonist of GPR120 did not block the anti-apoptotic action of DHA, suggesting a GPR120-independent mechanism. These results are in agreement with a recent study showing that a similar improvement on glucose homeostasis by ω-3 PUFAs in response to HFD in *Gpr120*-deficient mice as observed for wild type mice[5]. It has been shown that DHA can also be converted to N-acyl ethanolamines (NAEs) which could mediate their beneficial effect such as their anti-inflammatory properties[37–40]. Moreover, NAEs have been shown to be potent ligand of another G protein-couple receptor, CB1, which could modulate β cell function[41]. In this study, docosahexaenoyl ethanolamine, the NAE derivatives of DHA was unable to reproduce the anti-apoptotic action of DHA against gluco-lipotoxicity. Altogether, these results suggest that the Elovl2/DHA axis mediate its anti-apoptotic effect against gluco-lipotoxicity independently of GPCR in β cells.

Various stress signalling pathways have been shown to contribute to the loss of β cell secretory responsiveness and induction of apoptosis[42,43]. We and others provided evidence that gluco-lipotoxicity induced β-cell apoptosis through induction of the de novo ceramide synthesis[27,33]. In the present study, we found that Elovl2 over-expression inhibited accumulation of ceramide induced by gluco-lipotoxicity. Addition of DHA in the culture medium, diminished also β cell apoptosis. Gluco-lipotoxicity have been shown to induce the formation of ceramides with

specific N-acyl chain lengths rather than an overall increase of ceramide content[24,44]. Here, we found that DHA blocked accumulation of C18:0-cer, C22:0-cer, C24:0-cer and C24:1-cer species induced by gluco-lipotoxicity. In mammals, the variety of ceramide species relies on the existence of a family of ceramide synthases[45,46]. We previously found that gluco-lipotoxicity induced CerS4 in β cells which contribute to β cell apoptosis[24]. Nevertheless, DHA treatment has no effect on expression of ceramide synthase CerS4, suggesting that Elovl2/DHA axis is acting downstream of ceramide synthesis. However, it has been shown that lipid such as sphingosine-1-phosphate inhibits CerS2 activity[47]. Therefore, we can no exclude that DHA inhibits CerS activity involved in ceramide accumulation induced by gluco-lipotoxicity. In muscle, MUFAs such oleate, inhibits increase palmitate-induced DES1 expression, an enzyme responsible for converting dihydro-ceramide into ceramide[47]. However, neither palmitate nor DHA alter DES1 expression in β cells (unpublished data). Altogether, these results suggest that the Elovl2/DHA axis is not affecting enzyme involved in synthesis of ceramides but rather regulates ceramide metabolism to block their accumulation.

Ceramide synthesized in the ER is transferred to the Golgi where it is subsequently converted to sphingomyelin, glucosyl-ceramide and more complex glycosphingolipids[49]. Evidence to date indicates that there are two pathways by which ceramide is transported from the ER to the Golgi: a protein-mediated transport, by the soluble ceramide transfer protein CERT[34,50,51] and a CERT-independent vesicular traffic[50,52,53]. We previously showed that gluco-lipotoxicity impaired ceramide trafficking in β cells[25]. In the present study, we found that DHA restores partially the decrease of ceramide transporter CERT expression induced by gluco-lipotoxicity. Analysis of intracellular distribution of BODIPY-C5-Cer, which reflects intracellular movements of naturally occurring ceramides[53–55], showed that DHA could partially restore intracellular traffic of ceramides from ER to the Golgi apparatus in β cells under gluco-lipotoxic conditions. Therefore, this mechanism could contribute to reduce ceramide

accumulation at the ER in response to gluco-lipotoxicity. Interestingly, Guo et al. have recently shown that downregulation of CERT potentiated palmitate-induced inhibition of insulin gene expression in β cells[56], supporting a role of CERT in β cell dysfunction induced by lipotoxicity. At present, the role of CERT in β cell apoptosis induced by gluco-lipotoxicity is still unknown. Surprisingly, inhibition of SM and glucosyl-ceramide synthesis, enzyme which required transfer of ceramide from the ER to the Golgi apparatus, was unable to alter the beneficial effect of DHA on β cell survival. These results suggest that restoration of ceramide transport is no sufficient by itself to mediate the protective effect of the Elovl2/DHA axis on β cells under gluco-liptoxic conditions.

To explain the protective effect of the Elovl2/DHA axis, we raised the hypothesis that it could alter palmitate metabolism and thus modulate gluco-lipotoxic effects. In β cells, inhibition of mitochondrial β-oxydation of FFA has been shown to potentiate apoptosis induced by gluco-lipotoxicity[5]. In agreement, we found that AICAR, agent that activates AMPK and favours β-oxydation[36,57,58] dramatically prevented gluco-lipotoxicity-induced apoptosis. Interestingly, gluco-lipotoxicity has been shown to up-regulate expression of CPT-1a, the rate-limiting enzyme in β-oxydation of FFA, in β cells[10]. However, high glucose levels has been shown to decrease β-oxydation, through synthesis of malonyl-CoA, an allosteric inhibitor of CPT-1[59], supporting that FFA oxidation might be decreased in β cells even if the CPT-1a was increased by gluco-lipotoxicity. Nevertheless, etomoxir, a potent inhibitor of CPT-1 could still potentiate β cell apoptosis induced by gluco-lipotoxicity, suggesting only a partial inactivation of CPT-1 by gluco-lipoxicity. PUFAs such as EPA and DHA stimulate expression of CPT-1a in muscle cells[60,61]. In this study, we found that etomoxir totally prevented the protective effect of the Elovl2/DHA axis suggesting that it mediate its action by regulating β-oxydation of palmitate in β cells. PUFAs are known to prevented hepatic insulin resistance in an AMPKα2-

dependent manner through regulation of FFA β-oxydation[62]. In addition to AMPK, the peroxisome proliferator-activated receptor-α (PPARα) is a potent regulator of β-oxydation[63] and PPARα is essential for the insulin-sensitizing effects of PUFAs[64]. Recently, the PPARα agonist bezafibrate has been shown to reduce PA-induced β cell toxicity[65]. Therefore, it remains to establish the respective role of AMPK and PPARα in the protective effect of the Elovl2/DHA axis.

In conclusion, the present study demonstrates for the first time the existence of an intracellular Elovl2/DHA axis that could counteract the deleterious effect of gluco-lipotoxicity on β cells. This effect is mediated partly by inhibiting ceramide accumulation through the recovery of ceramide trafficking between ER and Golgi apparatus. The Elovl2/DHA axis also blocks ceramide accumulation by facilitating mitochondrial β-oxydation of palmitate. Interestingly, another elongase, Elovl5, appeared to control liver DHA levels[20,66]. Even if Elovl5 expression is not regulated by gluco-lipotoxicity in β cells (unpublished data), whether this elongase could mediate protective effect in β cells need to be examined. Finally, our work suggest that independently from intake of PUFAs, intracellular modulation of their intracellular levels could constitute a novel therapeutic strategy to prolong β cell survival and function.

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Figure Legends

Fig. 1 Palmitate and high glucose decrease Elovl2 expression in β cells. INS-1 cells were stimulated with 0.4 mM palmitate in the presence of low or high glucose concentrations. (A) Kinetic of Elovl2 transcript expression at time 0, 6 hours, 12 hours and 24 hours. (B) Elovl2 immunoblotting and quantification after 24hours of treatment. Results are represented as means ± s.e.m of minimum three independent experiments. A: $$ p<0.01 G5 vs G30; * p<0.05, **p<0.01, *** p<0.001 G5 vs G30P4; ## p<0.01, ### p<0.001 G5P4 vs G30P4; ££ p<0.01 G30 vs G30P4. B: *** p<0.001 G30 vs G30P4. G5: 5 mM glucose, G30: 30 mM glucose, G5P4: 5 mM glucose + 0.4 mM palmitate, G30P4: 30 mM glucose + 0.4 palmitate.

Fig. 2 Down-regulation of Elovl2 potentiates β cell apoptosis induced by palmitate. (A) Elovl2 mRNA quantification of INS-1 cells transfected with either siRNA-Control or siRNA-Elovl2. (B) Caspase-3/7 activity in INS-1 cells transfected with siRNA Control or Elovl2 and treated with 0.4 mM palmitate in the presence of low or high glucose concentrations for 24h. Results are represented as means ± s.e.m of minimum three independent experiments. Indicated conditions: (A) *** p<0.001; siRNA control vs siRNA Elovl2: (B) ** p<0.01 G5P4 in siControl vs siElovl2, # p<0.05 G5 vs G5P4 ### p<0.001 G30 vs G30P4, *** p<0.001 G30P4 in siControl vs siElovl2.

Fig. 3 Over-expression of Elovl2 partially inhibits β cell apoptosis induced by palmitate. INS-1 cells were transfected with GFP or Elovl2 adenoviral constructs prior to 24h treatment with 0.4 mM palmitate in the presence of low or high glucose concentrations. (A) human and mouse Elovl2 mRNA quantification by real time qPCR. (B) Overexpressed Elovl2-V5 was determined by western-blot using an anti-V5 antibody. (C and D) Caspase 3/7 activity and PARP cleavage were quantified in INS-1 cells over-expressing hElovl2 treated with 0.4 mM palmitate in the presence of low or high glucose concentrations for 24h. (E and F) Caspase 3/7 activity and PARP cleavage in INS-1 cells treated with 0.4 mM palmitate in the presence of low or high glucose concentrations with or without 10 μM DHA for 24h. Results are represented as means ± s.e.m of minimum three independent experiments. * p<0.05,

*** p<0.001 G30P4 in Ad-GFP vs Ad-Elovl2. G5P4: 5 mM glucose + 0.4 mM palmitate, G30P4: 30 mM glucose + 0.4 palmitate.

Fig. 4 DHA inhibits caspase-3/7 activity by palmitate independently of GPR120 stimulation and DHEA transformation in INS-1 cells. (A) Caspase activity was determined in INS-1 cells were incubated for 24h with 0.4 mM palmitate in the presence of low or high glucose concentrations with or without 10µM GPR120 agonists (Metabolex, AZ670, AZ423, GSK 137647) for 24h. (B) Caspase activity was determined in INS-1 cells were incubated for 24h with 0.4 mM palmitate plus low or high glucose concentrations with 10µM DHA in the presence of the absence of 10µM GPR120 antagonist (AH7614). (C). Caspase activity was determined in INS-1 cells were incubated for 24h with 0.4 mM palmitate in the presence of low or high glucose concentrations with or without 10µM DHEA 10µM. Results are represented as means ± s.e.m of minimum three independent experiments. *** p<0.001.

Fig. 5 Over-expressed Elovl2 and DHA inhibit ceramide accumulation induced by palmitate in β cells. INS-1 cells were transfected with Ad-GFP or Ad-Elovl2 upon 24h of treatment with 0.4 mM palmitate in the presence of low or high glucose concentrations low or high glucose with or without DHA (10µM or 100µM). (A) Total ceramide content was quantified as described in material and methods. TLC representing ceramide separation upon the DAG kinase assay; PA= phosphatidic acid, LPA= lysophosphatidic acid. (B) Quantification of ceramide levels. (C to F) Chain-length specific ceramide species were determined by LC–MS/MS as described in material and methods. Results are represented as means ± s.e.m of minimum three independent experiments. * p<0.05, * p<0.01, *** p<0.001 G30P4 in Ad-GFP vs Ad-Elovl2 and DHA. G5P4: 5 mM glucose + 0.4 mM palmitate, G30P4: 30 mM glucose + 0.4 palmitate.

Fig. 6 DHA partially restore the ER-to-Golgi ceramide trafficking defect in β cell treated with palmitate and high glucose. INS-1 cells were treated with 0.4 mM palmitate in the presence of low or high glucose concentrations low or high glucose with or without DHA (10µM) for 24h. (A) Ceramide transporter CERT protein levels was quantified by western-blot. (B) After treatment, cells were

incubated with 2.5 mM BODIPY-C5Cer as BSA complex 1:1 (m/m) in DMEM for 30 min at 4°C; labeled cells were incubated at 37°C for 30 min and analyzed. All images were processed and printed identically. (C). Caspase 3/7 activity was determined in INS-1 cells treated with 0.4 mM palmitate in the presence of low or high glucose concentrations low or high glucose with or without PPMP (10µM) or D609 (10µM) for 24h. (D and E) Caspase 3/7 activity and PARP cleavage were determined in INS-1 cells treated with C2-ceramide (25 µM) for 24h. Results are represented as means ± s.e.m of minimum three independent experiments. ** $p<0.01$, *** $p<0.001$.

Fig. 7 DHA inhibits β cell apoptosis induced by palmitate through activation of mitochondrial β-oxidation. (A and B) Caspase 3/7 activity and PARP cleavage were determined in INS-1 cells treated with 0.4 mM palmitate in the presence of low or high glucose concentrations low or high glucose with or without AICAR (1 mM) for 24h. (C and D) Caspase 3/7 activity and PARP cleavage were determined in INS-1 cells transfected with Ad-GFP or Ad-Elovl2 upon treatment with 0.4 mM palmitate plus low or high glucose concentrations low or high glucose with or without 10 µM DHA in the presence of the absence etomoxir (150µM) for 24h. Results are represented as means ± s.e.m of minimum three independent experiments. ** $p<0.01$, *** $p<0.001$.

Figure 1:

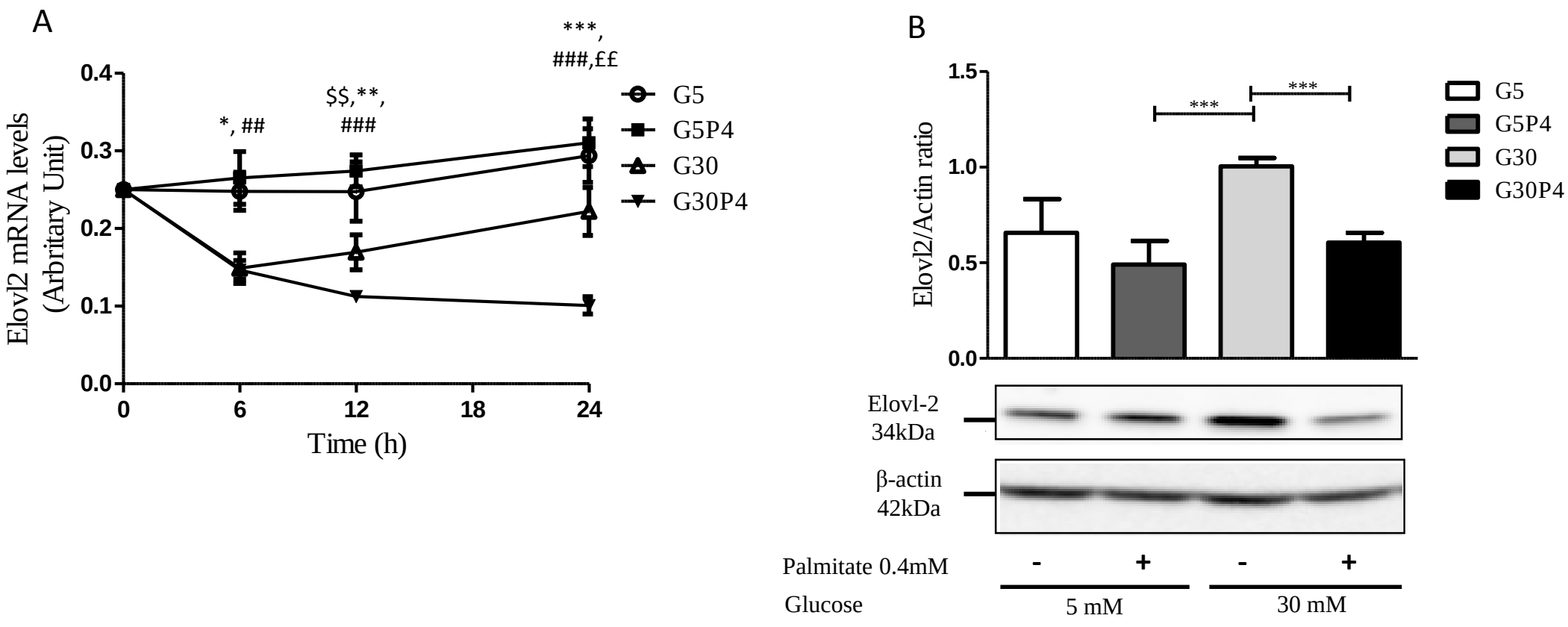

A
Elovl2 mRNA levels
(Arbitary Unit)
0.4
0.3
0.2
0.1
0.0
0
6
12
18
24
Time (h)
*, ##
$$,**,
###
***,
###,££
G5
G5P4
G30
G30P4
B
Elovl2/Actin ratio
1.5
1.0
0.5
0.0
***
***
G5
G5P4
G30
G30P4
Elovl-2
34kDa
β-actin
42kDa
Palmitate 0.4mM
- + - +
Glucose
5 mM
30 mM

Figure 2:

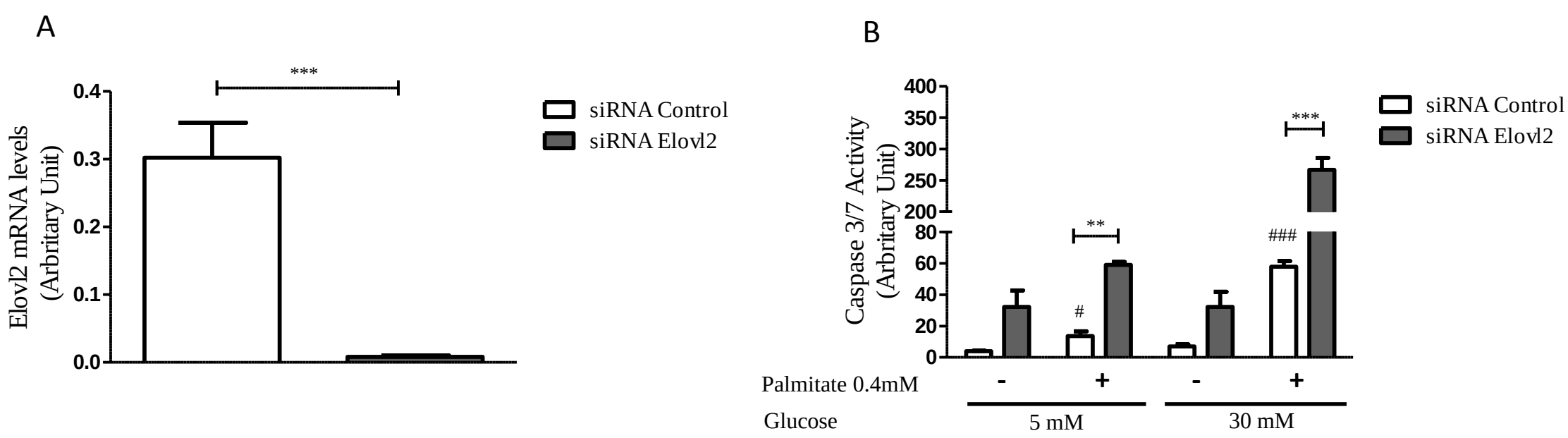
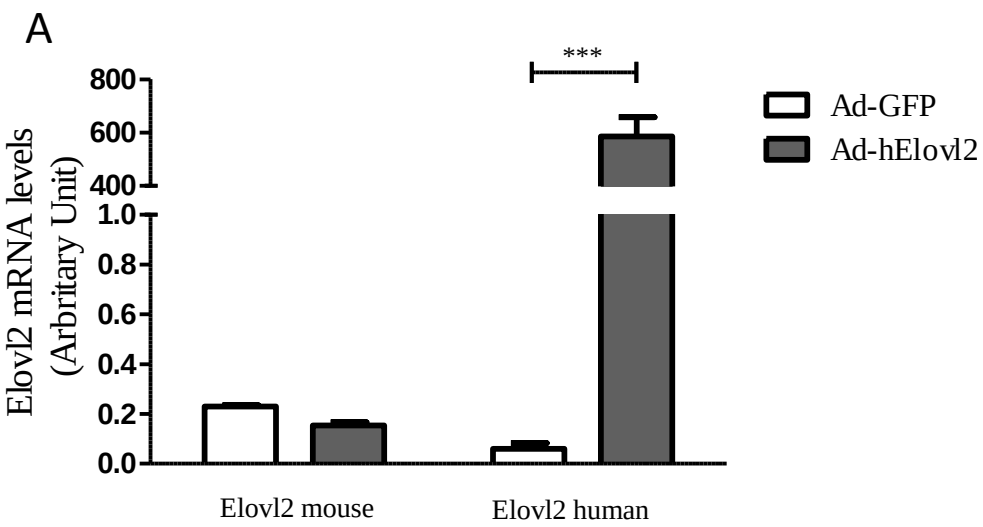
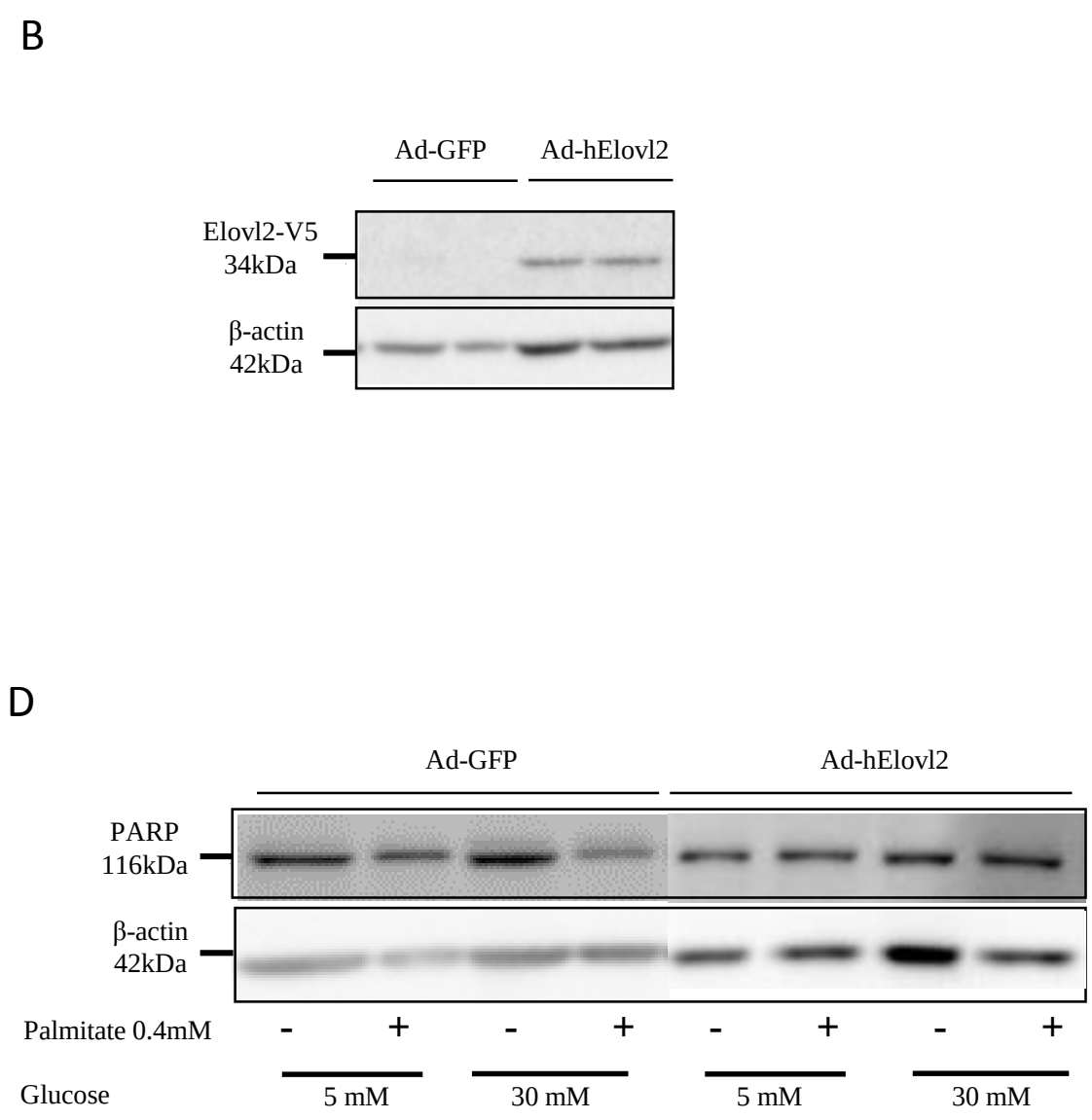
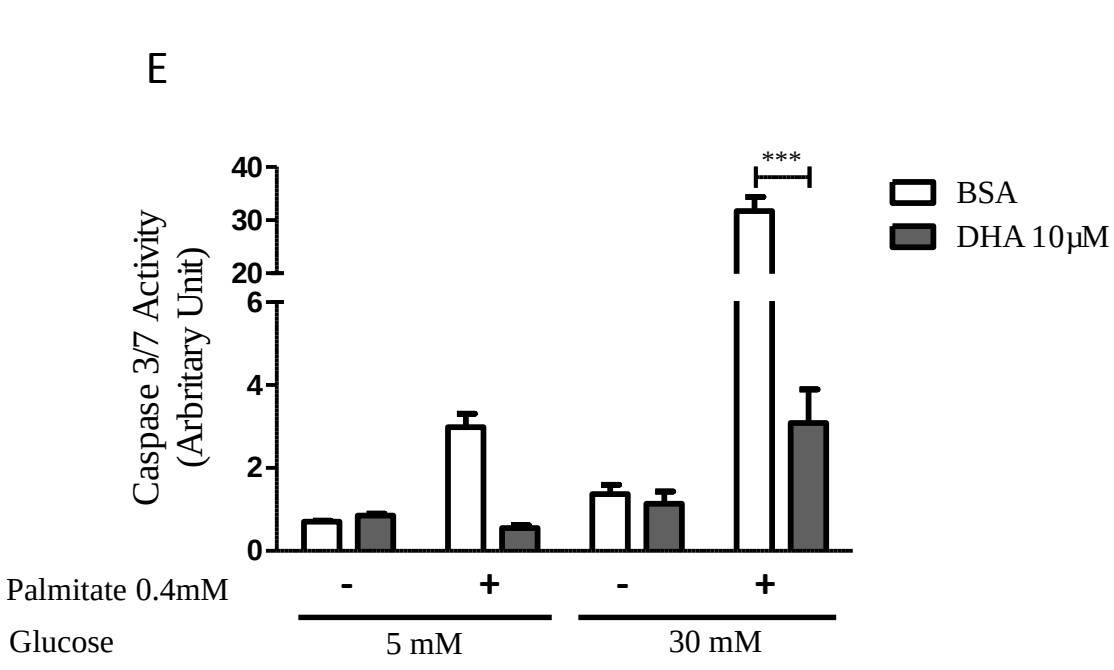

A
B
***
Elovl2 mRNA levels (Arbritary Unit)
0.4
0.3
0.2
0.1
0.0
siRNA Control
siRNA Elovl2
Caspase 3/7 Activity (Arbritary Unit)
400
350
300
250
200
80
60
40
20
0
**
#
###
***
siRNA Control
siRNA Elovl2
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
30 mM

Figure 3:


A
***
800
600
400
1.0
0.8
0.6
0.4
0.2
0.0
Elovl2 mRNA levels
(Arbritary Unit)
Ad-GFP
Ad-hElovl2
Elovl2 mouse
Elovl2 human


B
Ad-GFP
Ad-hElovl2
Elovl2-V5
34kDa
β-actin
42kDa
C
50
40
30
20
10
0
*
Caspase 3/7 Activity
(Arbritary Unit)
Ad-GFP
Ad-hElovl2
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
30 mM
D
Ad-GFP
Ad-hElovl2
PARP
116kDa
β-actin
42kDa
Palmitate 0.4mM
-
+
-
+
-
+
-
+
Glucose
5 mM
30 mM
5 mM
30 mM


E
40
30
20
6
4
2
0
***
Caspase 3/7 Activity
(Arbritary Unit)
BSA
DHA 10μM
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
30 mM

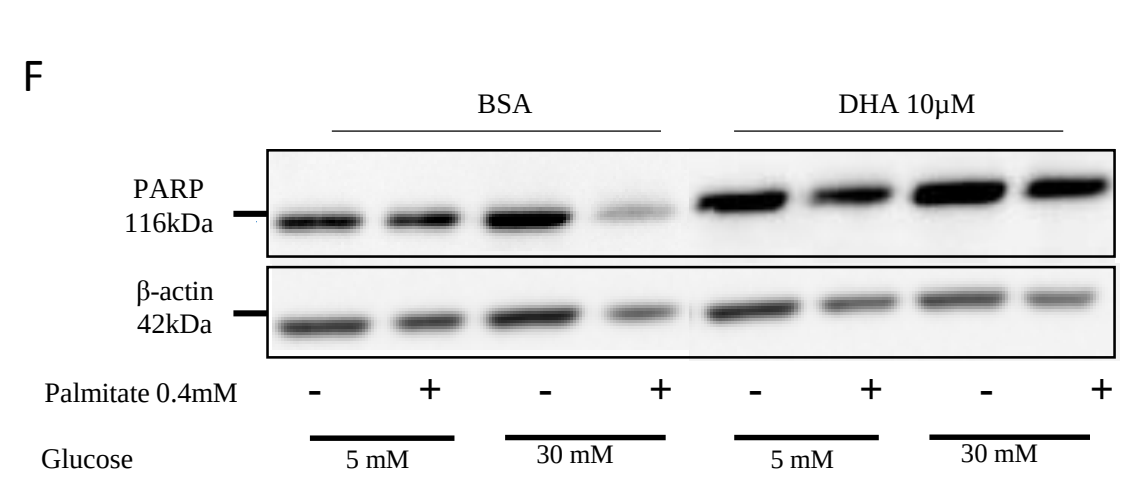
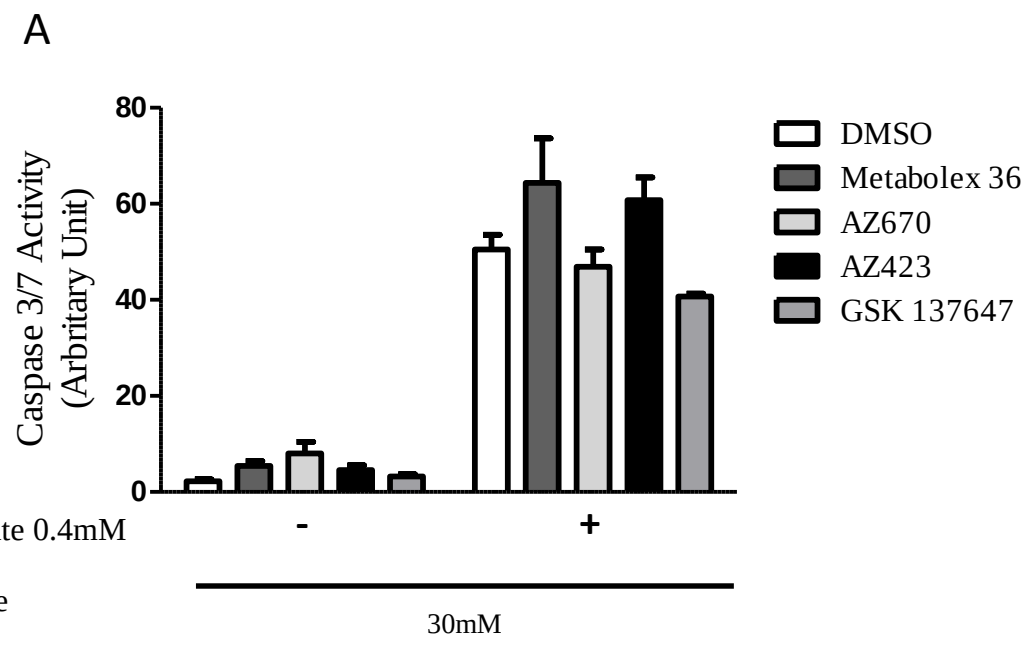
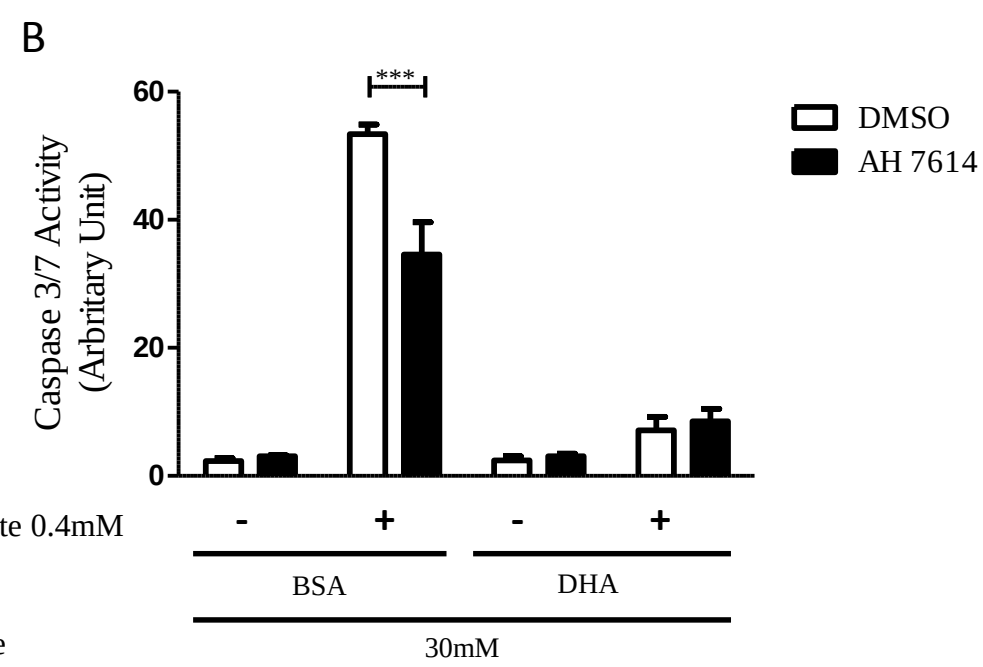

F
BSA
DHA 10μM
PARP
116kDa
β-actin
42kDa
Palmitate 0.4mM
-
+
-
+
-
+
-
+
Glucose
5 mM
30 mM
5 mM
30 mM

Figure 4:


A
Caspase 3/7 Activity (Arbitary Unit)
80
60
40
20
0
DMSO
Metabolex 36
AZ670
AZ423
GSK 137647
Palmitate 0.4mM
-
+
Glucose
30mM


B
***
Caspase 3/7 Activity (Arbitary Unit)
60
40
20
0
DMSO
AH 7614
Palmitate 0.4mM
-
+
-
+
BSA
DHA
Glucose
30mM

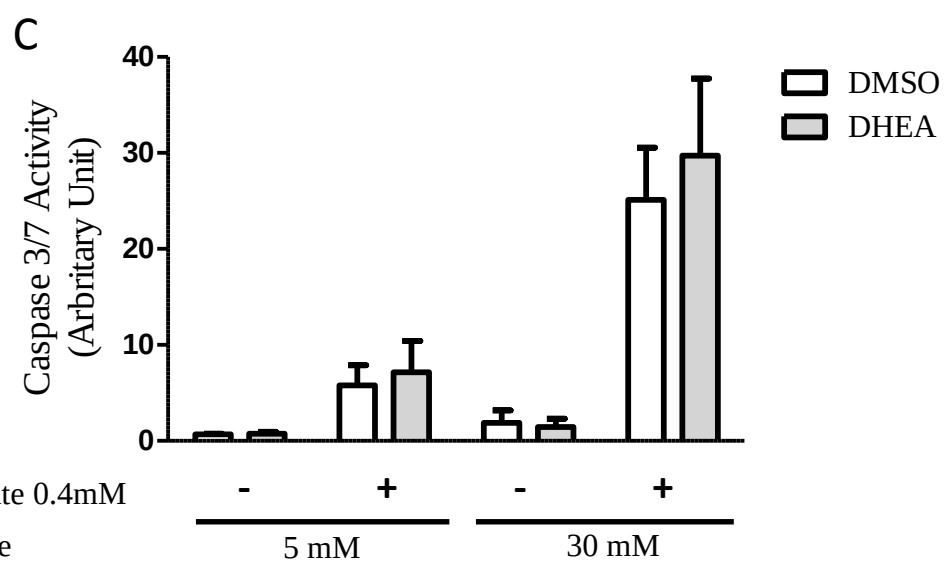

C
Caspase 3/7 Activity (Arbitary Unit)
40
30
20
10
0
DMSO
DHEA
Palmitate 0.4mM
-
+
-
+
Glucose
5 mM
30 mM

Figure 5:

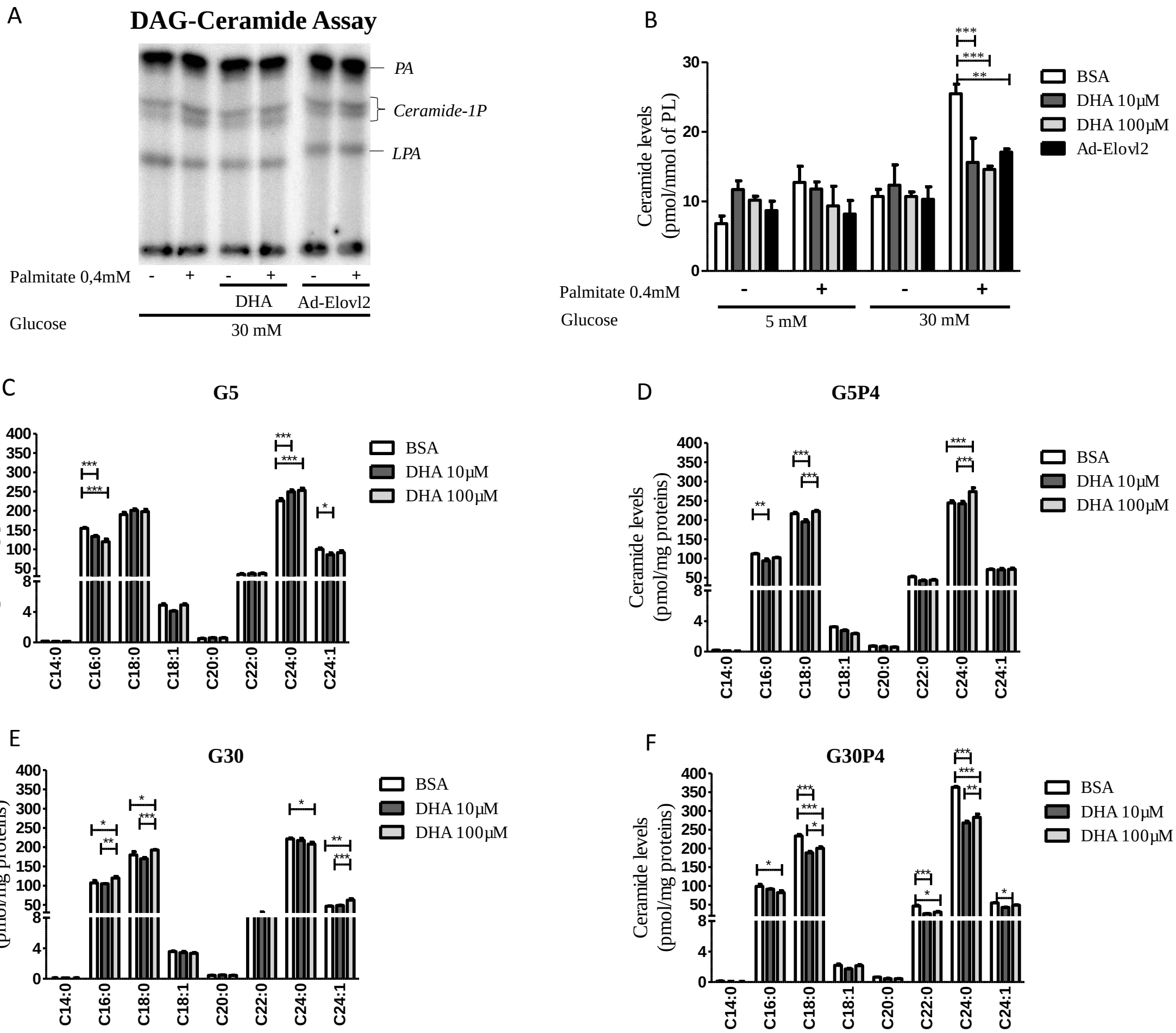
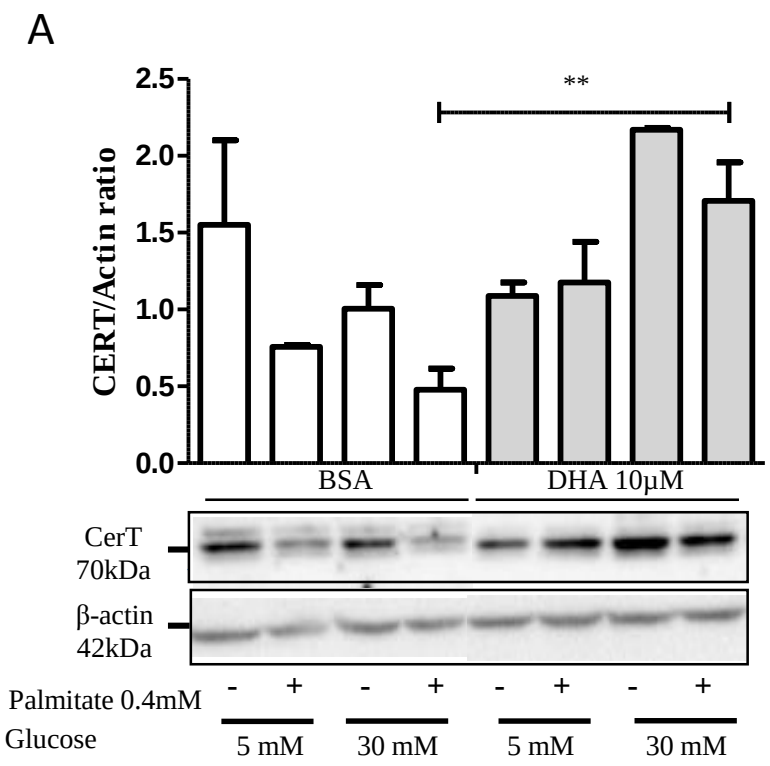
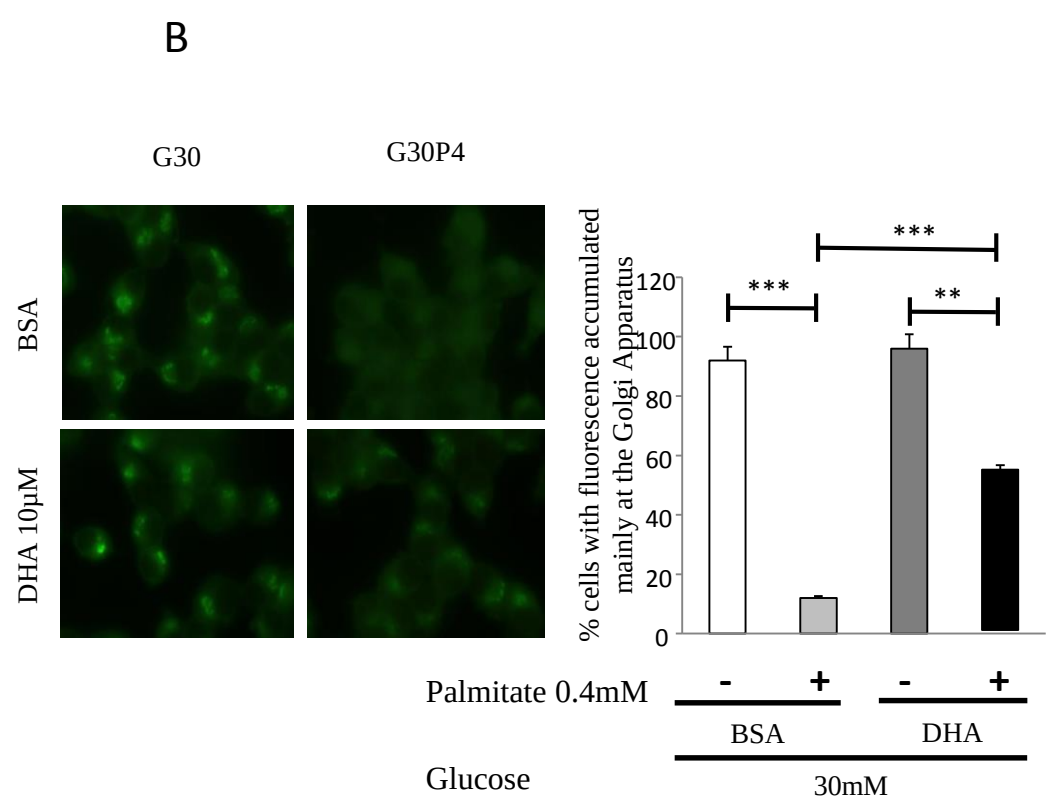
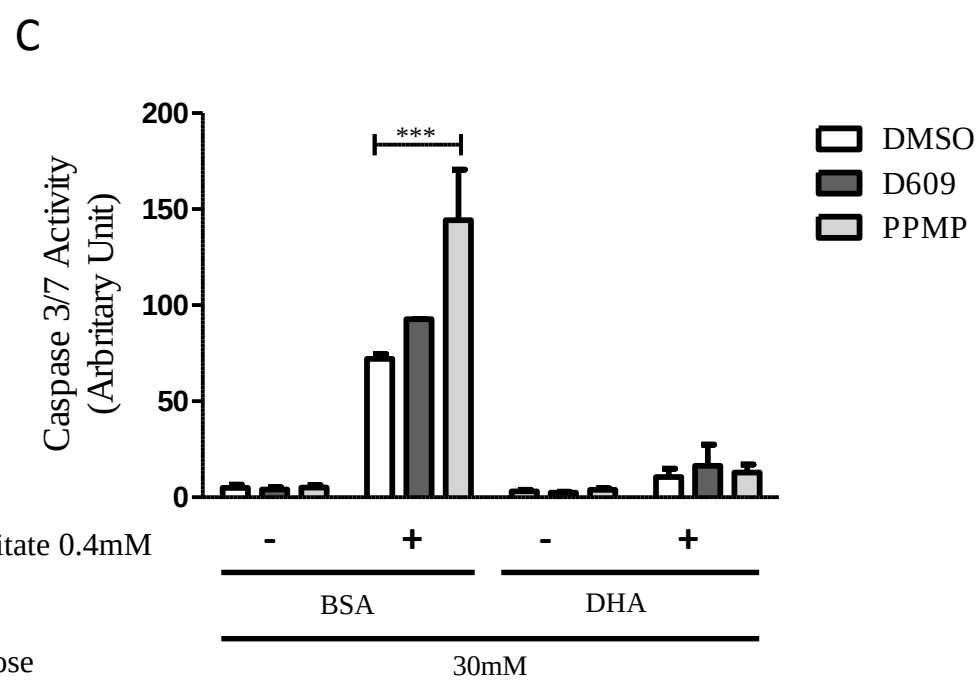

A
DAG-Ceramide Assay
PA
Ceramide-1P
LPA
Palmitate 0,4mM - + - + - +
DHA
Ad-Elovl2
Glucose
30 mM
B
Ceramide levels (pmol/nmol of PL)
Palmitate 0.4mM
Glucose
5 mM
30 mM
BSA
DHA 10µM
DHA 100µM
Ad-Elovl2
C
G5
Ceramide levels (pmol/mg proteins)
C14:0
C16:0
C18:0
C18:1
C20:0
C22:0
C24:0
C24:1
D
G5P4
E
G30
F
G30P4

Figure 6:


A
CERT/Actin ratio
2.5
2.0
1.5
1.0
0.5
0.0
**
BSA
DHA 10µM
CerT
70kDa
β-actin
42kDa
Palmitate 0.4mM - + - + - + - +
Glucose
5 mM
30 mM
5 mM
30 mM


B
G30
G30P4
BSA
DHA 10µM
% cells with fluorescence accumulated mainly at the Golgi Apparatus
120
100
80
60
40
20
0
***
***
**
Palmitate 0.4mM - + - +
BSA
DHA
Glucose
30mM


C
Caspase 3/7 Activity (Arbitary Unit)
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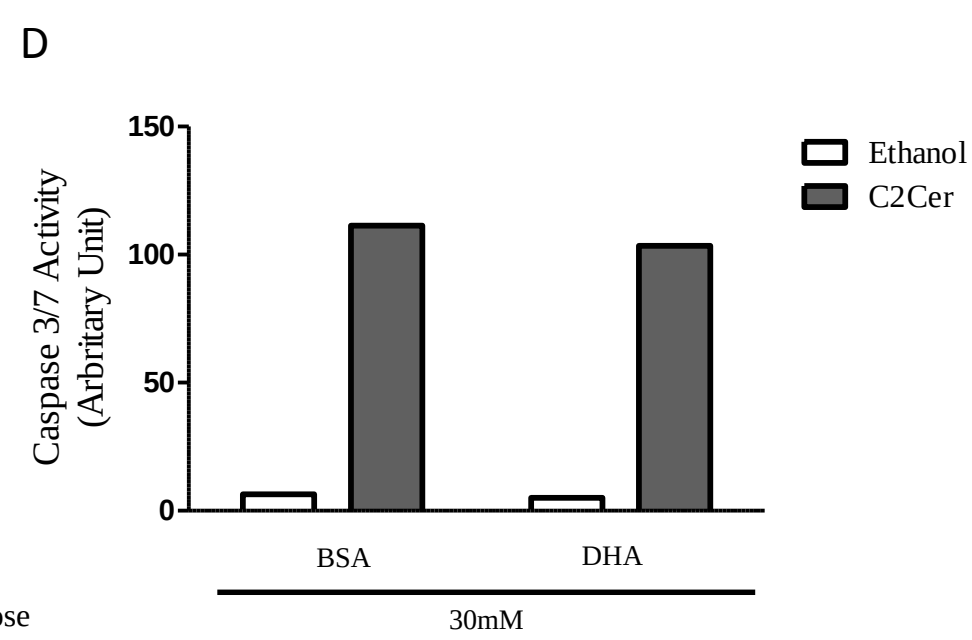
150
100
50
0
***
DMSO
D609
PPMP
Palmitate 0.4mM - + - +
BSA
DHA
Glucose
30mM


D
Caspase 3/7 Activity (Arbitary Unit)
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100
50
0
Ethanol
C2Cer
BSA
DHA
Glucose
30mM

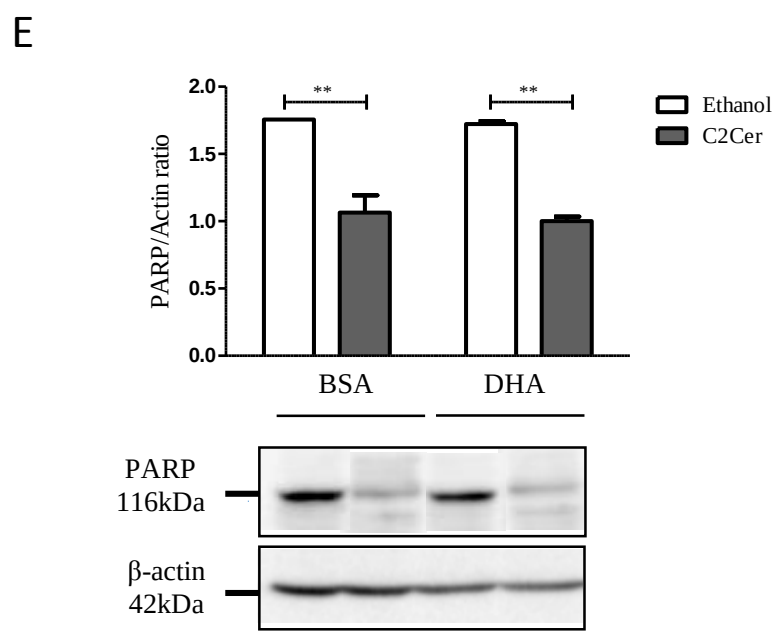
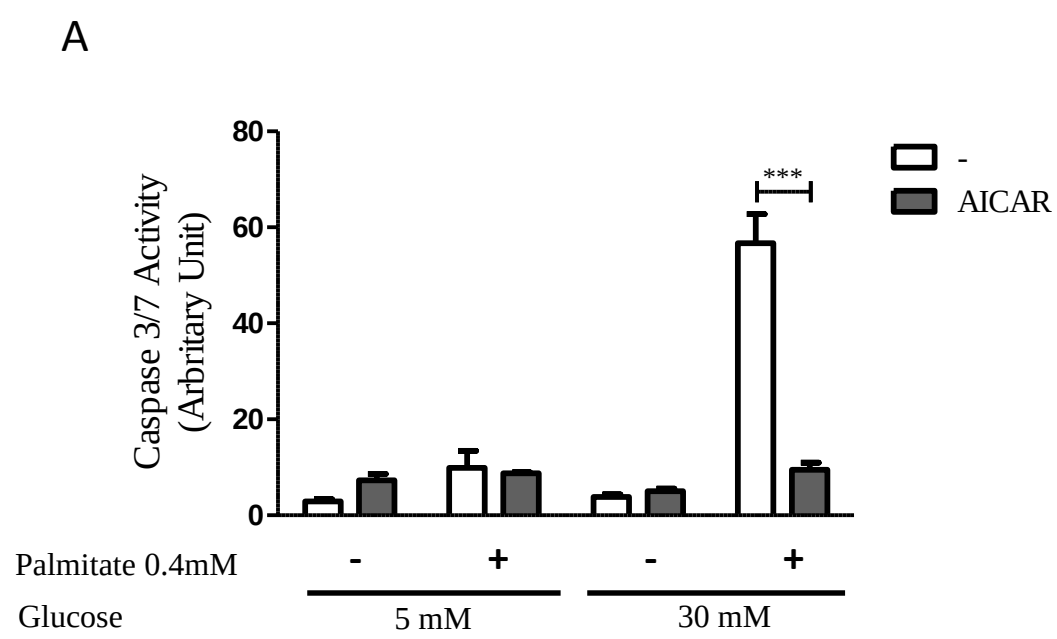
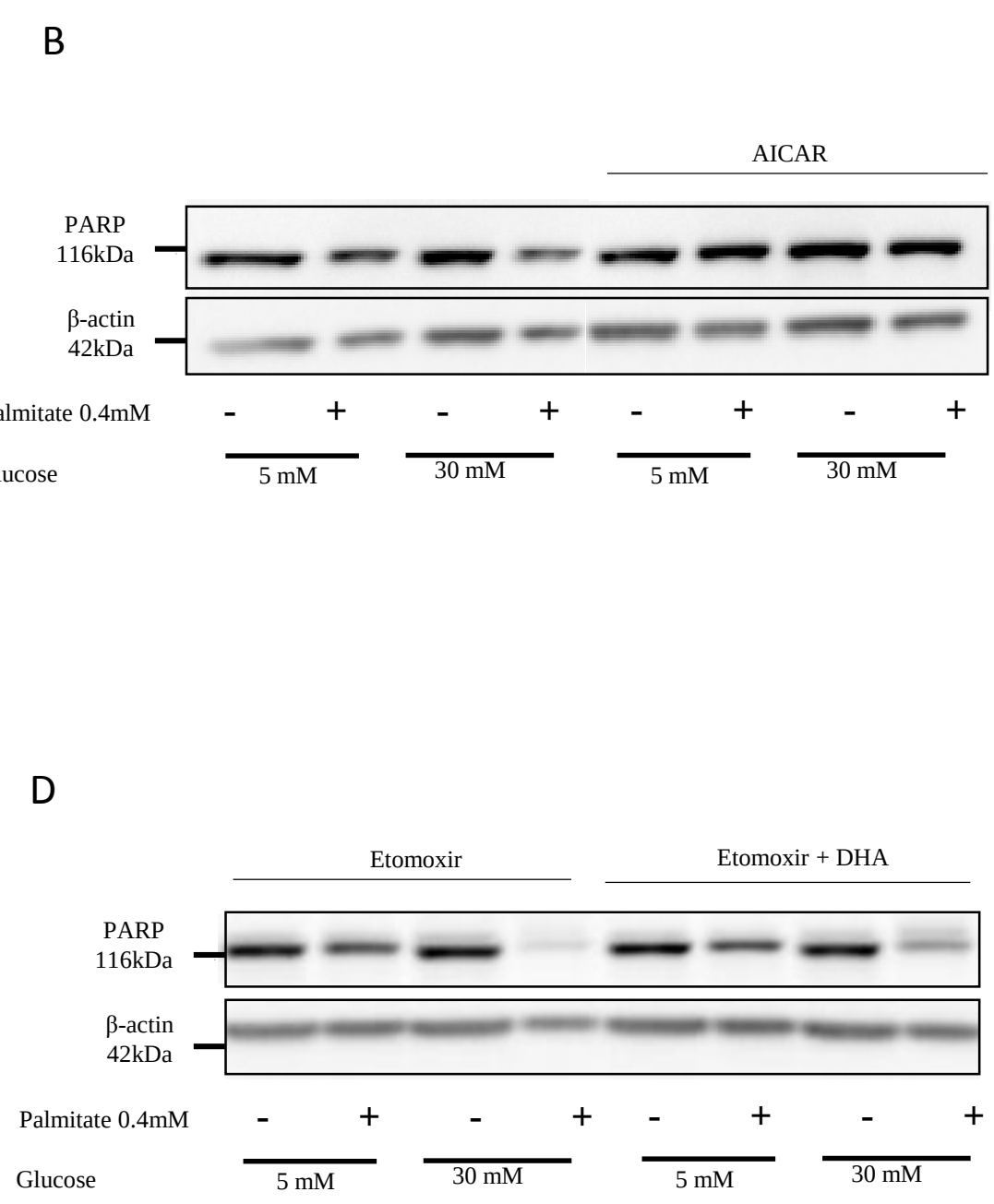
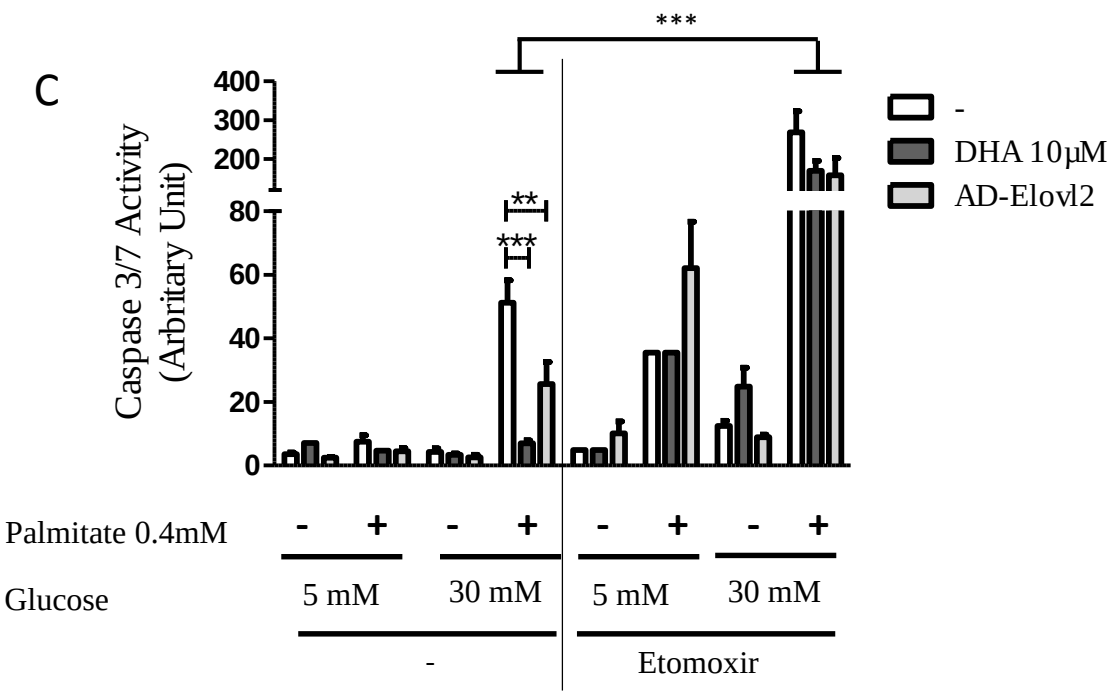

E
PARP/Actin ratio
2.0
1.5
1.0
0.5
0.0
**
**
Ethanol
C2Cer
BSA
DHA
PARP
116kDa
β-actin
42kDa

Figure 7:


A
Caspase 3/7 Activity (Arbitrary Unit)
80
60
40
20
0
***
-
AICAR
Palmitate 0.4mM
- + - +
Glucose
5 mM
30 mM


B
AICAR
PARP
116kDa
β-actin
42kDa
Palmitate 0.4mM
- + - + - + - +
Glucose
5 mM
30 mM
5 mM
30 mM


C
***
**
***
Caspase 3/7 Activity (Arbitrary Unit)
400
300
200
80
60
40
20
0
-
DHA 10µM
AD-Elovl2
Palmitate 0.4mM
- + - + - + - +
Glucose
5 mM
30 mM
5 mM
30 mM
-
Etomoxir

D
Etomoxir
Etomoxir + DHA
PARP
116kDa
β-actin
42kDa
Palmitate 0.4mM
- + - + - + - +
Glucose
5 mM
30 mM
5 mM
30 mM

III CONCLUSIONS

During my thesis work, I investigated for the first time the role of ELOVL2/DHA axis in pancreatic β-cell function. To mimics *in vitro* what happen *in vivo* during T2D installation associated with obesity, I used a condition defined as glucolipotoxicity (GL) with high glucose and high palmitate concentration. GL in pancreatic β cells induces impaired glucose-stimulated insulin secretion and cell apoptosis (Bellini et al. 2015). My results demonstrate that ELOVL2 over-expression and DHA supplementation ameliorate glucose-induced insulin secretion under GL, and DHA restores normal levels of Ca^{2+} influx. I also found that ELOVL2/DHA axis is involved on GL-induced apoptosis. In particular, silencing of *Elovl2* mRNA exacerbates GL-apoptosis, while *Elovl2* over expression or DHA supplementation protected cells from GL-induced apoptosis. I investigated which mechanisms are involved on ELOVL2/DHA protection from GL, and it seems to be not G-coupled protein receptor mediated (GPR120). GL-induced apoptosis is mediated by ceramide accumulation, I demonstrate that ELOVL2/DHA axis inhibits GL-induced ceramide accumulation, partially by restoring ER-to-Golgi ceramide transport mediated by CERT, even if the ameliorated flux seems not to be sufficient for the DHA's protection effects. Finally, I found that ELOVL2/DHA protection from GL-apoptosis needs the mitochondrial β-oxidation pathway, suggesting that ELOVL2/DHA axis is protecting pancreatic β-cells from apoptosis by modulating fatty acid partitioning in favour of non-toxic pathways.

1 ELOVL2/DHA AXIS AND INSULIN SECRETION

Lipid homeostasis plays an important role in the control of glucose homeostasis and its deregulation leads to important consequences, such as the development of metabolic syndrome. The disruption of lipid homeostasis triggers various toxic pathways and submits the body to deal with a continuous stress condition. In type 2 diabetes, two types of stresses are activated: lipid overload (lipotoxicity) and impaired control of glucose metabolism (glucotoxicity). During the first phases of the disease appearance, the tissues that play a primary role are adipose tissue, skeletal muscle and liver (Bugianesi et al., 2005). In normal conditions, these tissues play a central role in the metabolism and, in case of nutrient surplus, they function as "shock absorbers". Indeed, for some authors, it is believed that in metabolic disorders their function is to deal the lipotoxic stresses as longer as possible in way to protect the other tissues, which are much more sensitive to lipotoxicity, such as pancreatic β-cells, cardiomyocytes or retinal cells (Kusminski et al., 2009). When these tissues reach their maximal ability to handle the lipid

surplus, lipotoxic pathways overwhelm the beneficial ones; the adipose tissue starts to be subject to low grade of inflammation (Anghel et Wahi 2007), the liver develops steatosis and there is installation of a insulin resistance state. At this step of the disease progression, pancreatic β-cells play a primary role (Prentki et al., 2002; Prentki et al., 2006). These cells can adapt their function following the body needs: in case of IR they can increase their size and their number (Sharma et al., 2015). Unfortunately, much remains to discover about their function in normal and in diabetic condition. For this reason, our lab, in collaboration with other European groups inside the IMIDIA consortium, initiated a RNAseq analysis on isolated islet of Langerhans of different strain mice fed with chow or HFD diet. The database based on these analysis, allowed us to highlight new candidate genes important for β-cell function in normal and pathological conditions. Among these genes, the *elongase 2 (Elovl2)* has been chosen for further investigation on its role in pancreatic β-cells. Elongases are the rate limiting step enzymes for the very long chain fatty acid synthesis (VLCFA), and elongate FA with specific chain length. Regarding *Elovl2* little is known in literature. Generation of a knock out mouse for this protein determined that it is necessary for the endogenous synthesis of the ω-3 poly-unsaturated fatty acid docosahexaenoic acid (22:6,*n*-3) (DHA) (Pauter et al., 2014; Zadravek et al., 2011). Investigation of Elovl2 role in pancreatic β-cells allows us to study the endogenous function of DHA production in these cells. Especially, by determining the role of ELOVL2/DHA axis in β-cells dysfunction induced by glucolipotoxicity, which culminates with two effects: loss of capacity to secrete insulin in response to glucose, and cell apoptosis.

1.1 GLUCOLIPOTOXIC CONTROL OF ELOVL2 MRNA AND PROTEIN LEVELS

Palmitate is able to modify expression of many genes, so far different studies investigated palmitate-incubation effects on human pancreatic β-cells (Cnop et al., 2014; Hall et al., 2014). The outcome was a modification of the expression of many genes: 1325 genes on the first study, 1860 genes on the second. Moreover, Hall and colleagues determined that palmitate can altered global DNA methylation levels and DNA methylation levels of CpG island shelves and shores, 5'UTR, 3'UTR and gene body region. They determined that among the 1860 genes, whose expression is modify by palmitate, 290 of them are due to a change in DNA methylation (Hall et al., 2014).

We contacted the article's authors by personal communication to get more details about the palmitate modified genes expression and to investigate if *Elovl2* expression was modify in human islets. We found that *Elovl2* gene was not part of the 1325 or 1860 modified genes. This output could be explained by different factors: 1) the incubation time last for 48 hours and, in human islets, this incubation time is too short to have a real toxic effect due to palmitate, it is before the step of apoptosis induction (4 days of incubation are necessary (Maedler et al., 2003)). Indeed, in both studies apoptosis was not induced; 2) the incubation conditions were with high palmitate but low glucose concentrations, so they investigated the lipotoxicity and not glucolipotoxicity conditions (Cnop et al., 2014; Hall et al., 2014). It is already known that glucose levels strongly influence the effect of palmitate on β cells fate (Bellini et al., 2015) suggesting that gene expression modulated by palmitate could be different with or without glucose. In IMIDIA consortium, cohorts of human islets have been used to perform deep-sequencing analysis, in order to create a database similar to the one created with the 6 mice strains (Bellini et al., 2016 article 1). We analysed *Elovl2* correlation expression to T2D installation. Unfortunately, we did not find any correlation between *Elovl2* expression and diabetes installation. A limitation point of the study was that T2D patients formed a unique group and no distinction was made among lean, overweight and obese patient. A more accurate bioinformatics analysis could confirm or not *Elovl2* implication in T2D installation in human (Figure 13).

In order to validate or not a role for Elovl2 in β cells under gluco-lipotoxicity, we realized different experiments using different models (depending on the type of test performed). We checked the effects of glucolipotoxicity on INS-1 cells, MIN-6 cells and murine isolated islets of Langerhans. What emerged from these experiments, was that, Elovl2 mRNA and protein levels are decreased in all models when comparing high glucose with high glucose/palmitate conditions (glucolipotoxicity (GL)*). Some differences are present among INS-1 cells, MIN-6 cells and isolated islets. These differences are probably based on the fact that each of these models is cultured at different glucose concentration. In particular, MIN-6 are normally cultivated in high glucose condition (25 mM), and the low glucose (5 mM) represent a state of glucose deprivation for this cell line. In mouse isolated islets, ELOVL2 protein control is a little different, and palmitate seems to strongly inhibit ELOVL2 protein levels independently from glucose concentration. From these data, we highlighted that glucolipotoxicity is negatively influencing *Elovl2* mRNA and protein levels. We can hypothesize the existence of post-transcriptional controls that modify the protein levels and explain the lack of complete over-

lapping between the mRNA and proteins levels. As recently demonstrated, the yeast Elo2 protein activity is dependent on its phosphorylation (Zimmermann et al., 2013). Yeast Elo2 corresponds to the mammalian Elovl5 isoform (Zimmermann et al., 2013). Since ELOVL2 and ELOVL5 have redundant and complementary role on ω3 PUFAs synthesis, it would be interesting to investigate if the mammal ELOVL2 protein is also controlled by post-transcriptional modifications.

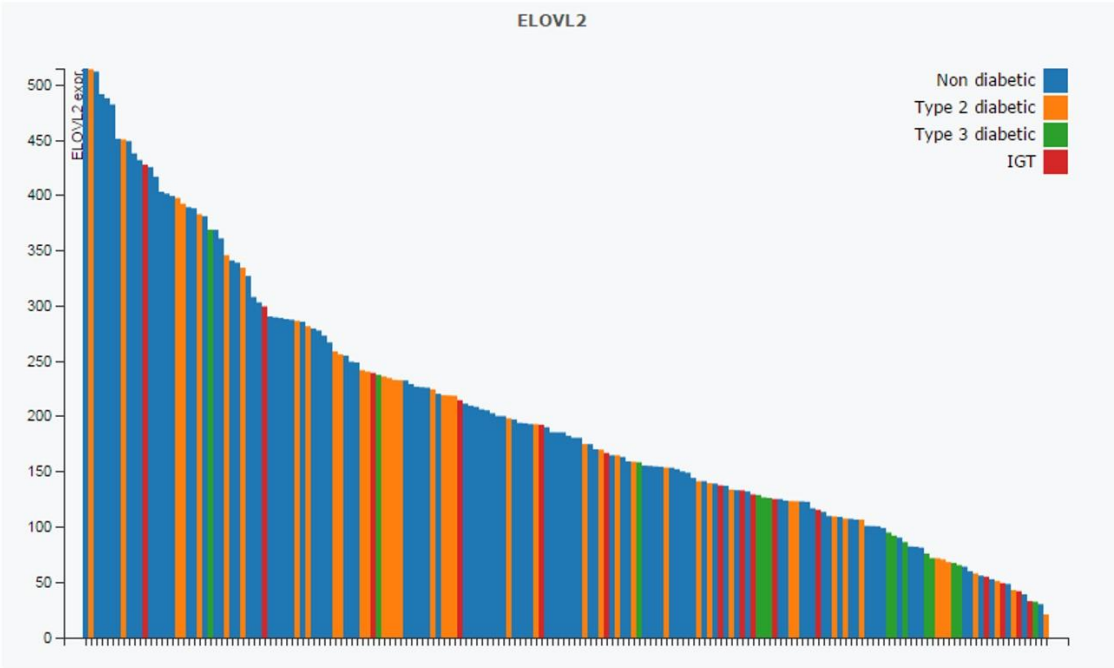
*On the following sections, we will talk about glucolipotoxicity referring to the condition high glucose/high palmitate. We are conscious that it is in part inappropriate to define glucotoxic the high glucose condition for MIN-6 cells, since this is the normal glucose concentration of culture. However, since this is the term used in literature to refer at the toxic effects caused by high glucose and palmitate in islets and INS-1 cells, for simplicity we will use a unique definition for this culture condition.

1.2 DHA/ELOVL2 AND INSULIN SECRETION

Since the IMIDIA database has been built on gene expression variation correlated to insulin secretion and glucose intolerance, our first investigation has been performed on this aspect of β-cell function. FFAs in β-cells are used as nutrient, and acute treatment with FFAs increase stimulates insulin secretion (McGarry et Dobbins 1999). Indeed, they are likely involved in β-cell adaptation to insulin resistance (Nolan et al., 2006), so far that some authors prefer to talk about lipoadaptation rather that lipotoxicity in pancreatic β-cells. Hyperlipidemia seems not to be toxic in pancreatic β-cells until occurs the hyperglycemia (Poitout et al., 2008). When GL occurs, pancreatic β-cells start to fail to properly respond to the body needs of insulin and start to enter in apoptosis.

Figure 13

A



B

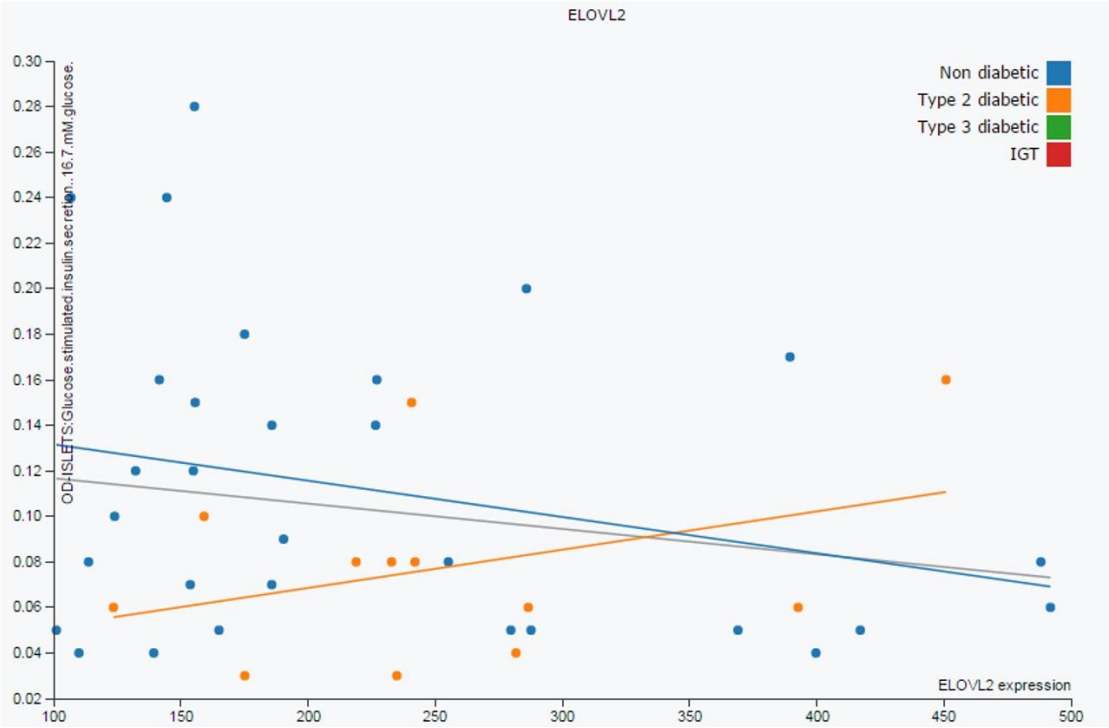


Figure 13. **Human islets RNAseq data**. (A) *Elovl2* expression levels of each patient: in blue are the non diabetic parients, in orange the T2D patients, in green T3D patients (is to indicate patients with T2D linked to neurodegenerative disease) and in red the Impaired Glucose Tolerance patients (IGT). (B) *Elovl2* expression levels correlated to glucose stimulated insulin secretion, in blue are the non diabetic patients, in orange the T2D patients and in gray is the correlation. It is possible to note that, in human, Elovl2 expression did not correlate with diabetes or to glucose stimulated insulin secretion. Taken from IMIDIA Database.

It is well established that monounsaturated fatty acids can counterbalance the negative effects of palmitate. A pioneer study, performed by Donath and colleagues, investigated the effects of different fatty acids (such as palmitate, palmitoleate, oleate) in *ex vivo* culture of human islets (Maedler et al., 2003) and demonstrated that palmitoleate and oleic acid can counterbalance palmitate-inhibited insulin secretion and the decreased of insulin content. Some years later, another study showed similar results using a ω3 PUFA, EPA (Kato et al., 2008). Regarding DHA effects, a study demonstrated that *in vitro* DHA supplementation enhanced glucose-stimulated insulin secretion via GPR40 receptor stimulation (Itoh et al., 2003). Our results also support these works and demonstrate that, under GL, another ω3PUFA, DHA, can partially counterbalance palmitate-inhibited insulin secretion in MIN-6 cells and *ex vivo* cultured islets of Langerhans from C57BL6 mice. Regarding the insulin content, we found a partial restauration of the insulin content only in MIN-6, which remain however far away from the normal values. In isolated islets, we demonstrated that DHA is not able to restore the insulin content under GL condition compared to controls.

Regarding ELOVL2, nothing is known in literature about its function in β-cells insulin secretion. Our data demonstrate that ELOVL2 over-expression in MIN-6 cells partially counteracts inhibition of insulin secretion under GL, even if in a lesser extent than DHA. Since ω-3 fatty acids represent a small portion of the fatty acids present into the cells, we hypothesized that the cells present limited DHA-precursors, such as EPA or DPA. For this reason, we provided some EPA to the adenoviral-infected cells. Insulin secretion resulted to be even better in Adeno-*Elovl2* infected cells supplemented with EPA under GL conditions, reaching comparable values with the DHA/palmitate and high glucose conditions. Regarding the other conditions, we evidenced a tendency to improve glucose-stimulated secretion in the presence of the adenovirus *Elovl2*. Nevertheless, insulin content was not restored by *Elovl2* over-expression under GL condition. Altogether, our results suggest for the first time that endogenous ω3 PUFAs synthesis by ELOVL2 could be beneficial to β cells to counteract the side effects of GL.

On the next sections, we will talk about the potential mechanisms involved in the effect ELOVL2/DHA on the controls of insulin gene expression and secretion.

1.2.1 G-coupled receptor and insulin secretion

Different publications demonstrated that beneficial roles of DHA are mediated by its interaction with G-coupled receptors, in particular with GPR120. So far, GPR120 has been proposed as a hot target for the development of anti-diabetic therapies (Liu et al., 2015; Reviewed in Moniri et al., 2015).

In pancreatic β-cells, insulin secretion can be enhanced through long chain fatty acid interaction with GPR40 (Itoh et al., 2003). GPR40 ligands are different lipids, included palmitate and DHA. However, different recent studies focused their attention on GPR120, which seems to be the main mediation of ω3 PUFAs beneficial effects (Moniri et al., 2015). GPR120 tissue distribution is not well defined, and its expression in pancreatic β-cells is still a matter of debate, since some authors believe that this receptor is not expressed on β-cells while others sustain that it is expressed (Taneera et al., 2012; Moran et al., 2014; Stone et al., 2014; Moniri et al., 2015). Groop and colleagues performed a gene expression analysis of human islets and their analysis correlated GPR120 expression to T2D. They demonstrated that EPA do not protect anymore from palmitate-induced apoptosis when GPR120 is knocked down (Taneera et al., 2011). Another study performed in BRIN-B11 pancreatic β-cells, GPR120 natural (ALA, EPA, DHA) and synthetic agonists (GW9508) have been tested demonstrating that GPR120 activation stimulates insulin secretion (Moran et al., 2014). However, these results are not conclusive. Indeed, in Moran and colleagues work, it has been used GW9508 as GPR120 agonist, but this molecule is mostly recognized as GPR40 agonist (Tikhonova et al., 2007). This pone a doubt regarding the fact that the results obtained are due to GPR40 or GPR120 or both receptors activation. In Taneera's study, 50 new genes has been proposed to be involved on β-cells secretion, and 11 of them has been knocked down and the insulin secretion has been quantify on INS-1 cells. Regarding GRP120, it has been investigated for β-cells apoptosis only (see discussed below), and no data exists on insulin secretion. To better define GPR120 expression pattern, transgenic mice Gpr120-knockout/β-galactosidase (LacZ) knock-in has been created (Stone et al., 2014). The β-galactosidase expression was controlled by endogenous GPR120 promotor. β-galactosidase activity has been detected on a small portion of islets cells, and immunofluorescent analysis co-localized β-galactosidase activity with somatostatin, suggesting that GPR120 is preferentially expressed in pancreatic δ cells (Stone et al., 2014).

Even if GRP120 role on pancreatic β-cells is still not well defined, we decided to test if the DHA protective role under GL conditions was G-protein receptor mediated. For this reason, we tested 4 different agonist and an antagonist of GPR120 on MIN-6 cells (Figure 14).

Figure 14

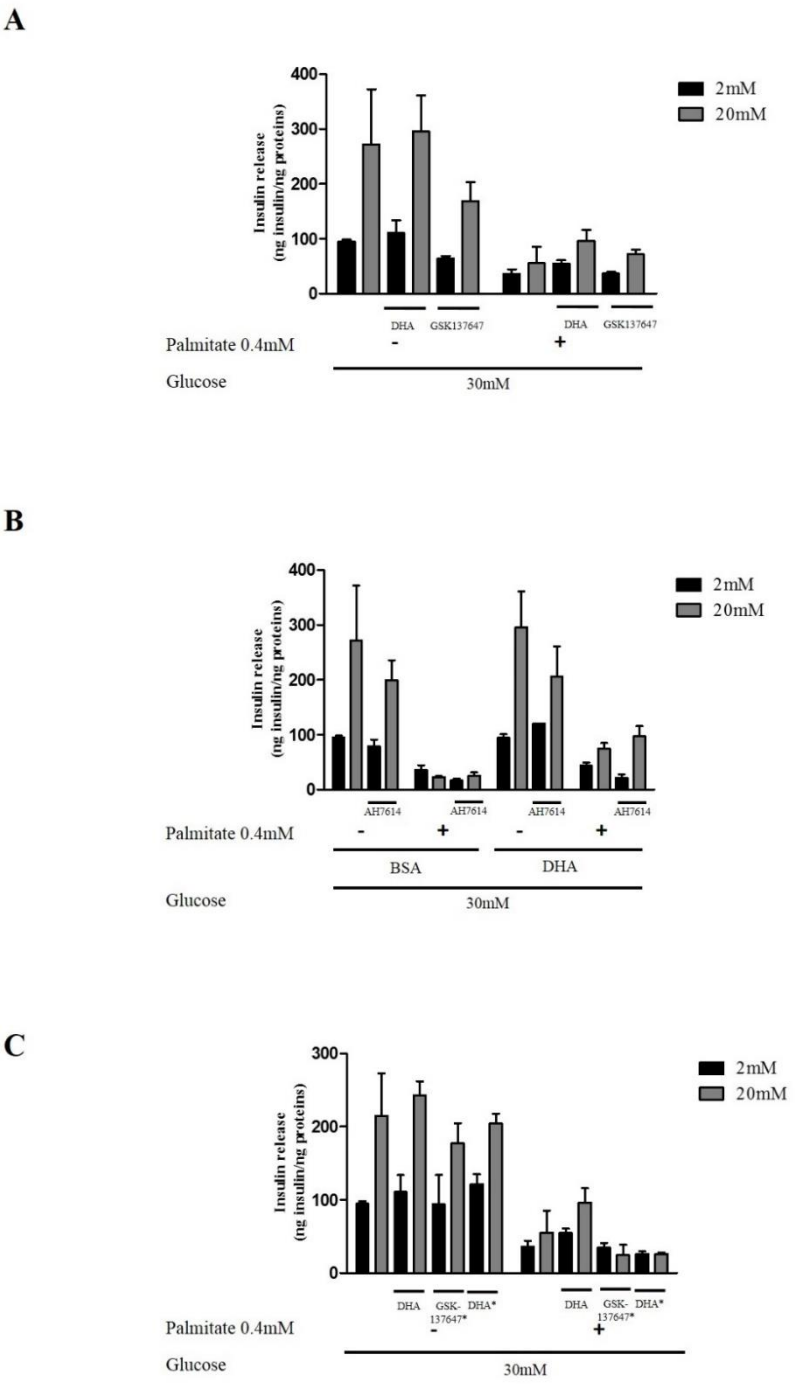


Figure 14. **MIN6 cells treated with GPR120 agonist and antagonist**. Cells were incubated for 24hours with high glucose concentration (25mM) in presence or not of palmitate (0.4mM) and DHA (10µM). Insulin secretion tests were performed as described in Materials and Methods. (A) GPR120 agonist, GSK 137647 (10µM), was added to the incubation medium in order to determine if it was able to reproduce the DHA effects on glucose-stimulated insulin secretion under GL. (B) GPR120 antagonist, AH7614 (10µM), was added to the incubation medium in order to determine if it was blocking the DHA ameliorated insulin secretion under GL. (C) 30minutes prior the incubation, cells were pre-incubated with GSK 137647 or DHA 10µM (condition with *) in order to determine if it is sufficient to activate a receptor-mediated cascade that reproduces DHA protecting effects from GL-impaired secretion. These preliminary experiments shows that GPR120 agonist is not able to ameliorate the insulin secretion under GL, and GPR120 antagonist is not blocking the DHA protective effects. Pre-incubation with DHA was performed to determine if DHA effects could be mediated by receptor, the results suggest that DHA need to be metabolized to generate its protecting effects. Statistics not shown.

We evidenced that the agonists were not able to reproduce the DHA protection and the antagonist did not inhibited DHA protection's effect. Our preliminary data suggests that DHA protection seems not to be a GPR120- mediated mechanism. However, to better define GPR120 involvement, it would be important to test the agonist and antagonist effects on isolated islets, or to directly modify GPR120 protein levels, by Knock-down or over-expression experiments. Another interesting experiment, that would exclude GPR120 involvement in DHA restauration of insulin secretion during GL, would be to test DHA effect on GPR120-KO mice isolated islets. It also remain to establish if the protective effect of endogenous production of DHA by ELOVL2 is mediated or not by a GPR120-mediated mechanism.

1.2.2 Protein Kinase C and insulin secretion

We also explored another mechanism to explain the effect of Elovl2 and DHA on insulin secretion. We performed a lipidomic analysis in INS-1 cells. The results evidenced a DAG accumulation under GL conditions. Interestingly, this accumulation was inhibited by addition of 100µM of DHA, but not with 10µM of DHA (Figure 15). We also saw that the partial restauration of insulin secretion under GL occurred only with elevated DHA concentration. This brought us to investigate a possible DAG role on impaired insulin secretion under GL. One of the most import effects of DAG is their ability to activate protein kinase C (PKC). In particular, the novel group of PKC, which include the δ, ε, η and θ isoforms, is activate by DAG and do not need Ca^{2+} influx for their activation (Schmitz-Peiffer et al., 1997). As already presented on the "Introduction section", different PKC isoforms have been implicated on insulin secretion. PKCε deletion *in vivo* prevented glucose intolerance on HFD-fed mice (Schmitz-Peiffer et al., 2007), while over-expression of kinase dominant negative PKCδ in pancreatic β-cells protected mice from HFD-induced glucose intolerance (Hennige et al., 2010). In C2C12 myotubes cells, palmitate activates PKCθ in order to inhibit Akt phosphorylation and insulin signalling. In these condition, addition of DHA inhibits partially palmitate-mediated PKCθ activation and recovered insulin signalling, through restored Akt phosphorylation (Capel et al., 2015).

Figure 15

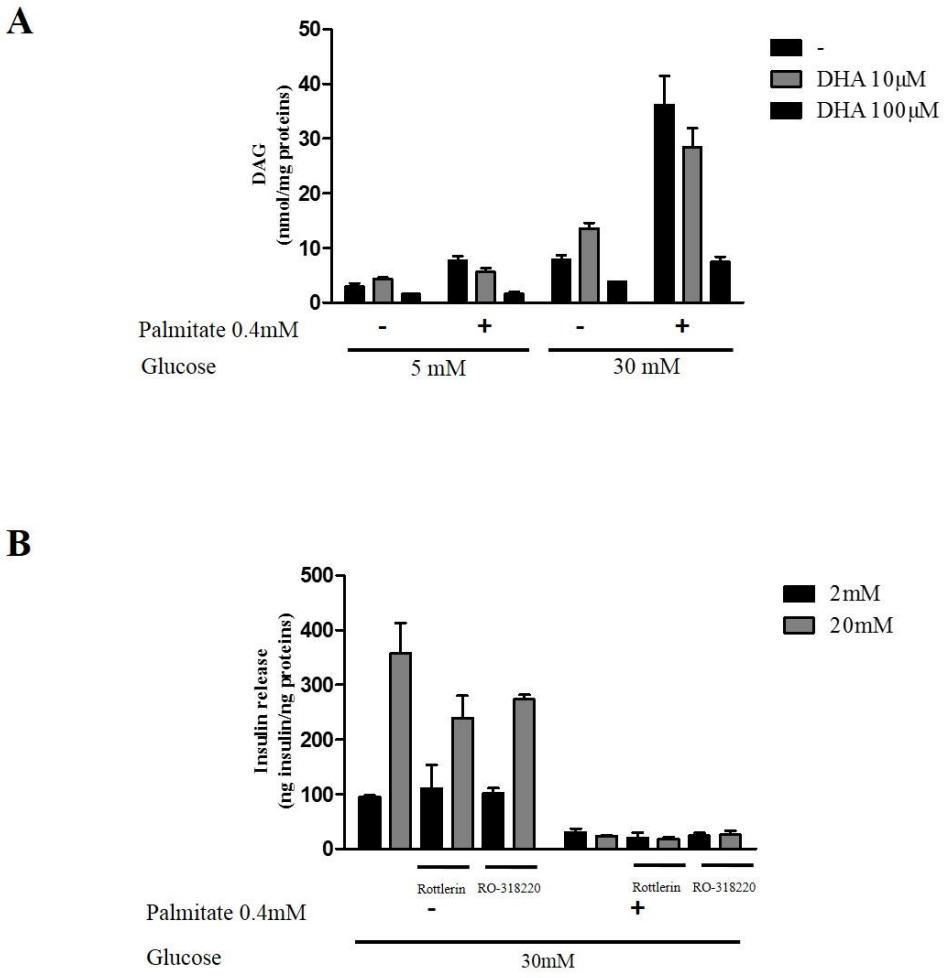


Figure 15. **DAG levels and PKC action in insulin secretion.** (A) INS-1 cells were incubated for 24hours with low or high glucose concentration (5mM or 30mM) in presence or not of palmitate (0.4mM). Total DAG were determined by LC-MS/MS (Materials and Methods) in presence of 10µM or 100µM of DHA. GL is inducing DAG accumulation and DHA 100µM and not 10µM is inhibiting this accumulation (B) MIN6 cells were incubated for 24hours with high glucose concentration (25mM) in presence or not of palmitate (0.4mM). Insulin secretion tests were performed as described in Materials and Methods. Different PKC inhibitors were tested: Rottlerin (10µM), PKCδ and PKCθ inhibitor, and RO-318220 (5µM), atypical PKC inhibitor. The 24h incubation with these inhibitors was toxic for the cells, so PKC inhibitors were added during the last 2h of the incubation. This preliminary experiments show that PKC inhibition is not sufficient to restaurate the GL-inhibited insulin secretion. Statistics not shown.

Therefore, we decided to investigate the role of PKC in the inhibition of insulin secretion in pancreatic β-cells. We used Rottlerin, a well-known PKCδ and PKCθ inhibitor, and RO31-8220 that inhibits all PKC (Soumura et al., 2010; Powell et al., 2004). The addition of these inhibitors, during GL stimulation, did not protected β-cells from a decrease of insulin secretion in response to glucose (Figure 15). However, it is possible to imagine that PKC activation occur relatively late or that the inhibitors have a determined half-life time, and after 24 hours they are not anymore active. We also tested the effects of these inhibitors during the last 30 minutes of GL

treatment and during the entire secretion test, but again these inhibitors did not increased insulin secretion in GL conditions (Data not shown). These preliminary data suggest that PKC are probably not involved in palmitate/high glucose inhibition of insulin secretion in MIN-6 cells. Nonetheless, it is possible that exogenous DHA or endogenous DHA production could controls PKC activation involved in other β-cells dysfunctions induced by GL, such as apoptosis. It also remains to establish the biological role of decreasing DAG levels by DHA in β cells under gluco-lipotoxic conditions. This could be related to the synthesis of TAG, neutral lipids that have been shown to detoxify palmitate's effect (see below section 2.7).

1.2.3 Insulin content

In agreement with previous study, we found that palmitate both at low and high concentration decreases the insulin content in MIN-6 cells. In literature, it is well established that palmitate decreased insulin gene expression, reducing PDX-1 nuclear translocation and blocking MafA mRNA translation (Kelpe et al., 2003; Hangman et al., 2005). These mechanisms seems to be mediated by ceramide accumulation. It has been quite surprising to see that DHA addition, which inhibits ceramide accumulation (and possibly ER stress), did not restored insulin content in MIN-6 cells under GL. Therefore, it is possible that insulin gene expression is inhibited by other mechanisms than the ceramide accumulation, or that this inhibition is performed by specific species of ceramide different from those involved in β-cell apoptosis. In addition, even if DHA is no playing a role on insulin gene expression, it could regulate insulin levels by acting at the levels of insulin maturation or stability, which are hampered by palmitate.

The experiments performed with *Elovl2* siRNA highlighted that *Elovl2* silencing decrease insulin content independently from any treatment in MIN6 cells. We still do not the reason why down-regulation of ELOVL2 affect the cell's reserve of insulin. A simple possibility could be that *Elovl2* knock down induce the β-cell death. Therefore, in the future, it would be interesting to further investigate the mechanisms involved in the control of insulin content by ELOVL2.

1.2.4 Insulin exocytosis

A mechanism that we still did not explored is the action of ELOVL2/DHA axis on insulin exocytosis machinery. Indeed, it has been demonstrated that long-time exposure of islets of Langerhans to GL inhibits the fusion steps of insulin granules with plasma membrane (Olofsson et al., 2007). Since we demonstrated that insulin content is only slightly restored with the

addition of DHA or the over-expression of *Elovl2*, it is possible that a better function of the exocytosis machinery could explain improved insulin secretion by the ELOVL2/DHA axis under GL conditions. It is extremely interesting that granuphilin, one of the proteins involved on the docking of the insulin secretory granules to plasma it is controlled at the expression levels by SREBP1-c (Kato et al., 2006). Granuphilin over-expression has been correlated to inhibition of insulin secretion, and knock down of the protein can restore insulin secretion (Kato et al., 2006). SREBP1-c is a well-known target of ω-3 PUFAs, and Pauter and colleagues demonstrated that *Elovl2*-KO mice presented up-activated SREBP1-c (Pauter et al., 2014). It is reasonable to hypothesize that DHA treatment or *Elovl2* over-expression inhibits SREBP-1c activation and could indirectly control granuphilin expression in β cells, improving in this way insulin granule exocytosis.

Insulin secretion is also tightly regulated by Ca^{2+} influx signals. We performed some experiments on Ca^{2+} influx, demonstrating that, during GL, glucose-stimulated Ca^{2+} influx is inhibited, and DHA is restorating the normal Ca^{2+} influx levels. Indeed, it is well established that Ca^{2+} influx plays a crucial role in the control of insulin secretion (Gilon et al., 2014). Our data demonstrate that DHA is able to partially restore normal Ca^{2+} glucose-stimulated concentrations, and this could explain the DHA ability to restore insulin secretion response. In future, it would be interesting to deeper analyse the mechanisms involved on DHA-ameliorated Ca^{2+} influx and its connection to insulin granules exocytosis.

2 DHA/ELOVL2 AXIS AND APOPTOSIS

DHA has been connected to many beneficial roles in cell function, particularly in adipose cells (Martínez-Fernández et al., 2015, Flachs et al., 2014). Regarding cell apoptosis, DHA induces apoptosis of cancer cells, but not in normal cells (Jeong et al., 2014; Kim et al., 2015). Interesting, Lewis lung cancer tumor cells implantation in *Fat-1* mice showed slower growth and higher levels of apoptosis than cells implanted into WT mice (Kim et al., 2015). Our data demonstrated for the first time that DHA and ELOVL2 play a protective role against palmitate-induced apoptosis of pancreatic β-cells. Indeed, DHA treatment totally protect INS-1 cells from the high glucose plus high palmitate-induced cell death, and *Elovl2* over-expression partially inhibits caspase activation by GL. We hypothesized that the partial protection of *Elovl2*-over-expressing cells from GL-induced apoptosis could be due to technical limitation: the infection

percentage and expression rate can be largely variable among the different cells, and, as occurred in MIN-6 cells, the concentration of EPA, a DHA precursor, are probably limited. We were not able to test the effects of EPA treatment since supplementation of EPA, even at low doses (10µM), is also protecting cells from apoptosis induced by GL. Another possibility would be to test the addition of docosapentanoic acid (22:5,*n*-3) (DPA), which is an intermediate involved on DHA synthesis (Figure 10, Pauter et al. 2014). If DPA by itself is not protecting β-cells from apoptosis induced by GL, it would be possible to determine the effects of endogenous DHA production from DPA through adenoviral *Elovl2* expression during GL.

Interestingly, knock-down of ELOVL2 protein, exacerbated toxic effects of GL. This result suggests that ELOVL2 and the endogenous production of ω3 PUFAs such as DHA might play a crucial role on the protection of the β-cell from the toxic effects of palmitate and high glucose. Our preliminary data show that addition of DHA to the *Elovl2* knocked-down cells partially protected cells from GL-induced apoptosis (data not shown).

Our results demonstrate that exogenous addition of DHA and its endogenous production is protecting pancreatic β-cells from apoptosis, and that *Elovl2* down-regulation makes cells more vulnerable to apoptosis induction. Regarding the mechanism involved, this will be discussed on the next session.

Even at basal levels, *Elovl2* knock-down also causes a significant increase in caspase activity, suggesting that ELOVL2 could be a protein implied in β cell survival. This is not completely surprising since *Elovl2* in different studies has been identify as human age prediction marker by looking at its methylation state; *Elovl2* gene possessed 7 CpG methylation sites that are linearly methylated depending on age (Garagnani et al., 2012; Zbiec-Piekarska et al., 2015; Kananen et al., 2016). We can speculate that maybe ELOVL2 protein expression is a survival signal, and its deregulation could be used by cells as an age indicator. Aging is accompanied by impaired glucose homeostasis and an increased risk of type 2 diabetes, culminating in the failure of insulin secretion from pancreatic β cells. Indeed, the incidence of type 2 diabetes is disproportionally high in the elderly, over 25%, by comparison to 4.1% in younger adults and 16.2% in the middle-aged (Centers for Disease Control and Prevention). Insulin resistance and β cell mass have been heavily studied as contributors to impaired glucose tolerance and diabetes in the elderly (Chang et Halter, 2003; Gunasekaran et Gannon, 2011; Kushner, 2013). Among the age-associated factors involved in β cells failure, reduced mitochondrial copy number (Cree et al., 2008) but also epigenomic changes affecting the expression of metabolic genes

(Avraharmi et al., 2015) have been already reported. Therefore, it would be really important to investigate if methylation of *Elovl2* gene could play a role in the appearance of type 2 diabetes linked to aging. Moreover, recent work has shown that palmitate treatment of human pancreatic islets gives rise to epigenetic modifications that together with altered gene expression may contribute to impaired insulin secretion and T2D (Hall et al. 2014). In this context, it will be interesting to determine if modulation of *Elovl2* gene methylation by GL in β cells is a regulatory mechanism of β cell dysfunction. In this context, it will be important to determine the proteins involved in the control of its expression and potentially in the control of its methylation state.

In GL conditions, nowadays there are not studies that investigated the DHA effects and connected them to cell apoptosis.

2.1 G-COUPLED RECEPTION AND APOPTOSIS

In order to determine the mechanisms involved in the protective effect of ELOVL2/DHA axis against GL, we decided to investigate the role of GPR120 receptor. As we made for insulin secretion, we tested four GRP120 agonists and one antagonist also on β-cell apoptosis induced by GL. As occurred for insulin secretion, GPR120 agonists are not able to protect INS-1 cells from GL. Moreover, addition of a GPR120 antagonist did not blocked DHA-mediated protection against GL-induced apoptosis. In these experiences, different type of agonists and antagonist incubations (24h or 30minutes before the end of evaluation apoptosis) were attempted. Our results suggest that, the inhibitory effects of DHA on GL-induced apoptosis, seems to be not mediated by DHA binding to the G-coupled receptor GPR120. Finally, we tested if an incubation with DHA for 30 minutes, which should be necessary and sufficient to activate the cascade of a receptor, was enough to protect β cells from GL. In these conditions, DHA is not able to protect cells from apoptosis induced by GL (data not shown). These evidences suggest that DHA effects seems not to be receptor-mediated, and DHA needs to enter inside the cells and to be potentially metabolized and fulfil his beneficial effects.

Among the different lipids derived from DHA, there is the docosahexaenoyl ethanolamide (DHEA), which is a potent endocannabinoids since it could activate cannabinoid CB(1) and CB(2) receptors in vitro (Brown et al. 2010). In cancer cells, DHEA has strong anti-proliferative effects (Brown et al., 2010). Activation of cannabinoid receptors expressed by islet cells can stimulate insulin secretion, which is obviously beneficial under conditions of impaired glucose

tolerance or type 2 diabetes (Li et al. 2011). However, the precise role of CB1 receptor in β cells is still not clear since others studies showed in human islets and diabetic mice, their inhibition attenuated diabetes and insulin resistance and protected β-cell loss. *In vitro*, studies confirmed that endocannabinoids promotes β-cell apoptosis (Jourdan et al., 2016). Our data demonstrated that DHEA was not able to reproduce DHA protective effects. Therefore, we can speculate that DHA is no acting through its conversion into DHEA and that problably the endocannabinoid pathway is probably not involved in the protective effects of the ELOVL2/DHA.

2.2 PROTEIN KINASE C AND APOPTOSIS

In renal proximal tubular cells, PKCθ has been demonstrated to be involved in palmitate-induced inflammation and apoptosis (Soumura et al., 2010). PKCθ activation was partially inhibited by oleate or EPA addition in presence of palmitate, and the PKCθ-dependent mitochondrial mediated apoptosis was prevented by oleate and EPA (Soumura et al., 2010). As already described, we detected an increase of DAG under GL, and its inhibition by high dose of DHA (100μM). DAG are well known to activate protein kinase C, especially the novel PKC isoforms. PKCθ and PCKδ inhibitor, rottlerin, was tested and resulted to be inefficient to prevent β-cell apoptosis induced by GL. Also in this case, as occurred for insulin secretion, PKCθ and PCKδ seemed to be not involved in the DHA effects against apoptosis induced by GL.

2.3 CERAMIDE AND APOPTOSIS

Ceramide accumulation were evidenced in pancreatic β-cells and islets of Langerhans stimulated with high palmitate and high glucose concentrations (Véret et al., 2011; Kelpe et al., 2003; Boslem et al., 2013). Among the lipids produced into the cells in response to palmitate, ceramide have been demonstrated to be the major lipids responsible for the toxic effect of palmitate (Véret et al., 2011; Biden et al., 2014; Poitout et al., 2008). Regarding the ω-3 fatty acids and ceramide levels, a restricted number of studies connected these two lipid species. *In vivo*, in skeletal muscles of mice fed with HFD-enriched with saturated fatty acids highly increase ceramide content, while PUFAs-enriched HFD did not (Blachnio-Zabielska et al., 2010). In mouse fibroblast cells, DHA resulted to attenuate multiple biochemical events

Figure 16

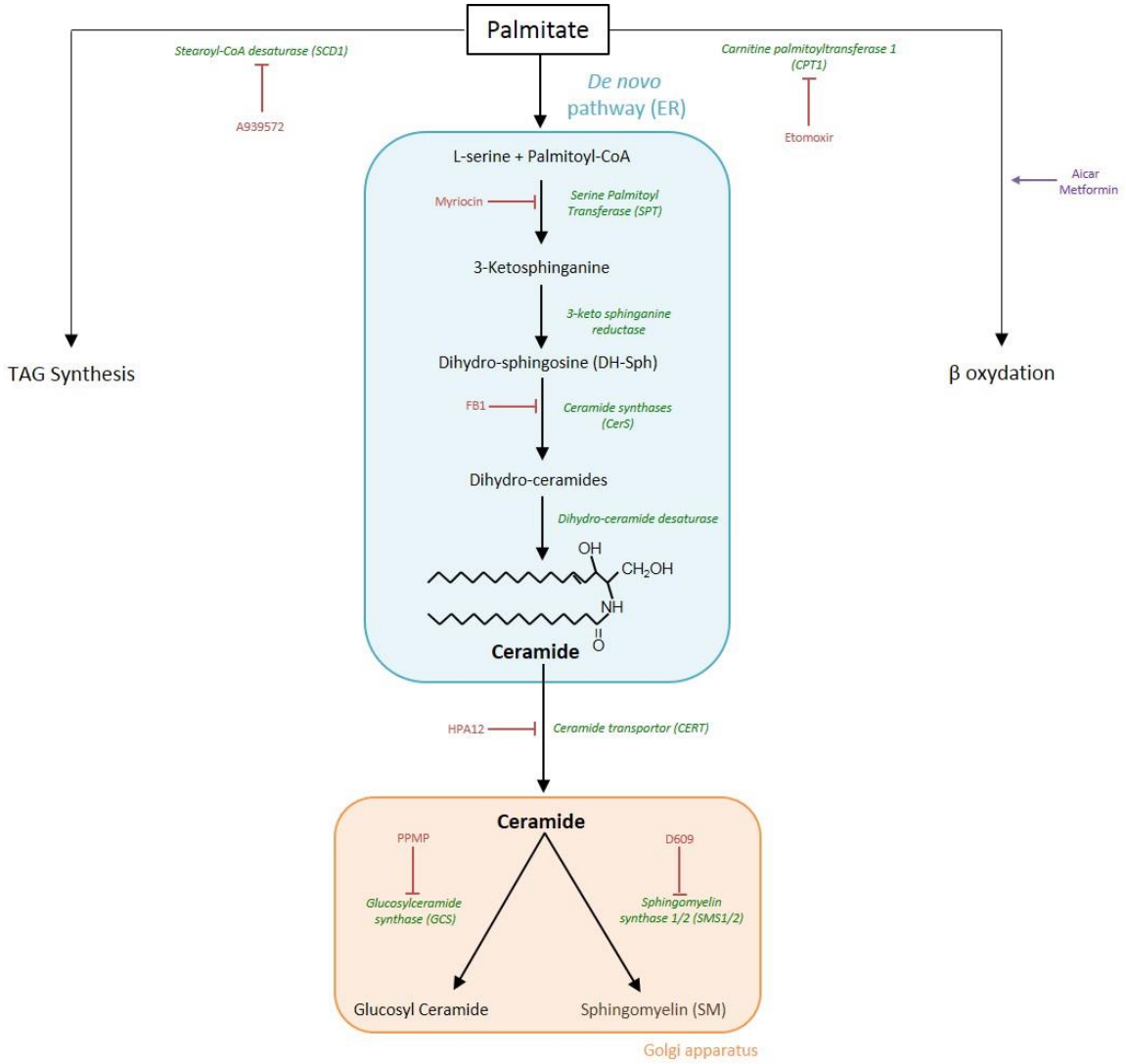


Figure. 16. Pathways involved on palmitate utilization. During GL, palmitate is preferentially used for the *de novo* ceramide synthesis. Alternative ways that can use FAs are TAG synthesis and β-oxidation. Here is represented the inhibitors that can be used to understand how FAs are partitioning into the cells, and which of these ways is involved.

associated with TNF-α-induced necroptosis, including ceramide accumulation (Pacheco et al., 2014).

We know that apoptosis in INS-1 cells is ceramide-mediated, so we decided to quantify ceramide accumulation into β cells treated with GL and DHA. Addition of DHA to the culture medium was preventing ceramide accumulation induced during GL. The same results were obtained in cells over-expressing *Elovl2*. To further investigate which type of ceramide species were down regulated, we performed a mass spectrometry lipid analysis. The lipidomic analysis determined that ceramide accumulation was due to the increase of specific ceramide species

(C18, C20, C22, and C24). Interestingly, DHA was inhibiting accumulation of these specific ceramide species.

Figure 17

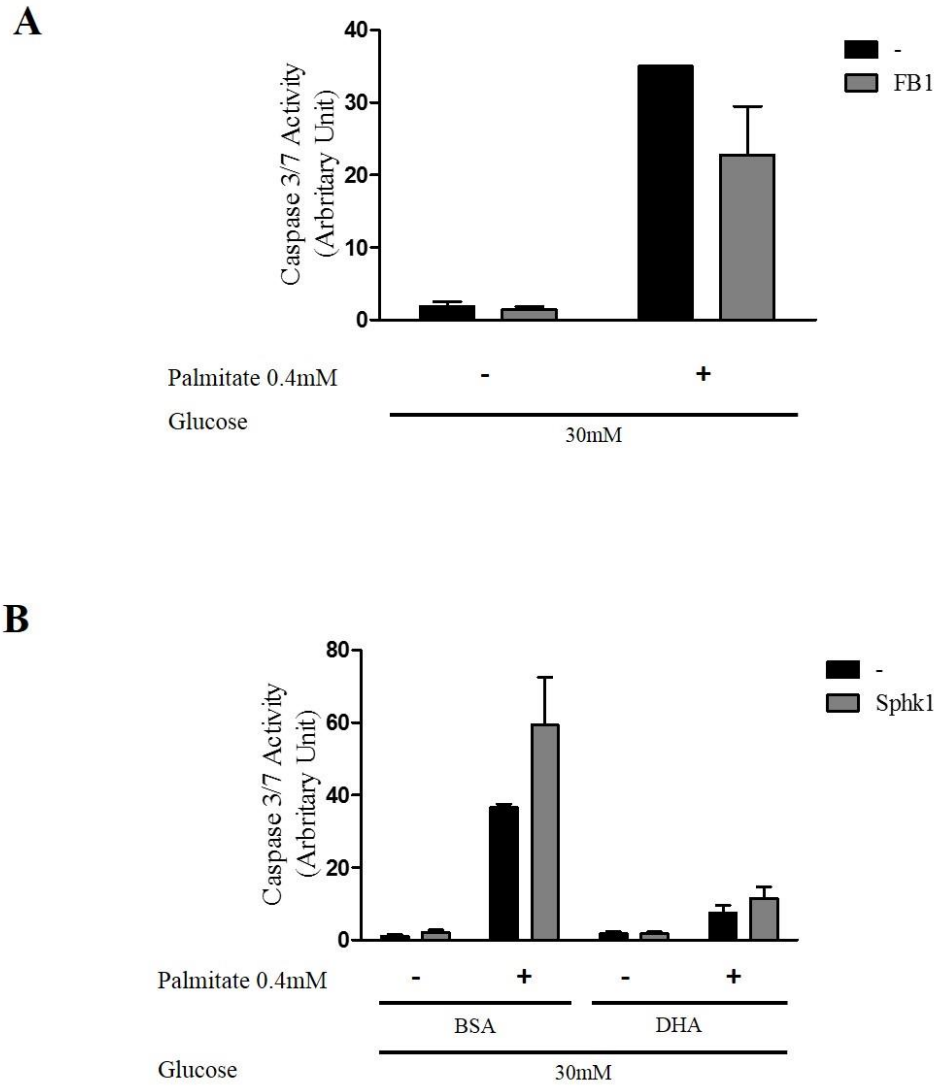


Fig. 17. Fuminisin B1 (FB1) and SphK1 inhibitor effects on glucolipotoxicity. INS-1 cells were incubated for 24hours with low or high glucose concentration (5mM or 30mM) in presence or not of palmitate (0.4mM). Apoptosis was determined by caspase 3/7 activity. (A) Fuminisin B1 (50µM) addition is partially protecting cells from GL-induced apoptosis. (B) Sphk1 inhibitor (10µM) is increasing the GL-apoptosis and in presence of DHA is not inhibiting the protecting effects. Statistics not shown.

In order to reproduce inhibition of the ceramide *de novo* synthesis but also the salvage pathway by DHA, we tested the effect of Fumonisin B1 (FB1) (Figure 16), a ceramide synthases inhibitor. FB1 partially inhibited the caspase activation (~50%) (Figure 17 and Véret et al.

2011). These results suggest that both pathways are involved on the deleterious effects of palmitate. These results have been strengthen by a recent study showing that palmitate is inducing both activation of the *de novo* and the salvage ceramide pathway in β-cells (Manukyan et al., 2015). Our previous data suggested a role of specific ceramide species in β cell apoptosis induced by GL (Véret et al. 2011). Diversity of ceramide species are based on the existence of various ceramide synthases (Wegner et al., 2016). Interestingly, it is known that mechanisms that control CerS are complex: transcriptional regulation and protein activity regulation are specific to each isoform (Wegner et al., 2016). This complicates the investigation on the role of CerS in deleterious effect of palmitate and therefore the action of DHA on them. To determine CerS involved on this effect it would be necessary to perform much more deep investigations, which goes over the simple gene expression. It could be interesting to use the recent rapid and reliable CerS assay that uses a fluorescent substrate made by Futerman's lab (Tidhar et al. 2015) in order to determine the impact of GL on CerS activity and the Elovl2/DHA effect.

Another unexplored mechanism is the activation of sphingomyelinase. Interesting, Lei and colleagues demonstrated that ER stress induced in pancreatic β-cells is mediated by ceramide accumulation. iPLA$_2$β-dependent induction of neutral SMase increased ceramide content and mediate β-cell apoptosis (Lei et al., 2010). iPLA$_2$β expression has been demonstrated to be induced by SREBP1-c activation. SREBP1-c activation is inhibited by DHA and potentially by *Elovl2* (since in Elovl2 KO mice SREBP1-c is up-regulated) (Pauter et al. 2014). However, the role of iPLA$_2$β is still controversial since a recent study suggested that over-expression of this enzyme mitigates palmitate-induced β-cell mitochondrial injury and apoptosis (Song et al. 2014). Nevertheless, it has been shown more recently that inhibition of neutral SMase partially protected against β cell apoptosis induced by palmitate (Boslem et al. 2013). These results suggest that loss of sphingomyelin in the ER could be also a major event in β-cell apoptosis induced by lipotoxicity. Consequently, it will be important to determine if ELOVL2/DHA axis regulate ceramide accumulation through the inhibition of SREBP1-c/SMase axis.

2.4 CERAMIDE METABOLISM AND APOPTOSIS

We hypothesized that ceramide flux could also be modified by DHA. *De novo* ceramide are transported to the Golgi apparatus by a specific transporter called CERT (Hanada et al. 2003), and we recently showed that CERT is inhibited in high glucose and palmitate condition (Gjoni et al. 2014) suggesting a possible role on ceramide accumulation during GL. In INS-1 cells,

DHA prevents CERT protein down regulation induced by GL. Moreover, when we determine ceramide trafficking between ER and Golgi, we found that DHA restore partially ceramide trafficking. These results suggests that ELOVL2/DHA axis could block ceramide accumulation by restoring its transport to the Golgi and therefore inhibiting β cell apoptosis.

In Golgi, ceramide could be transformed in other lipids, which are potentially less toxic for the cells. Sphingomyelin synthase (SMS) transfer the phosphocholine group from phosphatidylcholine to ceramide to generate sphingomyelin (SM) and diacylglycerol (DAG) (Delgado et al., 2006). This enzyme is known to directly regulate the balance between ceramide and DAG, and to control in this way opposite cellular processes such as cell proliferation, growth arrest and apoptosis (Delgado et al., 2006). Treatment with D609, a well-known inhibitor of SMS did not inhibited the DHA protective effects against GL-induced apoptosis. Ceramide could also be metabolized in glucosylceramide (GlcCer) by glucosylceramide synthase (Wegner et al., 2016). We tested a GlcCer synthases inhibitor, PPMP, to determine if DHA increased ceramide/GlcCer flux in order to inhibit apoptosis induced by GL. However, PPMP was not able to block the DHA's beneficial role against β cell lipotoxicity. Altogether, these results suggest that, even if the DHA is able to restore a normal CERT protein levels in conditions of GL, supporting a possible restauration of the ER-to-Golgi trafficking, this mechanism seemed to be insufficient for the DHA protecting effects. This conclusion let us to hypothesise that decrease of ceramide accumulation induced by DHA is not primarily due to an increase of ceramide trafficking between ER and Golgi.

Among the different ceramide derivate, sphingosine-1-phosphate (S1P) is one of the most beneficial ceramide's metabolite (Spiegel et al., 2003). Our lab demonstrated that over-expression of sphingosine kinase 1 (Sphk1) protects pancreatic β-cells from GL and restore the ER-to-Golgi protein trafficking (Véret et al., 2013). In cells, a ceramide/S1P biostat exists: the balance shifts toward one or the other lipid specie determine cell fate (Spiegel et al., 2003). For this reason, we tested if a Sphk1 inhibitor, and therefore inhibition of S1P formation, could counteract the protective effects of DHA under GL conditions. This inhibitor was no able to inhibit the protective effect of DHA (supplemental figure) suggesting that the ceramide/S1P biostat seemed to no be crucial on action of ELOVL2/DHA axis.

To conclude, our results demonstrated that DHA is inhibiting ceramide accumulation under GL conditions. The mechanism involved is not link to the inhibition of CerS, or the synthesis of S1P. ER-to-Golgi ceramide trafficking seems to be ameliorated with DHA addition, but it seem not to be sufficient for the DHA protection against β cell apoptosis. It could be interesting to

investigate the role of SMase inhibition, and more in details the salvage ceramide pathway, and determine if the inhibition of these pathways could contribute to the DHA effects.

Since in cells a balance is always maintained to regulate free palmitate levels, if it is not used for ceramide biosynthesis, it is probably used in other lipid pathways, such as the mitochondrial β-oxidation or TAG synthesis pathways.

2.5 ELOVL2/DHA AND B-OXIDATION

Under high concentration of palmitate, cell has to deal with a lipid surplus that need to be managed. High glucose concentration in β-cells causes malonyl-CoA accumulation (Poitout et al., 2008). Malonyl-CoA is a metabolic signalling molecule that regulates lipid partitioning. Indeed, this molecule inhibits CPT-1 function, which is a fatty acid transporter that catalyse the rate-limiting step of mitochondrial β-oxidation of fatty acids (Prentki et al., 2002). Consequently, in case of FFAs surplus with high glucose levels, FAs accumulates in cytoplasm and are used for complex lipid such as ceramides, or are esterified and incorporated into stable lipid droplets, which are harmful for the cells.

In condition of GL, fatty acids β-oxidation is partially inhibited in pancreatic β-cells (Prentki et al., 2002; Poitout et al., 2008). We determined if DHA and *Elovl2* over-expression were able to activate this pathway in order to detoxify the cells from the lipid surplus. For the reason, we tested the effect of an inhibitor of β-oxidation, etomoxir, and an activator of β-oxidation, AICAR (El-Assaad et al., 2003). Etomoxir is an indirect inhibitor since it acts at the levels of CPT-1, inhibition of this lipid transporter blocks fatty acid translocation into mitochondria and their utilization by the β-oxidation machinery. AICAR activates the AMPK kinase cascade, which positively controls the β-oxidation (El-Assaad et al., 2003).

One pioneer study of GL's effects on β-cell apoptosis and function, investigated the role of β-oxidation. The toxic effects of GL were mitigated by β-oxidation activation (El-Assaad et al., 2003). In this contest, AICAR protected β-cells from GL-induced apoptosis, while etomoxir exacerbated the lipotoxic effect (El-Assaad et al., 2003).

Regarding AMPK, it has been demonstrated that ω3 PUFAs protection effect against the induction of obesity, dyslipidemia and insulin resistance is AMPKα-dependent. Indeed, AMPKα-deficient mice fed with HFD- ω3 PUFAs supplemented diet were not anymore protected from low levels of hepatic and plasma triglycerides accumulation and developed IR

compared to the WT mice (Jelenik et al., 2010). Different studies demonstrated that ω3 PUFAs activate AMPK pathway. In cancer cell lines, DHA, through AMPK and AKT activation, controls autophagy activation and apoptosis induction. DHA also induced cell death via AMPK- and Akt-regulated mTOR inactivation (Kim et al., 2015). *In vivo,* endothelial function was ameliorated by EPA-treatment, through an increased AMPK phosphorylation and activation (Wu et al., 2012). Phospho-AMPK mediated nitric oxide production, which is involved on aorta endothelium-dependent vaso-relaxation. EPA mediated its beneficial effects through AMPK pathway since its effects were annihilated in aorta rings isolated from AMPKα1 KO mice (Wu et al., 2012).

AMPK activation is not the only pathway involved in β-oxidation regulation. PPARα has also been involved in the control of fatty acid oxidation pathway and in protection from lipotoxicity (Luquet et al., 2005; Flachs et al., 2005; Rao et Reddy 2004). In β-cells, a study investigated oleate-induced dysfunction and demonstrated that oleate inhibits glucose-stimulated insulin secretion. Modulation of PPARα by mRNA silencing or protein over-expression resulted on an aggravation of insulin secretion defect when PPARα was silenced and on a preserved insulin response when PPARα was up-regulated (Frigerio et al., 2010). This study show for the first time that PPARα modulation is important for β-cell function and insulin secretion. They also demonstrated in β-cells that PPARα is involved in both β-oxidation and fatty acid storage in neutral lipids such as TAG.

We tested the effects of β-oxidation modulation in GL conditions. As already present in literature, activation of β-oxidation resulted to protect the pancreatic β-cells from GL (El-Assaad et al., 2003). Interestingly, etomoxir strongly increased GL-induced caspase activation and blocked beneficial effects of *ELOVL2/DHA* axis. When β-oxidation is inhibited, ELOVL2/DHA are not anymore able to protect pancreatic β-cells from GL-induced apoptosis.

Different signalling pathways could be involved in the regulation of the β-oxidation by ELOVL2/DHA axis:

1- AMPK activation. We would like to confirm or not if AMPK activation is mediated by *ELOVL2/DHA* axis at different levels: by western blot analysis of phosphorylation state of AMPK and ACC; through molecular tools as the use of adenoviral over-expression of wild-type or dominant negative form (Ad-AMPK and Ad-DN-AMPK; and by the treatment of mouse AMPK-KO islets with DHA and Ad-*Elovl2*;

2- PPARα activation. We would like to investigate a possible involvement of PPARα in the *ELOVL2/DHA* protection against GL by molecular tools (siRNA and adenoviral overexpression) and by treatment of PPARα-KO mice islets with DHA and Ad-*Elovl2*.

Our actual data indirectly inhibits β-oxidation pathway by blocking fatty acid transport into mitochondria, mediated by etomoxir. By the use of additional inhibitors we would like to check if the effect of ELOVL2/DHA axis are mediated by enhancing lipid transport into mitochondria or directly by enhancing fatty acid β-oxidation. To achieve this goal we will test the effects of Perhexiline (CPT1 and CPT2 inhibitor) and Ranolazine (β-oxidation inhibitor) (Liu et al., 2016).

It would be also interesting to test if oxygen consumption in GL condition supplemented with DHA and Ad-*Elovl2*, to clearly demonstrate activation of fatty acid β-oxidation.

2.6 ELOVL2/DHA AND CARDIOLIPIN

Another pathway totally unexplored is the role of cardiolipin synthesis and remodelling. Oxidation of phospholipid caridiolipin has been involved on apoptosis induction. Indeed, oxidation of cardiolipin changes its interaction strength with cytochrome C, modulating cytochrome C retention into the mitochondria (Hardy et al., 2003). Interestingly, the susceptibility of cardiolipin to oxidation depends on its enrichment with PUFAs (Hishikawa et al., 2014). It would be interesting to determine in our system if cardiolipin level is increased, if cardiolipin is enriched by DHA residues and if it mediates the ELOVL2/DHA protection axis from GL in pancreatic β-cells.

2.7 ELOVL2/DHA AND TAG

A parallel axis of investigation has been the regulation of triglycerides production. Pancreatic β-cells have a limited capacity to stock triglycerides. However, the ability of normal β cells to accumulate triglycerides has been suggested as a cytoprotective mechanism against FFA-induced apoptosis (Figure 16) (Cnop et al. 2001). Moreover, an interesting study demonstrated that a selection of pancreatic β-cells resistant to GL demonstrated that cells more resistant to GL presented higher expression levels of stearoyl-CoA desaturase 1 (SCD-1) (Busch et al., 2005). Finally, β cell susceptibility to palmitate can be reduced by SCD-1 stimulation by LXR and PPARalpha agonists which favors their desaturation and subsequent incorporation in

neutral lipids (Hellemans et al. 2009). SCD-1 is involved on the conversion of saturated fatty acids (palmitate and stereate) in monounsaturated fatty acids (palmitoleate and oleate). Monounsaturated fatty acids are preferentially transported into mitochondria to be oxidized or are used for the TAG synthesis (Nolan et al., 2009). On the resistant pools, SCD-1 inhibition blocks the protection effect against GL-induced apoptosis (Busch et al., 2005). MUFAs have been demonstrated to be able to protect from palmitate-induced apoptosis by channelling palmitate into TAG synthesis (Cnop et al., 2001; Liestenberger et al., 2003). Soumura and colleagues demonstrated that oleate and EPA protect proximal tubular cells from the toxic effects of palmitate by channelling DAG incorporation into TAG (Soumura et al., 2010). Palmitate inhibit diacylglycerol acyltransferase 2 (DGAT) expression, and addition of oleate or EPA restored its expression. DGAT is the enzyme involved on the last step of the TAG synthesis (Nolan et al., 2009). Knock-down of DGAT by siRNA demonstrated that cells without this enzyme are not anymore protected from lipotoxicity by oleate or EPA (Soumura et al., 2010).

From these evidences, we tested if TAG accumulation is regulated in DHA-treated β cells under GL conditions. Staining of neutral lipid demonstrated that DHA is increasing TAG accumulation in β cells under GL (data not shown). Further experiments need to be performed to elucidate a possible role of SCD-1 or DGAT in the ELOVL2/DHA protection axis from GL dysfunctions.

3 *IN VIVO* EXPERIMENTS

3.1 DHA DIET SUPPLEMENTATION

Effect of DHA-supplementation have been largely investigated in many tissues (Martínez-Fernández et al., 2015, Flachs et al., 2014). Even if a clear consensus has still not be reached, it is mainly accepted that ω3 PUFAs supplementation is beneficial and could protect from IR installation associated to obesity in rodents and humans (Martínez-Fernández et al., 2015, Flachs et al., 2014). Only few study investigated pancreatic β-cell function in mice fed with HFD supplemented with ω3PUFAs (Winzell et al., 2006). Winzell et al. did not highlight a pancreatic β-cell protection in mice fed with HFD + ω3PUFAs supplemented diet. However, this study performed on female mice did not highlighted beneficial role of ω3 PUFAs on fat acid mass reduction or improved insulin response, suggesting to take these conclusions with caution (Winzell et al., 2006).

Figure 18

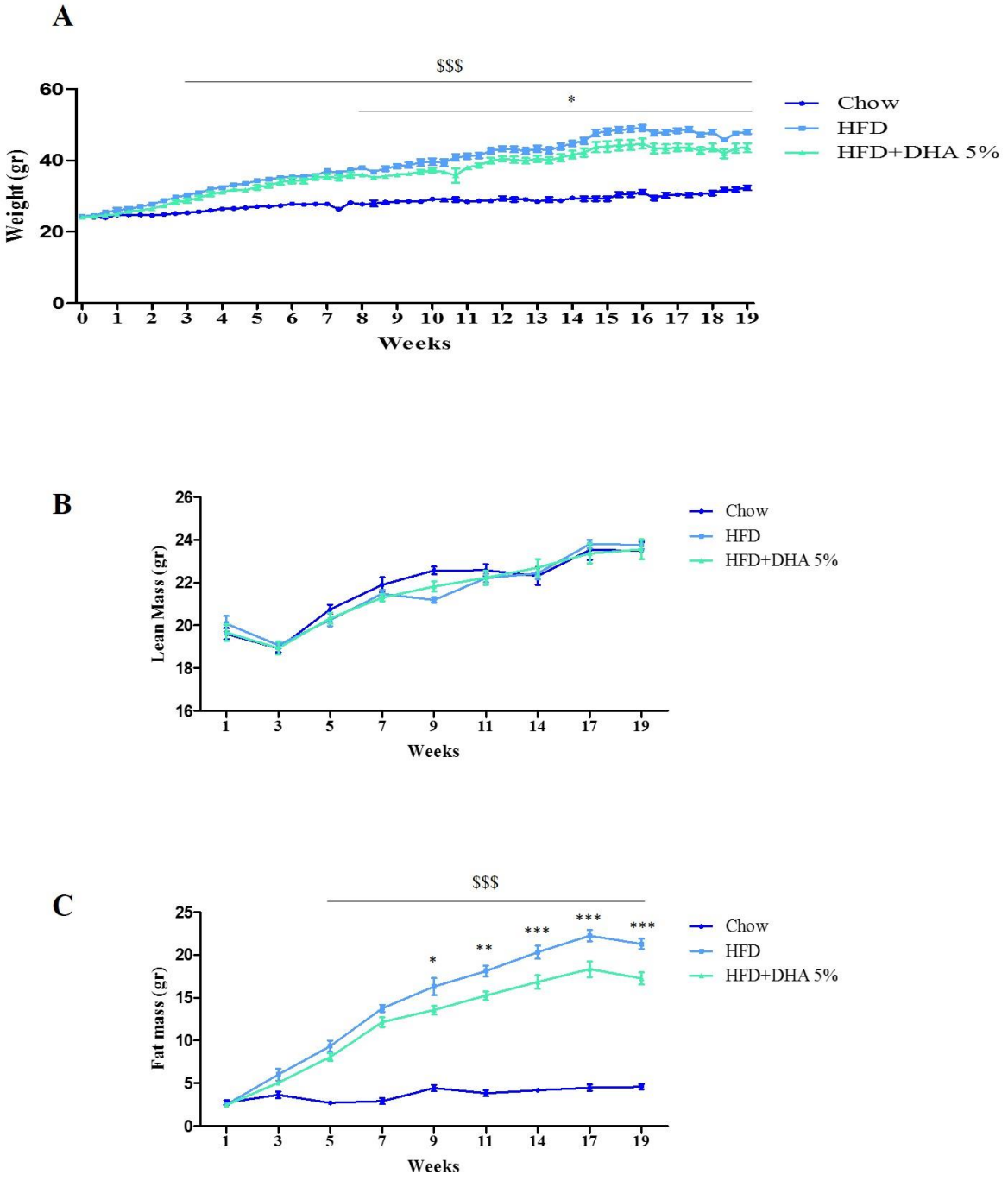


Fig. 18. DHA-supplementation effects on body weight and fat mass. C57BL/6j male mice were fed for 20 week with chow, HFD or HFD+5%DHA diet. (A) shown total mice weight. (B) lean mass determined by EchoMRI scan (Materials and Methods). (C) fat mass determined by EchoMRI scan (Materials and Methods). Results are represented as means ± s.e.m. of 6 different mice for group. two-way ANOVA test, Bonferroni post-test. *p<0.05, ** p < 0.01, *** p<0.001 HFD vs HFD+DHA; $$$p<0.001 Chow vs HFD and HFD +/- DHA.

Figure 19

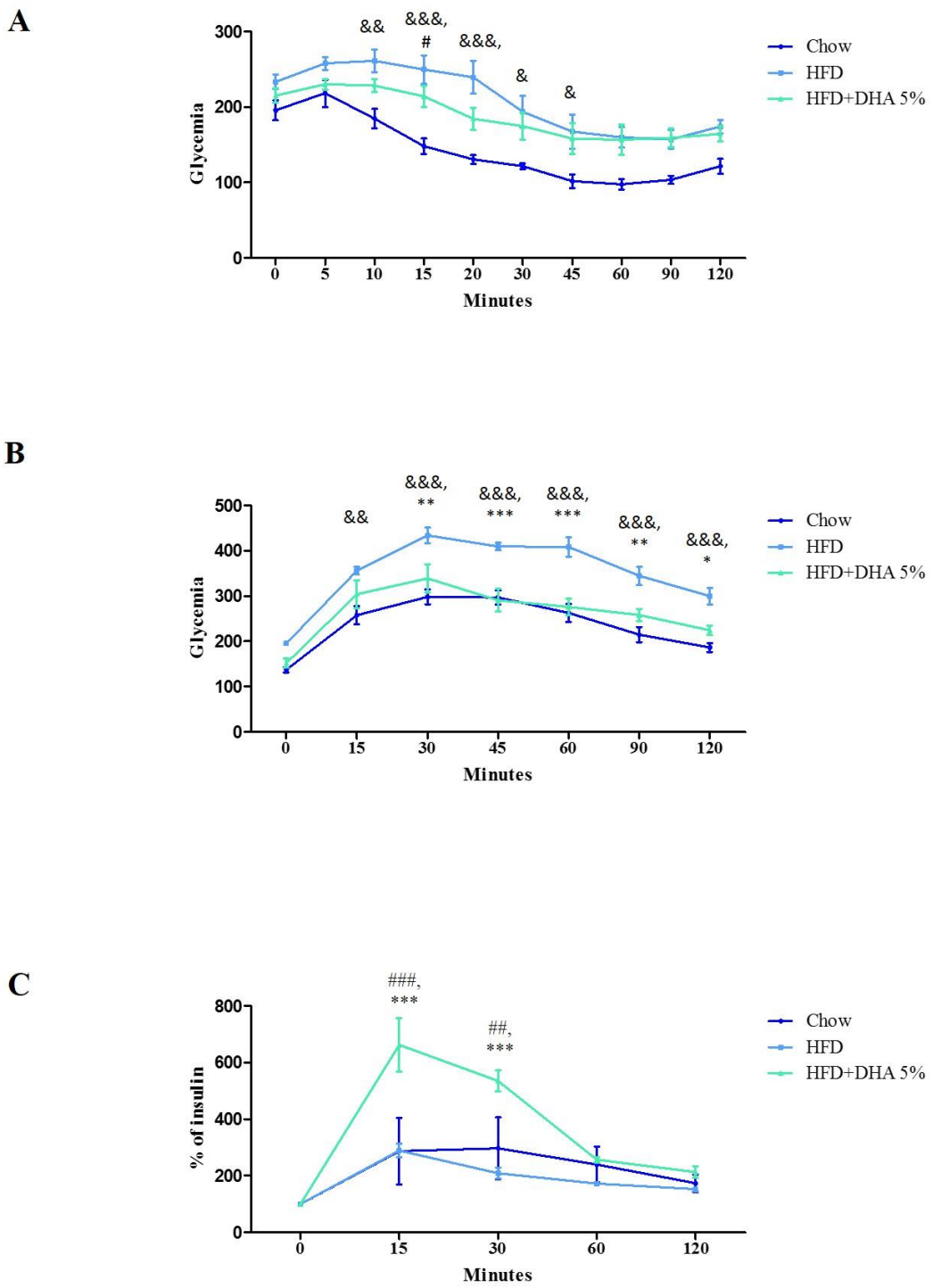


Fig. 19. DHA-supplementation effects on Glucose homeostasis. C57BL/6j male mice were fed for 20 week with chow, HFD or HFD+5%DHA diet. (A) Insulin tolerance test (ITT) at 17 weeks of diet. (B) Oral Glucose Tolerance Test (OGTT) at 18 weeks of diet. (C) % of Insulin secretion compared to basal insulin level quantify during OGTT test (See Materials and Methods). Results are represented as means ± s.e.m. of 6 different mice for group. One-way ANOVA test, Bonferroni post-test. *p<0.05, ** p < 0.01, *** p<0.001 HFD vs HFD+DHA; #p<0.05, ##p < 0.01 ###p<0.001 Chow vs HFD +/- DHA; && p<0.01, &&& p<0.001 chow vs HFD.

We decided to investigate the effects of DHA supplementation on C57BL/6j mice under HFD. To achieve this goal, we fed the mice with HFD diet supplemented with 5% fish oil (80% of DHA). In order to detect β-cells dysfunction and/or apoptosis, the experiment lasts for 5 months and half. The results showed a progressive installation of glucose intolerance and insulin resistance. At two months of diet, only slightly differences were evidenced between HFD- and HFD+DHA fed animals. After two months of diet, it starts to be a significant differences of body weight and fat mass: HFD+DHA fed mice accumulated less fat compared to the HFD-fed mice without differences in food intake (Figure 18). At fourth months, ITT highlighted that slightly differences were present between HFD and HFD+DHA fed mice, indicating IR installation in both mice groups. However, an oral glucose tolerance test (OGTT) revealed that HFD+DHA mice could manage their glycemia much better than HFD-fed mice (Figure 18). This amelioration was accompanied by a higher insulin secretion in response to glucose (Figure 19). At the end of the 5 months, in vitro insulin secretion tests showed that islets isolated from HFD+DHA fed mice have a better insulin secretion response to glucose (Figure 20). We're now analysis if β cell mass and apoptosis is modulated with DHA supplementation diet. Interestingly, a preliminary experiment with a HFD for only 28 days showed that ceramide levels in plasma was affected by DHA supplementation: there was a significant decrease of circulating ceramide species (data not shown). In this context, we're now analysing if we have a similar output after 5 months of HFD +/-DHA.

These experiments showed for the first time that *in vivo*, DHA supplementation ameliorated β cell function through up-regulation of insulin secretion in response to glucose. It remains to determine if it is a direct or indirect effect of DHA on islets function. Moreover, we will need to determine the molecular mechanism involved in these beneficial effect of DHA on islets.

Figure 20

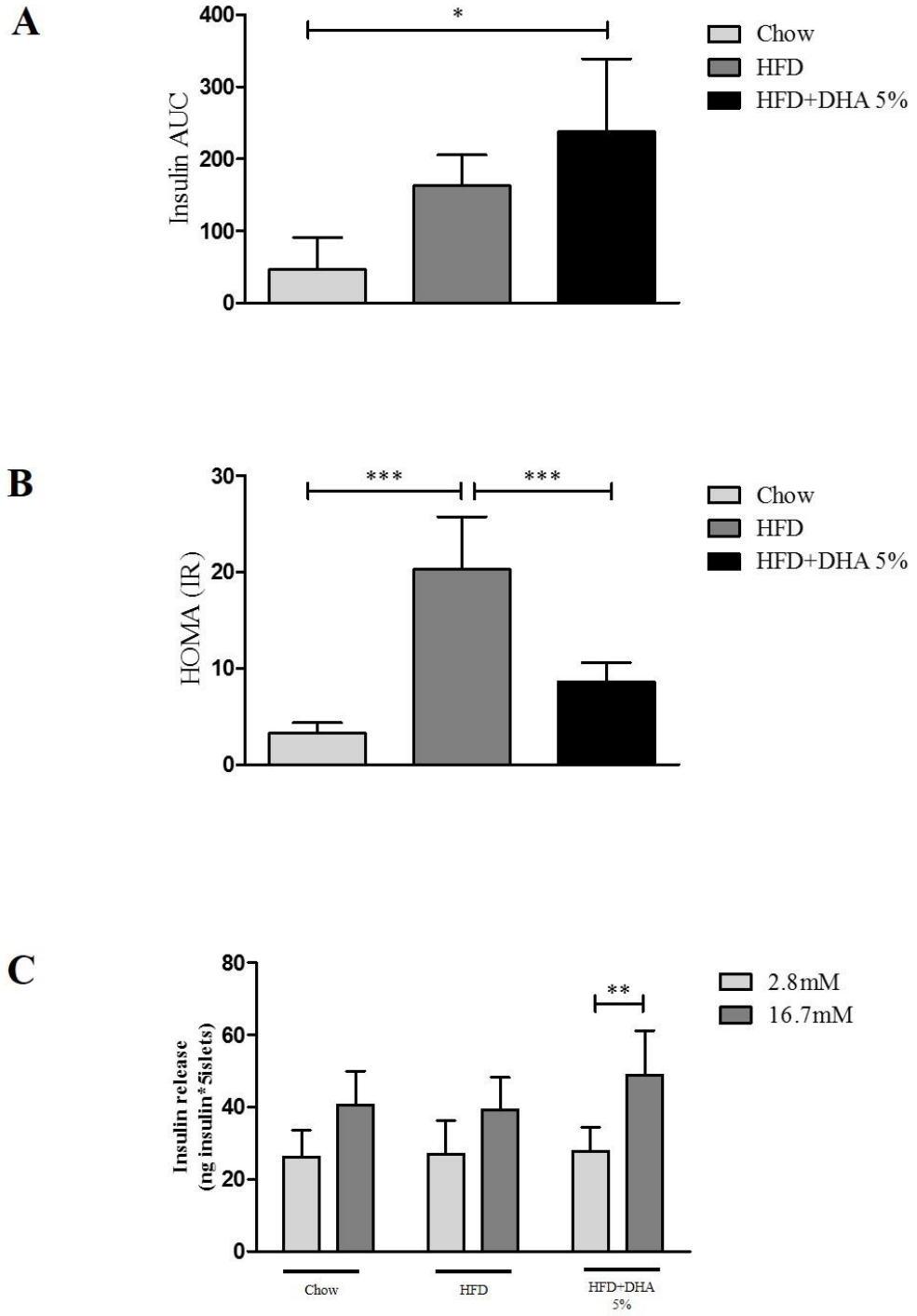


Fig. 20. DHA-supplementation effects on Glucose homeostasis. C57BL/6j male mice were fed for 20 week with chow, HFD or HFD+5%DHA diet. (A) Area under the curve (AUC) of insulin during OGTT test (Figure 18). (B) HOMA (IR) of OGTT test. (C) Insulin secretion test on isolated islets at the end of the experiment as described in material and methods. Results are represented as means ± s.e.m. of 6 different mice for group. *p<0.05, ** p < 0.01, *** p<0.001 one-way ANOVA test, Bonferroni post-test.

3.2 ELOVL2 KO MICE

An ELOVL2 conditional KO did not existed and we decided to generate an ELOVL2-Cre genetically modified mice. However, as presented on the introduction, a total ELOVL2-deficient mouse exists, but no experiments have been performed on islets of Langerhans (Pauter et al., 2014). In collaboration with Jacobsson's group, we performed some experiments on these Elovl2 KO mice. Preliminary data showed that isolated islets from *Elovl2* KO mice fed with one or two months of HFD do not properly response to glucose (Figure 21). These preliminary experiments are encouraging since they suggest that the lack of *Elovl2* could impact β-cell function and insulin secretion.

Figure 21

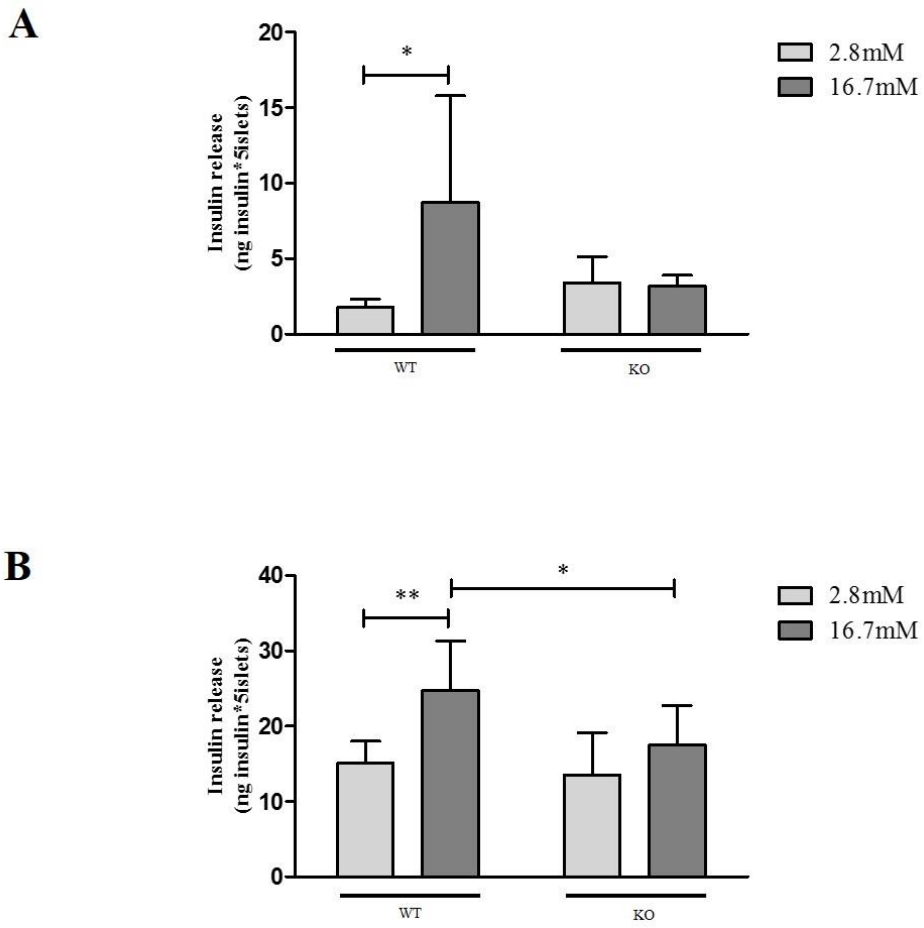


Fig. 21. HFD effects on *Elovl2*-KO mice islets. WT and *Elovl2*-KO mice were fed with HFD for 1 or 2 months (as described in Materials and Methods). (A) Insulin secretion test on isolated islets after 1 month of HFD (B) Insulin secretion test on isolated islets after 2 months of HFD. Results are represented as means ± s.e.m. of 3WT and 5KO mice for A and 7WT and 9KO for B. *p<0.05, ** p < 0.01, *** p<0.001 one-way ANOVA test, Bonferroni post-test.

4 FINAL CONCLUSIONS

During my thesis, I investigated the role of endogenous and exogenous role of DHA in pancreatic β-cell dysfunction under GL. I demonstrated that ELOVL2/DHA axis protects pancreatic β-cells from GL dysfunctions. I investigated the effects of cell's stimulation with different glucose concentrations and palmitate, demonstrating that GL negatively regulates *Elovl2* mRNA and protein levels in two pancreatic β-cell lines and in isolated islets of Langerhans.

ELOVL2/DHA axis ameliorates glucose-stimulated insulin secretion. Regarding the palmitate-decreased insulin content, DHA did not restored to normal insulin levels into these cells. Investigation of the possible involved pathways leads me to exclude an implication of the G-coupled protein receptors GPR40 and GPR120 and the proteins kinase PKCδ and PKCθ in the protective effect of DHA against GL-induced insulin secretion inhibition. However, further experiments are necessary to determine the mechanism involved on ELOVL2/DHA mediated protection.

I showed for the first time that ELOVL2/DHA axis protects pancreatic β-cells from GL-induced apoptosis. While the lack of ELOVL2 protein exacerbated GL-induced apoptosis. Mechanisms involved on the β-cells protection is linked to an inhibition of ceramide accumulation. The reduced ceramide content, in response to DHA under GL, seems to be due to inhibition of ceramide formation induced by GL. DHA seemed to no alter enzyme involved in *de novo* ceramide synthesis but rather it acts at the levels of ceramide trafficking between ER and Golgi apparatus. Indeed, DHA restored Ceramide transporter CERT protein levels under GL conditions, and partially inhibit the negative effect of GL on ceramide trafficking. This result suggest that ELOVL2/DHA axis could limit ceramide accumulation under GL by favouring their conversion into sphingomyelin and glucosyl-ceramide. However, inhibition of pathways involved in sphingomyelin ceramide and glucosyl-ceramide synthesis do not inhibit the DHA protective effects. I also demonstrated that DHA was not inhibiting ceramide's deleterious effect, since DHA is unable to counteract β-cell apoptosis induced by exogenous ceramide.

My data suggest that DHA is protecting pancreatic β cells through the enhancement of the mitochondrial β-oxidation pathway. Indeed, inhibition of CPT1 by the etomoxir was blocking

the protective effects of ELOVL2/DHA axis on GL-induced apoptosis. It would be interesting to determine if the β-oxidation is also mediating the ELOVL2/DHA protection from the GL-inhibition of the insulin secretion.

From this work, it appears that DHA could affect different metabolic/signalling pathways, but the most powerful effect is its ability to control fatty acid partitioning. DHA is able to completely change palmitate utilization in cell in order to block its transformation into toxic lipids such as ceramide and force its utilization by different pathways, such as TAG synthesis and β-oxidation. *In vivo*, DHA supplementation seems to have similar effects against the deleterious effect of obesity in other tissues since it decreases plasma circulating ceramide levels, fat mass, and enhanced glucose tolerance by enhancing and finer insulin secretion.

In future, it would be possible to test the *in vivo* ELOVL2 role in pancreatic β-cells, thanks to the generation of *Elovl2*-INS1-Cre mice by our lab. Further experiments will define precisely the role of β-oxidation in the effect of ELOVL2/DHA axis against GL, but also elucidate a possible involvement of TAG synthesis. In particular, we will determine the role of AMPK and PPARα in the effects of ELOVL2/DHA axis. Altogether, these data will help us to understand the mechanisms involved in the protective effects of ELOVL2/DHA axis and potentially propose new therapeutic targets to fight against the deleterious effects of gluco-lipotoxicity on β cells and the appearance of type 2 diabetes.

IV MATHERIALS AND METHODS

Materials

Tissue culture media and HBSS were from Life Technology. [γ^{32}P]ATP was purchased from Perkin Elmer. Palmitate, DHA, EPA, fatty acid-free BSA, AICAR, Fumonisin B1, anti β-Actin antibody and collagenase type V were from Sigma Aldrich. Diacylglycerol kinase, RO-318220 and Etomoxir were from Calbiochem. Apo-ONE homogenous caspase-3/7 assay was from Promega. All solvents were from Merck Eurolab or Fisher Scientific. C2-ceramide were from Avanti polar lipids. AH7614, GSK 137647, GW9508 and Rottlerin were purchased from Tocris Biotechnology. D609, PPMP were from Enzo. Anti-HA, anti-Elovl2, anti-PARP, anti-CERT and anti-β-actin antibodies were from Covance, InterChim, Cell Signalling and Abcam, respectively. siRNA against Elovl2 and control siRNA, lipofectamine LTX and RNAiMAX were purchased from Invitrogen. Metabolex 36, AZ670, AZ423 were kindly provided from AstraZeneca. Ad-Elovl2 were purchased from SignaGen Laboratories.

The experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40).

Elovl2 KO mice:

Elovl2 ($^{-/-}$) mice and WT mice (Zadravec et al., 2011) were housed on a 12-hour light/dark cycle and were fed a high-fat diet high-sucrose diet (D12451 Research Diets, 45% kcal fat expressed in Kcal/kg) for 1 or 2 months as already described in Pauter et al (Pauter et al., 2104).

DHA supplemented-diet:

8 weeks old C57BL6/J mice were housed on a 12-hour light/dark cycle and were fed a standard rodent chow (SAFE A04) or high-fat high-sucrose diet (SAFE 230, with 60% fat expressed in Kcal/kg) supplemented or not with 5% DHA oil (Polaris, Omegavie 80EE). The weight and the food intake were measured three times a week, ad libitum glycemia was measured once a week, fat mass and lean mass were determined by EcoMRI scan twice a month. ITT tests were performed at week 7, 12 and 17 of diet, OGTT tests were performed at week 8, 13, and 18 of diet. At the end of the treatment, mice were sacrificed and the islets of Langerhans isolated.

Islet isolation

Islet isolation was performed on 8-12 week old male C57BL/6j mice as previously described (Karaca et al. 2009). Briefly, collagenase solution were injected through bile duct, and pancreas

were incubated at 37°C for collagenase digestion. The digestion reaction was stopped by the addition of cold HBSS buffer supplemented with BSA. The digested pancreas were washed different times. A four layers density gradient were performed in order to isolate the islets from the exocrine tissue. Islets were handpicked under a stereomicroscope.

Cell and islet culture conditions

Rat Insulinoma INS-1 cells were grown in RPMI 1640 medium buffered with 10mM HEPES containing 10% fetal bovine serum (FBS), 2mM L-glutamine, 1mM sodium pyruvate, 50µM 2-β-mercaptoethanol and 100 U/ml penicillin/streptomycin. Prior to each experiment, cells were plated for 48 h in 96-well plates ($22x10^3$ cells/well) for MTT assay and caspase-3/7 activity; in 6-well plates ($1x10^6$cells/well) for RNA, protein and ceramide levels; in 50mm plates ($5x10^6$ cells/plate) for analysis of sphingolipids and fatty acid species by LC-MS/MS and GC-MS, respectively.

Mouse Insulinoma MIN-6 cells were grown in DMEM high glucose medium buffered with 15% fetal bovine serum (FBS), 50µM 2-β-mercaptoethanol and 100 U/ml penicillin/streptomycin. Prior to each experiment, cells were plated for 48 h in 96-well plates ($100x10^3$ cells/well) for insulin secretion test; in 6-well plates ($1x10^6$cells/well) for RNA and protein; in 50mm plates ($5x10^6$ cells/plate) for analysis of sphingolipids and fatty acid species by LC/MS/MS and GC-MS, respetively.

Fatty acids (Palmitate, DHA and EPA) were administered to the cells as a conjugate with fatty acid-free BSA. Briefly, dried aliquots of fatty acid in ethanol were dissolved in PBS containing 5% BSA to obtain a 4mM stock solution. The molar ratio of FFA to BSA was 5:1. The FFA stock solutions were diluted in RPMI supplemented with 1% FBS (described hereafter as incubation medium) to obtain a 0.4mM palmitate final concentration, and 0.1mM or 0.01mM DHA or EPA final concentrations.

Isolated islet where culture ex vivo in RPMI medium buffered with 10% FBS, 10mM HEPES, 1mM Sodium Pyruvate, 50µM β-mercaptoethanol and 100 U/ml penicillin/streptomycin. After one recovery night, islets have been stimulated with Palmitate 0.4mM and different glucose concentration for 24hours for RNA analysis, and for 48hours for protein and insulin secretion tests. Islets were maintained free floating at 37 °C in a humidified atmosphere of 95%O_2–5%CO_2 for 24h before use.

Cell transfection

INS-1 cells and MIN-6 cells were transiently transfected with 30 nmol of sequence-specific siRNA against Elovl2 and control siRNA using Lipofectamine RNAiMAX (Invitrogen). Cells and islets were transfected with Ad-Elovl2 provided from SignaGen Laboratories. Tests were performed 48 hours after infection.

Insulin secretion

MIN6 cells were seeded in 96-well plates, after 48 hours they were treated for 24 hours with fatty acids (palmitate, DHA and EPA) in the presence of various glucose concentrations, and inhibitors. Cells were then pre-incubated in DMEM without glucose for 30 minutes (deprivation phase), in KREBS containing 0.1% fatty-acid free BSA and 2 mM glucose for 30 min (stabilization phase). Insulin secretion was measured following a 30 min incubation in KREBS containing 0.1% fatty-acid free defatted BSA with 2mM glucose or 20mM glucose. Cells were extracted with protein lysis buffer and used for protein and insulin content quantification. Insulin concentration in medium was determined with Ultra Sensitive Mouse Insulin ELISA Kit (EUROBIO). Each condition was tested in triplicate.

Islets has been stimulated *ex vivo* for 48 h in culture medium supplemented with fatty acids (palmitate, DHA and EPA) in the presence of various glucose concentrations. Then, islets (batch of 5 islets in triplicate) in a 96-filtered-well plate were pre-incubated in KREBS—0.05% BSA with 2.8 mM glucose for 30 minutes, followed by 60 min incubation in KREBS —0.05% BSA with 2.8 or 16.7 mM glucose to measure glucose-induced insulin secretion. Each condition was tested in triplicate. Islets were extracted with protein lysis buffer and insulin concentration in the medium was quantified by ELISA kit (EUROBIO).

Western Blotting

Equal amounts of proteins were separated by 10% SDS-PAGE and then transblotted to nitrocellulose. Blots were probed with either anti-PARP (Cell Signalling), anti-Elovl2 (Interchim), anti-V5 (Invitrogen), anti-CERT (Abcam) antibody, stripped, and re-probed with a polyclonal anti-βactin antibody (Sigma). Immuno-reactive bands were visualized by enhanced chemiluminescence with appropriate horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch) and SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Measurement of caspase-3/7 activity

Caspase-3/7 activity assays were performed with the Promega Apo-ONE Homogeneous Caspase-3/7 Assay kit as described previously (Véret et al. 2011). Briefly, lysis buffer containing fluorogenic Z-DEVD-R110 substrate was added to each well and fluorescence was measured every 6 min over a 210 minutes period using a Fluostar plate reader set at 37°C (excitation at 485 nm and emission at 530 nm). Caspase-3/7-specific activity was expressed as the slope of the kinetic in arbitrary units. Each experimental condition tested was performed in triplicate.

Analysis of the Intracellular Distribution of Fluorescent Ceramides

INS-1 cells were plated in 6 well plate and grown on a glass coverslip and cultured as previously described (Gjoni et al. 2014). At the end of the treatments, cells were loaded with 2.5 µM BODIPY-C5-Cer or NBD-C6-Cer (as 1:1 complex with fatty acid free BSA) in RPMI 1640 at 4 °C for 30 min. After loading, cells were incubated 30 min at 37 °C in RPMI 1640 containing 5 mM or 30 mM glucose ± 0.4 mM palmitate with or without DHA and fixed with 0.5% glutaraldehyde solution in PBS for 10 min at 4 °C. Samples were immediately observed and analyzed with a fluorescence microscope (Olympus BX-50) equipped with a fast high resolution charge-coupled device camera (Colorview 12) and an image analytical software (Analysis from Soft Imaging System GmbH).

Oral glucose tolerance test (OGTT)

Mice were food deprived overnight prior to an oral administration of 2g/Kg of 30% glucose solution. Blood was sampled from the tail vein at 0, 15, 30, 60, 90 and 120 min in order to assay glucose concentration with a glucometer (A. Menarini Diagnostics, France). At 0, 15, 30, 60 and 120 min blood was also taken to measure plasma insulin with Ultra-Sensitive Mouse Insulin ELISA Kit (EUROBIO).

Insulin tolerance test (ITT)

Mice were food deprived for 5 hours prior to intra-peritoneal administration of insulin (Novorapid®) at 0.5UI/Kg. Blood was sampled from the tail vein at 0, 15, 30, 45, 60, 90 and 120 min in order to assay glucose concentration with a glucometer (A. Menarini Diagnostics, France).

Quantitative PCR

Total RNA was isolated from INS-1 cells using the RNeasy mini kit (Qiagen). Total RNA (4 µg) from each sample was reverse transcribed with 40 U of M-MLV Reverse Transcriptase (Invitrogen) using random hexamer primers. Real-time quantitative PCR amplification reactions were carried out in a LightCycler 1.5 detection system (Roche) using the LightCycler FastStart DNA Master plus SYBR Green I kit (Roche). Reverse transcribed RNA (10 ng) was used as the template for each reaction. All reactions were run in duplicate with no template control. The PCR conditions were: 95°C for 10 min, followed by 40 cycles at 95°C for 10 s, 60°C for 10 s and 72°C for 10 s. mRNA transcript levels of 4 housekeeping genes (rpL19, Tbp, cyclophilin a, 18S) were assayed. Since similar results were obtained with the 4 housekeeping genes, only rpL19 was retained for normalization of other transcripts. Primer sequences are: *Elovl2* rat gctacaacttgcagtgtcagaatc (forward) and accacaagaccttggctacc (reverse), *Elovl2* human tgtgtttccatctatgcacaagt (forward) and cagctgagcctgtgtgagat (reverse), rpL19 rat ccacaaactgaaggcag (forward) and ctccttggacagagtctt (reverse), rpL19 human tcaaaaacaagcggattctca (forward) and gcgtgcttccttggtcttag (reverse).

Lipid extraction for fatty acid measurement

Lipid extraction was performed as already described in Bligh and Dyer (Bligh et Dyer, 1959). Cell pellets were mixed with 800µL of boron trifluoride-methanol solution (BF3, 14% methanol, Sigma) and 10µg of hepradecanoic acid (C17:0) was added as internal standard. The mixture was heated at 100°C for 40minutes and cooled down to room temperature. 400µl of heptane and 800µl of H_2O were added, the solutions were vortex-mixed, and centrifuged. The fatty acid methyl esters (FAME) phase were extracted and concentrated by speedvac (Jouan, Saint-Herblain, France). The phase were successively diluted in 100µl heptane and the methyl formed fatty acid esters were analysed by gas chromatography-mass spectrophotometer single quadrupole-type (GCMS-QP2010, Shimadzu). 1µl of each sample were injected into a Suplcowax-type-10 column (30mm length x 0.25mm internal diameter x 0.25 µm thickness) A detector amplified and converted the signals. The FAME picks were identified by comparison of their retention time with the retention times of highly purified FAME standard.

Lipid extraction and sample preparation for LC/MS/MS

Cellular lipids were extracted by modified Bligh and Dyer procedure (Bligh et Dyer 1959) with the use of 0.1 N HCl for phase separation. C17-Cer (30 pmol) employed as internal standards,

was added during the initial step of lipid extraction. The extracted lipids were dissolved in methanol/chloroform (4:1, v/v) and aliquots were taken out to determine total phospholipid content. Samples were concentrated under a stream of nitrogen, redissolved in methanol, transferred into autosampler vials and subjected to consecutive liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

Enzymatic measurement of ceramide levels

Ceramide levels in cellular extracts were measured by the diacylglycerol (DAG) kinase enzymatic method as previously described (Véret et al. 2011). Briefly, aliquots of the chloroform phases from cellular lipid extracts were resuspended in 7.5% (w/v) octyl-β-D-glucopyranoside/5 mM cardiolipin in 1 mM DETPAC/10 mM imidazole (pH 6.6). The enzymatic reaction was started by the addition of 20 mM DTT, 0.88 U/ml E. coli DAG kinase, 5 μCi/10 mM [γ-^{32}P]ATP and the reaction buffer (100 mM imidazole (pH 6.6), 100 mM NaCl, 25 mM MgCl2, and 2 mM EGTA). After incubation for 1 h at room temperature, lipids were extracted with chloroform/methanol/HCl (100:100:1, v/v) and 1 M KCl. [γ-^{32}P]-ceramide phosphate was resolved by TLC with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, v/v) and quantified with a phosphorimager (Storm, Amersham). Known amounts of bovine ceramide standards were included in each assay. Ceramide levels are expressed as fmol by nmol of phospholipid (PL) levels. Each measurement was done in duplicate.

Measurement of total cellular phospholipids

Total phospholipids present in cellular lipid extracts used for ceramide analysis were quantified as described previously (Véret et al. 2011) with minor modifications. Briefly, a mixture of 10N H2SO4/70% perchloric acid (3:1, v/v) was added to lipid extracts which were incubated for 30 min at 210°C. After cooling, water and 4.2% ammonium molybdate in 4 N HCl/0.045% malachite green (1:3 v/v) was added. Samples were incubated at 37°C for 30 min, and absorbance was measured at 660 nm.

Analysis of ceramide species by LC-MS/MS

Analysis of ceramide species was performed by LC-MS/MS as described previously (Berdyshev et al 2006). Instrumentation employed was an API4000 triple quadrupole mass spectrometer (Applied Biosystems) interfaced with an automated Agilent 1100 series liquid chromatograph and autosampler (Agilent Technologies). Briefly, sphingolipids were ionized

via electrospray ionization (ESI) with detection via multiple reaction monitoring (MRM) in positive ions mode. Ceramide molecular species were resolved using a 3 x 100 mm X-Terra XDB-C8 column (3.5 µm particle size, Waters, Milford, MA) and a gradient from methanol/water/formic acid (61:39:0.5, v/v) with 5 mM ammonium formate to acetonitrile/chloroform/water/formic acid (90:10:0.5:0.5, v/v) with 5 mM ammonium formate at a flow rate of 0.5 ml/min. MRM transitions monitored for the elution of ceramide molecular species were as follows: m/z 510>264, 14:0-Cer; m/z 538>264, 16:0-Cer; m/z 540>284, 16:0-DHCer; m/z 552>264, 17:0-Cer (internal standard); m/z 564>264, 18:1-Cer; m/z 566>284, 18:1-DHCer; m/z 566>264, 18:0-Cer; m/z 568>284, 18:0-DHCer; m/z 594>264, 20:0-Cer; m/z 596>284, 20:0-DHCer; m/z 624>284, 22:0-DHCer; m/z 650>264, 24:1-Cer; m/z 652>284, 24:1-DHCer; m/z 652>264, 24:0-Cer; m/z 654>284, 24:0-DHCer; m/z 680>264, 26:1-Cer; m/z 682>264, 26:0-Cer; m/z 708>264, 28:1-Cer; m/z 710>264, 28:0-Cer.

Standard curves for each of ceramide molecular species were constructed via the addition of increasing concentrations of the individual analyte to 30 pmol of the structural analogs of the sphingolipid classes used as the internal standards. Linearity and the correlation coefficients of the standard curves were obtained via a linear regression analysis. The standard curves were linear over the range of 0.0 – 300 pmol of each of the sphingolipid analytes with correlation coefficients (R2) >0.98. Parameters of declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were determined for each individual analyte by the infusion of the corresponding standards. Turbo-V ion source was operated at 550°C, GS1=40, GS2=50, and Curtain Gas=20. Correction for ion suppression by the matrix was controlled by creating standard curves in the presence of total lipid extract from human pulmonary artery endothelial cells (4 nmol total lipid phosphorus).

Statistical analysis

Data are expressed as means ± S.E.M. Significance was assessed by the Student's t test unpaired and two-tailed. P values less than 0.05 were considered as significant.

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Review

Roles of Sphingolipid Metabolism in Pancreatic β Cell Dysfunction Induced by Lipotoxicity

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Abstract: Pancreatic β cells secrete insulin in order to maintain glucose homeostasis. However, various environmental stresses such as obesity have been shown to induce loss of secretory responsiveness in pancreatic β cells and pancreatic β cell apoptosis which can favor the development of type 2 diabetes (T2D). Indeed, elevated levels of free fatty acids (FFAs) have been shown to induce β cell apoptosis. Importantly, the chronic adverse effects of FFAs on β cell function and viability are potentiated in the presence of hyperglycaemia, a phenomenon that has been termed gluco-lipotoxicity. The molecular mechanisms underlying the pathogenesis of gluco-lipotoxicity in pancreatic β cells are not completely understood. Recent studies have shown that sphingolipid metabolism plays a key role in gluco-lipotoxicity induced apoptosis and loss of function of pancreatic β cells. The present review focuses on how the two main sphingolipid mediators, ceramides and sphingoid base-1-phosphates, regulate the deleterious effects of gluco-lipotoxicity on pancreatic β cells. The review highlights the role of a sphingolipid biostat on the

dysregulation of β cell fate and function induced by gluco-lipotoxicity, offering the possibility of new therapeutic targets to prevent the onset of T2D.

Keywords: obesity; type 2 diabetes; gluco-lipotoxicity; islet of Langherans; ceramide; sphingosine-1-phosphate; sphingolipids; apoptosis; insulin; pancreas

Abbreviations

FFA, free fatty acid; LC-CoA, long chain acyl-CoA; S1P, shingosine-1-phosphate; CPT-1, carnitine palmitoyl-CoA transferase 1; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; ER, endoplasmic reticulum; SPT, serine palmitoyl-transferase; DH-Sph, dihydrosphingosine; CerS, ceramide synthases; PASK, serine/threonine Per-Arnt-Sim domain-containing kinase; SMS1, sphingomyelin synthase 1; SphK, sphingosine kinase.

1. Introduction

In mammals, the levels of plasma glucose are tightly regulated to maintain normo-glycemia, and chronic hypoglycemia or hyperglycemia can result in injuries to various tissues. Insulin secreted by pancreatic β cells plays a major role in the maintenance of normo-glycemia. The biological machinery of pancreatic β cells enables them to regulate the rate of insulin secretion in response to variations in plasma glucose levels. Diabetes is a disease characterized by a chronic abnormal elevation of plasma glucose concentrations. Type 1 diabetes is an autoimmune disease against pancreatic β cells whereas type two diabetes (T2D) is a multi-factorial disease due to two principal dysfunctions: (1) a loss of insulin secretion and (2) insulin resistance, which is defined by the failure of insulin to elicit anabolic effects and stimulate glucose uptake in targeted tissues.

The etiology of T2D is not well established but it is certain that loss of insulin secretion is directly linked to a loss of function and apoptosis of pancreatic β cells [1,2]. The insulin secretory capacity and reduction in β cell mass, observed during T2D, are thought to be amplified by the development of chronic hyperglycaemia, a phenomenon that has been termed "glucotoxicity" [2]. In addition to hyperglycaemia, accumulated evidence suggests that T2D is often associated with abnormalities in lipid metabolism and excessive levels of circulating lipids [3,4]. Indeed, it has been shown that 44% of diabetic patients are also obese [5], suggesting a link between obesity and T2D. Free fatty acids (FFAs) are important physiological fuels for islets, and act as supplemental nutrients able to potentiate insulin secretion in response to glucose [6,7]. However, chronically elevated levels of FFAs in circulation have been postulated to cause peripheral insulin resistance and impairment of β-cell insulin secretion, a phenomenon that has been termed "lipotoxicity" [8,9]. The molecular mechanisms underlying the pathogenesis of lipotoxicity in pancreatic β cells are not completely understood. Usually, low levels of FFAs are readily degraded by β-oxidation and are therefore non-toxic for β-cells. In contrast, chronic elevation of FFA levels raised the levels of long chain acyl-CoA (LC-CoA), which serve for complex lipid synthesis [8,10]. Interestingly, it has been proposed that a specific class of lipids, namely sphingolipids, and in particular ceramides, are important mediators of

FFA-induced β cell dysfunction and apoptosis [8]. In the present review, we will illustrate the mechanisms involved in pancreatic β cell lipotoxicity with a special focus on the role of ceramide metabolism at the molecular, cellular and systems levels. In addition, we will also discuss the role of other sphingolipid metabolites, such as sphingosine-1-phosphate (S1P) in the control of pancreatic β cell lipotoxicity. A better understanding of the role of sphingolipid metabolism involved in pancreatic β cell lipotoxicity may open up the potential for identification of pharmacological targets for the prevention and/or treatment of obesity associated T2D.

2. Lipid Metabolism in Pancreatic β Cells

Lipids have many biological functions; they serve as energy reserves, signalling molecules, and as major membrane components. In pancreatic β cells, lipids are extracted from plasma lipoproteins through lipoprotein lipases secreted by pancreatic β cells [11]. Lipids are converted into long chain acyl-CoA (LC-CoA) by the Acyl-CoA synthase in the cells [12]. LC-CoA may be then exported to mitochondria through carnitine palmitoyl-CoA transferase 1 (CPT-1) before being channeled into the β-oxidation pathway in order to provide energy. Interestingly, the fate of LC-CoA in pancreatic β cells is determined by intracellular glucose levels. At low glucose concentrations, FFAs are used to produce energy through the β-oxidation pathway [13]. In contrast, at high glucose concentrations, FFA metabolism will be preferentially oriented to lipid esterification. The main metabolites able to control the lipid metabolism in response to glucose appear to be malonyl-CoA and the LC-CoA [10]. High glucose concentrations accelerate its metabolism and increase intra-cytoplasmic citrate concentration through the Krebs cycle. The resulting citrate is then converted into malonyl-CoA by the acetyl-CoA carboxylase (ACC) [14–16]. Normally, malonyl-CoA is used as a substrate by FFA synthase (FAS) to produce FFAs; however in pancreatic β cells, the activity of FAS is lower than the activity of ACC [17], favouring the accumulation of malonyl-CoA. Interestingly, increased malonyl-CoA levels can inhibit CPT-1 activity leading to a reduction in mitochondrial transport of LC-CoA and its subsequent β-oxidation. The consequences of cytoplasmic FFA accumulation are the production of lipids derived by various metabolic pathways, such as esterification or diglyceride synthesis [14,18], or phospholipid synthesis [19,20]. In addition to these effects, the glucose also induces the expression of genes involved in lipogenesis via the transcriptional factor SREBP1c, which activates *de novo* lipogenic genes [21].

3. The Phenomenon of Pancreatic β Cell Gluco-Lipotoxicity

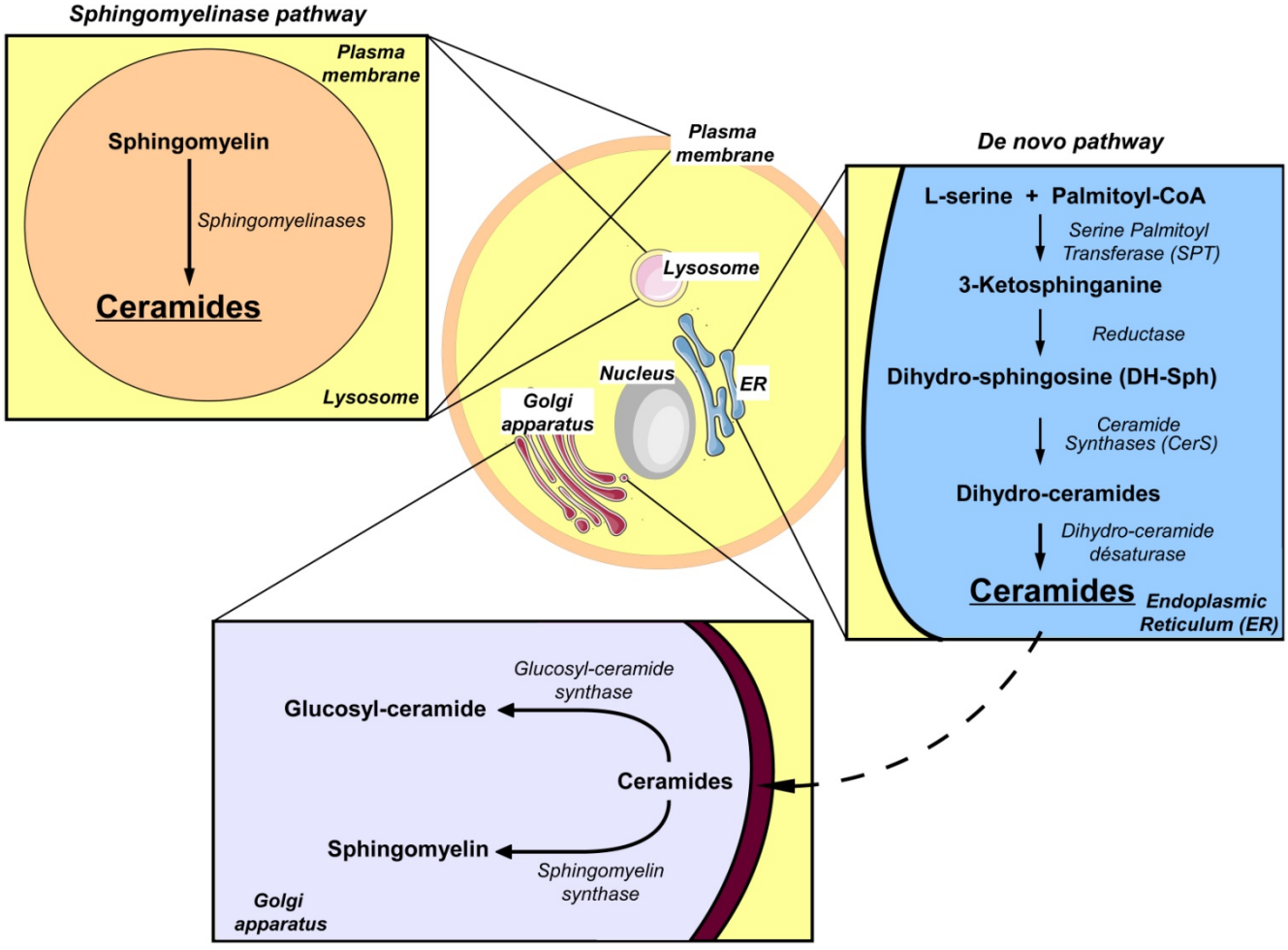
It has been estimated by the World Health Organization that obesity is the main epidemic of the century, affecting about 1 billion people around the world [5]. Obesity has many deleterious physiological effects like cellular energy imbalances that can result in impairment of insulin secretion and the development of insulin resistance. Many hypotheses suggest a link between insulin resistance and obesity and T2D. Among these hypotheses, it has been proposed that hypoxia, fibrosis, and inflammation observed during obesity will impair adipose expansion and therefore the storage of excess FFAs. This phenomenon leads to ectopic storage of lipids [22–24]. The FFAs which accumulate in non-adipose tissues such as muscle and liver induce the activation of non-oxidative metabolic pathways. These pathways can lead to the production of toxic lipids. This phenomenon is

called lipotoxicity [22–24]. It has been shown that lipotoxicity can result in insulin resistance of skeletal muscle, hepatic steatosis [25,26] and impairment of insulin secretion by β-cells [8,9]. Indeed, palmitate, one of the most abundant FFAs in plasma, has detrimental effects on β-cell function, including impairment of glucose-induced insulin release [27,28], defective insulin gene expression [29–31], and induction of β-cell apoptosis [32–36]. Importantly, the chronic adverse effects of FFAs on β cell function and viability are potentiated by hyperglycaemia, a phenomenon that has been termed "gluco-lipotoxicity" [37,38]. It has been observed that β cell apoptosis induced by palmitate is highly potentiated in the presence of elevated glucose concentrations [32,39]. As proposed above, when glucose and lipid levels are simultaneously elevated, glucose inhibits FFA oxidation and stimulates the incorporation not only of endogenous LC-CoA but also excess FFAs into the synthesis of complex lipids [8,10,39]. Interestingly, among these lipids produced, ceramides have been suggested to be important mediators of FFA-induced β-cell dysfunction and apoptosis [8,39,40].

4. Sphingolipid Metabolism

It is well established that sphingolipids are structural components of cellular membranes. However, several studies have now shown that sphingolipids have important roles in the regulation of some cellular processes [41,42]. Moreover, the deregulation of these processes plays a key role in the onset of pathologies affecting cellular proliferation and apoptosis [42–44]. Several pathways for sphingolipid metabolism have been described [45]. One of these pathways is the *de novo* synthesis pathway which is initiated on the cytoplasmic face of the endoplasmic reticulum (ER) (Figure 1). The first step of this pathway is the condensation of L-serine with palmitoyl-CoA to form 3-ketosphinganine. This reaction is catalyzed by serine palmitoyl-transferase (SPT) [41]. 3-Ketosphinganine is then reduced to dihydrosphingosine (DH-Sph) by 3-ketosphinganine reductase. The resulting DH-Sph acts as a substrate for ceramide synthases (CerS) leading to the production of dihydro-ceramides. Dihydro-ceramides can be transformed into ceramides by dihydroceramide desaturases [46]. Depending on the LC-CoA attached to the DH-Sph by CerS, the dihydroceramides and the ceramides are classified into different species with varying FA chain lengths (14–32 carbons in mammals) and saturation of the carbon chain [47]. Ceramides can be transported to the Golgi apparatus [48] where they are converted into sphingomyelin by sphingomyelin synthase or into glucosyl-ceramides by glucosyl-ceramide synthase. In addition to *de novo* synthesis, another metabolic pathway also leads to ceramide production and this involves the degradation of sphingomyelin into ceramide by sphingomyelinases. This process takes place in the lysosomal membrane and the cytoplasmic membrane [41]. In this web of sphingolipid metabolism, ceramides are considered as central players in the metabolism of sphingolipids. Indeed, ceramides are the precursors of many lipid messengers. For example, ceramides can be metabolized into sphingosine which is in turn phosphorylated to yield sphingosine-1-phosphate (S1P) [41]. Ceramides can also be phosphorylated by ceramide kinase to form ceramide-1-phosphate [41].

Figure 1. Synthesis of sphingolipids in mammalian cells. Two main pathways to produce sphingolipids exist in mammals. The *de novo* pathway of synthesis starts on the cytoplasmic face of the endoplasmic reticulum (ER). The first step of this pathway is the condensation of L-serine with palmitoyl-CoA to form a 3-ketosphinganine. This reaction is catalyzed by serine palmitoyl-transferase (SPT). 3-Ketosphinganine is then reduced to form dihydrosphingosine (DH-Sph) by 3-ketopshinganine reductase. DH-Sph is a substrate of ceramide synthases (CerS) which produce dihydro-ceramide. Dihydroceramides are transformed into ceramides by dihydroceramide desaturase. Ceramide can be transported to the Golgi apparatus to be transformed into more complex sphingolipids such as sphingomyelin and glucosyl-ceramides. A second synthesis pathway is the catabolic pathway that leads to the degradation of sphingomyelin (SM) into ceramides by sphingomyelinases. This process takes place in the lysosomal and plasma membranes.



5. Ceramide and Pancreatic β Cell Gluco-Lipotoxicity

As noted above, among the lipids produced during lipotoxicity, ceramides have been implicated in the deleterious effects of gluco-lipotoxicity on pancreatic β cells and in a more general manner, on T2D and obesity. For example, patients with T2D present an increase in sphingolipid levels in plasma, muscle, adipose tissue and liver [25,49]. The accumulation of sphingolipids has been correlated with an increase in insulin resistance [50,51], loss of function and death of pancreatic β cells [8].

Additionally, sphingolipids also appear to also play a role in the complication of obesity and T2D. Interestingly, the two forms of SPT (SPT 1 and 2) have a Km for L-serine and the palmitoyl-CoA near to their physiological concentrations [52,53], and as such, are sensitive to relatively small variations in the concentrations of their substrates. Moreover, condensation of L-serine and palmitoyl-coA is the rate-limiting step in *de novo* synthesis of ceramides [54]. These two characteristics of SPTs suggest that excessive concentrations of palmitate and its activated form palmitoyl-CoA in a cell can induce the aberrant production of ceramides. It has been shown that diminution of pancreatic β cell mass and loss of insulin secretion are correlated to an increase in intracellular ceramide levels and SPT expression in islet of Langerhans of obese Zucker diabetic fatty (ZDF) rats [35].

5.1. Effect of Ceramide on Pancreatic β Cell Function

Previous studies have demonstrated that cellular permeable analogues of ceramide impaired insulin production in pancreatic β-cells [55]. Under gluco-lipotoxic conditions, several studies showed a correlation between *de novo* ceramide synthesis and defective insulin expression in islet of Langerhans [8]. In islet of Langerhans and pancreatic β cell lines, ceramide dependent inhibition of insulin expression is mediated by the dephosphorylation of protein kinases, ERK1/2 by PP2A [56,57]. Moreover, the *de novo* ceramide pathway inhibits the nuclear translocation of two important transcription factors, PDX-1 and Mafa, for insulin-induced gene expression [8]. However, the intracellular signaling pathways mediating these effects are mostly unknown. Previous studies have shown that glucose regulates insulin-induced gene transcription via the serine/threonine Per-Arnt-Sim domain-containing kinase (PASK) [56]. Interestingly, palmitate decrease glucose-induced expression of PASK mRNA and protein levels [56], suggesting that ceramides may function as negative regulators of PASK. It appears that in pancreatic β cells, inhibition of ceramidase, which increased intracellular levels of ceramides, also leads to a reduction in insulin expression [57]. More recently, genetic deletion of sphingomyelin synthase 1 (SMS1) has been shown to induce accumulation of ceramides in islet of Langerhans, dysfunction of mitochondria and the subsequent inhibition of insulin secretion [58]. In Yano's study, accumulation of ceramides affected mitochondria membrane integrity and induced an excessive production of ROS which were partially responsible for the defect in insulin secretion in pancreatic β cells [58]. Pharmacological inhibition of SMS or down-regulation by siRNA of the ceramide transporter (CERT), which regulate ceramide transport between the ER and Golgi apparatus, increased dysfunction of pancreatic β cells induced by palmitate by affecting binding of transcription factors to the insulin promoter [59]. Altogether, these results suggest that ceramide accumulation, either through the induction of *de novo* synthesis or reduced conversion into more complex sphingolipids, is involved in loss of function in pancreatic β cells.

5.2. Effect of Ceramide on Pancreatic β Cell Survival

It has been shown that ceramide analogues can block proliferation and induce apoptosis of β cells and islets of Langerhans in culture [60,61]. *De novo* ceramide synthesis induced by palmitate has also been implicated in pancreatic β cell apoptosis [8,39]. Indeed, inhibition of *de novo* ceramide synthesis abrogates pancreatic β cell apoptosis induced by palmitate [36,39,62]. More importantly, treatment of ZDF rats with a potent inhibitor of SPT, cyclo-serine, attenuated islet of Langerhans apoptosis

and prevented the appearance of hyperglycemia [35]. Ceramide accumulation induced by (gluco)-lipotoxicity induces pancreatic β cell apoptosis through different pathways. FFAs have been shown to induce accumulation of ceramides in ER of pancreatic β cells [40,63]. To date, the mechanisms used by (gluco)-lipotoxicity to block the transport of ceramides remain unknown. However, ceramide accumulation leads to ER stress which plays a key role in lipotoxic induced apoptosis [40,63,64]. Biden and co-workers showed that localized ceramide production and the associated decrease of SM levels in the ER in response to lipotoxicity is important to initiate ER stress in β cell [40,63]. Ceramide induced pancreatic β cell apoptosis is mediated by mitochondrial dysfunction and ROS production [60,61]. This result is also supported by the observation that ceramide dependent ROS production induced by gluco-lipotoxicity inhibits insulin secretion [8]. Evidence to date suggests that ceramide induce apoptosis through mitochondria injuries and subsequent production of ROS [65]. Nevertheless, the role of ROS in gluco-lipotoxicity is still unclear because in some cases, ROS have been excluded from ceramide-induced loss of function or apoptosis of pancreatic β cells [40,66]. Moreover, other hypotheses have been proposed about cellular signals involved in apoptosis of pancreatic β cell induced by ceramides. For example, it has been proposed that β cell apoptosis is induced through the activation of PP2A and the subsequent dephosphorylation of AKT [67], as seen in muscle tissues [68].

5.3. Role of Different Ceramide Species in Gluco-Lipotoxicity Induced Pancreatic β Cell Apoptosis

Ceramide species are distinguishable by the length and/or saturation of their *N*-acyl chains [47,69,70]. Because ceramide species have many biological roles depending on the cell type and on the inducing signals for their production [47], it is therefore important to determine changes in ceramide species in response to biological stimuli. Indeed, recent studies have reported distinct cellular functions for ceramides with specific *N*-acyl chain lengths [47,70]. The emergence of lipidomic analysis in the field of sphingolipid metabolism allows us to determine the effect of various treatments, not only on ceramide accumulation but also on changes in levels of ceramide species within the ceramide pool [52]. In pancreatic β cells, it appears that gluco-lipotoxicity modifies the levels of specific ceramide species, specifically C18:0, C22:0 and C24:1 [39]. In mammals, the variety of ceramide species relies on the existence of a family of enzymes, the ceramide synthases (CerS) [47,70]. Six CerS have been identified and they possessed a characteristic substrate preference for particular fatty acyl-CoA [47,70]. Interestingly, this increase in ceramide species is associated with an augmentation of CerS4 expression, which was found to play a critical role in pancreatic β cell apoptosis [39]. Collectively, the results suggest that gluco-lipotoxicity induces β cell apoptosis not only through the induction of *de novo* ceramide biosynthesis, but also through the formation of ceramides with specific carbon chain lengths rather than an overall increase in ceramide content. This hypothesis is supported by a recent study showing that pancreatic β cell apoptosis is potentiated when high glucose levels increased expression of FA elongase 6 (Elovl6) [71], an enzyme which catalyzes FFA elongation [72]. Importantly, the activity of Elovl-6 is mostly involved in elongation of *de novo*-synthesized palmitate to produce stearate, suggesting that gluco-lipotoxicity could mediate β-cell apoptosis at least through ceramide C18:0 by a concomitant up-regulation of CerS4 and Elovl-6. Recently, Prentki and co-workers have shown that gluco-lipotoxicity induced early changes in lipid partitioning in order to

induce β-cell dysfunction and apoptosis [73]. Indeed, gluco-lipotoxicity induced the expression of proteins which favored FFA esterification, such as SCD-1 through FFA desaturation, and decreased the expression of enzymes involved in lipid oxidation, such as the β subunit of AMP-kinase. These results suggest that in addition to downstream lipid partitioning induced by gluco-lipotoxicity, the synthesis of specific ceramide species could also play a critical role in β cell apoptosis. However, the precise role of specific ceramide species in β cell dysfunction induced by gluco-lipotoxicity remains to be clarified. Indeed, a recent study showed that the inability of CerS2 knock-out mice to synthesize C22–C24 ceramide species did not result in defective insulin secretion [74]. Therefore, it will be important, in the future, to validate these observations *in vivo* by using rodent models of T2D associated to obesity, and ultimately in human islets of Langerhans in order to identify potent therapeutic targets to limit pancreatic β cell gluco-lipotoxicity.

6. Role of Sphingoid Base Phosphates in T2D

During the past decade, other sphingolipid metabolites, namely sphingoid base phosphates, have been shown to have a role in T2D. The sphingoid base phosphates are S1P and its analogue dihydroS1P, which are respectively produced by phosphorylation of Sph and DH-Sph by sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2) [45]. S1P is known to induce proliferation, survival, migration and calcium mobilization in many cell types [42]. In T2D patients and rodent models of T2D, the activity of SphK1 and/or mRNA of SphK1 are increased in myocardium [75]. Interestingly, palmitate [76] or glucose [77,78], taken separately, not only increased sphingoid base phosphate levels but also SphK1 activity in different cell types such as muscle and endothelial cells. Moreover, treatment with S1P analogs protected cells from apoptosis [79,80] induced by hyperglycemia. In addition, *in vivo* over-expression of SphK1 gene in KK/Ay type 2 diabetic mice [81] or treatment mice under high fat diet with S1P analog [82] reduced the deleterious appearance of insulin resistance and T2D. Nevertheless, the role of the SphK1/S1P axis in T2D is still unclear. Indeed, induction of diabetes by an injection of streptozotocin leads to the activation of SphK1 associated to an inflammatory phenotype in vascular endothelium and retina [77,83]. Moreover, the SphK1/S1P axis appears to stimulate the expression of cytokine by adipocytes isolated from ob/ob mice [84] and in obese T2D patients [85].

6.1. Sphingoid Base Phosphates and Insulin Secretion

In pancreatic β cells, SphK1 and SphK2 and four of the five S1PR are expressed [85–87]. Expression and activity of SphK1 and SphK2 are stimulated by cytokines such as IL1β, TNFα and interferon-γ, but also by glucose and acetylcholine [77,87]. The role of S1P in insulin secretion is still unclear. Indeed, it has been shown that adding S1P to culture medium of pancreatic INS-1 β cell line and islets of Langerhans inhibits insulin secretion in response to glucose potentiated by GLP-1. The S1P1 receptor, coupled to Gi appears to be involved in this phenomenon [86]. In contrast to this study, S1P was found to stimulate secretion of insulin in islet of Langerhans [88] and in pancreatic HIT-T 15 β cell line [89,90]. Interestingly, Ozcan and colleagues showed that SphK2 plays a critical role in S1P production and insulin secretion induced by glucose [90]. To date, SphK1 has not been implicated in the control of insulin secretion. However, most of these studies have been conducted in pancreatic β cell

lines or isolated normal islets. It will thus be important to investigate the *in vivo* role of sphingoid base phosphates by analyzing insulin secretion in available knock-out mice for SphK1 and SphK2 [91,92].

6.2. Sphingoid Base Phosphates Pancreatic β Cell Survival

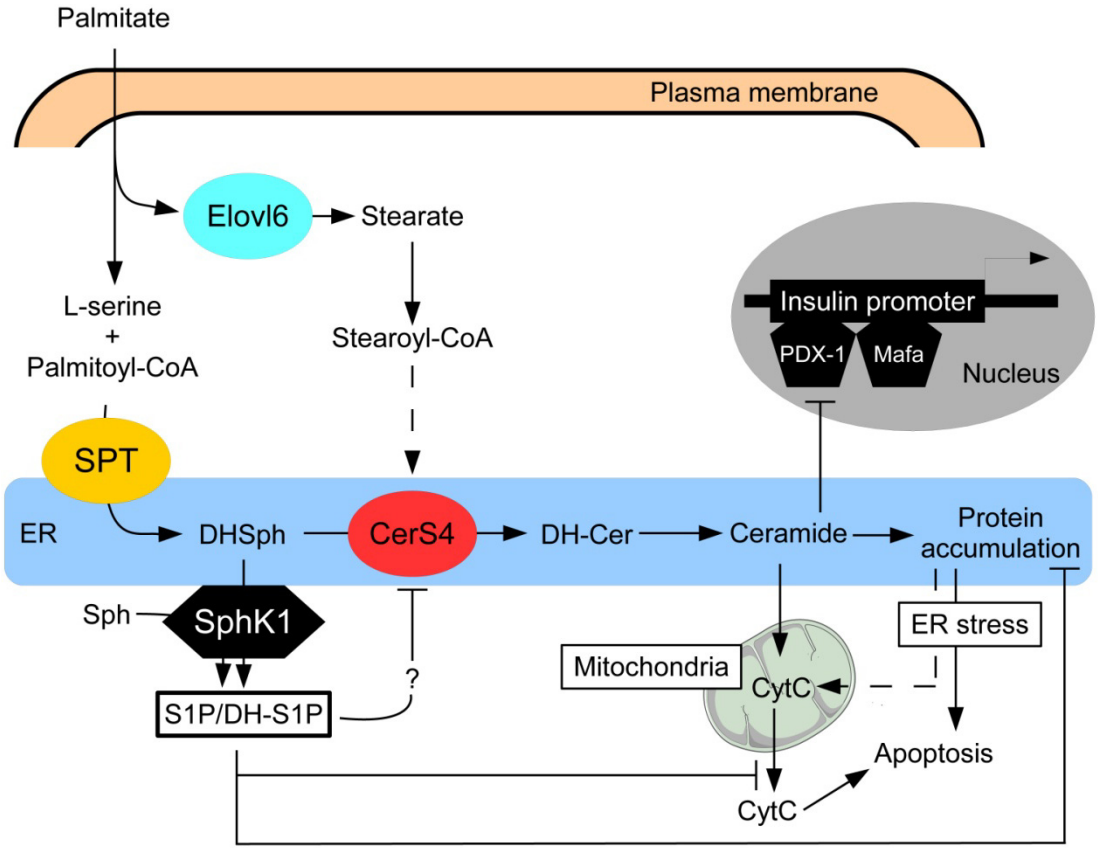
In pancreatic β cells, the SphK1/S1P axis plays a critical role in survival. Indeed, adding S1P to culture medium of human or murine islets of Langerhans inhibited apoptosis induced by cytokines [93]. In this case, the anti-apoptotic effect of S1P is mediated by the S1P2 or S1P3 receptors, and the activation of the PKC pathway [93,94]. Interestingly, activation of SphK by glucose has also been implicated in β cell proliferation [76]. It was observed that gluco-lipotoxicity increased sphingoid base phosphates via up-regulation of the SphK1 pathway in pancreatic β cells [88]. Pharmacological inhibition of SphK activity drastically potentiates β cell apoptosis whereas over-expression of SphK1 partially inhibits β cell apoptosis induced by gluco-lipotoxicity. The anti-apoptotic effect of sphingoid base-1-phosphates are likely to be mediated by opposing signaling pathways to ceramide-induced apoptosis. Indeed, over-expression of SphK1 not only inhibited lipotoxicity-induced loss of mitochondrial transmembrane potential and cytochrome c release in pancreatic β cells [95] but also impaired protein trafficking between ER and Golgi which contributed to lipotoxicity-induced ER stress [88]. Another anti-apoptotic effect of sphingoid base phosphates involved the alteration of lipotoxicity-induced ceramide synthesis in pancreatic β cells [88]. While the mechanisms for this latter process remain to be established, it could potentially involve inhibition of CerS by S1P [96]. The *in vivo* protective role of SphK1 against pancreatic β cell apoptosis induced by gluco-lipotoxicity was supported by a recent study by Qi and co-workers who showed that while a high fat diet-fed wild-type (WT) mice developed glucose intolerance, all high fat diet-fed Sphk1 knock-out mice manifested evident diabetes, a phenomenon due to a drastic decrease in pancreatic β cell mass [95]. Nevertheless, the *in vivo* role of SphK1 still remains unclear since a recent study showed that under high fat diet-fed mice, SphK1 deficiency was associated with enhanced insulin signaling in adipose and muscle tissues and improved systemic insulin sensitivity and glucose tolerance [85]. Despite this recent study, the anti-apoptotic role of sphingoid base phosphates in pancreatic β cells are in agreement with the work of Boslem *et al*., who showed that ceramide removal from the ER, by favoring their conversion into glucosylceramides [40], is critical for β cell survival under gluco-lipotoxic conditions. Our findings suggest that the SphK1/sphingoid base phosphates axis could constitute a new therapeutic strategy for preventing pancreatic β cell death and thus the onset of T2D associated with obesity. In the future, it will be important to identify potent activators of the SphK1/sphingoid base phosphates axis in pancreatic β cells. In support of this, a recent work by Holland *et al*. [97] showed that adiponectin can preserve pancreatic β cell function by shifting the ceramide/S1P ratio towards higher relative levels of S1P.

7. Conclusions

Because of their inter-convertibility and opposing effects, the dynamic balance between sphingoid base phosphates and ceramides has been proposed to be an important factor that determines cell fate [42], a concept called the sphingolipid biostat. The results of recent *in vitro* and in some cases *in vivo* studies lend credence to the observation that pancreatic β cell apoptosis induced by gluco-lipotoxicity is regulated by such a sphingolipid biostat (Figure 2). The existence of this biostat

offers the possibility to find new therapeutic targets in order to limit pancreatic β cells failure observed during obesity, and therefore to prevent the appearance of T2D.

Figure 2. Role of sphingolipid biostat in β-cell apoptosis during gluco-lipotoxicity. Palmitate has been shown to induce ceramide accumulation by a dual mechanism involving serine palmitoyl-transferase (SPT) and the formation of ceramides with specific *N*-acyl chain lengths by ceramide synthase 4 (CerS4). Fatty acid elongase 6 (Elovl-6) provides preferential substrate for CerS4 by converting palmitate into stearate. Ceramides reduces insulin expression through inhibiting the binding of pancreatic and duodenal homeobox 1 (PDX-1) and Mafa transcriptional factors to insulin promoter. Ceramide accumulation can also induce pancreatic β cell apoptosis. However, palmitate also induces sphingosine kinase 1 (SphK1) expression, which will channel the preferential metabolic flow of newly formed dihydrosphingosine (DHSph) towards its phosphorylation into dihydrosphingosine-1-phosphate (DHS1P) and increase S1P levels. Accumulation of these sphingoid base phosphates in the ER will play a protective role against palmitate-induced ceramide-dependent apoptotic β cell death. Sphingoid base phosphates inhibit ceramide synthesis by acting probably on CerS4 activity and restore protein trafficking in the endoplasmic reticulum (ER), alleviating ER stress and attenuating cytochrome C (CytC) release from mitochondria. DH-Cer: dihydro-ceramides; Sph: sphingosine.



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EXPERT OPINION

1. Introduction
2. Ceramides and lipotoxicity
3. Ceramide metabolism and lipotoxicity: role of S1P
4. Conclusion and expert opinion

Targeting sphingolipid metabolism in the treatment of obesity/type 2 diabetes

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Introduction: Obesity is a major factor that is linked to the development of type 2 diabetes (T2D). Excess circulating fatty acids (FAs), which characterize obesity, induce insulin resistance, steatosis, β cells dysfunction and apoptosis. These deleterious effects have been defined as lipotoxicity.

Areas covered: FAs are metabolized to different lipid species, including ceramides which play a crucial role in lipotoxicity. The action of ceramides on tissues, such as muscle, liver, adipose tissue and pancreatic β cells, during the development of T2D will also be reviewed. In addition, the potential antagonist action of other sphingolipids, namely sphingoid base phosphates, on lipotoxicity in skeletal muscle and β cells will be addressed.

Expert opinion: Ceramide is a critical mediator to the development of T2D linked to obesity. Targeting proteins involved in ceramide's deleterious action has not been possible due to their involvement in many other intracellular signaling pathways. A possible means of counteracting ceramide action would be to prevent the accumulation of the specific ceramide species involved in both insulin resistance and β-cell apoptosis/dysfunction. Another possibility would be to adjust the dynamic balance between ceramide and sphingoid base phosphate, both known to display opposing properties on the development of T2D-linked obesity.

Keywords: adipose tissue, apoptosis, ceramide, insulin, islet of Langerhans, lipotoxicity, liver, muscle, obesity, pancreatic β cells, sphingolipids, sphingosine-1-phosphate, type 2 diabetes

Expert Opin. Ther. Targets [Early Online]

1. Introduction

1.1 Type 2 diabetes

In most of the developed and developing countries, type 2 diabetes (T2D) is one of the major causes of immature illness and death, which imposes a substantial financial burden to the society [1]. During diabetes, an imbalanced regulation of glucose levels occurs, resulting in a chronic and abnormal elevation of plasma glucose levels that lead to heart disease, kidney failure, limb amputation and blindness. In healthy people, the balance between glucose production and its utilization is tightly controlled. Normoglycemia is mainly maintained through insulin secretion by pancreatic β cells and inhibition of glucose production by the liver. The biological machinery of these cells promotes a response to threshold glucose circulating levels, by releasing insulin when required. The insulin released decreases glucose level by two major actions: it increases glucose uptake by tissues, mainly by the skeletal muscle, and it inhibits glucose release by the liver. The etiology of T2D is not well established but the pathophysiology of this disease is associated with both genetic and environmental factors, as well as with lifestyle, diet and obesity. To explain the

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Article highlights.

- Ceramide accumulation plays a critical role in the development of lipotoxicity induced by obesity in several tissues.
- Potential role of specific ceramide species in the deleterious effect of lipotoxicity.
- Another sphingolipid, sphingosine-1-phosphate (S1P) seems to have a protective role against lipotoxicity.
- Pushing ceramide metabolism toward the synthesis of less harmful lipid, such as S1P, could represent a potential target to counteract lipotoxicity.

This box summarizes key points contained in the article.

defects observed in both the insulin sensitivity and the insulin secretion in T2D, several mechanisms have been highlighted in the different tissues [2,3].

1.2 Lipotoxicity

Evidence suggests that T2D is often associated with excessive levels of circulating lipids as well as abnormalities in lipid metabolism [4]. World Health Organization showed that 44% of diabetic patients are also obese, suggesting a link between T2D and obesity. Prospective epidemiological studies performed on populations with low or high risk of developing T2D showed that high free fatty acid (FFA) concentration in plasma is associated with the incidence of T2D [5]. Until 15 years ago, the 'Randle cycle hypothesis' seemed the best explanation for understanding the relationship between carbohydrate metabolism and lipid metabolism, particularly the deleterious role of fatty acids (FAs) on the action of insulin on glucose metabolism in insulin-sensitive tissues. In 1963, Philip Randle proposed the existence of a glucose/FA cycle in cells that could play a crucial role in the development of diabetes [6]. Indeed, a substantial increase in FFA oxidation would result in an inhibition of hexokinase, leading to inhibition of glycolysis and to a rise of intracellular glucose. In the 1990s, new studies challenged this cycle. Contrary to Randle's proposition, they showed that the accumulation of FFAs in the muscle decrease intracellular glucose concentrations [7]. Obviously, it appears that the Randle cycle is only one of the many mechanisms by which FAs can influence insulin action and glucose metabolism in insulin-sensitive tissues. FFAs, in particular triglycerides, are the main energy source for cells and are used by tissues through the oxidative pathway. In normal conditions, the unconsumed fraction of FFA is stored into adipocytes within lipid droplets as reserve of energy and is released in fasting conditions. However, persistent elevated levels of circulating FFAs can lead to a saturation of the adipose tissue (AT) and to an ectopically storage of FFAs in other peripheral tissues. Chronically elevated levels of FFAs in circulation have been postulated to cause peripheral insulin resistance and impairment of insulin secretion by pancreatic β cells. This phenomenon has been defined as 'lipotoxicity' [8,9]. Among the lipids that accumulate in these tissues, diacylglycerol, phosphatidic acid and ceramides have been shown to play a crucial role in mediating lipotoxicity.

In the present review, we will illustrate the mechanisms that are activated during lipotoxicity in different tissues, such as muscle, liver, AT, pancreas and even the brain, with a special focus on the role of ceramides at the molecular and cellular levels as well as in animal models. In addition, we will also present the role of other ceramide metabolites, such as sphingosine-1-phosphate (S1P), in the control of lipotoxicity. A better understanding of role of ceramide and its metabolism in lipotoxicity may lead to the identification of pharmacological targets for the prevention and/or treatment of T2D-associated obesity.

2. Ceramides and lipotoxicity

It has been proposed that ceramides are important mediators of lipotoxicity [8]. In obesity, ceramides have been shown to accumulate in insulin-sensitive tissues and in pancreatic β cells [10]. Ceramide levels increase in both plasma and skeletal muscle of diabetic patients, as well as in the liver, plasma and muscle of obese mice [11]. In addition, a relationship between ceramide levels and insulin resistance has been clearly demonstrated *in vivo* [11]. In the context of obesity-associated FA overload, *de novo* ceramides are mainly produced from saturated FAs (palmitate). This synthesis occurs in the endoplasmic reticulum (ER) (**Figure 1**) via different reactions that begin with the condensation of L-serine with palmitoyl-coenzyme A (CoA) by serine palmitoyltransferase (SPT), generating 3-ketosphinganine, which is then reduced to form dihydrosphingosine (DH-Sph) via 3-ketosphinganine reductase. DH-Sph is then acylated by ceramide synthase (CerS) isoforms to form dihydroceramide (DH-Cer). In mammals, six CerS isoforms are expressed: CerS 1 – 6 [12]. They carry out the same chemical reaction, but display distinct specificities for the acyl-CoA chain length they use for *N*-acylation (**Figure 1**). Thus, CerS isoforms are responsible for the FA composition of ceramides. The chain base moiety of the lipid is finally desaturated by dihydroceramide desaturase (DES1) at the 4 – 5 position of the sphingoid base backbone to form ceramide. Once synthesized, ceramides can be transported to the Golgi apparatus [13] where they are converted into sphingomyelin by sphingomyelin synthases (SMS) or into glucosylceramides through glucosyl-CerS. The *de novo* synthesis pathway is not the only way to produce ceramide; the other metabolic pathway involves the degradation of sphingomyelin into ceramide by sphingomyelinases (SMase) and this process takes place in the lysosomal and the cytoplasmic membrane [14].

In this web of sphingolipid metabolism, ceramides are considered as central players in the synthesis of sphingolipids. Indeed, ceramides are the precursors of many lipid

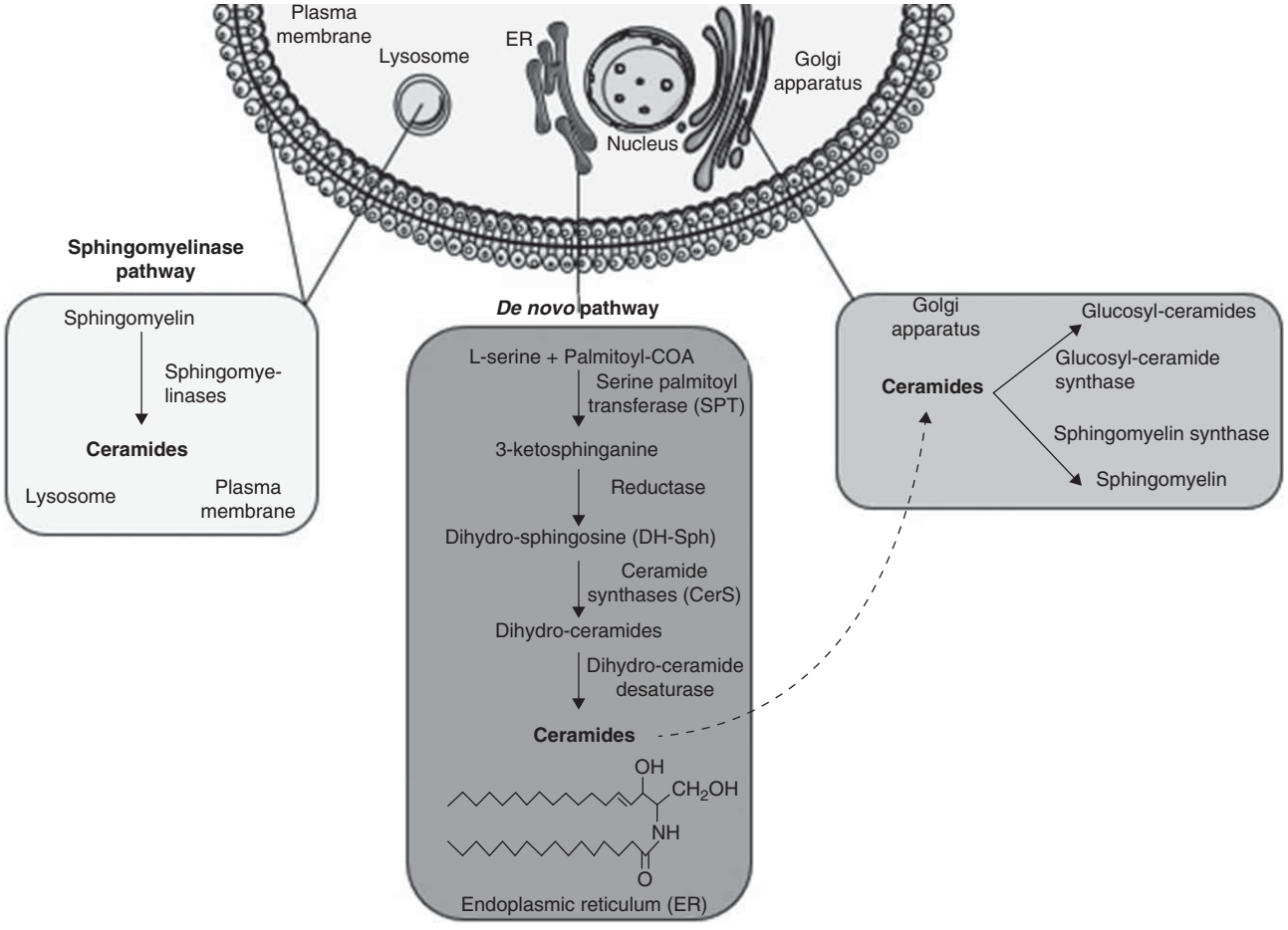

Figure 1. Illustration showing the sphingolipids metabolism in mammalian cells. In mammals, there are two main pathways to produce sphingolipids. The first one is the *de novo* synthesis pathway which starts on the cytoplasmic face of the ER. The first step of this pathway results in the condensation of palmitoyl-CoA and L-serine to form 3-ketosphinganine. This reaction is mediated by SPT. 3-Ketosphinganine is then reduced to produce DH-Sph which is transformed by CerS on dihydroceramides. These are finally desaturated into ceramides by dihydroceramide desaturase. Ceramides can be transported to the Golgi apparatus to be metabolized into more complex sphingolipids such as glucosylceramides and sphingomyelin. The second synthesis pathway is the sphingomyelinase pathway that takes place in the lysosomal and plasma membrane. This pathway leads to the degradation of SM into ceramides by sphingomyelinase.

CerS: Ceramide synthase; DH-Sph: Dihydrosphingosine 1-phosphate; ER: Endoplasmic reticulum; SM: Sphingomyelin; SPT: Serine palmitoyltransferase.

messengers. For example, ceramides can be metabolized into sphingosine which is in turn phosphorylated to yield S1P [14].

2.1 Ceramides and muscle

During obesity, ceramide is the most toxic lipid that accumulates in the muscle [15,16]. The association between increasing ceramide concentration in the muscle and insulin resistance has been well demonstrated in muscles of both rodents and humans [17-19]. The inverse relationship between increased intramuscular ceramide content and insulin sensitivity has also being demonstrated *in vitro* by exposing muscle cell lines to either saturated FAs (such as palmitate) or to ceramide analogs directly [20,21]. Interestingly, studies have shown that, in human skeletal muscle, ceramide concentration was higher in type I than in type II fibers in patients with T2D and in obese subjects [22]. The capacity to oxidize fat is higher in type I compared to type II muscle fibers; thus intracellular FFA availability should be greater in type I muscle fibers. Since muscles of patients with T2D contain < 30% mitochondria than normal subjects [23], higher *de novo* ceramide synthesis may be facilitated in these fibers.

There is a current consensus that protein kinase B (PKB/Akt) is ceramide's main target for inducing insulin resistance. PKB/Akt is an insulin signaling intermediate that plays a pivotal role in inducing the insulin-dependent uptake and utilization of glucose in insulin-sensitive tissues [21]. Ceramides alter PKB/Akt in muscle cells via two nonexclusive mechanisms. The first involves the activation of PP2A phosphatase (**Figure 2**).

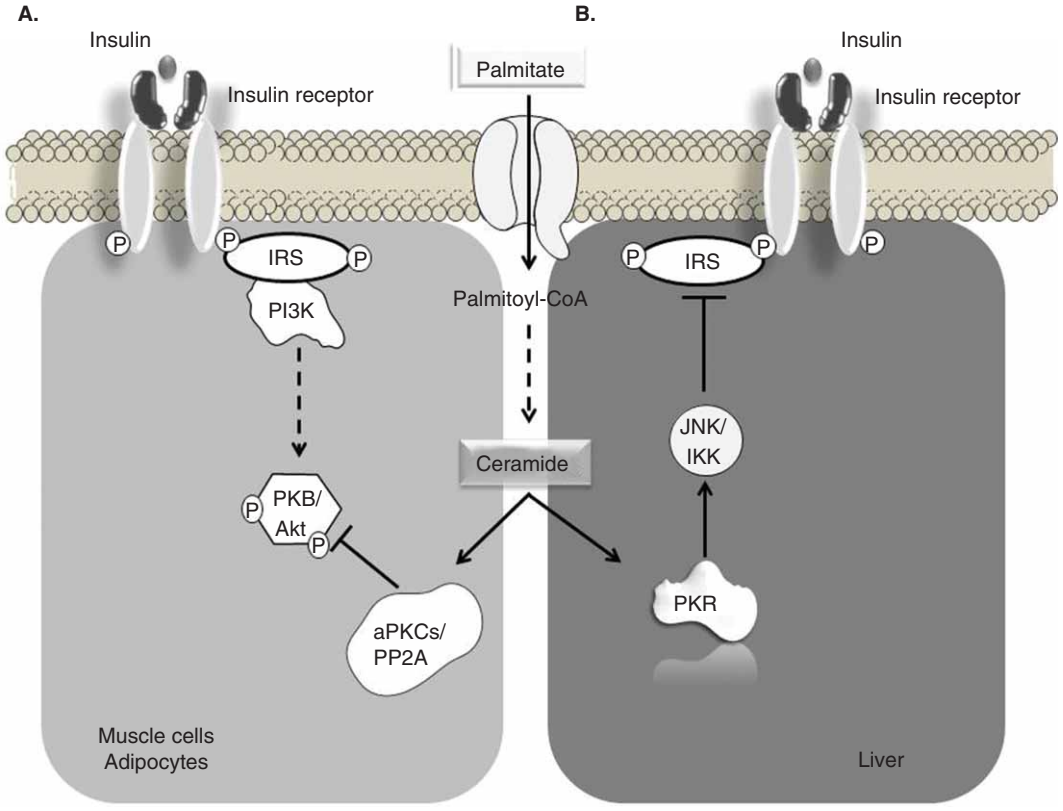

Figure 2. Illustration showing the mechanisms mediating ceramide inhibitory action on insulin signaling. Binding of insulin to its receptor at the plasma membrane promotes the activation of PKB/Akt via tyrosine phosphorylation of IRS and activation of PI3K. The concomitant generation of phosphatidylinositol-3,4,5-triphosphate (not shown) by PI3K facilitates PKB/Akt recruitment to the plasma membrane where it is activated by its upstream kinases. Activated PKB/Akt promotes glucose uptake in adipocytes and skeletal muscle, as well as glycogen synthesis in skeletal muscle and liver. In liver, insulin promotes lipogenesis and inhibits gluconeogenesis. Palmitate-generated ceramide alters insulin signaling through different mechanisms. (**A**) In insulin-sensitive cells, ceramide inhibits PKB/Akt through the activation of aPKCs that bind, phosphorylate and sequester PKB/Akt into caveolin-enriched microdomains or through the activation of PP2A that dephosphorylates PKB/Akt. (**B**) In liver, ceramide also acts negatively on insulin signaling by inhibiting IRS through the activation of the 'PKR/JNK-IKK' pathway.

IRS: Insulin receptor substrate; JNK: Janus kinase; PI3K: Phosphatydilinositide-3 kinase; PKB: Protein kinase B; PKR: RNA-dependent protein kinase.

PP2A is a cytoplasmic serine/threonine phosphatase expressed ubiquitously. PP2A dephosphorylates many substrates *in vitro* and is involved in the regulation of almost all cell activities. Ceramide-activated PP2A has been shown to prevent the activation of PKB/Akt in response to insulin through the dephosphorylation of serine 473 and threonine 308 PKB/Akt sites [18]. Treatment of different cell types such as the C2C12 muscle cell line, PC12 nerve cells and brown adipocytes with okadaic acid has demonstrated that PP2A mediates the negative action of ceramide on PKB/Akt in these cells [24]. The second mechanism involves a family of PKCs [25], atypical PKCs (aPKCs) which inhibit the PKB/Akt translocation to the plasma membrane to be phosphorylated in response to insulin (**Figure 2**). This process depends on the activation of aPKCs by ceramides. Indeed, pharmacological inhibition of aPKC activity prevents the deleterious effect of ceramides on the activation of PKB/Akt by insulin in both adipocytes and L6 myotubes [26]. Ceramides directly activate aPKCs through a putative binding region [27]. It has been demonstrated in L6 myotubes that ceramide-activated aPKCs bind and phosphorylate PKB/Akt on a threonine 34/serine 34 residue (depending on the PKB/Akt isoform) located in the PH domain of the kinase, making it impossible for phosphatydilinositide-3 kinase (PI3K)-produced phosphatidylinositol-3,4,5-triphosphate to bind to it in response to insulin [26]. More recently, it has been shown that ceramides inhibit the activation of PKB/Akt by insulin via the activation of aPKCs in human myotubes [19]. Moreover, inhibition of aPKCs activation by ceramides in myotubes obtained from diabetic patients restores insulin signaling pathway in these cells [19]. Reports also show that aPKCs can also be activated by insulin and growth factors in a PI3K-dependent manner. In these conditions, aPKCs

play a positive role in cell signaling. For example, insulin-induced aPKC activation has been shown to be crucial for GLUT4 translocation in insulin-sensitive tissues [28]. Such diversity in aPKC function is now well documented, and there is a growing recognition that aPKCs act as molecular switches which, depending on their subcellular localization and the activating stimuli, could either promote or inhibit cell signaling.

Why do ceramides alter insulin signaling via two different mechanisms? The answer to this question may be held in membrane composition variability of the different cells studied. Indeed, it has been shown that ceramides induce the recruitment of PKCζ-PKB/Akt complex in specific membrane areas called caveolin-enriched microdomains (CEMs) [29]. These lipid microdomains are special platforms that are the origin of numerous signaling pathways [30]. These CEMs are enriched with not only various sphingolipids (ceramides, sphingomyelins) and cholesterol but also an important structural protein called caveolin [30]. Interestingly, caveolin can bind to aPKC [31] and it has further been found that ceramides inactivate PKB/Akt by inducing the sequestration of the PKCζ-PKB/Akt complex in the CEM of these CEM-rich cells. In CEM-poor cell types, however, ceramides act negatively on PKB/Akt via PP2A [32].

2.2 Ceramide and liver

The liver plays an important role in obesity-related insulin resistance, with excessive hepatic glucose production contributing to fasting hyperglycemia. Studies suggest that the liver is a key site for ceramide production [33], but its contribution to the onset of insulin resistance is still under debate. Whereas some studies found no real correlation between high hepatic ceramide levels and insulin sensitivity [34], others demonstrated that high-fat diet (HFD)-induced hepatic insulin resistance was reversed by inhibiting ceramide synthesis or by increasing ceramide degradation selectively in hepatocytes [35]. Liver-produced ceramides also play a role in the progression of hepatic steatosis. Indeed, blocking *de novo* ceramide synthesis using myriocin-reduced HFD induced macrovesicular fat accumulation and hepatic triglyceride content [36,37]. These results were recently supported by a study using hepatic-specific CerS6 knockout mice [38]. In that study, reduced C16-ceramide concentration was observed in the liver of these mice and they were protected from diet-induced obesity and displayed improved glucose tolerance [38]. Concomitantly, similar conclusions were obtained using CerS2 knockout mice. CerS2 is the dominant isoform in the liver and its disappearance led to an increase in C16-ceramide content in the liver and conferred mice susceptibility to diet-induced steatohepatitis and insulin resistance [39].

Interestingly, some studies suggest that hepatic ceramides could also act indirectly on other peripheral tissues (muscle, heart and AT) after being secreted into the bloodstream. The liver is considered as a primary source of plasma ceramide since in physiological conditions most of the ceramides synthesized in the liver are excreted into the circulation as components of very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) [40]. Addition of palmitate to rat hepatocytes induces an increase in ceramide secretion, whereas incubation of hepatocytes with Fumonisin B1, an inhibitor of CerS, reduces both sphingolipid synthesis and secretion by 90% without affecting the overall lipoprotein synthesis and the secretion of apolipoprotein B [40]. A study compared the levels of VLDL- and LDL-associated ceramides in obese patients with T2D [41]. In this latter study, only LDL-containing ceramides were found to be elevated in the blood of T2D patients compared to lean insulin-sensitive individuals [41]. In agreement with this treatment of cultured myotubes with LDL-containing C16- and/or C24-ceramides *in vitro* promoted insulin resistance in these cells, demonstrating that liver-produced LDL-containing ceramides are deleterious for peripheral tissues [41]. Altogether, these data suggest that, in lipotoxic conditions, liver-produced ceramides are exported out of the liver mainly as LDL and affect glucose metabolism in other insulin-sensitive tissues. However, on increased hepatic saturated FA influx, as observed in insulin resistance state, the rate of increase in FAs exceed their secretion, resulting in a progressive accumulation of ceramides in the liver, leading then to steatosis and insulin resistance.

In contrast to what has been described in muscle and adipocytes, ceramides do not seem to induce insulin resistance through direct inhibition of PKB/Akt in the liver. Interestingly, a very recent study has shown that hepatic ceramides impair PKB/Akt activity and the insulin signaling pathway through activated aPKCs, thus inducing increased glucogenesis and lipogenesis in liver [42]. Moreover, a study by Yang *et al.* [36] has shown that ceramides inhibit insulin signaling through the activation of the double-stranded RNA-dependent protein kinase (PKR) (**Figure 2**). Originally, PKR was identified as an IFN-induced kinase that responds to viral infection [43] by promoting apoptosis [44]. Interestingly, PKR has been shown to be activated in liver of obese human subjects [45]. In 2010, Yang *et al.* [46] demonstrated in HepG2 hepatic cells that ceramide-induced PKR activation upregulates the phosphorylation of insulin receptor substrate 1 (IRS1) at Ser312, which in turn suppresses insulin-induced tyrosine phosphorylation of IRS1. The effect of PKR on IRS1 has been shown to be mediated by both Janus kinase (JNK) and Inhibitor of Kappa B Kinase [46]. Since then, involvement of PKR in mediating lipid-induced insulin resistance has been reinforced recently by using PKR inhibitors in obese and diabetes mice (*ob/ob*). Moreover, it has been found that PKR inhibitors improved both insulin sensitivity and glucose intolerance in these mice [47].

2.3 Ceramides and adipocytes

Previous studies have also demonstrated a key role of ceramide in adipocytes. Ceramide concentration is largely increased in AT of human obese diabetic patients and this lipid concentration correlates positively with Homeostatic Model Assessment-Insulin Resistance [48]. In addition, diet-

induced obesity in mice is also associated with increased AT ceramide concentration and insulin resistance [49]. The involvement of ceramides in the onset of insulin resistance in adipocytes has been demonstrated *in vitro* by using both 3T3-L1 and human adipocytes. Treatment of these cells with ceramides analogs prevents insulin-induced glucose uptake completely through the inhibition of PKB/Akt (**Figure 2**) [32]. Moreover, exposure of 3T3-L1 adipocytes to ceramide, downregulates mRNA expression of the insulin-regulated glucose transporter GLUT4 [50].

In adipocytes, contrary to liver and skeletal muscle, ceramide biosynthesis does not involve the *de novo* pathway [20] but rather comes from sphingomyelin. Indeed, studies have shown that TNF-α induces sphingomyelin hydrolysis through the activation of SMase [50]. In fact, the *de novo* ceramide biosynthesis pathway is rather impaired in insulin resistance state in white AT. This is due to a decrease in expression of DES1, the enzyme that converts inactive DH-Cer into active ceramide [51]. Interestingly, adipocytes that do no express DES1 display impaired adipocyte differentiation and lipid accumulation [51]. As a consequence, in lipotoxic condition, adipocytes cannot buffer lipid excess efficiently, thus enhancing lipid uptake by other peripheral tissues such as liver and muscles.

As observed in muscle cells, PKB/Akt has also been found to be the main target of ceramide in adipocytes. Indeed, treatment of either 3T3-L1 adipocytes or human adipocytes with a short-chain ceramide analog C2-ceramide blunts insulin-induced PKB/Akt activation, specifically through a mechanism involving aPKCs and sequestration of PKB/Akt into CEM.

2.4 Ceramide and pancreatic β cells

Ceramide has an important role on β-cell fate and function. Indeed, it has been demonstrated that ceramide analogs can induce apoptosis of β cells and islets of Langerhans in culture by blocking proliferation [52]. It has also been shown that *de novo* ceramide synthesis pathway is implicated on pancreatic β-cell apoptosis [8,53]. Interestingly, it has been shown that diminution of pancreatic β-cell mass and loss of insulin secretion are correlated with an increase in intracellular ceramide levels and SPT expression in islet of Langerhans of obese Zucker diabetic fatty rats [54]. Lipotoxicity induces an accumulation of ceramides that is responsible for pancreatic β-cell apoptosis through different pathways. Ceramide-induced β-cell apoptosis is mediated by reactive oxygen species (ROS) production and mitochondrial dysfunction [52]. Some data suggest that ceramides induce apoptosis through mitochondria injuries and subsequent ROS production [55]. Pancreatic β-cell apoptosis could be induced through the activation of PP2A and the subsequent dephosphorylation of PKB/Akt [56] like it has been observed in muscle. ER stress has also been shown to be an important regulator of β-cell apoptosis in T2D [57]. In agreement, the group of T. Biden has shown ceramide-mediated β-cell apoptosis through the induction of ER stress [58]. In mammals, several ceramide species exist. In pancreatic β cells, it appears that depending on lipotoxicity, the levels of specific ceramide species are modified, specifically Cer-C18:0, Cer-C22:0 and Cer-C24:1 [53]. The variety of ceramide species relies on an enzyme family, the CerS [13]. This family is composed of six components each of which has been identified to possess a characteristic substrate preference for a particular fatty acyl-CoA. Interestingly, β-cell lipotoxicity is associated with an augmentation of CerS4 expression, which has been found to play a critical role in pancreatic β-cell apoptosis (**Figure 3**) [53]. However, the specific role of each ceramide species in β-cell dysfunction induced by lipotoxicity remains to be clarified. Indeed, a recent study showed that CerS2 knockout mice are unable to synthesize C22 – C24 ceramide species and did not result in defective insulin secretion [59]. More recently, it has been shown that lipotoxicity could increase ceramide levels by affecting ceramide transport [60]. In mammals, two pathways are known by which ceramides are transported from the ER to the Golgi apparatus: a protein-mediated transport, by the soluble ceramide transfer protein ceramide transporter (CERT) [61], and by a CERT-independent vesicular trafficking [14,60]. Lipotoxicity has been shown to impair both vesicular- and CERT-mediated ceramide transport by decreasing of phospho-PKB/Akt levels, which in turn possibly inhibit vesicular traffic, and by reducing the amount of active CERT (**Figure 3**) [60]. It remains important to determine if this defect of ceramide transport contribute to β-cell apoptosis induced by lipotoxicity.

Ceramide also have a critical role in insulin gene expression and could cause insulin secretion defects. Ceramide generated from lipotoxicity inhibits the insulin gene expression [8]. The mechanism involved in the insulin expression inhibition is not related to insulin mRNA instability but to the inhibition of glucose-induced insulin promoter activity [62]. This inhibition is associated with a decreased binding activity of the transcription factors MafA and pancreas-duodenum homeobox 1 (PDX-1) [63]. MafA is affected at the level of its expression, whereas PDX-1 is affected by its ability to translocate to the nucleus [63]. The mechanisms through which ceramide impairs PDX-1 subcellular localization and MafA expression are unknown, even if some studies have identified potential candidates. The c-jun NH2-terminal kinase JNK is known to be a target of ceramide [64] and can repress insulin gene expression via c-jun-dependent inhibition of E1-mediated transcription and via c-jun-independent inhibition of PDX-1 binding [65]. Another study demonstrated that in islet of Langerhans and β-cell line, ceramide-dependent inhibition of the insulin gene expression is mediated by the dephosphorylation of protein kinases, ERK1/2 by PP2A [66]. The inhibition of insulin gene expression seems also to be correlated with the inhibition of the ceramidase, which increases the intercellular levels of ceramide [66]. Also, ceramide transport between ER and Golgi apparatus has been demonstrated to play a role in insulin gene expression, pharmacological inhibition of SMS

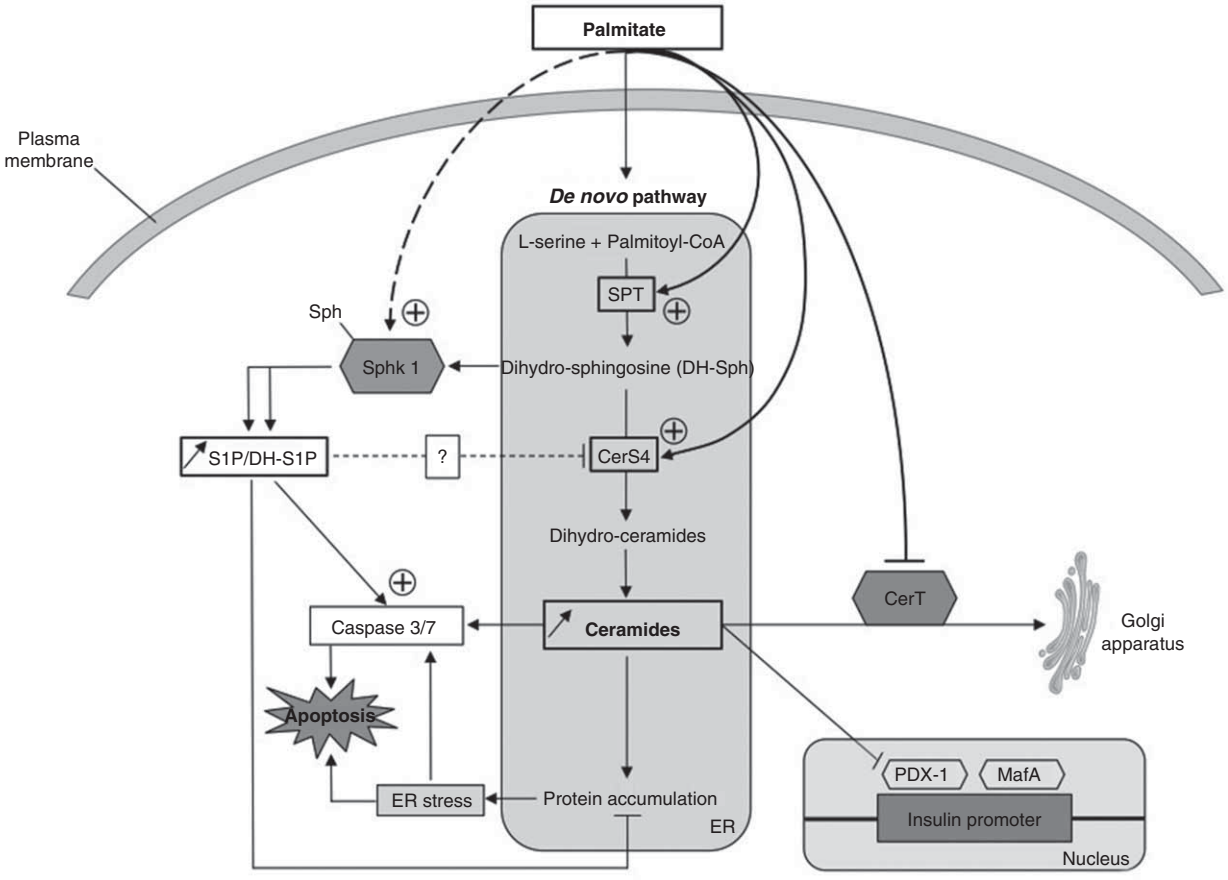

Figure 3. Illustration showing the role of sphingolipids in β-cell apoptosis during lipotoxicity. Lipotoxicity has been shown to create an accumulation of ceramide by stimulating enzymes from *de novo* synthesis pathway such as SPT and CerS4. It can also create an accumulation of ceramides inhibiting ceramide translocation from the ER to the Golgi apparatus by blocking CerT. These ceramides can also inhibit insulin expression through inhibiting the binding of PDX-1 and MafA transcriptional factors to insulin promoter. Moreover, palmitate is also capable of inducing the production of S1P and DH-S1P by phosphorylation of DH-Sph by Sphk1. Accumulation of these sphingoid base phosphates in the ER will play a protective role against palmitate-induced ceramide-dependent apoptotic β cell death. It was proposed that S1P and DH-S1P inhibit ceramide synthesis by acting on CerS4 activity and restore protein trafficking in the ER, alleviating ER stress and attenuating caspase-3/7 activation.
CerS: Ceramide synthase; CerT: Ceramide transporter; DH-Sph: Dihydrosphingosine 1-phosphate; ER: Endoplasmic reticulum; PDX1: Pancreas-duodenum homeobox 1; S1P: Sphingosine 1-phosphate; Sphk1: Sphingosine kinase 1; SPT: Serine palmitoyltransferase.

or downregulation by siRNA of the CERT increase dysfunction of pancreatic β cells induced by palmitate by affecting the binding of transcription factors to the insulin promoter [67]. Ceramide seems to also act at the insulin secretion level. In Yano *et al*. study, genetic deletion of SMS1 has been shown to induce accumulation of ceramide in islet of Langerhans, dysfunction of mitochondria and the subsequent inhibition of insulin secretion [68]. Indeed, ceramide accumulation was found to affect mitochondria membrane integrity and induced an excessive production of ROS, which were partially responsible for the defect in insulin secretion in pancreatic β cells [68]. Taken together, these results suggest that ceramide accumulation, either through the reduced conversion into more complex sphingolipids or the induction of the *de novo* synthesis is involved in pancreatic β cells loss of function.

2.5 Ceramides and hypothalamus

Several studies demonstrated that the brain, and especially hypothalamus, is able to detect variations in the circulating FA concentration [69]. FFAs are not used by the hypothalamus as nutrients but as cellular messengers. Indeed, they play a major role in the control of energy balance [70], including the regulation of the orexigenic hypothalamic neuropeptides NPY/AgRP release. However, in the case of hyperlipidemia, it has recently been suggested that the brain could be a new target of lipotoxicity [69]. Indeed, circulating FAs would be able to accumulate in the hypothalamus causing long-term changes in sympathetic nervous activity that could result in deregulation of glucose homeostasis. The accumulated FAs become toxic and especially induce a hypothalamic insulin and leptin resistance that is responsible for a deregulation of

the energy balance. This would block the inhibitory action of insulin on production/secretion of AgRP neuropeptides and neuropeptide Y [70], contributing to obesity and also to the installation of T2D. Interestingly, it has been shown that an exogenous ceramide administration on the lateral ventricule of the hypothalamus of rats is associated with hypothalamic inflammation and ER stress [71]. Different studies also demonstrated that one of the pathological mechanisms of leptin resistance is mediated by hypothalamic ER stress [72]. These data suggest that ceramides accumulation under lipotoxic conditions in the hypothalamus could play a key role on the deregulation of the energy balance controlled by the brain. However, further experiments remain to be done to establish if endogenous ceramide synthesis in the hypothalamus could be a regulator of lipotoxicity.

3. Ceramide metabolism and lipotoxicity: role of S1P

Once generated, ceramides are the common precursor of several sphingolipid derivatives. They can produce a wide range of different metabolites such as S1P, glucosylceramides, ceramide-1-phosphate and sphingomyelin, all of which possess patent properties. Interestingly, like ceramides, most of these metabolites were found in human plasma at significant levels and the increase in concentration of some of them is often associated with important metabolic disturbances.

In the present review, we will focus on the role of S1P in lipotoxicity. Ceramide degradation to give S1P is initiated by ceramidases to produce sphingosine which is then phosphorylated to give S1P by sphingosine kinases (SphKs) [73]. Two SphK isoforms has been characterized and are called SphK1 and SphK2. Both isoforms are predominantly localized in the cytosol, and to a lesser extend in membranes. SphK1 and SphK2 display different kinetic properties, tissue distribution and temporal expression, suggesting that they perform distinct cellular functions and may be regulated differently [74]. SphK1 is expressed predominantly in epithelial and bronchial smooth muscle cells, whereas the expression of SphK2 is ubiquitous [75]. Following synthesis, S1P is exported by carriers belonging to the ATP-binding cassette family [76] and binds to its S1PR receptors (G-protein-coupled) present at the cell surface. In human plasma, 65% of S1P is transported by lipoproteins (particularly high-density lipoprotein) [77]. S1P is transported to endothelial cells to bind S1PR membrane receptors and to stimulate growth and survival. So far, five S1P receptors (S1P1 – 5) have been identified. They are expressed ubiquitously and are coupled to different G proteins (Gi, Gq, G12/13) that regulate many signals (Rac, PI3-kinase, ERK, JNK) [74]. S1P, which circulates in mammals at high concentrations (> 200 nM), plays an important role in controlling both cell survival and growth (opposite effects to those of ceramides) and SphKs have emerged as important signaling enzymes. As such, SphKs control the balance between ceramide and S1P and serve as 'sphingolipid rheostat'.

3.1 In muscle and liver

In the muscle, several studies demonstrated a positive action of S1P on glucose metabolism. *In vitro* studies showed that S1P stimulates glucose transport through transphosphorylation of the insulin receptor in muscle cells [78]. Using SphK1 activators to push the metabolism of ceramide toward S1P production, insulin sensitivity is enhanced in muscle cells [79]. In addition, moderate overexpression of SphK1 in mice improves insulin sensitivity in skeletal muscle [80]. Similarly, injection of an adenovirus encoding for SphK1 improves glucose metabolism in obese and diabetic mice [81], or treating mice under HFD with an S1P analog prevents the appearance of insulin resistance [82]. However, SphK1-deficient mice fed with HFD do not seem to be more insulin-resistant than wild-type (WT) animals [83]. Finally, Cowart and coworkers showed that activation of the SphK1/S1P axis is involved in the production/secretion of the proinflammatory IL-6 cytokine in response to palmitate in muscles [84]. These evidences reinforce results from an older study that also shows a deleterious effect of S1P on insulin signaling pathway in epithelial cells [85]. In liver, some studies have shown that S1P displays protective functions, opposite to those of ceramides [86,87]. Surprisingly, a very recent study shows that S1P production in response to palmitate in isolated hepatocytes induces insulin resistance [88]. From these data, it appears that the beneficial role of S1P in regulating insulin sensitivity during obesity is still unclear, especially in *in vivo* studies [88].

3.2 In pancreatic β cells

The role of S1P role in insulin secretion is still unclear. A study showed that adding S1P to the culture medium of pancreatic INS-1 β-cell line and islets of Langerhans inhibits insulin secretion in response to glucose potentiated by GLP-1, mediated by S1P1 receptor coupled to Gi [89]. In contrast to this study, S1P was found to stimulate the secretion of insulin in islets of Langerhans [90] and in pancreatic hamster insulinoma tumor-T15 15 β-cell line [91,92]. Interesting, Ozcan and coworker showed that SphK2 plays a critical role in S1P production and insulin secretion induced by glucose [92]. So far, SphK1 has not been implicated in the control of insulin secretion. However, the *in vivo* role of sphingoid base phosphates remains to be investigated by analyzing insulin secretion in available SphK1 and SphK2 knockout mice [93].

The SphK1/S1P axis also plays a critical role in β-cell survival. Indeed, adding S1P to the culture medium of human or murine islets of Langerhans inhibits apoptosis induced by cytokines, mediated by the activation of the PKC pathway, through S1P2 or S1P3 receptors [89,94]. Pharmacological inhibition of SphK activity drastically potentiates β-cell apoptosis, whereas overexpression of SphK1 partially inhibits β cells

induced by lipotoxicity [90]. The antiapoptotic effects of sphingoid base-1-phosphate are likely to be mediated by opposing signaling pathways to ceramide-induced apoptosis (**Figure 3**). Indeed, SphK1 overexpression not only inhibited lipotoxicity-induced loss of mitochondrial transmembrane potential and cytochrome C release in pancreatic β cells [83] but also impaired protein trafficking between the ER and the Golgi apparatus which contributed to lipotoxicity-induced ER stress in pancreatic β cells [90]. It has also been shown that another antiapoptotic effect of sphingoid base phosphates involved the alteration of lipotoxicity-induced ceramide production in pancreatic β cells [90]. Although the mechanism remains to be established, it has been proposed as hypothesis for CerS inhibition mediated by S1P [95]. Regarding the *in vivo* protective role of ShpK1 against pancreatic β-cell apoptosis induced by lipotoxicity, Qi *et al.* showed that whereas a HFD-fed WT mice developed glucose intolerance, all the HFD-fed SphK1 knockout mice manifested evident diabetes, due to a drastic decrease in pancreatic β-cell mass [83]. However, a recent study demonstrates that under HFD SphK1-deficient mice showed enhanced insulin signaling in adipose and muscle tissues and improved systemic insulin sensitivity and glucose tolerance [96]. Moreover, the SphK1/S1P axis appears to stimulate the expression of cytokines by adipocytes isolated from *ob*/*ob* mice and in obese T2D patients [96]. Altogether, these data maintain that the *in vivo* SphK1 role in lipotoxicity is unclear.

4. Conclusion and expert opinion

Our modern society displays a crucial need: to eat quickly. Fast foods satisfy this need and their services meet the expectations of active customers. Consequently, the concept of fast food gradually replaces a traditional balanced and healthy diet. Among the consequences of this mode of nutrition, two pathophysiological aspects are seen in our society: obesity and T2D. Indeed, uncontrolled consumption of a food rich in saturated FAs (mostly palmitate) increases the risk of storage in non-adipocyte tissue of a lipid-derivative called ceramide that is known to alter cell function in insulin-responsive or -producing tissues such as skeletal muscle, liver, AT and pancreatic β cells. FFAs will induce a cellular lipotoxicity that leads to insulin insensitivity at the level of muscle, AT and liver and to β cells apoptosis and inhibition of insulin secretion.

Among lipid metabolites, increasing evidence indicates ceramides as the principal actor of lipotoxicity. Lipotoxicity has been demonstrated to activate *de novo* ceramide synthesis or the sphingomyelin pathway, causing not only ceramide accumulation but also inhibition of ceramide translocation from the ER to Golgi apparatus (at least in pancreatic β cells). As presented on this review, ceramide accumulation activates many cellular pathways that induce cellular stresses and dysfunctions. Indeed, ceramide accumulation induces ER stress, ROS production and mitochondrial dysfunction that induce β-cell apoptosis. Ceramide acts also at the gene expression level, by inhibiting insulin gene expression in pancreatic β cells. In muscle and, ceramide is responsible for the installation of insulin resistance. Given the fact that ceramides contribute importantly to the development of diabetes, the next question to resolve is whether proteins that have been demonstrated to mediate ceramide's deleterious signal in tissues could serve as potential therapeutic targets with the final aim of counteracting ceramide action. Because of the role of aPKCs in several intracellular signaling pathways, the answer will be probably negative. However, recent reports suggest that inhibition of aPKC *in vivo* results in correction of hepatic abnormalities and correction or improvement of hepatosteatosis [28]. In hepatocytes, PKR seems to be an important kinase that mediates ceramide action. However, as with aPKCs, considering that PKR is involved in numerous pathways, it would be difficult to target PKR specifically in order to restore insulin sensitivity. Indeed, PKR is also involved in the immune response. Therefore, inhibiting its expression weakens the antiviral response.

Thus, it seems that the only way to fight ceramide action would be to prevent its intracellular accumulation. Two possibilities exist to achieve this goal: i) blocking ceramide biosynthetic pathways; or ii) pushing ceramide metabolism toward other less-harmful sphingolipid derivatives.

The first possibility seems easier to achieve since many inhibitors of the *de novo* ceramide biosynthesis pathway are already commercially available. However, ceramide constitutes essential membrane components, necessary for the correct cell functioning. Ceramides are important for membrane fluidity and are ubiquitarily present. Indeed, if the *de novo* ceramide synthesis pathway is blocked, as occurs with CerS (2 and 3) knockout mice, the phenotype results are lethal [97,98]. Furthermore, ceramides inhibition may only be effective in the short term, but the long-term side effects are unknown. Currently, pharmacological inhibitors of *de novo* ceramide synthesis exist, as myriocin. Myriocin is also an anti-inflammatory drug, and its usage could lead to an immunity imbalance and to the installation of other pathologies. Moreover, a recent study has shown the limits of this approach. Indeed, they generated mice in which the gene-encoding DES1 enzyme transforms inactive DH-Cer into active ceramide [99]. Placed under HFD to induce insulin resistance, these mice did not produce ceramides and were much less insulin-resistant than control mice under the same conditions. However, these DES1 knockout mice exhibited major growth retardation, many physiological abnormalities and died 8 – 10 days after birth [100]. To conclude, blocking ceramide *de novo* biosynthesis pathway completely has a beneficial effect on insulin sensitivity but also kills them rapidly; the complete absence of ceramide synthesis (and of resulting sphingolipid derivatives) is probably very harmful to membrane structures and to the transduction of countless intracellular signals.

Another possibility is to focus on specific ceramide species. Accumulating works demonstrate that the toxic ceramide effect is not due to the total ceramide increase but rather to specific ceramide species, such as CerS4 in pancreatic β-cell lipotoxicity [53]. Moreover, Turpin *et al.* [38] have shown a strong correlation in AT between CerS6 expression, its major product, C16-ceramides, and the level of insulin resistance in human patients. At the same time, these results were supported by Raichur *et al.* [39] who used mice deficient of CerS2, where they observed a dramatic compensatory increase in intracellular concentrations of C16-ceramides in the liver, predisposing the animals to steatohepatitis and insulin resistance [39]. A better comprehension of which ceramide species are toxic and the development of pharmacological inhibitor of specific CerS could constitute a good strategy to inhibit lipotoxicity mediated by ceramides. Currently, CerS inhibitors do exist, but they are either toxic (like fumonisin B1) or not potent enough (like the FDA-approved FTY720). In addition, these inhibitors are not specific to a defined CerS isoform. Interestingly, Schiffmann *et al.* have recently developed potent selective CerS inhibitors [101]. However, there is presently no information available about whether diminishing the synthesis of specific ceramide species *in vivo* would prevent the deleterious effect of lipotoxicity in human. Therefore, more studies are needed in this field to understand the precise role of CerS in lipotoxicity.

The second possibility to try to counteract the deleterious action of ceramide may be to study the regulation of their metabolic fate. Indeed, once synthesized, ceramides are then converted into other sphingolipid derivatives such as S1P. Sphingoid base phosphates and ceramides are interconvertible and have opposing effects, and it has been proposed that a dynamic balance between these two lipids could be an important factor that determines cell fate [74]. This concept is defined as sphingolipid biostat. The existence of this biostat offers the possibility to find new therapeutic targets in order to limit lipotoxicity observed during obesity. In pancreatic β cells it has recently been demonstrated that by shifting the ceramide:S1P ratio using adiponectin, β cells functioning is preserved [102]. The toxic effects of ceramides could be counteracted metabolizing them into less toxic lipid species as S1P or sphingomyelin. Pushing ceramide metabolism into the S1P pathway could represent a potential target to counteract the ceramide lipotoxicity induced during T2D-linked obesity. But as stated above, since most of these ceramide derivative lipids seem to play divergent role (positive or negative) depending on the tissue, additional investigations on the function of S1P and its synthesizing enzymes will be necessary before clarifying their role in lipotoxicity.

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Declaration of interest

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