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Monoacylglycerol as a metabolic coupling factor in glucose-stimulated insulin secretion

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Résumé

Les cellules beta pancréatiques sécrètent l'insuline lors d'une augmentation post-prandiale du glucose dans le sang. Ce processus essentiel est contrôlé par des facteurs physiologiques, nutritionnels et pathologiques. D'autres sources d'énergie, comme les acides aminés (leucine et glutamine) ou les acides gras potentialisent la sécrétion d'insuline. Une sécrétion d'insuline insuffisante au besoin du corps déclanche le diabète. Le rôle que joue l'augmentation du calcium intracellulaire et les canaux K^+/ATP dans la sécrétion d'insuline est bien connu. Bien que le mécanisme exact de la potentialisation de la sécrétion d'insuline par les lipides est inconnu, le cycle Glycérolipides/Acides gras (GL/FFA) et son segment lipolytique ont été reconnu comme un composant essentiel de la potentialisation lipidique de la sécrétion d'insuline. Le diacylglycérol, provenant de la lipolyse, a été proposé comme un signal lipidique important d'amplification. Cependant, l'hydrolyse des triglycérides et des diacylglycérides a été démontrée essentielle pour la sécrétion d'insuline stimulée par le glucose, en suggérant un rôle du monoacylglycérol (MAG) dans ce processus.

Dans cette étude, on démontre que la réduction de la sécrétion d'insuline stimulée par le glucose, lors d'une inhibition de la lipolyse, est restaurée par l'addition de MAG. Dans les cellules beta pancréatiques, le niveau de MAG augmente en présence des concentrations élevées du glucose, et également lorsqu'on inhibe l'enzyme MAG hydrolase abhydrolase-6 (ABHD6) avec l'inhibiteur spécifique WWL70.

L'analyse lipidomique a démontré qu'après la stimulation des cellules beta pancréatiques avec le glucose et aussi avec le WWL70, l'espèce la plus accumulée de MAG était le 1-stearoylglycérol (1-SG). L'addition de 1-SG, de 1-palmitoylglycérol (1-PG) ou de WWL70 augmente la sécrétion d'insuline stimulée par le glucose, et cette augmentation est indépendante de la génération de acides gras à partir de MAG. Cela suggère que le MAG est un signal lipidique pour la potentialisation de la sécrétion d'insuline stimulée par le glucose. De plus, la surexpression du gène d'ABHD6 dans les cellules INS832/13 cause une réduction de la sécrétion d'insuline, due probablement à la diminution des niveaux intracellulaire de MAG.

Avec le but de comprendre le mécanisme moléculaire impliqué dans la potentialisation de la sécrétion d'insuline par le MAG, on a bloqué l'action du récepteur vanilloid-1 (TRPV1) liant le MAG par l'agent pharmacologiste, AMG9810. Le traitement des cellules beta pancréatique par AMG9810 entraîne une diminution de la potentialisation de la sécrétion de l'insuline induite par le MAG. Il est a noter que le MAG pourrait activer TRPV1 par une liaison physique dans la membrane cellulaire interne; ce qui entraînerai l'entrée du calcium dans la cellule, et ensuite la stimulation de l'exocytose des granules à insuline. En soutien de cette hypothèse, on a trouvé une diminution du calcium intracellulaire lorsqu'on traite au AMG9810 des cellules beta pancréatique de rat (provenant des îlots dispersés) stimulées au glucose et au WWL70.

L'ensemble des résultats suggère que le MAG est un médiateur de la potentialisation lipidique de la sécrétion d'insuline stimulée par le glucose. Vu que l'inhibition pharmacologique d'ABHD6 augmente la sécrétion d'insuline, on pourra conclure que cette enzyme représente une cible thérapeutique potentielle dans le développement des médicaments anti-diabétiques, visant une augmentation de la sécrétion d'insuline.

Mots-clés : diabète, sécrétion d'insuline stimulée par le glucose, MAG, ABHD6, TRPV1.

Abstract

Insulin secretion by the pancreatic □-cell in response to post-prandial increase in blood glucose levels is an essential physiological process that is governed by cellular, nutritional and pathological factors. Other fuels including amino acids like leucine and glutamine and also fatty acids contribute to further augment insulin secretion. Failure to secrete adequate amount of insulin according to the changing demands of the body by □-cell is a key determinant of diabetes. The role played by the elevated Ca^{2+} influx and K^{+}-ATP channels in insulin secretion is well known. Even though the precise mechanism of the lipid amplification of insulin secretion and the involved molecular signals are not clear, Glycerolipid/Free fatty acid (GL/FFA) cycle and its lipolytic segment have been recognized as essential components in the lipid amplification pathway of insulin secretion. Diacylglycerol produced by lipolysis was proposed as an important lipid amplification signal. However, hydrolysis of triglycerides and also of diacylglycerols is shown to be essential for glucose stimulated insulin secretion (GSIS), indicating a possible role for monoacylglycerol (MAG) in this process.

In the present study we demonstrate that the obliterated GSIS due to lipolysis inhibition in □-cells can be restored by providing exogenous MAG. In the □-cells MAG levels increase significantly in the presence of high glucose concentration and specific inhibition of the major MAG hydrolase, abhydrolase-6 (ABHD6), in □-cells and islets with WWL70 leads to accumulation of MAG with concomitant increase in insulin secretion. Lipidomics analysis

indicated that the major MAG species that is elevated by high glucose as well as WWL70 addition is 1-stearoylglycerol (1-SG). Exogenously added 1-SG and also 1-palmitoylglycerol (1-PG) strongly enhanced GSIS and this augmentation is not dependent on the generation of FFA by these MAGs. This indicates that MAG is a potential candidate for being the lipid signal for GSIS amplification. Further evidence for this was provided by the observation that overexpression of the MAG hydrolase ABHD6 in INS832/13 cells, resulted in decreased insulin secretion, probably owing to the lowered MAG level inside the □-cells.

Pharmacological studies using AMG9810, a specific antagonist of transient receptor potential vanilloid-1 (TRPV1) receptor that binds MAG, revealed that a blockade of TRPV1 strongly attenuated the MAG-augmented insulin secretion. Since MAG is a potential activator of TRPV1, it is likely that MAG binds on the inner surface of the cell membrane to TRPV1, which in turn triggers rapid influx of Ca^{2+} thereby promoting insulin granule exocytosis. Thus, AMG9810 was found to lower Ca^{2+} influx into dispersed rat islet cells that was induced by high glucose and also WWL70.

These results collectively suggest that MAG is the potential mediator of the lipid amplification of glucose-stimulated insulin secretion. Our results also indicate that pharmacological intervening at the ABHD6 hydrolysis step enhances insulin secretion; this enzyme protein can be a promising thrapeutic target for the development of anti-diabetic drugs that promote insulin secretion.

Keywords: Diabetes, glucose-stimulated insulin secretion, monoacylglycerol, ABHD6, TRPV1

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1-OG	1-oleoylglycerol
1-PG	1-pamitoylglycerol
1-SG	1-stearoylglycerol
2-AG	2-arachidonoyl glycerol
2-OG	2-oleoylglycerol
ABHD6	alpha/beta-hydrolase domain 6
ABHD12	alpha/beta-hydrolase domain 12
ADA	American Diabetes Association
ADPR	ADP ribose
ATF6	activating transcription factor 6
ATGL	adipose tissue triglyceride lipase
CHOP	C/EBP homologous protein
DAG	diacylglycerol
GAD65	glutamic acid decarboxylase
GDM	gestational diabetes mellitus
GIP	glucose-dependent insulinotropic peptide
GL/FFA cycle	glycerolipid/ free fatty acid cycle

GLP-1	glucagon-like peptide-1
GLUT2	glucose transporter 2
GSIS	glucose-stimulated insulin secretion
HSL	hormone sensitive lipase
IRE1	inositol requiring 1
IRSs	insulin receptor substrates
JNK	c-JUN NH2-terminal kinase
MAG	monoacylglycerol
MAGL	monoacylglycerol lipase
MCF	metabolic coupling factor
MDP	morphologically docked pool
NSF	soluble *N*-ethylmaleimide-sensitive factor
PDX-1	pancreas-duodenum homobox-1
PERK	PKR-like kinase
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
ROS	reactive oxygen speices
SNAP-25	synaptosomal-associated protein 25
SNARE	soluble NSF attachment protein receptor

RRP	readily released granules
SRP	signal recognition particle
T1D	type 1 diabetes
T2D	type 2 diabetes
TG	triacylglycerol
TRP channel	transient receptor potential channels
UPR	unfolded protein response
VAMP-2	vesicle-associated membrane protein 2
ZDF	Zucker Diabetic Fatty
ZnT7	zinc transporter 7
ZnT8	zinc transporter-8

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1. Introduction

1.1 Obesity and Diabetes

1.1.1 The current situation in the world

Economic development and industrialization in many countries offer a wide variety of choice of foods to people. This has led more and more people to choose foods with elevated levels of saturated fatty acids, sugar-sweetened beverages and starchy food and reduced consumption of fibre. In conjunction with more sedentary lifestyle of people the energy from food intake has often considerably surpassed the daily energy expenditure. The surplus energy is predominantly stored as fat in the adipocytes and other tissues, therby contributing to obesity among these people and this is particularly marked for individuals who are genetically predisposed to obesity (Alappat and Awad, 2010).

Obesity has become a major socio-economic problem all over the world, especially in the developed countries. According to the American Obesity Association, nealy one third of the total US population is affected by obesity with a similar situation in Canada. A report from the National Diabetes Surveillance System indicated that approximately 25% of all Canadian adults are obese as are 10% of the children (Anis et al., 2010).

The global obesity epidemic is a primary cause for the increasing world-wide incidence of diabetes in the recent years. According to the World Health Organization and the International Diabetes Federation, the number of diabetes patients has increased from 100–135 million in 1994–1995 to approximately 246 million in 2007, worldwide. With a further

7 million people developing diabetes each year, this number is expected to hit 438 million by 2030. In Canada, approximately 1.9 million men and women had been diagnosed with diabetes in 2005-2006, which represents about 1 in 17 Canadians - 5.5 % of all women and 6.2 % of all men. And now in 2010, more than 9 million Canadians live with undiagnosed or diagnosed diabetes, or prediabetes.

Diabetes is defined in simple terms, as the pathological situation where inadequate amount of insulin is secreted to meet the needs of body, with the resultant hyperglycemia, the hallmark of diabetes. However, diabetes is a highly complex metabolic disorder with a multitude of mechanisms for its causes, including both genetic and environmental factors depending on different types of diabetes. Diabetes, on the basis of its causes, could be divided into three major types- type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes as well as other types, such as permanent neonatal diabetes (Gloyn et al., 2004).

1.1.2 Type 1 Diabetes (T1D)

T1D that is also named Juvenile diabetes, accounts for about 5 -10% of all cases of diabetes. In the United-States, 1 million people have been diagnosed with T1D in 2000. There are potentially another million patients, mostly adults, who have been possibly misdiagnosed as having type 2 diabetes (Prevalence of Diabetes in the United States based on US Population:

275 million in 2000). Additional studies predict that the incidence of T1D may be increasing at a rate of 3 - 4% a year (Dahlquist, 1998; Gloyn et al., 2004).

Type 1 diabetes is in the vast majority of its cases a chronic autoimmune disease, in which T cells and macrophages invade pancreatic islets and specifically target and kill beta cells in genetically predisposed individuals causing near complete loss of insulin secretion with the resultant hyperglycemia (Sera et al., 1999; Winter, 1996); At present, the exact mechanisms underlying the causes whereby the body's immune system is triggered to attack the beta cells is still not well understood. Some studies indicated that autoimmune, genetic, and environmental factors, possibly viruses, are involved in this process. Generally, T1D most often affects children and young adults, but can also appear at any age. Symptoms include increased thirst and urination, constant hunger, weight loss, blurred vision, and extreme fatigue. If not treated with insulin, a person with type 1 diabetes can lapse into a life-threatening diabetic coma, also known as diabetic ketoacidosis. Although the symptoms of T1D generally develop within a short period, the beta cell destruction maybe occurring over the span of several years prior the development of overt T1D. Thus, this allows for the possible prevention of T1D transition to overt diabetes. More than 30 years ago, it was observed that certain antibodies (named as islet cell antibodies) in TID patients could specifically bind to pancreatic islets. Presently, three major autoantigens: glutamic acid decarboxylase (GAD65) (Baekkeskov et al., 1989), a protein tyrosine phosphatase-like molecule (IA-2) (Notkins et al., 1996) and insulin (Palmer, 1987) have been identified. Using assays that employ islet cell autoantibodies, it is possible to

accurately identify people at higher risk for T1D. Once the risk is identified, these people can have time to use different strategies to delay or prevent the incidence of T1D, such as taking oral insulin capsules (Skyler et al., 2005). For people who are already suffering with T1D, the secure and relatively convenient way is to treat the disease by daily administration of insulin, because presently there is no “cure” for T1D. Even though islet transplantation has shown to be a promising therapy for T1D for many years (Gruessner et al., 2004; Rickels et al., 2006), it is not widely used due to its high price, graft immuno-suppression associated problems, and limitation of islet donors. Another promising strategy for T1D is the regeneration of beta cells from stem cells, precursor cells or the remaining beta cells (Meier et al., 2005). Thus, murine adult pancreatic precursor cell likely exists that could differentiate into cells with the characteristics of ¦Âcells (Seaberg et al., 2004). Another study showed that the prinicipal source of new beta cells is from the pre-existing beta cells, rather than pluripotent stem cells (Dor et al., 2004).

1.1.3 Gestational diabetes

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance that is first detected during pregnancy (Jovanovic et al., 1998). According to the American Diabetes Association (ADA), about 7% of all the pregnancies in USA are diagnosed with having GDM (Simmons et al., 2010). Another report indicates that the total incidence of the GDM can be as high as 17.8% (Metzger et al., 2010). Once diagnosed with GDM, the mother as well as its offspring, who has been exposed to hyperglycemic intrauterine

environment, has increased risk of developing type 2 diabetes mellitus in the future (Bellamy et al., 2009; Clausen et al., 2008). Because of the lack of uniform diagnostic criteria (Karagiannis et al., 2010), the global incidence of GDM is difficult to estimate. But it is well-accepted that GDM is a common disorder in pregnancy (Yogev et al., 2004).

The mechanism related to incidence of GDM has not been fully elucidated, but at least three different factors have been shown to be implicated in this process: 1) auto-immune destruction of beta cell; 2) genetic abnormalities leading to defective insulin secretion; 3) beta cell dysfunction related to insulin resistance. As a consequence, the incidence of GDM is not only influenced by environmental factors, but also by genetic factors, and it shares some similarities to both T1D and T2D. So treatment of GDM may be different based on the individuals. The importance of nutrition in the management of GDM is well established (Amann-Gassner and Hauner, 2008). Since glucose is the primary fuel for the fetus and the characteristic of GDM is hyperglycemia, a healthy pregnancy outcome has to be met in the context of aberrant regulation of glucose metabolism. The main objective of nutritional recommendation for women with GDM is to maintain maternal normoglycemia and to reduce inadvertent accelerated fetal growth. The dietary advises are expected to have a lifelong positive impact for women with GDM so that maternal health is maintained and the risk for developing type 2 diabetes mellitus in the future is alleviated.

1.1.4 Type 2 diabetes (T2D)

T2D is characterized with inadequate insulin secretion by pancreatic beta cells leading to hyperglycemia in individuals with a long history of insulin resistance and is predominantly due to the dysfunction of beta cells. T2D is thought to be a complex syndrome of polygenic nature (Fig 1) and many genes, such as CAPN10, ENPP1, HNF4A, ACDC, SLC308A, IDE-KIF11, EXT2-ALX4 that contribute to the development of T2D have been identified (Perry and Frayling, 2008; Sladek et al., 2007) with the help of the new high-density array technology that permits the simultaneous genotyping of thousands of polymorphisms. Some of the identified susceptible genes, such as zinc transporter SLC308A, are exclusively expressed in beta cells (Potapov et al., 2010). Many other genes that are also related to T2D still need to be identified. Discovery of new T2D risk genes will help in identifying novel players in T2DM pathogenesis, and will unravel novel mechanisms and lead to more efficient therapeutics.

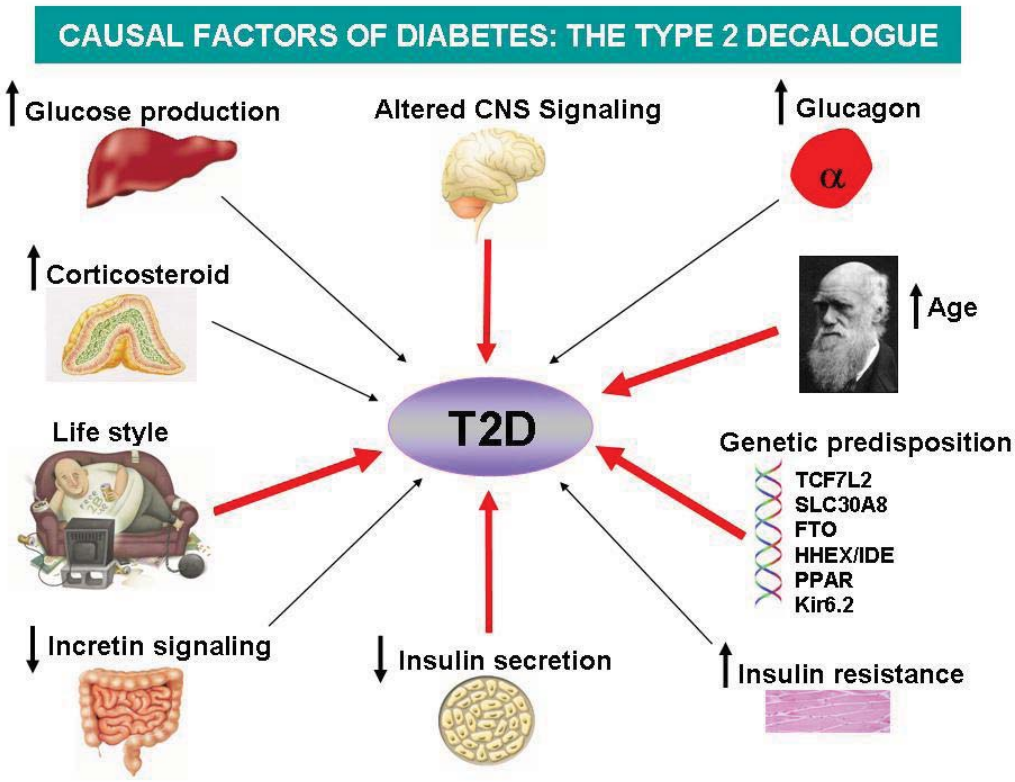

Figure 1 The possible causal factors of T2D. The onset of T2D is complex, not only related to the environmental factors, such as change of life style, but also the genetic background. Several risk factors have been linked with onset of T2D and four factors, including life style, decreased insulin secretion, altered CNS signaling and genetic predisposition shown in red arrow play crucial roles in T2D.

T2D is the more common type of diabetes and accounts for approximately 90 to 95 percent of all cases of diabetes. Approximately 150 million people worldwide were affected by T2D in the year 2000 but this number has climbed to ~250 million in 2010. According to the Canadian Diabetes Association, it is estimated that nearly 3 million Canadians suffer from T2D in 2010. Because of the global obesity epidemic and increasing number of

people with insulin resistance, it is predicted that this number could increase to 370 million by 2030. Incidence of T2D is strongly associated with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity and ethnicity (Al-Adsani et al., 2009). There has been an alarming increase in childhood obesity associated with T2D (Amed et al., 2010). However, nationally representative data on prevalence of type 2 diabetes in youth are not available.

Unlike T1D, the symptoms of type 2 diabetes develop gradually. T2D is actually considered as a syndrome rather than a disease such as anemia and hypothyroidism. Even though there are no overt symptoms for T2D such as fever, pain etc., if the hyperglycemia of these patients is not corrected soon with pharmacological, nutritional or physical intervention, gradually the function of several organs, such as kidney, foot as well as eyes and brains, can be compromised. Thus untreated T2D can become a serious health and social problem due to secondary complications (Renard, 2009), affecting the quality of life and even threatening life. Even though our knowledge related to the onset of T2D is still poor, some crucial factors have been identified and proven to be implicated. Numerous studies show that most individuals with T2D have insulin resistance (Lillioja et al., 1993; Prentki and Nolan, 2006) and more importantly, insulin resistance appears before the development of hyperglycemia in subjects that eventually develop T2D (Martin et al., 1992; Prentki and Nolan, 2006). Furthermore, it is now recognized that T2D develops in insulin-resistant subjects with the onset of beta cell dysfunction (Prentki and Nolan, 2006). So

insulin resistance and beta cell dysfunction are two crucial factors that play significant role in the onset of T2D as shown in Fig 2.

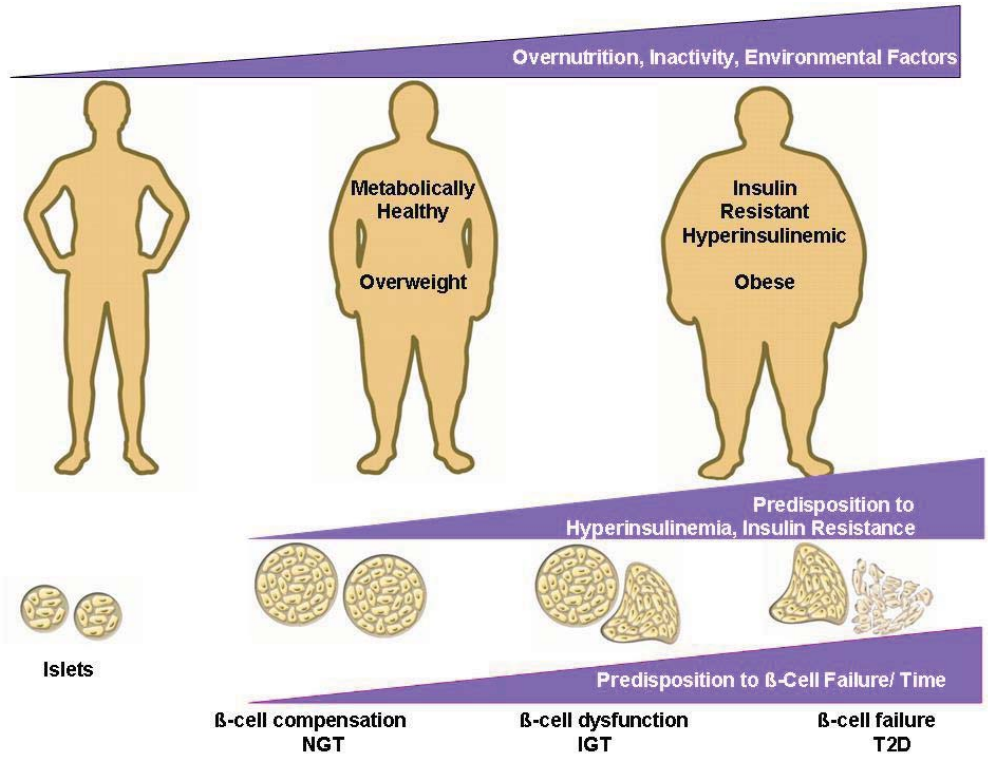

Figure 2 Islet beta cell failure and natural history of T2D. T2D develops in response to overnutriton and lack of physical activity in subjects that have underlying genetic and acquired predispositions to both insulin resistance (and/or hyperinsulinemia) and beta cell dysfunction. Over time, islet beta cell compensation for the insulin resistance fails, resulting in a progressive decline in □-cell function. As a consequence, subjects progress from normal glucose tolerance (NGT) to IGT and finally to established T2D. Even after diagnosis of T2D, □-cell function continues to worsen such that subjects progress from needing changes in diet/exercise only to requiring oral hypoglycemic agents and eventually insulin for achievement of adequate glycemic control. Future therapies will be directed not only to achievement of euglycemia, but also changing the course of the disease by reversing the processes of □-cell failure (Nolan et al., 2006b).

1.1.4.1 Insulin resistance

Insulin sensitivity is commonly described as the ability of insulin to lower plasma glucose levels by suppressing hepatic glucose production and stimulating glucose utilization in skeletal muscle and adipose tissue (Fujimoto, 2000). Reduced insulin sensitivity makes the insulin-target tissues to respond poorly to insulin, leading to insulin resistance, which is an important link between obesity and T2D. Development of insulin resistance depends both on inherited and acquired factors. The inherited factors are most evident in the off-springs of T2D, who have more chance to be affected by insulin resistance (Warram et al., 1990). Some inherited defects have been identified, such as mutations in insulin receptors, glucose transporters and signaling proteins, but these mutations are relatively rare, and cannot explain the most common forms of insulin resistance, which is usually closely related to T2D. More work needs to be done to identify these inherited defects. With regard to the acquired causes of insulin resistance, some of the responsible factors include less physical activity, fuel surfeit, aging, hyperglycemia, hyperlipidemia and high level of plasma free fatty acids (FFA) as well as some medications. The mechanisms whereby these factors lead to insulin resistance are largely unknown. Some evidences indicate that impaired insulin signal transduction may be responsible. Insulin signaling involves a cascade of events initiated by insulin binding to its cell surface receptor, followed by receptor autophosphorylation, and activation of receptor tyrosine kinases, which result in tyrosine phosphorylation of insulin receptor substrates (IRSs)(Choi and Kim, 2010). IRSs further activate phosphatidylinositol 3-kinase (PI3K) and as a consequence, Akt will be activated and then phosphorylate its downstream targets, such as AS 160, which is implicated in the

process of glucose transport. IRS-1/PI3K/Akt signaling pathway is crucial in the regulation of glucose transport in response to insulin in skeletal muscle, the major site of glucose disposal (Koopmans et al., 1997). Loss of insulin signaling leads to severe insulin resistance in hepatocytes and progressive liver dysfunction (Pessin and Saltiel, 2000). Restoration of IRS-1/PI3K/Akt signaling pathway using insulin-sensitizer agents, such as metfomin, troglitazone and other thiazolidinediones could reverse insulin resistance and delay, or even prevent the onset of T2D. Interestingly, a polymorphism associated with T2D has been found within the IRS1 gene locus (Rung et al., 2009).

Insulin resistance is closely related to lipid metabolism. FFA derived from lipid metabolism (both do novo synthesis and lipolysis) is an important inducer of insulin resistance. From one side, high levels of FFA could directly interrupt the insulin signaling and decreased plasma membrane translocation of Glut4 in muscle and therefore lead to lower glucose disposal (Boden et al., 1991). Decreasing the FFA level in obese nondiabetic individuals is helpful to ameliorate insulin sensitivity in skeletal muscle (Santomauro et al., 1999). Blood levels of FFA are influenced by dietary intake and also by contributions from lipolysis of depot fats in adipose and other tissues. Adipose tissue triglyceride lipase (ATGL) is an important enzyme in lipolysis and its deficiency leads to TG accumulation and decreased FFA release, as a result, increasing insulin sensitivity and glucose tolerance (Hoy et al., 2010). On the other hand, increased level of FFA causes DAG and ceramide accumulation and DAG has been shown to disrupt normal insulin signaling by serine phosphorylation of

IRS1, through the activation of some atypical protein kinase C isoforms (Itani et al., 2002). Ceramides could activate protein phosphatase 2A, leading to dephosphorylation of Akt, thereby blocking insulin signaling and inhibiting Glut4 translocation to the cell membrane, thus disrupting insulin-mediated glucose uptake into skeletal muscle (Bonen et al., 2004). Besides the level of circulating FFA, intracellular content of FFA is also an important determinant of insulin resistance that acts independently of cellular energy balance and stress (Matsuzaka et al., 2007). Additional metabolic signaling pathways that could be central to insulin resistance are the AMP-kinase/malonyl-CoA signaling network and the glycerolipid/FFA cycle. Thus, elevated glucose and FFA will lead to AMPK inhibition, acetyl-CoA carboxylase activation and enhanced malonyl-CoA accumulation leading to reduced FFA oxidation and a rise in DAG that can activate C-kinase enzyme and thus cause reduced insulin signaling (Prentki and Corkey, 1996; Ruderman and Prentki, 2004). The evidence has been reviewed recently that altered GL/FFA cycling, with resulting accumulation of particular GL, such as DAG, MAG and phosphatidate, is implicated in insulin resistance and tissue malfunction during fuel surfeit (Prentki and Madiraju, 2008).

Besides FFA, Sphingolipids metabolism, such as glucosylceramides are also implicated in the pathogenesis of insulin resistance. It has been shown that pharmacological inhibition or genetic ablation of enzymes controlling sphingolipid synthesis in rodents ameliorates insulin resistance (Holland and Summers, 2008; Summers and Nelson, 2005).

It is widely accepted that insulin resistance plays crucial role in pathogenesis of T2D and other diseases associated with the metabolic syndrome. Amelioration of insulin resistance is necessary to delay or prevent the onset of T2D. Some evidence showed that angiotensin-converting-enzyme inhibitors could prevent the onset of T2D (Solski and Longyhore, 2008). And also physical activity could improve insulin sensitivity and glucose tolerance in the skeletal muscle, and therefore it is also a good measure to prevent T2D. Certainly, our current knowledge related to insulin resistance is not enough to prevent T2D. More efforts are needed to completely understand the molecular mechanisms, and to find better treatments to prevent and cure insulin resistance and T2D.

1.1.4.2 Beta cell dysfunction

Beta cell is the only cell that synthesizes insulin and is responsible for insulin release, which plays key role in glucose homeostasis. Multiple evidences indicate that beta cell dysfunction is present in individuals with T2D and acts as an integral part in the pathogenesis of T2D. The identified mechanisms responsible for beta cell dysfunction include glucotoxicity, lipotoxicity, glucolipotoxicity, inflammation and ER stress. We will separately introduce these different aspects.

1.1.4.2.1 Glucotoxicity

Glucose toxicity for beta cells is defined as dysfunction induced by the exposure of these cells to chronically high concentrations of glucose, which causes impaired glucose-

stimulated insulin secretion and insulin gene expression as well as apoptosis under some conditions (Gleason et al., 2000). After chronic high glucose treatment, beta cell lines, such as HIT-T15 and INS-1 have reduced glucose-stimulated insulin secretion and also reduced insulin content (Olson et al., 1993). Studies showed that the insulin promoter activity and pancreas-duodenum homobox-1 (PDX-1) and MafA binding activity to the insulin promoter also decreased (Olson et al., 1993; Olson et al., 1995). Similar results have been obtained in rat islets cultured ex vivo for up to six weeks (Briaud et al., 1999).

The mechanism regarding glucose toxicity is closely related to the remodeling of glucose metabolism in the beta cell. Instead of glucose being metabolized via glycolysis to produce pyruvate that enters the Krebs' cycle, additional pathways are activated that include glyceraldehyde autooxidation, lipogenesis with DAG formation and PKC activation, sorbitol metabolism leading to the accumulation of reactive oxygen speices (ROS) (Poitout and Robertson, 2002). Due to poor defense mechanisms against ROS in the beta cells (low expression of superoxide dismutases and virtually no expression of catalase or glutathione peroxidase) ROS eventually cause beta cell dysfunction. Interestingly, beta-cell specific overexpression of glutathione peroxidase preserves intranuclear MafA and reverses diabetes in db/db mice (Harmon et al., 2009).

By virtue of glucotoxicity leading to oxidative stress, some antioxidants may be beneficial in protecting beta cell from this insult. This hypothesis has been supported in studies using

the anti-oxidants N-acetylcysteine and aminoguanidine. In the Zucker Diabetic Fatty (ZDF) rats, treatment with these antioxidants has been shown to decrease markers of oxidative stress and improve glycemia in this T2D model (Briaud et al., 1999). In db/db mice, treatment with N-acetylcysteine enhanced insulin secretion, ameliorated glycemia, reduced apoptosis and increased beta cell mass (Kaneto et al., 1999).

1.1.4.2.2 Lipotoxicity

The concept of lipid toxicity derived from the phenomenon that T2D is commonly linked with elevated levels of triacylglycerol (TG) and FFA in the plasma. Similar to glucotoxicity, lipotoxocity refers to chronically elevated level of FFA eventually causing beta cell dysfunction (Gremlich et al., 1997). Different fatty acid species have differential effect in inducing beta cell apoptosis. Saturated fatty acids, such as palmitate and stearate have strong effect in inducing cell apoptosis. By contrast, un-saturated fatty acids, such as oleate, have been shown to protect beta-cells from apoptosis (Cnop et al., 2001; El-Assaad et al., 2003; Maedler et al., 2003; Morgan et al., 2008). And this protective effect may be due to the strong ability of unsaturated fatty acid in inducing TG synthesis (Cnop et al., 2001). But this conclusion has been under debate, as other results indicate the protective effect is due to reasons other than the metabolism of FFA (Morgan et al., 2008).

Even though the concept of lipotoxicity has been there for many years, it is still short of strong experimental support. Our and other people's results indicate that FFA can induce

beta cell dysfunction only in combination with elevated levels of glucose. In the presence of low glucose, high FFA (at reasonable concentrations) does not change both in vitro and in vivo the total insulin content of the ß-cell and only slightly affects glucose-stimulated insulin secretion. So from this standpoint, the concept of glucolipotoxicity (Prentki et al., 1998), combination of chronic effect of both high glucose and FFA, is more reasonable to explain the toxic effect of FFA in beta cells and therefore gained more focus in recent research.

1.1.4.2.3 Glucolipotoxicity

Glucolipotoxicity is a combination of glucotoxicity and lipotoxicity, but importantly incorporates the concept of synergy of the toxicity of these fuels when present in excess simultaneously. This concept has been initially advanced advanced by our lab together with Dr B Corkey (Prentki and Corkey, 1996) and has been widely accepted. Glucolipotoxicity indicates the synergistic effects in chronic effect of glucose and fatty acid in inducing beta cell dysfunction, and therefore glucolipotoxicity shows some similar features as glucotoxicity, such as decreased glucose-stimulated insulin secretion and decreased total insulin content of beta cells. Glucolipotoxicity also induces beta cell dysfunction in a unique fashion. When INS832/13 cells, derived from rat beta cells were incubated in different conditions with high glucose (20mM glucose, no FFA), or high FFA (0.4mM palmitate, 5mM glucose), or high glucose (20mM) plus high FFA (0.4mM), high FFA alone has no effect in comparison to high glucose, indicating only high FFA *per se* has no

toxic effect. Of special importance, the combination of high glucose and high FFA showed highest apoptosis, compared to the group with low glucose (El-Assaad et al., 2003). Further study indicates that this strong apoptosis inducing effect was closely related to lipid esterification processes, TG accumulation and ceramide deposition as well as activation cascade-3 pathway in the cells (El-Assaad et al., 2010). Similar results were also reported in dispersed rat and human islets (Buteau et al., 2004). Some in vivo results also favor the concept of glucolipotoxicity. A 72 h infusion of glucose and intravenous fat emulsion in 6-month-old rats leads to insulin resistance and reduced insulin secretion in vivo. This was associated with diminished glucose-stimulated second-phase insulin secretion and proinsulin biosynthesis and lower insulin content as well as reduced expression of typical beta cell genes in isolated islets (Fontes et al., 2010).

The mechanisms related to glucolipotoxicity are strongly associated with glucose and FFA metabolism. As proposed by our lab, glucose functions as the main determinant of fatty acid partitioning inside the beta cells. When glucose concentration is in the low to normal range, fatty acids are transported into mitochrondria through CPT-1, for beta oxidation without causing any toxic effect. When glucose and fatty acids are both in elevated concentration, glucose is converted to citrate through TCA cycle and then leads to the synthesis of malonyl-CoA, which inhibits CPT-1 activity. Inhibition of beta oxidation, at the CPT-1 step causes fatty acid partitioning from beta oxidation to esterification. High glucose also leads to enhanced lipolysis, and if the rates of esterfication and lipolysis are

the same, there is little toxic effect even in the presence of high glucose and high fatty acid. But if these rates of esterfication and lipolysis are not balanced, many lipid derivatives may accumulate and induce beta cell dysfunction (Poitout and Robertson, 2008; Prentki et al., 2002; Prentki and Madiraju, 2008).

1.1.4.2.4 Endoplasmic reticulum (ER) stress

The main function of pancreatic beta cell is insulin synthesis according to the body's demand. As estimated, beta cell can produce 18-75 pg insulin per minutes per islets under basal conditions. This large amount underscores the special "insulin factory" characteristic of beta cells with a highly developed ER, which is the major site responsible for post-translational modification, folding and assembly of newly synthesized secretory proteins, and a cellular calcium store. ER is also an organelle that controls cell survival. A myriad of pathological and physiological factors, such as impairment of protein transport from the ER to the Golgi, and calcium depletion from the ER lumen, can compromise the function of the ER, termed as ER stress (Laybutt et al., 2007). Beta cells have to employ certain cytoprotective mechanisms to mitigate ER stress, referred to as the unfolded protein response (UPR), also named as ER stress signaling, that is elicited by ER stress (Eizirik and Cnop, 2010). The UPR can reduce ER stress and maintain ER function to produce and process proper amounts of proteins. In the event that the UPR cannot maintain ER homeostasis, cells activate at least three apoptosis pathways to induce cell apoptosis (Kim et al., 2006): the transcriptional induction of the genes for CHOP (C/EBP homologous

protein)/ GADD153 pathway (Oyadomari and Mori, 2004), the c-JUN NH2-terminal kinase (JNK) pathway (Kaneto et al., 2005), and the ER-localized cysteine protease caspase-12 pathway (Shiraishi et al., 2006),. Multiple studies have shown that ER stress is implicated in beta cell apoptosis and may be responsible for the reduction of beta cell mass in individuals with T2D.

Three ER membrane-associated proteins, inositol requiring 1 (IRE1), PKR-like kinase (PERK), and activating transcription factor 6 (ATF6) (Yoshida, 2007), have been identified as master regulators in ER stress signaling and shown to regulate glucose homeostasis. Pancreatic beta cells deficient in PERK are more susceptible to ER stress-induced apoptosis. And PERK-deficient mice develop severe hyperglycemia soon after birth in virtue of defects in islet proliferation and increased apoptosis (Harding et al., 2001).

However, our understanding of the UPR in beta-cells is far from complete. The complexity of UPR pathways as well as its master regulators has not been completely investigated. We need to identify the cross talk between UPR pathway and other signaling pathways, such as mTOR, which have been shown to decrease ER stress (Qin et al., 2010), and also focus on identifying endogenous molecules and chemical compounds that could modulate ER stress and protecting beta cells from metabolic stress-induced apoptosis.

1.1.4.2.5 Inflammation

It has been recognized that cytokines are one of the main “players” in inducing T1D (Mandrup-Poulsen et al., 1996). The main types of cytokines are interleukin-1beta (IL-1beta), tissue necrosis factor 1alpha and interferon-¦Ã But the role of cytokines in the pathogenesis of T2D is still under debate. It has been reported that high glucose can induce human islets to produce IL-1beta, which causes beta cell dysfunction (Donath et al., 2003). But these results could not be reproduced by others. Most recently, it has been shown in a clinical trial that a drug blocking binding of IL-1beta to its receptor improved beta cell function and ameliorated the hyperglycemia in human T2D, indicating the important roles of IL-1beta exerting negative effects on beta cells (Mandrup-Poulsen et al., 2010). However, large-scale clinical studies in human T2D should be conducted to confirm these results.

1.2 Insulin secretion

As indicated in the first chapter, despite the huge variations in beta cell dysfunction in each individual affected with diabetes, the common hallmark for all types of diabetes is hyperglycemia, due to insufficient insulin secretion after stimulation. Insulin was discovered by Banting and Best in 1922 and its primary structure was deduced in 1951 by Fred Sanger, who was awarded the Nobel Prize (Sanger, 2001). Insulin is composed of two polypeptide chains referred to as the A chain and B chain, which are linked together by two disulfide bonds, and an additional disulfide is formed within the A chain. In most species, the A chain consists of 21 amino acids and the B chain of 30 amino acids. Although the amino acid sequence of insulin varies among species, certain segments of the molecule are

highly conserved, including the positions of the three disulfide bonds, both ends of the A chain and the C-terminal residues of the B chain. These similarities in the amino acid sequence of insulin lead to a three dimensional conformation of insulin that is very similar among different species, and thus insulin from one animal is very likely biologically active in other species.

1.2.1 Insulin biosynthesis and insulin exocytosis

Insulin biosynthesis is a complex process and involves the formation of several intermediates, including preproinsulin, proinsulin and other intermediate cleavage products (Halban, 1991). The initial precursor of insulin from translation of insulin mRNA is preproinsulin, which is synthesized as a single peptide in the cytoplasm and contains a characteristic signal peptide with hydrophobic N-terminal 24 amino acid residues. This signal peptide can be recognized by the signal recognition particle (SRP) (Egea et al., 2005) and then cotranslationally translocated into the lumen of the ER. Then the signal peptide of preproinsulin is cleaved by a specialized signal peptidase located on the inner surface of the rough ER membrane (Patzelt et al., 1978) and produces proinsulin with three disulfide bonds to stabilize the structure. The ER is an important site for insulin biosynthesis and the folding of newly synthesized proinsulin. IRE1a, an ER-resident protein kinase, has a crucial function in insulin biosynthesis. IRE1a phosphorylation is coupled to insulin biosynthesis in response to transient exposure to high glucose; inactivation of IRE1a signaling by siRNA

or inhibition of IRE1a phosphorylation inhibits insulin biosynthesis (Lipson et al., 2006). After the formation of properly folded proinsulin, it is then transported into the Golgi apparatus for packaging into secretory granules, where the mature insulin is produced. Convertases are responsible for conversion of proinsulin to insulin in the secretory granules. At least two convertases, named PC2 and PC1/3 have been identified in the beta cells. Using immunostaining, PC2 and PC1/3 have been shown to colocalize with proinsulin, indicating its role in cleavage of proinsulin (Ugleholdt et al., 2006). The difference between PC2 and PC1/3 is the cleavage site in the proinsulin. PC2 specifically cleaves the proinsulin at the A-chain junction, and PC1/3 is shown to cleave B-chain junction. After cleavage, the final products are equal amount of mature insulin and C-peptide, and therefore, C-peptide is a good indicator of insulin secretion and insulin stability.

Calcium and Zinc play important roles in regulating insulin biosynthesis. By pulse-chase radiolabelling of isolated rat islets of Langerhans, it has been shown that the Ca2+ ion is required for prohormone processing in the granules. Depletion of calcium from ER inhibits the conversion of proinsulin to insulin (Guest et al., 1997). Zinc co-crystallizes with insulin in dense core secretory granules. Cellular zinc homeostasis is largely maintained by zinc transporters and intracellular zinc binding proteins. Over-expression of zinc transporter 7 (ZnT7) in beta cells leads to an increase of insulin mRNA expression and subsequent insulin protein synthesis in the cells by modulating Mtf1 transcriptional activity (Huang et al., 2010). Using Zinc transporter-8 (ZnT8) deficient mice, ZnT8 was shown to be essential

for the formation of insulin crystals and the efficient packaging of insulin in beta cells (Lemaire et al., 2009).

The mature insulin is stored in secretory granules, and awaits the signal to release the hormone into the blood. The release process is referred as insulin exocytosis. Insulin vesicle exocytosis is a complex process involving many steps, including vesicle movement, docking, priming, and finally fusion with the plasma membrane and each step requires careful regulation in order to release appropriate amounts of insulin in response to diffevarious stimuli. In order to easily explain the vesicle exocytosis, the vesicles have been divided into three different groups depending on their release competence following beta cell stimulation: the readily released granules (RRP), the morphologically docked pool (MDP) and the reserve pool (Bratanova-Tochkova et al., 2002). As its name implies, RRP is the pool of granules that are immediately available to be released after stimulation. MDP means that the granules have already docked on the plasma membrane and some of the granules are already primed and others not. With regard to the reserve pools, they are larger and more complex than RRP. Several reports indicated that some newly synthesized insulin is stored in the reserve pools and is released preferentially. Capacitance studies showed that in mouse beta cells, the total number of granules is estimated as13000, of which 0.3-0.7% belong to the RRP group, 1-7% to the MDP, and the rest are comprised in the reserve pool. That is, the total number of RRP granules ranges from 40 to 100, while the total number of granules in the MDP and reserve pools is approximately 1,000 and 11,600, respectively

(Bratanova-Tochkova et al., 2002). Insulin secretion is a biphasic process (Rorsman et al., 2000). As RRP is responsible for the first phase of glucose stimulated insulin secretion, it is obvious that the sustained second phase of glucose-stimulated release must involve translocation of granules from reserve pools to the readily releasable pool or transformation of morphologically docked granules to release competency before exocytosis.

Several hypotheses have been provided to explain the process of insulin vesicle exocytosis. The soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) hypothesis has attracted more attention (Lang, 1999). This hypothesis proposes the formation of complex between the proteins located in the plasma membrane, syntaxin and synaptosomal-associated protein 25 (SNAP-25) and the protein from the vesicle-associated membrane protein 2 (VAMP-2)/synaptobrevin-2 (Lang and Jahn, 2008). At least 5 protein super-families have been identified to be involved in this process, such as SNAREs, Sec1/Munc18 (SM) proteins, Synaptotagmins, Rab proteins and Endocytic proteins. Different proteins participate at different steps and play crucial roles. Loss of the fusion between the membrane protein and vesicle completely blocked vesicle exocytosis. Knocking down the protein “Munc18-1” decreased the second phase of insulin secretion (Tomas et al., 2008). Rab plays a critical role in the formation, trafficking, and tethering of vesicles to the target compartment in endocrine and nonendocrine cells. Rab27a deficient mice showed decreased number of docked granules and insulin secretion (Kasai et al., 2005). Besides these core proteins, the remodeling of F-actin also plays important roles in

insulin exocytosis. F-actin negatively regulates exocytosis via binding and blocking Syntaxin 4 accessibility (Jewell et al., 2008). But further studies indicate that metabolic amplifying pathway increases both phases of insulin secretion and is independent on actin microfilaments (Mourad et al., 2010).

The mechanism that underlies the biphasic nature of the insulin secretion process are still poorly understood, but two well-established pathways have been characterized: the K_{ATP}-dependent/triggering pathway and the K_{ATP}-independent/amplification pathway(Gilon et al., 2002)

1.2.2 Inhibitors and stimulators of insulin secretion

It is well-accepted that insulin vesicle exocytosis needs some signal to switch from "quiescence state" to "release state". The signals that are implicated in stimulating insulin secretion, named as stimulators (Fig 3), include mainly glucose, amino acids, FFA, Glucagon, GLP-1, GIP, and acetylcholine. At the same time, the beta cells also employ some signals to inhibit/ slow down insulin secretion, referred to as "inhibitors" (Fig 3). Many inhibitors have been identified, in particular somatostatin, epinephrine and galanin. In the following part, we will separately discuss the effect of each stimulator and inhibitor on insulin secretion.

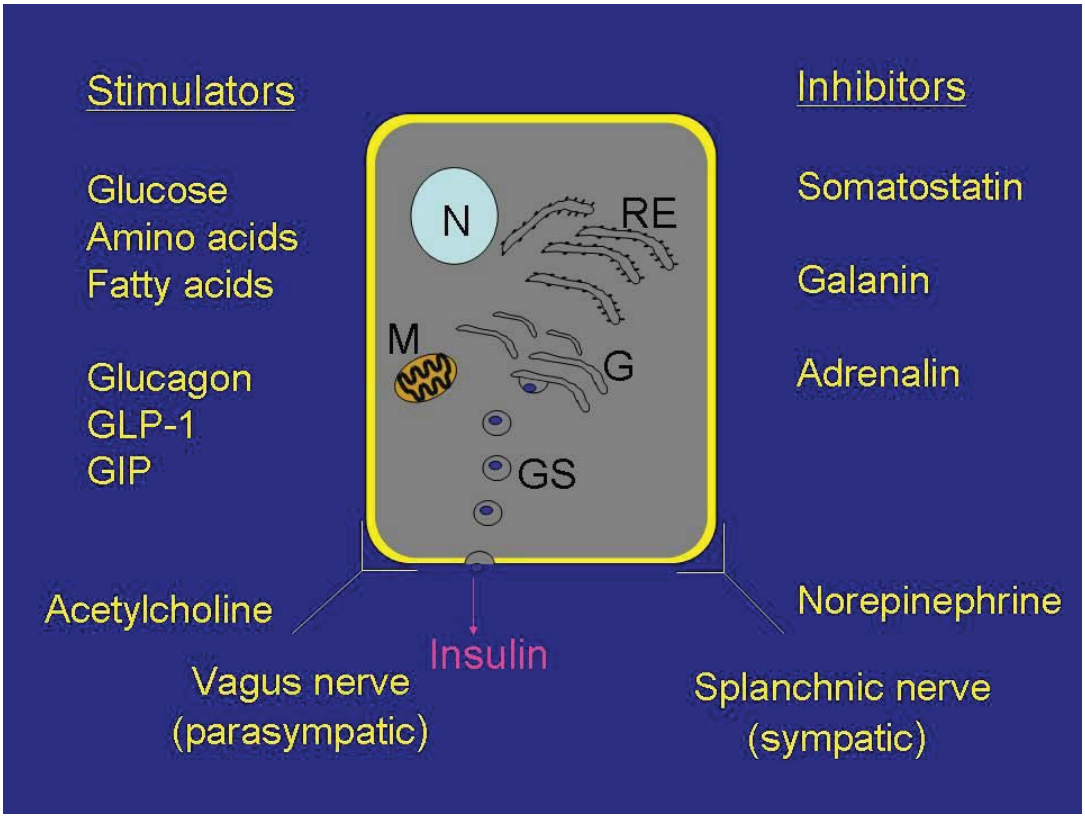

Figure 3 Stimulators and inhitors of insulin secretion. Insulin vesicle exocytosis needs some signal to switch between "quiescence state" and "release state". As its name implied, stimulators promote insulin secretion and inhibitors shut down insulin secretion. Several stimulators, such as glucose, amino acids, fatty acids, glucagons, GLP-1, GIP and acetylcholine have been identified and shown to enhance insulin secretion, while some inhibitors, such as somatostatin, galanin, adrenalin and norepinephrine have been shown to inhibit insulin secretion through different mechanisms as indicated in the text.

1.2.2.1 Glucose

Glucose is the most potent nutrient signal for the beta cell. The mechanism concerning glucose as the potent signal is closely related to its metabolism inside the cells (Fig 4). Following the increase in blood glucose concentration, the glucose sensing mechanisms located in the beta cell drive glucose transporter 2 (GLUT2) to transport glucose into the

cells, where it is oxidized via glycolysis and the TCA cycle to produce ATP. An increase in the ratio ATP-to-ADP closes the ATP-sensitive K^+ (K^+_{ATP}) channels, causing depolarization of the plasma membrane, which in turn opens L-type voltage dependent Ca^{2+} channels. Along with Ca^{2+} efflux from ER, it would take part in priming the secretory vesicles and also exocytosis. Additional mechanisms implicated in glucose signaling to insulin secretion are discussed below.

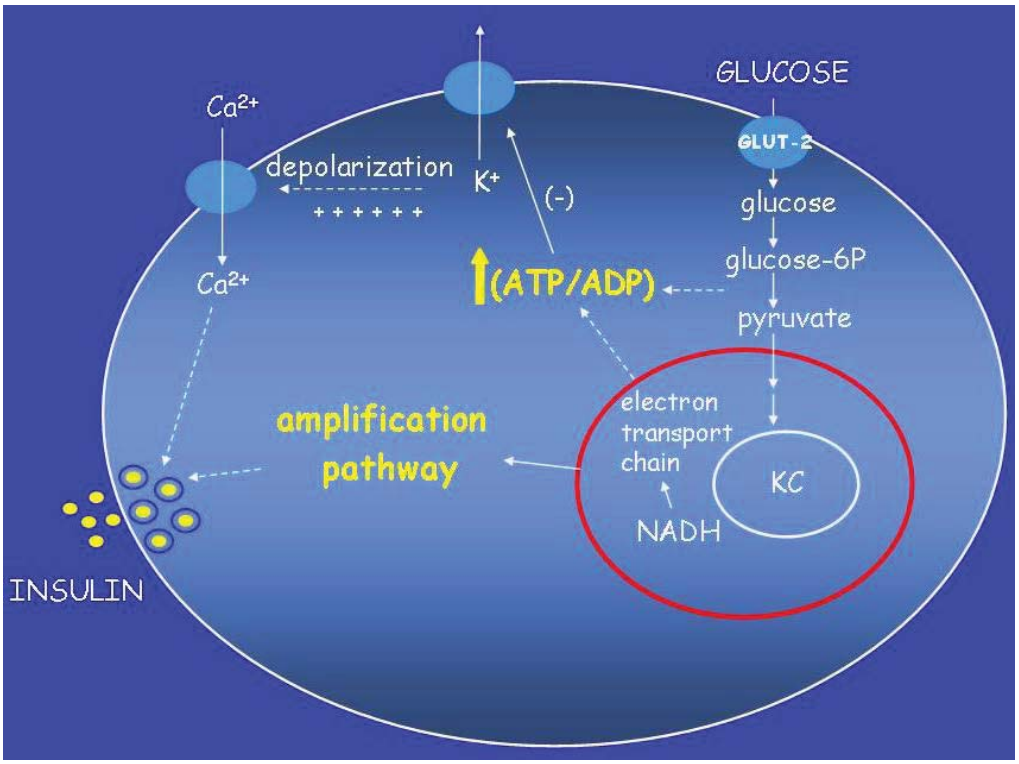

Figure 4 Triggering and K+-ATP independent/ amplification pathways in beta cell metabolic signaling. Multiple signals take part in inducing insulin secretion. After glucose enters the cytosol through the Glut-2 transporter, it is converted into pyruvate. Pyruvate enters mitochondrial and is oxidized in the tricarboxylate (TCA) cycle generating NADH through electron transport chain that eventually results in ATP production. A rise in cytosolic ATP causes the K+ channels of the plasma membrane to close with resultant influx of Ca2+. Increased Ca2+ in the cytosol triggers the release of insulin from secretory

granules into the extracellular space. Metabolites derived from TCA cycle also act as signaling molecules in the amplification pathways of insulin secretion.

1.2.2.2 FFA

At basal glucose concentration, FFA barely induces insulin secretion. But at high glucose concentration, FFA potentiates glucose-stimulated insulin secretion. The mechanism related to the effect of FFA can be explained by the "trident model" (Fig 5) (Nolan et al., 2006b). The first two arms of this model related to the intracellular metabolism of FFA, and the third arm implicates the FFA receptor (also known as GPR40) located in the plasma membrane. The AMP-activated protein kinase/malonyl-CoA/long-chain acyl-CoA (LC-CoA) signaling network is the first arm. At high glucose the level of malonyl-CoA increases causing the switch in FFA metabolism from beta oxidation to esterification, resulting in the availablitity of LC-CoA and acylglycerol (DAG, lysophosphatidic acid, phosphatidic acid) intermediates for signaling purpose. The second arm is related to glycerolipid/ free fatty acid (GL/FFA) cycling, which will be introduced in detail in the next chapter. The third arm is related to signaling via the activation of the highly expressed receptor GPR40, by medium to long chain FFA. GPR40 deficient mice have decreased FFA potentiation of glucose-stimulated insulin secretion (Kebede et al., 2008).

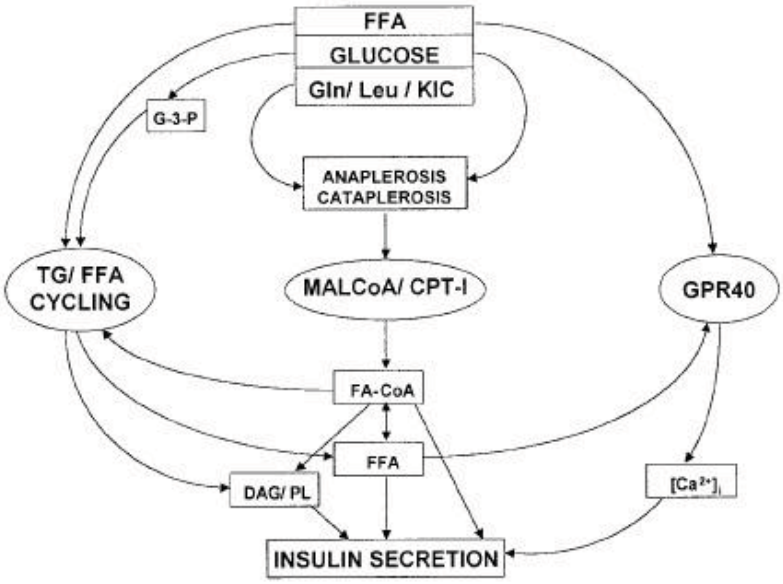

Figure 5 The trident model of beta cell lipid signaling. Three interdependent arms of lipid signaling are proposed by which FFAs augment insulin secretion. First, glucose and other nutrient secretagogues such as glutamine (Gln), Leucine (Leu), and α-ketoisocaproate (KIC) contribute to anaplerosis, which allows cataplerotic efflux of citrate from mitochondria. This results in malonyl-CoA (MALCoA) formation, inhibition of CPT-1 activity, and FA oxidation, and accumulation of LC-CoAs that stimulate insulin secretion directly or by the formation of complex lipids such as DAG and various phospholipids (PL). The second arm involves glucose-responsive TG/FFA cycling, due to the effects of glucose to concomitantly promote FA esterification (provision of glycerol-3-phosphate [G-3-P] and Mal-CoA inhibition of FA oxidation) and lipolysis processes. This allows, particularly in the presence of exogenous FFAs, for the accumulation of cycle intermediates (LC-CoA, DAG, PL, and FFA) that have signaling roles. Third, exogenous FFAs activate the cell surface FA receptor, FFAR1/GPR40, which causes an increase in intracellular Ca2+. FFAs formed from the hydrolysis of TG can cross the cell membrane and, together with exogenous FFAs, activate FFAR1. Thus, the "trident pathways" of lipid amplification intercommunicate and synergize to promote insulin secretion.

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1.2.2.3 Amino acids

Individual amino acids alone do not induce insulin secretion at their physiological concentrations. However, combination of some amino acids at high concentrations, such as

L-alanine and glutamine and leucine (Newsholme et al., 2007), show strong stimulation of insulin secretion. The mechanisms vary differently dependent on the type of amino acids. L-Arginine, a cationically charged amino acid, can directly depolarize the plasma membrane and causes insulin secretion; but this effect is dependent on the presence of glucose. L-alanine has a more complex mechanism to stimulate insulin secretion. On one hand, L-alanine can be co-transported with Na^+, and thus can depolarize the plasma membrane; on the other hand, L-alanine can be metabolized, to produce ATP, leading to KATP channel closure. Both patways appear to promote Ca2+ influx and insulin secretion. Leucine is known to activate glutamate dehydrogenase and thus strongly potentiates the effect of L-glutamine in inducing insulin secretion via anaplerotic input (see below) into the Krebs cycle (Yang et al., 2010b).

1.2.2.4 GLP-1 and GIP

Glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) are two important gluco-incretins, and play crucial roles in inducing insulin secretion. GLP-1 and GIP contribute to more than 50% of the total post-prandial insulin secretion (Vilsboll and Holst, 2004). GLP-1 is produced from enteroendocrine L- cells of the intestinal mucosa and is released into the portal circulation in response to meal ingestion (Dalla Man et al., 2010). GIP is produced from endocrine K-cells in the duodenum after ingestion of carbohydrate and fat. The effect of GLP-1 in inducing insulin is not dependent on intracellular fuel metabolism in the beta cell (Peyot et al., 2009a). Both GLP-1 and GIP

exert their effects on insulin secretion through binding to their respective cell surface receptors in beta cells and elevating the production of cAMP and Ca2+, which activate downstream signaling patways that trigger insulin exocytosis.

1.2.2.5 Acetylcholine

Acetylcholine (Ach) is synthesized using the substrates acetyl-CoA and choline by choline acetyltransferase in the cytoplasm. Then ACh is transported from the cytoplasm into the vesicles by an antiporter (Su et al., 2007). Multiple evidences have shown that Ach released by cholinergic nerve terminals that innevate the pancreas plays important roles in stimulating insulin secretion, particularly during the “cephalic phase” of insulin secretion that occurs prior to food intake when one sees a meal.

1.2.2.6 Somatostatin

Somatostatin, a growth-hormone-release inhibiting factor initially isolated from the hypothalamus, inhibits insulin secretion. Somatostatin directly acts on beta cells, and inhibit the initial and the late sustained phase of insulin secretion (Alberti et al., 1973). This inhibitory effect is mediated by a G-protein receptor, which can decrease Ca2+ entry through voltage-dependent Ca2+ channels. (Hsu et al., 1991). The somatostatin receptor participates in the regulation of insulin secretion and glucose homeostasis. Somatostatin receptor subtype 5 deficient mice show decreased blood glucose and plasma insulin and increased leptin and glucagon concentrations (Strowski et al., 2003).

1.2.2.7 Galanin

Galanin is a 29-amino-acid peptide, and is widely expressed in the peripheral and central nervous systems. Galanin strongly inhibit insulin secretion in vitro and its in vivo administration results in sustained hyperglycemia (McDonald et al., 1985). Further studies have indicated that galanin inhibits insulin release, through a pathway involving a pertussis-toxin-sensitive, guanine-nucleotide-binding regulatory protein, negatively coupled to adenylate cyclase (Amiranoff et al., 1988). Galanin also inhibits exocytosis at a site distal to and independent of the gating of K+ channels and the inhibition of adenylate cyclase activity (Ullrich and Wollheim, 1989).

1.2.2.8 (Nor) epinephrine

Epinephrine in the blood and norepinephrine released by adrenergic nerve terminals play a role in regulating insulin secretion and action during stress and exercise. Epinephrine, acting primarily through a ¦Âadrenergic receptor, markedly reduce tissue insulin sensitivity and this effect results from both peripheral and hepatic resistance to the action of insulin (Deibert and DeFronzo, 1980) These catecholamines via α-adrenergic receptors reduce insulin secretion and are particularly important to prevent hypoglycemia when muscle contraction promotes glucose uptake by tissues, and when enhanced whole body glucose oxidation is required (Gerich et al., 1991).

1.2.3 Metabolic signals promoting insulin secretion

Nutrient secretagogues induce activation of beta cell metabolism and ATP production, which precedes the rise in Ca2+ and secretion. The availability of substrates and their metabolism causes the production of metabolic coupling factors (MCF) leading to the insulin release (Fig 6). The initial metabolism of fuels in the beta cells appears to be governed by a "push" (substrate availability) than a "pull" (Ca2+ influx driving mitochondrial metabolism), unlike other tissues (Peyot et al., 2009a).

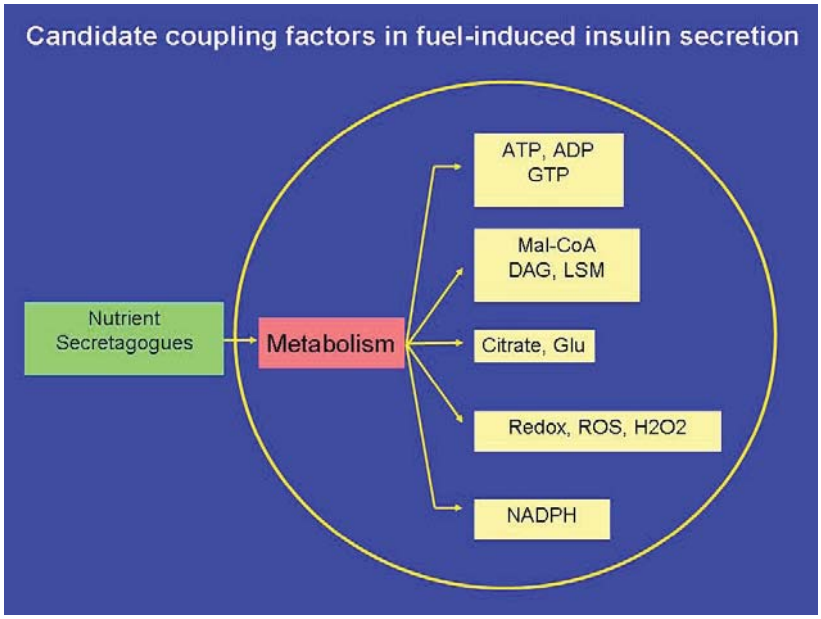


Figure 6 Candidate coupling factors in fuel-induced insulin secretion. Nutrient secretagogues induce activation of beta cell metabolism and ATP production. The availability of substrates and their metabolism causes the production of metabolic coupling factors (MCF) leading to the release of insulin. Several MCF, including ATP, ADP, GTP, Malonyl-CoA, DAG, citrate, ROS, NADPH have been proposed to play key role in promoting insulin secretion.

1.2.3.1 Mitochondrial signals: anaplerosis, cataplerosis and pyruvate cycling

Cataplerosis and anaplerosis are processes that represent the efflux of Krebs' cycle intermediates from mitochondria into the cytosol, and their replenishment, respectively (Farfari et al., 2000; Schuit et al., 1997). Both processes are quantitatively similar because the Krebs' cycle is not a sink for glucose carbons (Brun et al., 1996; Schuit et al., 1997). In the beta cells, anaplerosis and cataplerosis pathways are thought to provide the carbon precursors (eg citrate) of MCF for insulin secretion (Fig 7). Cytosolic citrate increases at high glucose concentration in the beta cell, and it has been shown that pyruvate cycling allows for high glycolytic flux via NADH reoxidation and the production of two MCF, malonyl-CoA (MalCoA) and NADPH (Guay et al., 2007). Altered flux through anaplerosis, in response to glucose correlates with MCF production and insulin secretion (Jensen et al., 2008). The expression of pyruvate caboxylase (PC), malic enzyme (ME) and ATP-citrate lyase (ACL), enzymes of the pyruvate/citrate cycle is defective in islets from humans with T2D (Jitrapakdee et al., 2010). PC expression is reduced in islets of the diabetic GK and ZDF rats (MacDonald et al., 1996). RNAi-knockdown of PC (Hasan et al., 2008), ME (Guay et al., 2007; Ronnebaum et al., 2008) and ACL (Guay et al., 2007) curtailed GSIS. Although some studies have questioned the role of ACL and ME in beta cell signaling (Ronnebaum et al., 2008), there is a general consensus that pyruvete cycling processes are implicated in the regulation of beta cell fuel signaling. Thus, inhibition of one cycling process may in some studies be compensated by enhanced flux through another pyruvate cycling pathway.

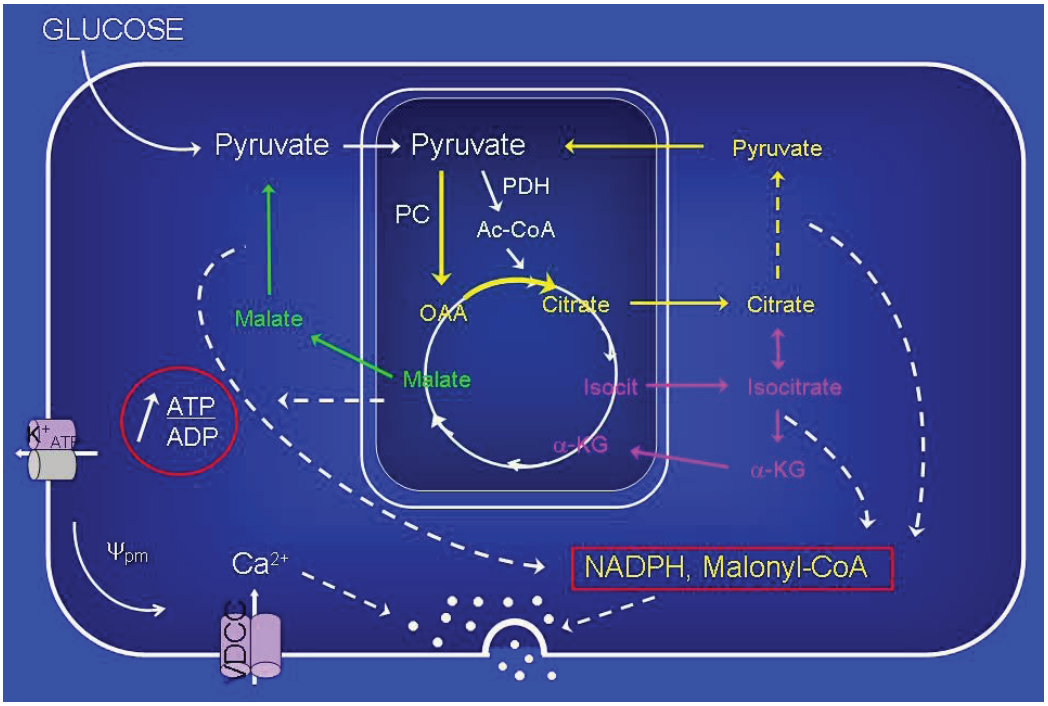


Figure 7 pyruvate cycling and insulin secretion. Pyruvate-derived from glucose metabolism enters in the tricarboxylic acid cycle via its carboxylation by PC, producing oxaloacetate (OAA). This anaplerotic process leads to the formation and accumulation of citrate within the mitochondria. At increasing levels, mitochondrial citrate is exported into the cytoplasm in exchange with malate. Once in the cytosol, citrate is cleaved by ACL, producing pyruvate, leading to the production of NADPH. Pyruvate re-enters the mitochondria via the pyruvate transporter. The pyruvate/citrate cycling together with glucose-derived pyruvate leads to the net synthesis of malonyl-CoA, NAD^+, NADPH, and indirectly ATP, which increased insulin secretion.

1.2.3.2 NADPH, Adenine and Guanine Nucleotides and Glutamate

Ample evidence implicates cytosolic NADPH as a MCF. GSIS positively correlates with the NADPH/NADP ratio and NADPH directly induces exocytosis in single beta cells. Recent studies (Reinbothe et al., 2009) indicate that NADPH acts via glutaredoxin-1 that may control exocytotic effectors and/ or voltage K+ channels Kv 2.1 to promote the rise of

Ca2+. Several studies demonstrated that ATP and ADP act as MCF in the beta cell. It has also been suggested that mitochondrial GTP positively regulates GSIS via mitochondrial Ca2+ handling (Kibbey et al., 2007). Glucose-derived glutamate, via the glutamate dehydrogenase reaction, was proposed to act as MCF. Thus, activating mutations in GDH are associated with hyperinsulinemia (Kranendijk et al., 2010). However, the view that glutamate as a MCF has been challenged (Malaisse et al., 1979), and the evidence indicates that anaplerotic oxidative deamination of glutamate via GDH, producing alpha-ketoglutarate in the Krebs cycle (Agarwal et al., 2006), is implicated in this type of hypersulinemia.

1.2.3.3 Lipid metabolism dependent amplification of beta cell metabolic signaling

Glucose metabolism leads to a rise in MalCoA, which inhibits carnitine palmitoyltransferase-1 (CPT-1) and blocks beta-oxidation and thus causes a fuel shift in beta cells from fatty acid to glucose utilization (figure 8). Overwhelming support for the MalCoA/CPT-1 interaction and fatty acyl-CoA, or their derivations such as diacylglycerol (DAG), in beta cell fuel stimulation was provided by our lab and others. Thus, overexpression of MalCoA decarboxylase (MCD) in the cytosol or a MalCoA-insenstive CPT-1 mutant in the beta cell curtailed GSIS (Roduit et al., 2004). Nutrient inhibition of fatty acid oxidation is accompanied by elevated glycerolipid (GL) formation and significant increases in the total mass of DAG, triacylglycerol (TG) and phosphatidic acid (PA) were shown to occur in glucose-stimulated beta cells. Inhibition of beta oxidation by MalCoA

leads to a rise in cytosolic FACoA, and, as far as signaling is concerned, FACoA, FFA themselves and complex lipids (DAG, lysophophatidic acid and PA) are potential MCF, as these compounds directly influence signaling pathways and secretion. MalCoA/lipid signaling is important not only in beta cells as we initially proposed, but also in the control of insulin action, appetite and body weight.

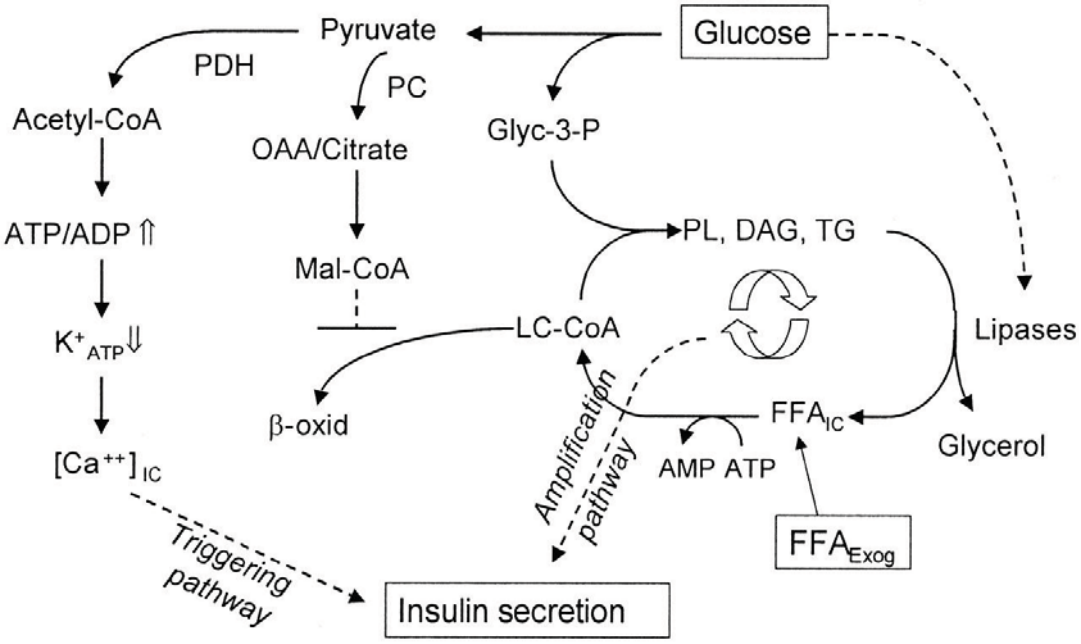


Figure 8 Interactions between glucose and fatty acid metabolism in nutrient-secretion coupling. This model illustrates the synergistic interaction between glucose and FA metabolism and the generation of lipid signaling molecules that augment GSIS. Glucose gives rise to pyruvate, which, when channeled through pyruvate dehydrogenase (PDH), contributes to induction of insulin secretion via ATP production and the K_{ATP}-dependent triggering pathway. Pyruvate alternatively can be channeled via pyruvate carboxylase (PC) into the anaplerosis/cataplerosis pathway, which contributes to increases in cytosolic oxaloacetate (OAA) and citrate. Glucose increases malonyl-CoA (Mal-CoA), which blocks FA oxidation by inhibiting CPT-1. Inhibition of FA oxidation allows LC-CoA esters to accumulate in the cytosol. LC-CoAs are formed from FFAs supplied externally or produced by the lipolysis of endogenous TG. LC-CoAs can be esterified with glycerol-3-phosphate (Glyc-3-P) to form complex lipids such as TG, DAG, and phospholipids (PL). Glucose also

activates lipolysis, which favors TG/FFA cycling from endogenous lipids. Exogenous FFAs (FFA_{Exog} contribute by amplifying the levels of cycle intermediates (LC-CoAs, TG, DAG, PL, and intracellular FFAs [FFA_{IC} themselves), all of which may be implicated in lipid signaling effector pathways. ¦Âoxid, ¦Âoxidation; $[Ca^{++}]_{IC}$, intracellular calcium.

1.3 Glycerolipid/free fatty acid cycle (GL/FFA cycle)

GL/FFA cycle, previously named as triacylglycerol/ free fatty acid cycle (TG/FFA cycle), did not draw great attention since its description in 1965. With the rapidly increasing knowledge of the mechanisms of obesity and diabetes, it is gradually becoming evident that this cycle located at the heart of the metabolic network, tightly linking glucose and FFA metabolism together, with important roles in churning out a plethora of signaling molecules controlling numerous biological processes (Prentki and Madiraju, 2008).

1.3.1 The process of GL/FFA cycle and related enzymes

GL/FFA cycle (figure 9) is a continuous active process and shown to exist in almost every cell type. The biological process of this cycle encompasses esterification of FFA onto a glycerol backbone to synthesize glycerolipids followed by their hydrolysis with the release of the FFA that can be re-esterified. The full cycle contains two continuous steps- lipogenesis and lipolysis. Lipogenesis starts from glycerol-3-phosphate generated from glycolysis or gluconeogenesis and fatty acid-CoA from fatty acid synthesis or exogenous fatty acid, to synthesize lysophosphatidic acid (LPA) in the presence of glycerolphosphate acyltransferace (GPAT). LPA can be further esterified into phosphatidic acid (PA), which is converted into diacylglycerol (DAG) and triacylglycerol (TG), the main energy form stored in the lipid droplets. LPA, PA and DAG arising from GL/FFA cycle can also be

incorporated into phospholipids. Following lipogenesis, the final product TG and some phospholipids enter the "lipolysis" process to produce DAG, monoacylglycerol (MAG), and glycerol as well as free fatty acids, released at each step. Relative to the full cycle, in some conditions to produce certain special signaling molecule, the cells may hold several short product/ substrate cycles, such as from PA to DAG, which will converted to PA instead of incorporating into TG (figure 9).

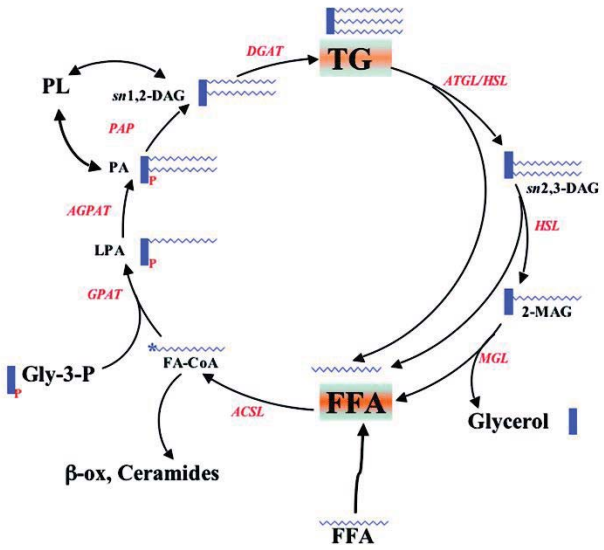


Figure 9 Enzymes and intermediates of the triacylglycerol/free fatty acid cycle. Fatty acyl-CoA (FACoA) is formed by long-chain acyl-CoA synthase (ACSL) from free fatty acids (FFA) supplied externally or produced by the hydrolysis of triacylglycerol (TG), diacylglycerol (DAG) and monoacylglycerol (MAG). The accumulating FACoA is partitioned into the formation of complex lipids through the condensation with glucose-derived glycerol-3-PO4 (Gly3P) by glycerol-3- phosphate acyltransferase (GPAT) to form lysophosphatidic acid (LPA). LPA is further converted by acyl glycerol-3-phosphate acyltransferase (AGPAT) to phosphatidic acid (PA), which eventually gives rise to DAG by the action of phosphatidic acid phosphatase (PAP), or phospholipids (PL). DAG thus formed is acylated by diglyceride acyltransferase (DGAT) to form TG. DAG derived from TG by adipose triglyceride lipase (ATGL) and/or hormone sensitive lipase (HSL), is hydrolyzed to 2-MAG by HSL. 2-MAG is hydrolyzed by MAG lipase (MGL) to FFA and glycerol, which is secreted out of the cell. In some cells the released glycerol is recycled

back into TG/FFA cycling by conversion to Gly-3-P by glycerokinase. ¦Âox, oxidation.

It is now well-accepted that the GL/FFA cycle is under stringent control via separate enzymes responsible for forward and backward reactions. The rate-limiting step in this cycle lies in the initial step involved in glycerol-3-phosphate and fatty acid-CoA to produce LPA. So the enzyme glycerol-3-phophate transferate (GPAT) involved in this step is of high importance. Till today, four enzymes have been cloned and identified, viz., GPAT1 to GPAT4. GPAT1 and GPAT2 are localized in the mitochondria, while GPAT3 and 4 are in the ER. Mitochondrial GPAT1 has been characterized more thoroughly than the other isoforms. GPAT1 deficient mice displayed markedly lower hepatic TG and DAG concentration, and were protected from hepatic steatosis (Xu et al., 2006) and hepatic insulin resistance possibly due to a lower DAG-mediated PKC activation. Further study using the metabolomics analysis showed that several metabolites, including palmitate, 1-mono-16:0-glycerol, 1,2-di-16:0-glycerol were decreased, and other metabolites involved in carbohydrate metabolism were increased in the knock out mice, which may provide important clues to explain high insulin sensitivity in this kind of mice. The second enzyme involved in this cycle is 1-acyl-sn-glycerol-3-phosphate acyltransferase, also named as lysophophatidic acid acyltransferase (LPAAT), catalyzing the reaction from LPA to PA. Several isoforms of LPAAT have been identified, of which only alpha and beta show high activity. *Agpat2*$^{?\tilde{a}?b}$ mice develop severe lipodystrophy affecting both white and brown adipose tissue, extreme insulin resistance, diabetes, and hepatic steatosis associated with high expression of lipogenic genes and high rates of de novo fatty acid biosynthesis. PA

phosphatase (PAP) is responsible for converting PA to DAG. In mammals, two types of PAP (PAP1 and PAP2) have been recognized. PAP-1 is found mainly in the ER and believed to be involved in the synthesis of TG and PL. PAP-2 is located near the plasma membrane and may be producing DAG form PA released from membrane.

The next important enzyme involved in acylating DAG to TG is diacylglycerol acyltransferase (DGAT), with important roles in regulating energy storage and metabolism. Two type of DGAT (DGAT1 and DGAT2) exist in the mammals. DGAT1 has been reported to be highly expressed in skeletal muscle, intestine as well as other tissues, whereas DGAT2 is mostly expressed in the adipocytes and liver. Overexpression of DGAT1 in rat islets has been shown to increase palmitate incorporation into TG resulting in a modest accumulation in TG content and decreased glucose-stimulated insulin secretion, which may be due to "glucolipotoxicity". DGAT-1 knock out mice were shown to have increased insulin sensitivity and enhanced glucose tolerance, and protected from diet-induced obesity (Yamaguchi et al., 2008); DGAT2 also plays a fundamental role in mammalian triglyceride synthesis and is required for survival based on the fact that DGAT2-deficient (*Dgat2*$^{-/-}$) mice are lipopenic and die soon after birth, apparently from profound reductions in substrates for energy metabolism and from impaired permeability barrier function in the skin. Also in these KO mice, DGAT1 was unable to compensate for the absence of DGAT2, supporting the hypothesis that the two enzymes play fundamentally different roles in mammalian triglyceride metabolism.

The process of lipolysis is also highly dependent on several important enzymes, depending on different reactions. The first well-known TG lipase is adipose TG lipase (ATGL; also named desnutrin and patatin-like domain containing phospholipase A2, PNPLA2 or PLA2¦Æ, which is proved to function in catalyzing TG to produce 2,3-DAG instead of 1,2-DAG. It is becoming evident that ATGL prefers to hydrolyse TG at sn-1 position. Hormone sensitive lipase (HSL) was shown to exhibit activities to hydrolyse TG, DAG and MAG in vitro, but its main role in vivo was revealed to hydrolyse DAG to MAG better than MAG to glycerol. Other DAG lipases, such as sn1-DAG lipase, have been identified, but their relative contribution for DAG hydrolysis is still unclear. sn-1 DAG lipases show high activity in brain and pancreatic beta cells compared to other tissues. This enzyme shows remarkable specificity to DAG containing arachidonic acid (AA) at position 2 to release 2-arachidonic glycerol (2-AG), which is an important signaling molecule in stimulating CB-1 and CB-2 receptors. The MAG produced by DAG lipase is further hydrolysed by MAG lipase. Several enzymes have been proven to show MAG lipase activity in vitro. Of special interest, MAG lipase (MAGL), ABHD6 and ABHD12 contribute to almost the whole MAG hydrolytic activity in brain (Blankman et al., 2007). It is well-known that all the MAG lipases are capable of hydrolyzing both 1-MAG and 2-MAG. We will discuss the MAG lipases in detail in the following part.

1.3.2 MAG lipases

The first classical MAG hydrolyzing enzyme identified was MAG lipase, which was for a long time recognized as the main MAG lipase in all cell types (Farooqui et al., 1984). MAG lipase has been reported to increase several fold in some cancer cells during cancer progression. Further studies indicated that MAG lipase controls a MAG-FFA network to regulate cancer cell migration and progression. Knockdown of this protein could inhibit cancer cell migration, which could be reversed by adding exogenous stearic acid and palmitic acid, independent of CB1 and CB2 pathway (Nomura et al., 2010). In 2007, another two enzymes ABHD6 and ABHD12 were identified using activity based protein profiling methods in the brain tissue (Blankman et al., 2007), and these two enzymes were shown to contribute to 15% of the total MAG lipase activity in the brain, as classical MAG lipase is responsible for the remaining 85%. Recent studies have shown that ABHD6 is highly expressed in several tumor cell lines, such as breast cancer cell line MCF7, but the significance of this high expression is not clear (Max et al., 2009). Another report demonstrated that inhibition of ABHD6 in brain produced the cannabnoid effect via accumulation of 2-AG, indicating the important roles of ABHD6 in regulating cell function (Marrs et al., 2010). Even though it has been demonstrated that efficient lipolysis takes place in beta cells in response to glucose stimulation, resulting in the production and release of both glycerol and FFA, the terminal lipase involved in MAG hydrolysis has not been characterized. The relative contributions of classical MAG lipase (inhibited specifically by JZL184), ABHD6 (inhibited specifically by WWL70) and ABHD12 to MAG hydrolysis in beta cells are yet to be worked out.

1.3.3 Functions of GL/FFA cycle

As indicated above, GL/FFA cycle consists of multiple steps, catalyzed by separate enzymes also in the forward and backward directions. Such a complex enzyme system offers several "regulating points" to better and more conveniently control this cycle, in order to meet the diverse needs of signaling metabolite supply for the biological functions of the cell. Any disturbance of this cycle leads to a wide range of pathophysiological phenomena, including type-2 diabetes and cancer (figure 10).

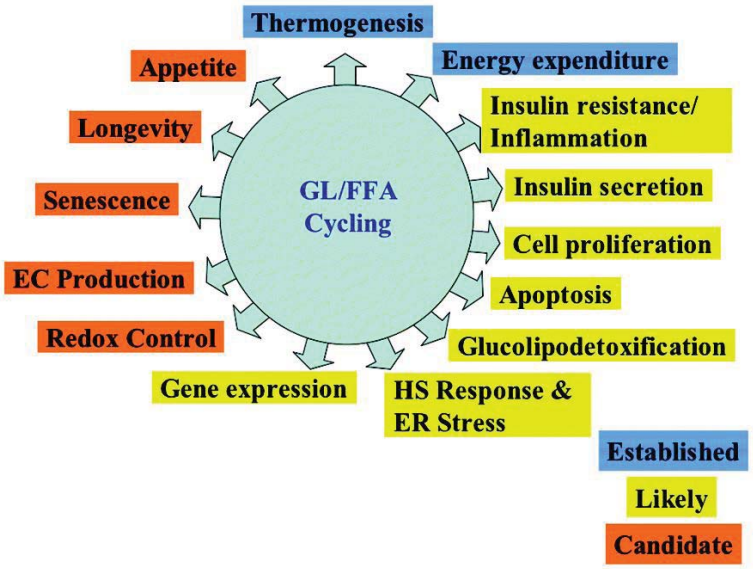


Figure 10. Biological processes regulated by GL/FFA cycling. Operation of GL/FFA cycling generates various metabolites and signals involved in the control of multiple biological processes. The figure illustrates established and candidates processes as well as those clearly emerging from recent studies. ER, endoplasmic reticulum; HS, heat shock; EC, endocannabinoid (Prentki and Madiraju, 2008).

The GL/FFA cycle is capable of producing a plethora of signaling molecules controlling numerous biological processes. Their roles could be divided into three categories: established roles, emerging roles as well as candidate roles. The established role was related to energy homeostasis and thermogenesis. In mammals, maintenance of body temperature is at least in part, if not largely dependent on lipolysis, an integral part of GL/FFA cycle. FFA produced from lipolysis can activate mitochondrial uncoupling proteins (UCPs), which act to uncouple the respiration from oxidative phophorylation and help in dissipating the respiratory energy as heat. The decreased ability to mount adequate thermogenic response by enhancing lipolysis may be responsible for the reduced tolerance to cold of ob/ob mice, ATGL-KO mice and Zucker fatty rats.

Besides the established roles, there are many emerging roles of GL/FFA cycle, which encompasse a whole range of regulatory functions and include detoxification of fuel oversupply, regulation cell survival and proliferation, regulation of gene expression as well as insulin secretion (Prentki and Madiraju, 2008).

It has been hypothesized (Nolan and Prentki, 2008; Prentki and Madiraju, 2008)(figure 11) that GL/FFA cycle allows for fuel detoxification because it directs the glucose carbon to glycerol, which leaves beta cell that express little glycerol kinase. Furthermore, FFA released from lipolysis are also transported out of the cells or transported in to mitochondria for beta oxidation. As GL/FFA cycle is a high energy-consuming pathway,

several ATP molecules are hydrolyzed (7 per cycle) and the energy is released in the form of heat during one full cycle. Some steps in the cycle have been shown to involve in ATP usage, such as FFA conversion into fatty acid-CoA and LPA synthesis. The released heat is beneficial to maintain mammal body temperature as well as for fuel detoxification.

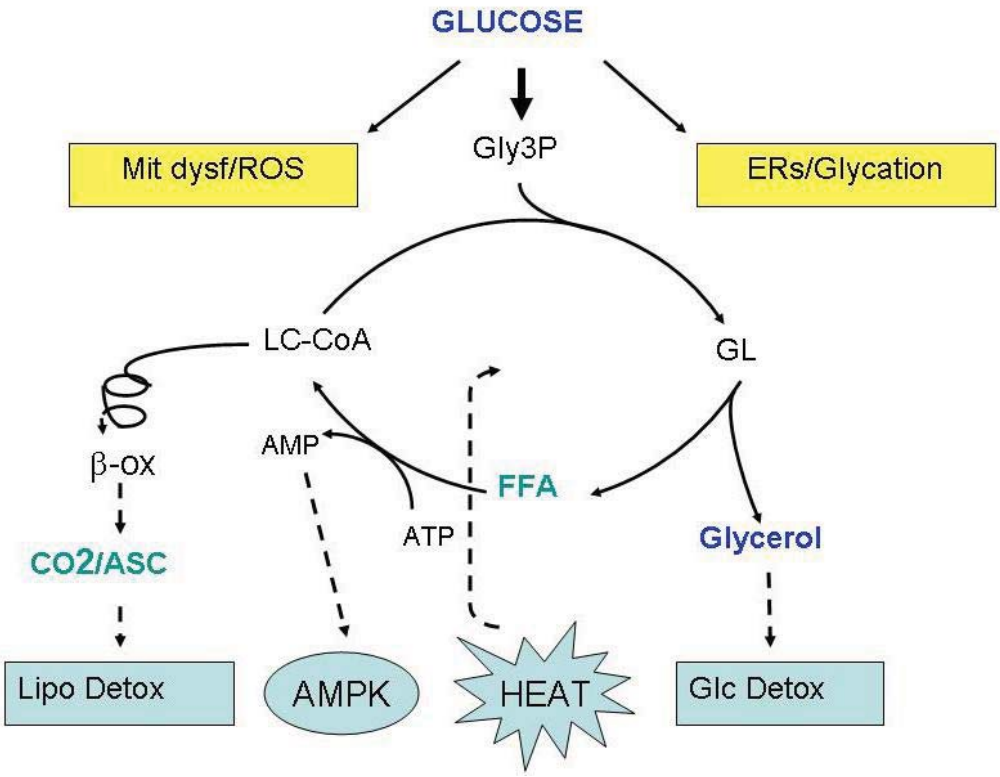


Figure 11 GL/FFA cycling and nutrient gluco-lipo-detoxification. Glucose metabolism via the GL/FA cycle provides an alternative pathway to mitochondrial oxidation for glucose metabolism in the ¦Âcell that also produces important coupling factors for secretion. This pathway can also act to detoxify excess nutrients. Glucose metabolised via glycerol-3-phosphate (Gly3P) in the GL/FA cycle has its carbons released from the cell in the glycerol form (GL) and not oxidized, termed gluco-detoxification (Glc Detox). Activation of the lipolysis arm prevents accumulation of complex GL and steatosis. ¦Âoxidation (¦Âox) of fatty acid to CO_2 and acid soluble products (ASP) is supported by maintenance of long chain acyl-CoA (LC-CoA) substrate levels and the fact that AMP produced by FFA activation promotes AMP-Kinase (AMPK) activation. AMPK upregulates ¦Âoxidation in

many cell types, including the ¦Âcell, allowing for lipo-detoxification. The cycle also utilizes ATP, which dissipates the mitochondrial membrane potential, lessening the likelihood of cellular damage by mitochondrial dysfunction (Mit dysf) and reactive oxygen species (ROS) production. Diversion of glucose metabolism via GL/FA cycling is also likely to reduce effects of glucose toxicity on endoplasmic reticulum stress (ERs) and cell damage (Nolan and Prentki, 2008).

Regulation of gene expression is also one of the most important roles of GL/FFA cycle. Some metabolites, such as DAG are produced during cycling could directly or indirectly activate or elevate the level of hypoxia inducible factor 1alpha (HIF-1alpha), a vital transcription factor that regulates the expression of various glycolytic enzymes, vascular endothelial growth factor (VEGF) and also favors cell survival. Also protein lysine acetylation has emerged as a key posttranslational modification in celluar regulation, and some reports showed lysine acetylation is a prevalent modification in enzymes that catalyze intermediate metabolism according to cellular nutrition conditions. GL/FFA cycle is a very active metabolic pathway, which continuously uses NADH and regenerates NAD, which is a substrate for the protein deacetyltion enzymes, sirtuins, which regulate a wide range of cellular functions including gene expression. Thus it is possible that GL/FFA cycle controls gene expression by regulating sirtuin activity and protein lysine deacetylation. Also, because of the ability of GL/FFA cycle to activate sirtuins, which are well known for their involvement in promoting longevity, the possibility that GL/FFA cycle indirectly contributes to longevity exists.

GL/FFA cycle may participate in the regulation of cell survival and proliferation through TG deposit in non-adipose cells. High concentration of FFA in combination with high concentration of glucose is really toxic to non-adipose cells, such as beta cells, which could interfere with many cellular processes, in particular mitochondrial function and energy homeostasis. Several reports have shown that TG accumulation in non-adipose cells could be regarded as a defective mechanism against acute FFA toxicity. TG build-up, together with high active GL/FFA cycle could protect the cells as long as the cells retain the ability to hydrolyse TG to maintain the amount of TG in a reasonable extent. The roles related to insulin secretion will be discussed in detail in next chapter.

1.3.4 Regulation of insulin secretion by the GL/FFA in beta cells

The significance of GL/FFA cycling for insulin secretion became evident from studies showing curtailed GSIS in rat islets upon lipolysis inhibition by the pan-lipase inhibitors 3,5-dimethylpyrazole (Masiello et al., 2002) and orlistat (Mulder et al., 2004). In rat and mouse islets, the lipase activity is proportional to insulin secretion. ATGL and HSL have been implicated in the process of insulin secretion. Knockdown of these two proteins using siRNA has been shown to decrease insulin secretion in cell lines. Furthermore, mice deficient in ATGL have shown decreased insulin secretion in vivo and ex vivo (Peyot et al., 2009b). Studies with the whole body HSL-deficient mice showed inconsistent results based on genetic background of the KO mice. While some reports showed no influence on insulin secretion in HSL-KO mice other reports showed clear reduction in insulin secretion

especially in fasted male mice (Mulder et al., 2003; Peyot et al., 2004). This discrepancy was resolved in the beta cell-specific HSL-knockout mice, which showed significantly reduced insulin secretion (Fex et al., 2009). Although the lipases ATGL and HSL are implicated in insulin secretion, little is known about the role of MAGL, ABHD6 and ABHD12 in insulin secretion. Definition of their roles in the beta cell is of interest because these enzymes are indispensable for completing lipolysis, and we hypothesized that they may also have an important role in regulating insulin secretion.

The other arm of the GL/FFA cycle (lipogenesis) may also take part in insulin secretion. Some studies have shown that rapid lipogenesis and lipid remodeling from secretagogue carbons support insulin secretion in INS832/13 cell line (MacDonald et al., 2008). Glucose carbon rapidly incorporates into different classes of lipids in the beta cell (Prentki and Madiraju, 2008), indicating the high active of lipogenesis, important part of GL/FFA cycle.

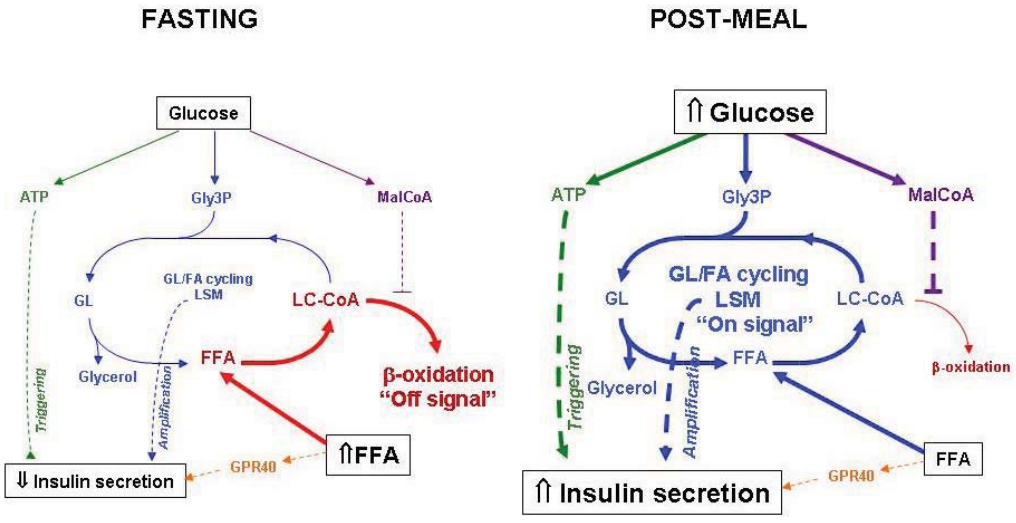


Figure 12 Model illustrating the role of the free fatty acids (FFA) and the glycerolipid/fatty acid (GL/FA) cycle in the regulation of insulin secretion as a function of the nutritional state. During fasting **(a)**, the ¦Âcell triggering pathway of glucose signaling is inactive, ensuring that insulin secretion remains low even though FFA are elevated under this nutritional condition. Furthermore, FFA are preferentially oxidized rather than cycled, as the level of glucose-derived malonyl-CoA (MalCoA) is low. FFA signaling via GPR40 is insufficient to promote insulin secretion when the triggering pathway is inactive. After a meal **(b)**, increased ¦Âcell glucose metabolism promotes insulin secretion by activating both the triggering and GL/FA cycle amplification pathways. Triggering occurs as a result of glucose oxidation and the K^{+}_{ATP} channel dependent pathway. Glucose promotes GL/FA cycling by increasing MalCoA which blocks ¦Â oxidation which allows long chain acyl-CoA (LC-CoA) availability for the cycle, providing glycerol-3-phosphate (Gly3P) for synthesis of GL such as lysophosphatidic acid, diacylglycerols and triacylglycerols, and activating lipolysis. In the presence of these glucose-derived signals, direct signaling of FFA via GPR40 is now able to amplify secretion. The 'off signal' for the GL/FA cycle amplification pathway is ¦Âoxidation, since it depletes lipid metabolites from the cycle. The 'on signal' is the cycling itself that is promoted by elevated glucose (Nolan and Prentki, 2008).

1.4 TRP channels

Transient Receptor Potential (TRP) channels constitute a large and diverse family of proteins, which are classified into at least three main subfamilies of closely related channels called canonical (TRPC), vanilloid (TRPV) and melastatin-related (TRPM) channels, as well as four additional groups of more distantly related TRP subfamilies named TRPA (ankyrin), TRPP, TRPML and TRPN) (Venkatachalam and Montell, 2007). TRP channels are present almost in every organ. They are higly expressed in brain but also located in the heart, heart, kidney, testis, lung, liver, spleen, pancreases, ovaries, intestine, prostate, placenta, uterus, and vascular tissues. They are also reported to be widely expressed in different cell types, such as neural cells, muscle cells and endothelial cells. TRP channels show very similar structures, conserved from one to another species (Moiseenkova-Bell

and Wensel, 2009). The TRP channel proteins possess six transmembrane domains, which anchor the channel to the lipid bilayer of the membrane. Compared to the classical voltage-gated channels, TRP channels do not have a voltage sensor domain, and are regarded as a non-selective cation channels. All the TRP channels show varying degrees of permeability to cations; of special interest, some of the TRP channels are highly permeable to calcium (Birnbaumer, 2009).

Different approaches, such as over-expression of dominant-negative variants, antisense oligonucleotides, RNAi and targeted deletion of the gene using homologous recombination both in the cells and in the mice, have been widely used to uncover the roles of TRP channel proteins. It has been widely accepted that TRP families play important roles in regulating cell functions, such as thermosensation, pheromone reception, magnesium homeostasis as well as regulation of vascular tone. Loss of activity of TRP channels have been implicated in severe human diseases, including some cancers (Venkatachalam and Montell, 2007).

The activation of TRP channels can occur in several different ways: in some cases, they can be activated by background currents, whereas in others, activation needs some special stimulators, such as endogenous agonists. In either case, a functional unit of TRP channel requires tetrameric assembly of homomeric or heteromeric TRP peptides.

1.4.1 TRP channels identified in beta cells

As a non-selective cation channels, some TRP channels may be permeable to calcium and then regulate beta cell function. Till now, three subfamilies (TRPC, TRPM and TRPV) have been identified in beta cells.

1.4.1.1 TRPC subfamily

The subfamily of TRPC consists of at least seven subtypes: from TRPC1 to TRPC7. Three subtypes- TRPC1, TRPC4 and TRPV6 have been identified in the beta cell. TRPC1 channel was the first cloned TRP channel in mammals based on the high sequence similarities to Drosphila melaogaster, and it is also the first TRP protein identified in the beta cells. TRPC1 is highly expressed in the brain, heart, testes, ovary, bovine aortic endothelial cells, and salivary glands. (Song and Yuan, 2010). TRPC1 was reported to form functionally distinct heteromers with its close relatives TRPC3 and TRPC5 (Clapham et al., 2001). As an assumed store-operated channel, it is not very clear that the activation of TRPC1 is mediated by release of stored calcium or through phospholipase c (PLC) dependent pathways.

The functions of TRPC1 in the beta cells have not yet been reported. TRPC4 is structurally similar to TRPC1, and it is reported to be highly expressed in the brain and adrenal grand. In the beta cells, TRPC4 transcripts are easily detectable in mouse islets using RT-PCR. TRPC4 is activated by G-protein families and receptor tyrosine kinases. The role of TRPC4

in beta cells is not known because TRPC4-deficient mice did not show any difference in glucose tolerance (Freichel et al., 2004). TRPC6 is sensitive to DAG in a manner that is independent of protein kinase C (PKC) activity. It was postulated that it form heteromers with TRPC3 and TRPC7. TRPC6-deficient mice have been shown to have 3 fold increase in the mRNA of TRPC3, indicating a functional redundancy (Dietrich et al., 2005). The mRNA level of TRPC6 in the beta cells is very low and its role in this cell type is not known.

1.4.1.2 TRPM channels

The TRPM subfamily consists of at least eight subtypes: TRPM1 to TRPM8. TRPM2, TRPM3 and TRPM5 have been identified in the beta cells (REF). TRPM2, also named LPRPC2, binds ADP ribose (ADPR) and nicotinamide dinucleotide (NAD). TRPM2 activity is modulated by variations in the cellular redox state (Togashi et al., 2006). TRPM2 is thought to regulate cell death induced by changes of the cellular redox state. In the beta cells, TRPM2 has been detected in the mouse, rat and human islets, and activation of TRPM2, by the change of the temperature, could strongly stimulate insulin secretion (Uchida et al., 2010). TRPM3 was also identified and cloned from beta cells, and it was proposed to function as ionotropic steroid receptors in pancreatic ¦Âcells (Wagner et al., 2008).. TRPM3 channels are highly permeable and conductive for $Zn2^{+}$. Of special importance, TRPM3 channels mediate Zn2+ uptake in pancreatic ¦Âcells independently of VGCCs and the uptake could take place even in the very low concentration of $Zn2^{+}$ outside

the cells, and consequence, TRPM3 constitutes a regulated entry for $Zn2^{+}$ ions in beta cells (Wagner et al., 2010).

TRPM5 seems to have a wide range of tissue distribution, including beta cells. This channel could mediate calcium-activated, non-selective cationic currents, and therefore, may regulate many biological processes inside the cells. Using TRPM5-deficient mice, this channel has been shown to be essential for sweet, umami, and bitter taste reception directly or indirectly through PLC pathway (Riera et al., 2009; Sugita, 2006). In the beta cells, TRPM5 may play an important role in regulating insulin secretion, but till now, there is still no direct evidence to support this hypothesis.

1.4.1.3 TRPV subfamily

The subfamily of TRPV consists of at least six subtypes: from TRPV1 to TRPV6. And in the beta cells, TRPV1 and TRPV2 are already identified. TRPV1 is highly expressed in dorsal root ganglia (DRG) as well as neurons. As a non-selective cation channel, it has been shown to cause calcium influx in different cell types, as a result, it could regulate many processes, such as relieve of pain. Activation of TRPV1 by dietary capsaicin improves endothelium-dependent vasorelaxation, and more importantly, it prevents hypertension (Yang et al., 2010a). TRPV2 also has a wide tissue distribution, and mainly in the neurons, neuroendocrine cells in the gastrointestinal tract and blood cells such as macrophages (Vennekens et al., 2008). As a heat-sensitive channel, TRPV2 channel could be activated

by a noxious temperature (>52°C) - the physiological significance of which is the possible to link with nociception. Besides heat, it also could be activated by growth factors and other ligands. One characteristic of TRPV2 channel protein is that it could translocate from cytoplasm to plasma membrane after different stimulation, such as IGF-1. Because of its identification in beta cells, such as mouse islets, special interest has been paid to the effect of insulin secretion and TRPV2. Now it is clear that TRPV2 functions as an insulin-mediated regulator of calcium entry (Hisanaga et al., 2009). Further studies implicated TRPV2 in the regulation of the first phase of insulin secretion (Aoyagi et al., 2010).

1.4.2 TRPV1 and beta cell function

TRPV1 has been identified in the beta cells, and also has been shown to play a role in regulating beta cell function, such as insulin secretion (Akiba et al., 2004). Many stimuli have been shown to activate TRPV1, which included heat (>43°C) and capsaicin (Caterina et al., 1997), protons (<pH 6) (Latorre et al., 2007) and cations (Ahern et al., 2005); some lipids and their derivatives, including *n*-arachidonoyldopamine (NADA), *n*-oleoyldopamine, anandamide, oleoylethanolamide and other *N*-acylethanolamines (and lipoxygenase derivatives. And more recently MAG was shown to activate TRPV1 and cause calcium influx in HEK cell line (Iwasaki et al., 2008). We think this finding is highly important, because MAG is a common metabolite from the sequential hydrolysis TG. MAG could activate TRPV1, which has been shown to play important role in calcium influx causing insulin secretion.

TRPV1 has been detected using western blotting in INS-1 cell line as well as rat islets and its agonists could induce insulin secretion, even in the basal condition (1mM glucose) (Akiba et al., 2004). Systemic use of capsaicin could increase glucose tolerance in the Zucker fatty rat (Gram et al., 2007). TRPV-1 deficient mice have been generated and proved to be resistant to obesity after high fat feeding (Motter and Ahern, 2008). And TRPV-1 has been shown not only located inside the beta cells, and also in the nerve fibres in the islets (Razavi et al., 2006).

1.5 Hypothesis

Glycerolipid/ free fatty acid cycling with its lipolysis and lipogenesis segments, contributes to the lipid-amplification pathways of promoting GSIS in □-cells. GL/FFA cycle is suggested to produce different lipid signaling molecules that enhance insulin secretion (Prentki and Madiraju, 2008). The significance of lipolysis in this signal molecule production has been well-documented by the studies showing curtailed GSIS in □-cells upon lipolysis inhibition by the pan-lipase inhibitors orlistat (Winzell et al., 2006) and 3,5-dimethylpyrazole (Masiello et al., 2002). Orlistat was also shown to antagonize palmitate-augmented GSIS (Nolan et al., 2006b). However, the precise lipid molecule(s) or the enzyme involved has not been identified. Even though the role of HSL, which conducts DAG hydrolysis in the GL/FFA cycle, in insulin secretion has been debated (Mulder et al., 2003), Prentki group provided convincing evidence for the importance of HSL (Peyot et al., 2004; Roduit et al., 2001) in the regulation of GSIS both *in vivo* and *in vitro* and this has

been confirmed recently by others (Fex et al., 2009) using □-cell specific HSL-knockout mice. Specific inhibition of another DAG hydrolyzing enzyme, *sn1*-DAG lipase, by RHC80267 in □-cells was also found to reduce GSIS (Guenifi et al., 2001; Konrad et al., 1994). Besides DAG hydrolysis, lipolysis of triglycerides by ATGL is also shown to be important by a study from Prentki lab, in fuel and non-fuel stimulated insulin secretion (Peyot et al., 2009b). It was proposed that DAG is a potential signaling molecule that is involved in the lipid-amplification of GSIS, as DAG can activate munc-13, which is essential for insulin exocytosis and also PKC enzymes that are important for GSIS. Inasmuch as either the lack or inhibition of ATGL, HSL or *sn1*-DAG lipase in □-cells, leads to reduced insulin secretion and because orlistat also inhibits palmitate-enhanced GSIS, we hypothesize *that the lipolytic product subsequent to DAG hydrolysis i.e., monoacylglycerol (MAG), but not DAG as was initially thought (Prentki and Matschinsky, 1987) is the major lipolysis-derived signal for insulin secretion.* Thus, in this study, we want to test the hypothess that MAG, generated from GL/FFA cycling, may be the necessary signal for the lipid amplification of GSIS.

2. MATERIALS AND METHODS

2.1 Materials

Cell culture supplies were from Corning (Corning, NY) and Fisherbrand (Canada). 2-Arachidonylglycerol was from Sigma Chemicals. WWL70 was obtained from Cayman Chemical Company and AMG9810 was from Tocris Bioscience. Both WWL70 and AMG9810 were dissolved in dimethylsulfoxide (DMSO) before their use in insulin secretion experiments. D-[U-^{14}C] glucose was from GE Healthcare (Canada) and palmitate sodium salt was from Nu-Check Prep (Elysian, MN). The bicinchoninic acid protein assay from Pierce (Rockford, IL) was used. Stock unlabelled palmitate was prepared at 4 mM concentration in 5% defatted BSA as described elsewhere (Roduit et al., 2004). Orlistat was purchased from Sigma and JZL184 was from Cayman. Antibodies were from Abcam.

2.2 Cell culture

INS 832/13 cells (Hohmeier et al., 2000) were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium with sodium bicarbonate, supplemented with 10% (v/v) fetal calf serum (Wisent), 10 mM HEPES, pH 7.4, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM □-mercaptoethanol (complete RPMI). Cells were grown to 80% confluence. Media were changed to RPMI 1640 containing 3 mM glucose supplemented as the complete RPMI 24 h prior to the experiments. Insulin secretion incubations were conducted in Krebs-Ringer bicarbonate buffer containing 10 mM HEPES, pH 7.4 (KRBH).

2.3 Islet isolation

All procedures were approved by the Institutional Committee for the Protection of Animals at the Centre Hospitalier de l'Université de Montréal Research Center. Wistar rats, C57Bl6 or CD1 mice (all male) from Charles River (St-Constant, QC, Canada) were anaesthetized with Somnotol® (MTC Pharmaceuticals, Canada) and sacrificed by exsanguination. Pancreatic islets were isolated by collagenase (type XI from Sigma) digestion of total pancreas (Gotoh et al., 1987), followed by separation (centrifugation at 1,040 x *g*) on a Histopaque 1091 or 1077 (Sigma) gradient. Isolated islets were handpicked and cultured overnight in a Petri dish at 37°C in a humidified atmosphere containing 5% CO_2 in regular RPMI 1640 medium with sodium bicarbonate, supplemented with 10% fetal calf serum, 10 mM HEPES pH 7.4, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin.

Human islets were obtained from the Human Islet Isolation Core of the Montreal Diabetes Research Center, using appropriate ethical guidelines. The donors were males without any known disease. Islets were hand-picked and cultured overnight before use. Both rodent and human islets were also treated with trypsin to disperse islet cells and then cultured overnight in RPMI complete medium. These overnight cultured dispersed islets cells were then used for examining the effect of various pharmacological agents.

2.4 Insulin secretion measurement

INS 832/13 cells were washed in KRBH containing 1 mM glucose and 0.5% defatted BSA (KRBH 1G/0.5%BSA) and pre-incubated for 45 min in KRBH 1G/0.5%BSA in presence of pharmacological agents (at indicated concentrations) or vehicle. For examining the effect of WWL70 (ABHD6 inhibitor), AMG9810 (TRPV1 antagonist), orlistat (pan-lipase inhibitor), and JZL184 (MAG lipase inhibitor), drugs were added first in pre-incubation media and then during incubations. 2-AG was added to cells at concentrations up to 1 μM, and other MAG species at concentrations up to 400μM. Insulin secretion from INS 832/13 cells was measured by either 45-min or 2h static incubations in KRBH containing various glucose concentrations, 0.5% defatted BSA and pharmacological agents or vehicle, with or without 35 mM KCl or 0.3 mM palmitate, as specified.

For insulin secretion from rat, mouse or human islets, batches of 10 islets were washed in KRBH containing 2.8 mM glucose and 0.5% defatted BSA, and pre-incubated for 45 min in KRBH containing 2.8 mM glucose, 0.5% defatted BSA and different pharmacological agents (at the indicated concentrations) or DMSO. Islets were then incubated for either 45 min or 2h in KRBH containing various glucose concentrations or 2.8 mM glucose plus 35 mM KCl, 0.5% defatted BSA in the presence or absence of drugs (at indicated concentrations) or DMSO. At the end of the incubations, media were collected and insulin was extracted from cells or islets in acid-ethanol (1.5% HCl, 75% ethanol). Total insulin contents and insulin released into medium were determined by radioimmunoassay using human insulin standard (Linco Research, MO).

2.5 Effect of Lipase inhibitors on the incorporation of [U-^{14}C]-*glucose into lipids*

INS832/13 cells were incubated overnight at 11 mM [U^{14}C]-glucose in RPMI medium in a CO_2-incubator in order to pre-label lipids in the cells. Then the cells were washed in KRBH (1 mM [U^{14}C]-glucose; same specific activity as with 11mM glucose) and pre-incubated for 45 min in KRBH at 1 mM [U^{14}C]-glucose to bring metabolism to basal level. After this, cells were further incubated for 2h in KRBH at 1 & 10 mM [U^{14}C]-glucose with and without the following inhibitors: Orlistat (50 μM), WWL70 (10 μM) and JZL184 (1 μM). Then the cells were washed rapidly with PBS, flash-frozen in liquid nitrogen and extracted for lipids. Lipids were separated by TLC and the associated radioactivity was quantified. Neutral lipids were separated on boric acid/silica gel-TLC, with a two dimentional two solvent systems; first, with petroleum ether: diethyl ether: acetic acid (70:30:1); then using a second solvent system up to half of the plates, with chloroform: acetone: acetic acid (60:40:1). Phospholipids were separated with a solvent system of chloroform: methanol: water (65:25:4).

2.6 LC/MS/MS measurement of MAG and DAG species

INS832/13 cells were seeded in 12-well plates at a density of 0.3 million cells per well and cultured for 36 h. Then the cells were starved in RPMI 1640 with 10% FBS at 1mM glucose for 2 h. Cells were then washed in KRBH with 0.5%BSA and 1mM glucose and pre-incubated for 45 min in the same KRBH to bring metabolism to basal level. Then the cells were further incubated for 2h in KRBH at 1 or 10 mM glucose and the indicated inhibitors. WWL70 was used at 10 μM, where shown. Then the cells were rapidly washed

and flash-frozen in liquid nitrogen and extracted for lipids. The lipid extracts were sent to Kansas Lipidomics Research Center, Kansas State University for analysis by LC-MS/MS.

2.7 ABHD6 Over-expression

The pCMV-based plasmids expressing human ABHD6 and Green Fluorescent Protein (GFP) were obtained from Origene. pCMV-AC plasmids coding for either ABHD6 or GFP (used as control and also to monitor transfection efficiency) were introduced into INS832/13 cells using the Amaxa Nucleofector method (Program T-27, solution V; Amaxa, Inc., Gaithersburg, MD). Five micrograms of DNA were used for each transfection of 6 million cells. Transfection efficiency was more than 85%, as assessed by cytometry and confocal microscopy of GFP-transfected cells. After transfection, cells were seeded in 12-well and six-well plates for insulin secretion and Western blot analysis, respectively, and cultured for 72 h before collecting for Western blot analysis or performing insulin secretion experiments.

2.8 Quantitative real-time RT-PCR

Total RNA was extracted from INS 832/13 cells, islets and rodent tissues using the Rneasy Mini kit (Qiagen) with Rnse-free Dnase (Qiagen). RNA (3 µg) was reverse transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen, Canada) and hexamers as previously described (Delghingaro-Augusto et al., 2009). Gene expression was determined by the standard curve method and normalized to the expression of ¦Âactin mRNA. Real-time PCR analysis was performed using a Rotor-Gene R3000 (Corbett Research, Australia)

and the LCR Faststart DNA masterplus SYBR Green reagent (Roche, Canada). Primers for MAG lipase, ABHD6 and TRPV1 were designed using Primer3 software. Results are expressed as the ratio of target mRNA to ¦Âactin mRNA.

2.9 Immunoblot analysis

INS 832/13 cells, islets and rodent tissues were processed for SDS-PAGE and Western blotting. Cells or tisuses were washed with cold PBS and lysed using a lysis buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, protease inhibitors, 1 mM Na_3VO_4 and 2.5 mM $Na_4P_2O_7$. Lysates were sonicated, aliquots were taken for protein assay and samples were stored at -80°C. Proteins from total cell extracts (20 µg protein) were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes (Scheicher & Schuell, Germany) for Western blotting. Blotted proteins were probed using monoclonal antibodies (Abcam) against MAG lipase and ABHD6 according to suppliers' protocols. Horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules) was used as second antibody with SuperSignal West Pico chemiluminescence (Pierce) for detection.

2.10 Cytosolic Ca^{2+} measurement in rat islets

Cytosolic Ca^{2+} was measured by adapting a previously described procedure (Jahanshahi et al., 2009). Briefly, dispersed rat islet cells were plated on a 42 mm coverslip and incubated overnight at 37°C in complete medium at 11 mM glucose. Cells were starved in RPMI complete medium at 2.8 mM glucose for 2h and loaded in KRBH containing 2.8 mM

glucose, 1% BSA (defatted), 2.5 mM probenicid, 0.2 mM sulfinpyrazone and 6 ¦ÌM Fluo-4AM + pluronic acid F-127 (1:1) (Invitrogen, USA) for 75 min at 37°C. Coverslips were then mounted in a closed perifusion chamber, and cells perifused with KRBH containing 1% BSA, 2.5 mM probenicid, 0.2 mM sulfinpyrazone and 2.8 mM or 16.7 mM glucose or 2.8 mM glucose + 35 mM KCl using a syringe pump (New Era pump system). The microscope, perifusion chamber and solutions were maintained at 37°C. Cells were incubated for 180 s at 2.8 mM glucose and images were recorded for baseline determination. Images were then recorded every second for a period of 10 min in the presence of 16.7 mM glucose, and for a period of 2 min in the presence of KCl using a Leica TCS SP5 inverted confocal microscope. Incubation with 16.7 mM glucose was started at time 20 s, but the analyses were started at 150 s to exclude baseline fluctuations (caused by the autofocus stabilization device of the microscope scanner and false-positive fluorescence due to osmolarity changes in the perifusion chamber). Inhibitor of ABHD6 (WWL70, 10 μM) and of TRPV1-R (AMG9810, 10 μM) were included in the perifusion medium at both glucose concentrations. Perifusions were conducted sequentially, with initially 2.8 or 16.7 mM glucose alone, and then with WWL70 followed by AMG9810, in the same perifusion chamber.

2.11 Statistical analysis

Values are expressed as means ± SEM. Statistical analysis was performed using one-way ANOVA with Dunnett's post-test for multiple comparisons or two-way ANOVA with

Bonferroni's post-test for multiple comparisons using GraphPad Prism (GraphPad Software).

3. Results

3.1 Restoration of GSIS by MAG in INS832/13 □-cells after inhibition by the lipolysis inhibitor orlistat

Since the lipolysis inhibitor orlistat reduces GSIS, we examined if the GSIS can be restored by the addition of MAG to □-cells. We initially assessed the effect of 2-AG (1μM) in INS832/13 cells at low and high glucose and the results indicated that the orlistat-inhibited GSIS was almost completely restored by 2-AG (Fig. 13A) and 2-AG was able to enhance insulin secretion even in the absence of orlistat inhibition. However, since 2-AG is a known endocannabinoid, which may act by activating CB1/CB2 receptors, and also because CB1/ CB2 receptors were earlier implicated in GSIS (Matias et al., 2006), we checked if blocking the CB1 receptor antagonizes the 2-AG effect. However, AM251, an antagonist of CB1-receptor was found to have no significant influence on 2-AG mediated effects on insulin secretion either at 2 mM or 10 mM glucose (Fig. 13A). We further ascertained that the MAG-restoration of orlistat inhibited GSIS in INS 832/13 □-cells was not specific to 2-AG and that 1-stearoylglycerol (1-SG, 100μM), 1-palmitoylglycerol (1-PG, 100μM) were also able to restore GSIS (Fig 13B). 1-SG and 1-PG could completely restore GSIS, inhibited by 25 μM orlistat, at 5mM glucose, and partially at 10mM glucose. Both 1-SG and 1-PG were also found to significantly stimulate GSIS in the absence of orlistat (Fig. 13B). Thus orlistat inhibition of lipolysis, which results in reduced formation of MAG due to the inhibition of

TG and DAG hydrolysis, is associated with lowered GSIS and replenishing cells with exogenous MAGs could restore secretion, suggesting that MAG is the likely mediator of GSIS.

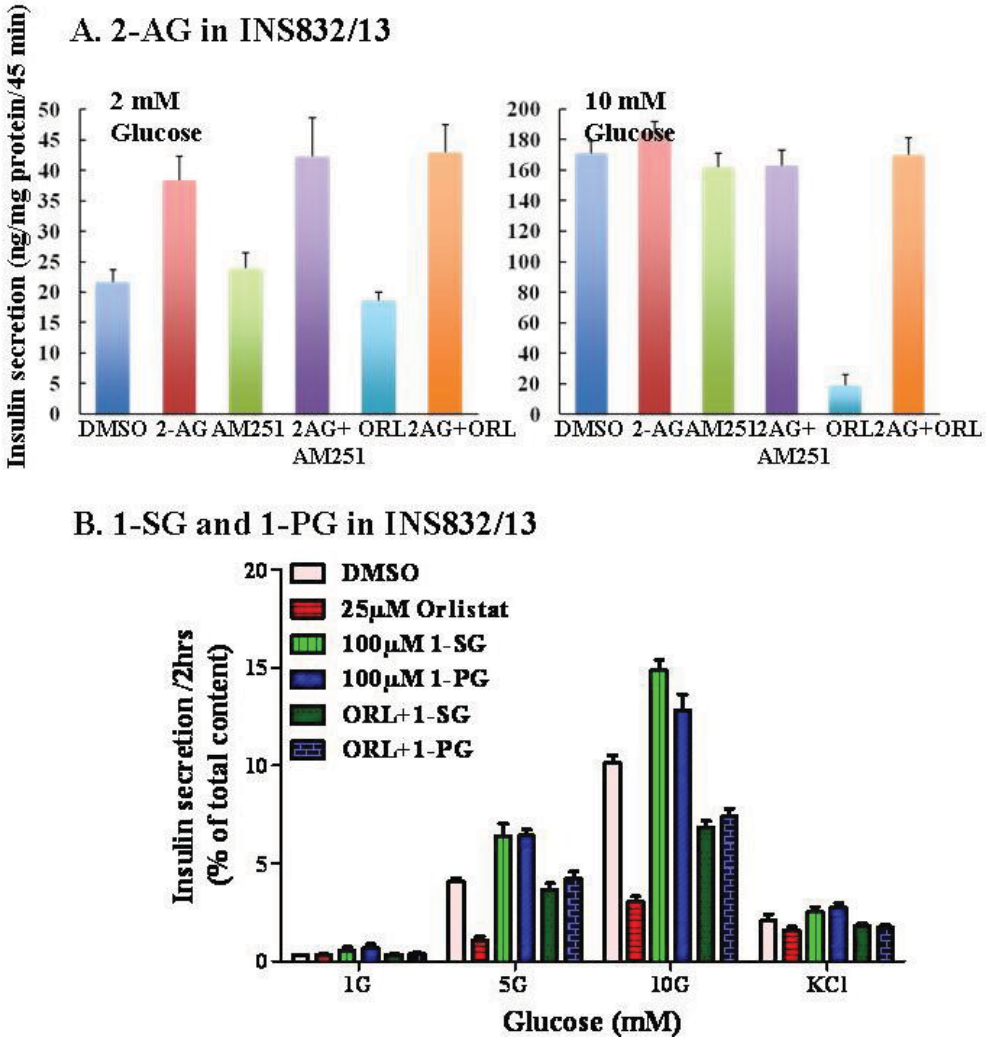


Figure 13 Various MAG species restore GSIS in INS832/13 cells treated with the panlipase inhibitor orlistat. INS832/13 cells were pre-incubated in KRBH with different MAGs without orlistat for 45 min, and then the cells were incubated at various glucose concentrations, or at 1 mM glucose plus 35 mM KCl, with or without different MAGs, in the absence and presence of orlistat for 45 min (A) or 2 h (B). Fig 4A shows studies using

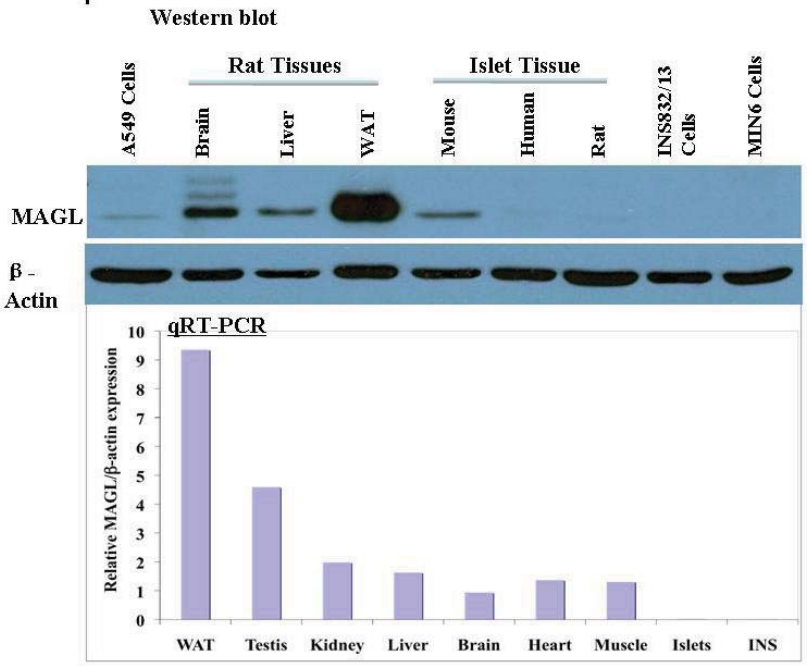
2-AG, orlistat and the CB1 receptor antagonist AM251 (1μM). Fig 4B shows studies using 1-SG and 1-PG. Results are means ± SE of 3 separate experiments, in triplicate wells.

3.2 Expression of MAG-lipase and ABHD6 in different tissues, islets and □-cell lines:

After observing that MAG can restore GSIS inhibited by orlistat, we assessed the expression of MAG hydrolases in □-cells as there have been no studies regarding MAG lipase expression in islets or □-cells in comparison to other tissues. Expression of MAG-lipase in different rat tissues and in islets from mouse, rat and humans as well as two beta cell lines was examined by Western blotting and RT-PCR. Comparatively, MAG-lipase expression in white adipose tissue was much higher at both mRNA and protein levels (Fig. 14A), while its expression is either undetectable in the beta cell lines (INS832/13 and MIN6) and human and rat islets or much less in the mouse islets (Fig. 14A).

Expression of plasma membrane-bound ABHD6, which has been proposed as a novel MAG lipase in brain and other tissues and hydrolyzes MAG to glycerol and FFA, was also examined. As indicated in the Figure 14B, expression of ABHD6 was found to be highest in liver, while its expression in INS832/13 and MIN6 cells and in mouse, rat and human islets is also significant.

A. MAG lipase



B. ABHD6

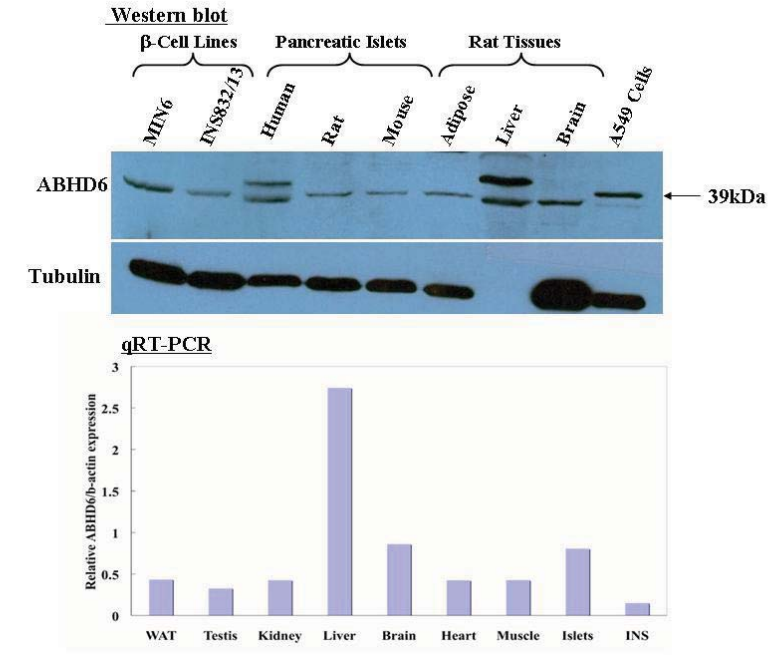


Figure 14 Expression of MAGL (A) and ABHD6 (B) in rat tissues, islets and beta cells. Protein and mRNA were extracted indicated in the "Materials and Methods.

3.3 The effect of inhibition of MAGL and ABHD6 on GSIS.

Hydrolysis of MAG either by MAG lipase was shown to be specifically inhibited by JZL184 in brain (Long et al., 2009). Similarly, WWL70 was described as a specific inhibitor of ABHD6 in brain neuronal cells, with an EC_{50} of 70nM (Blankman et al., 2007). Since orlistat is a pan-lipase inhibitor, we tested the effects of specific inhibition of MAG lipase and ABHD6 on GSIS. There was no significant change in GSIS in INS832/13 cells incubated in the presence of JZL184 (1μM) for 2h, which is consistent with the poor expression of MAGL in these cells (Fig 15A).

Inhibition of ABHD6 in INS832/13 cells, which is expressed at relatively higher levels than MAG lipase, by 10μM WWL70, resulted in significantly enhanced GSIS at 5 and 10 mM glucose (Fig. 15B). WWL70 effects on insulin secretion also occurred in the presence of 0.3mM palmitate (data not shown). Under similar incubation conditions, 25 μM orlistat near completely inhibited GSIS. These results are compatible with the possibility that reduction of total lipolysis by orlistat likely reduced the levels of MAGs, whereas, inhibition of ABHD6 probably caused MAG to build-up, and that there is a reciprocal relationship between MAG levels of □-cells and GSIS.

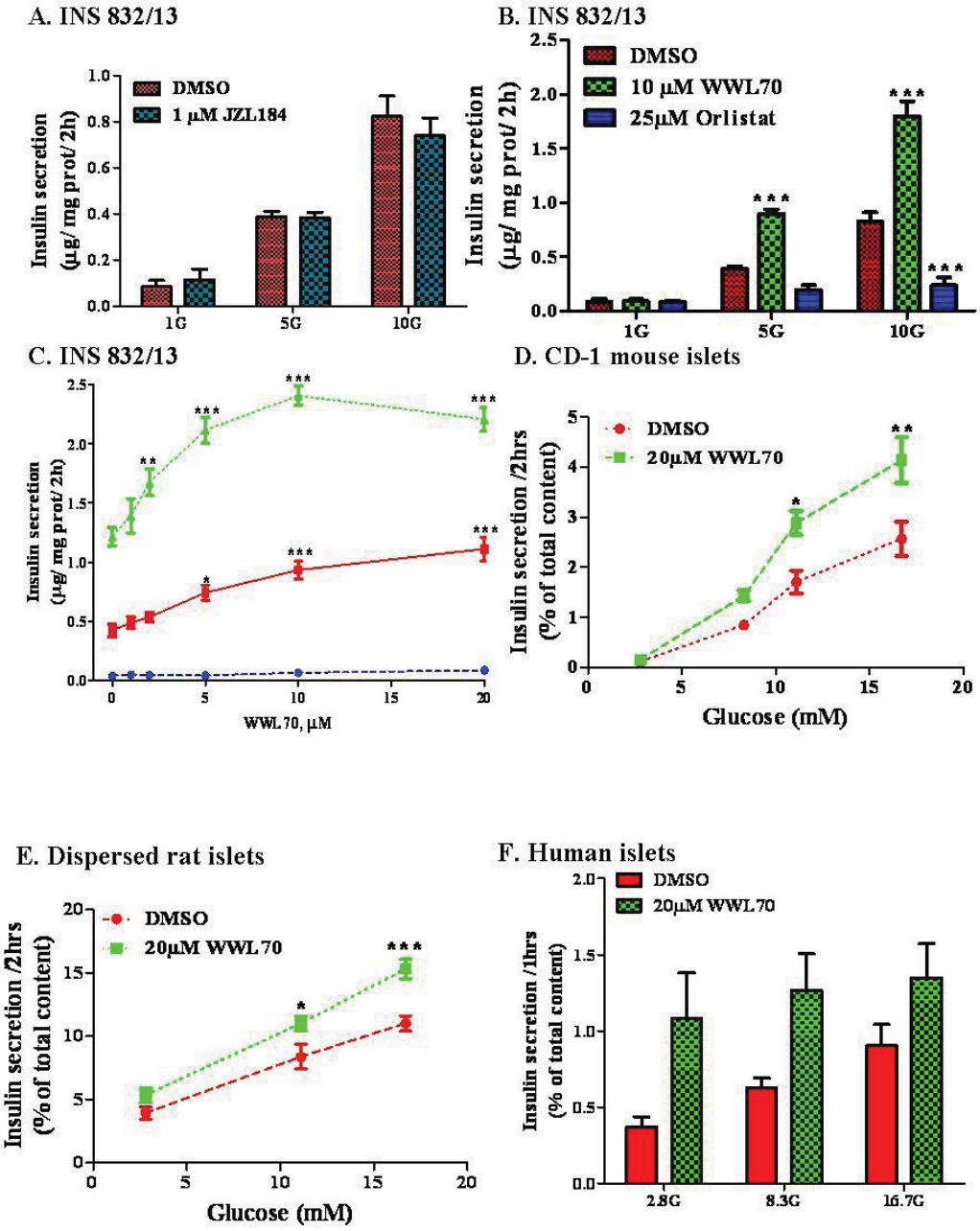


Figure 15 The effect of MAGL and ABHD6 inhibitor on insulin secretion in beta cells. INS832/13 cells or mouse, rat and human islets were pre-treated with KRBH with 0.5%

BSA, either with MAGL inhibitor JZL184, or with ABHD6 inhibitor WWL70 for 45 min, and then the cells were treated with inhibitors at different glucose concentration as indicated in the figures. Figure 15A shows the effect of MAGL inhibitor JZL184 on insulin secretion in INS832/13 cells; Figure 15B shows the effect of ABHD6 inhibitor WWL70 and pan-lipase inhibitor orlistat on insulin secretion in INS832/13 cells. Figure 15C indicates the dose-dependence effect of WWL70 on insulin secretion in INS832/13 cells; Figure 15D indicates the effect of the inhibitor WWL70 on insulin secretion in dispersed rat islets; Figure 15E indicated the effect of inhibitor WWL70 on insulin secretion in CD-1 mouse islets.; Figure 15F shows the effect of the inhibitor WWL70 on insulin secretion in human islets. Results are means ± SE of 3 separate experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the corresponding DMSO group.

We then examined the dose-dependent effects of WWL70 on insulin secretion. In this experiment, we assessed the effect of WWL70 at different concentrations (1, 2, 5, 10 & 20 μM) on insulin secretion. As indicated in the Figure 15C, WWL70 dose-dependently increased insulin secretion at 10mM glucose, with the maximal effect at 10μM.

To further confirm the results obtained in INS832/13 cells, which are established immortalized cells, we have checked the effect of WWL70 in mouse (Fig. 15D), rat (Fig. 15E) and human islets (Fig. 15F). Inhibition of ABHD6 by WWL70, in islets from rat, mouse or human, also significantly stimulated insulin secretion (Fig. 15D-F), indicating that the results seen with WWL70 in INS cells are reproducible in islets.

3.4 ^{14}C-Glucose incorporation into neutral lipids: Effect of orlistat and WWL70 at low and high glucose concentrations

We then checked if either enhancing or inhibiting the GL/FFA cycle lipolytic segment, respectively, with glucose or by orlistat and WWL70 in INS832/ 13 cells, results in altered

profile of lipid molecules, in particular, MAG. INS832/13 cells were incubated as indicated in the “Materials and Methods” under equilibrium labeling conditions. After separation of different lipids using TLC, the associated radioactivity was quantified by software. Results are shown as the amount of glucose used for lipid synthesis. Even though the mass of individual lipids was not quantified directly, the radioactivity-derived results give an approximate idea about the relative quantities of these lipid species. Consistent with previous reports, different neutral lipids, including TG, DAG and MAG, increased with increasing glucose concentration as shown in Figure 16A. Also there was marked TG accumulation in the presence of orlistat, while it increased slightly in the presence of WWL70. DAG levels did not show any significant differences due to inhibitor treatments (Fig. 16A). The results also shows that glucose stimulation of the □-cells increased both 1-MAG and 2-MAG levels and that WWL70 further amplified the glucose effect. However, the pan lipase inhibitor orlistat reduced the MAG levels (Fig. 16A), probably because of its effects on ATGL, HSL and *sn1*-DAG lipases and increased TG content. The changes in the MAG levels paralleled the changes in insulin secretion, indicating a possible direct relationship between cellualar MAG content and insulin secretion.

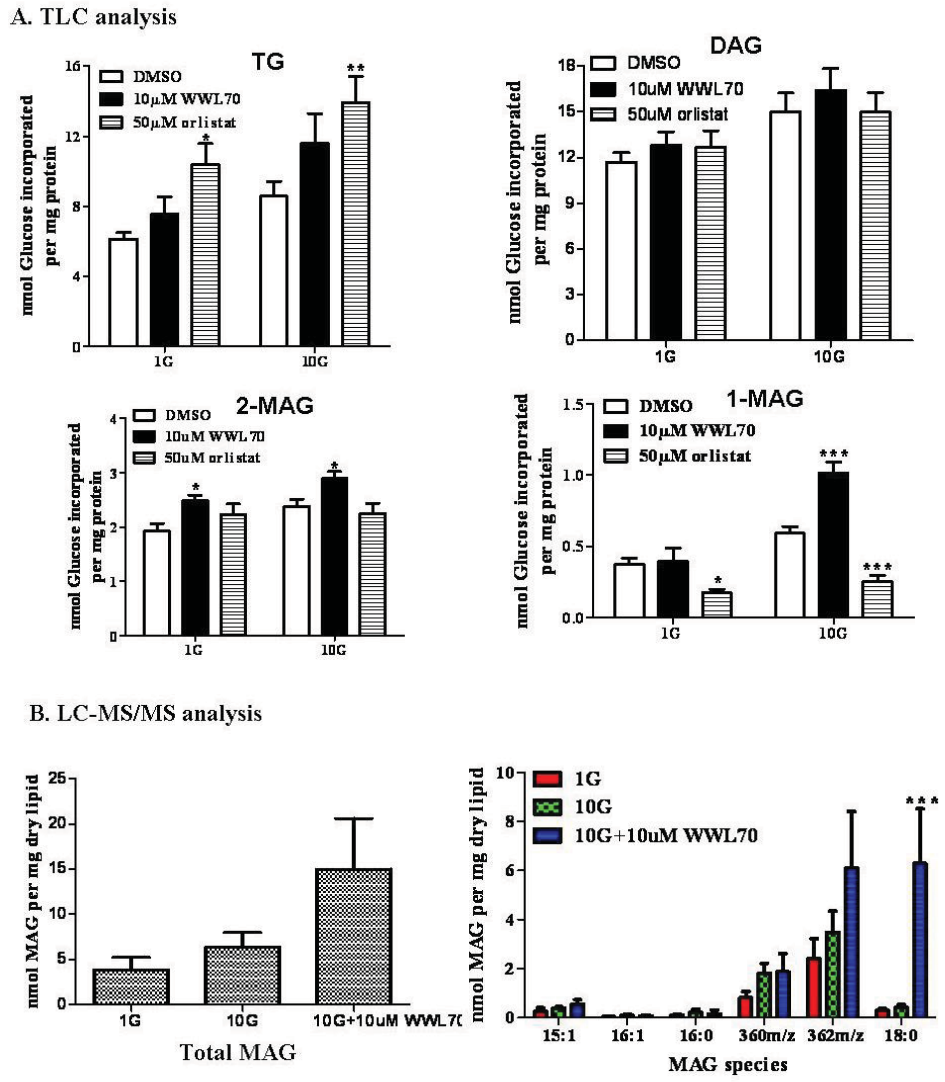


Figure 16 Lipid profiles measured by TLC (A) and LC-MS/MS analysis (B). INS832/13 cells were treated as indicated in the 'materials and methods". Figure 16A shows the change of different neutral lipids, including TG, DAG and MAG after treatment with WWL70 and orlistat; Figure 16B shows the change of total MAG and each MAG species after treatment with 1mM and 10mM glucose as well as with WWL70. *p<0.05, **p<0.01, ***p<0.001 when compared to the corresponding glucose group.

3.5 Profile of different MAG species, measured by LC-MS/MS analysis

INS832/13 cells were incubated as described “Materials and Methods”. Lipid extracts were processed for LC-MS/MS for profiling MAG and DAG species. Results indicated that high glucose concentration elevates MAG levels and the presence of ABHD6 inhibitor WWL70, leads to much higher levels of various species of MAG (Fig. 16B). In particular, we noticed that 1-stearoylglycerol (1-SG) is elevated quite significantly in the presence of WWL70 (Fig. 16B, right panel). We also noticed significantly elevated levels of two unknown species of MAG. The total DAG content increased with increasing glucose concentration, but WWL70 had relatively little further effect on DAG levels (data not shown).

3.6 The effect of exogenous MAG on insulin secretion

Inasmuch as MAG levels increased with WWL70 addition and correlated with elevated insulin secretion, we examined whether there is any direct effect of different MAGs on insulin secretion. In these experiments, we tested 5 different MAGs (1-stearoylglycerol, 1-SG; 1-palmitoylglycerol, 1-PG; 1-oleoylglycerol, 1-OG; 1-linoleoylglycerol, 1-LG; 1-arachidonoylglycerol, 1-AG; 2-oleoylglycerol, 2-OG) for their dose-dependent effects in INS832/13 cells at 5mM glucose. Results indicated that different MAGs show varying effects in inducing insulin secretion (Fig. 17). We found that 1-SG and 1-palmitoylglycerol (1-PG) maximally stimulated insulin secretion, as compared to 1-OG, 2-OG and 1-LG (Fig. 17B).

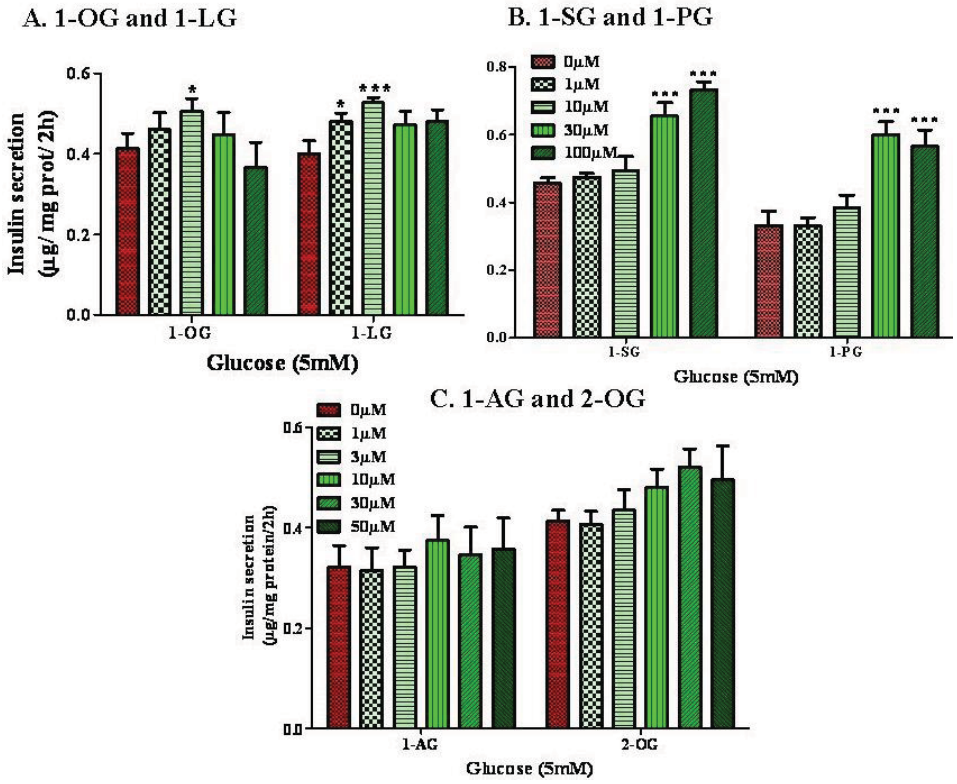


Figure 17 The effect of exogenous MAG species on insulin secretion. INS832/13 cells were preincubated with KRBH with 2mM glucose and 0.5%BSA plus different MAGs for 45min, and then INS832/13 cells were incubated in KRBH with 5mM glucose plus different MAGs for 2h. Results are means ± SE of 3 separate experiments, in triplicate wells. *p<0.05, **p<0.01, ***p<0.001 when compared to the corresponding control group.

3.7 The effect of overexpression of ABHD6 on GSIS

Since the inhibition of ABHD6 in the □-cells enhances GSIS, probably via augmenting the MAG levels, we decided to examine whether overexpression of ABHD6 has opposing effects (i.e., inhibitory) on GSIS. Overexpression of ABHD6 in INS832/13 cells (transfected with pCMV-ABHD6 plasmid; origene) was conducted by the Amaxa Nucleofector method (Program T-27, solution V; Amaxa, Inc., Gaithersburg, MD). The

tranfection efficiency was evaluated to be more than 85% (data not shown) by cotransfecting the cells with a GFP expression plasmid and by cytometry and confocal microscopy of GFP in the transfected cells. After 72h transfection, the cells were collected for Western blotting and insulin secretion. Results showed that ABHD6 protein level increased about 2-fold in the cells transfected with the expression plasmid (Fig. 18A). Overexpression of ABHD6 led to a significant decrease in both glucose stimulated and also palmitate-amplified insulin secretion (Fig.18B). The enhanced ABHD6 activity in the INS832/13 cells reduced GSIS, probably by lowering the MAG levels, which are elevated in the presence of glucose and palmitate. These results support the view that MAG mediates or is involved in the augmented insulin secretion at high glucose ± palmitate, as overexpression of ABHD6 dampens the insulin secretion stimulating effect of high glucose ± palmitate (Fig. 18B).

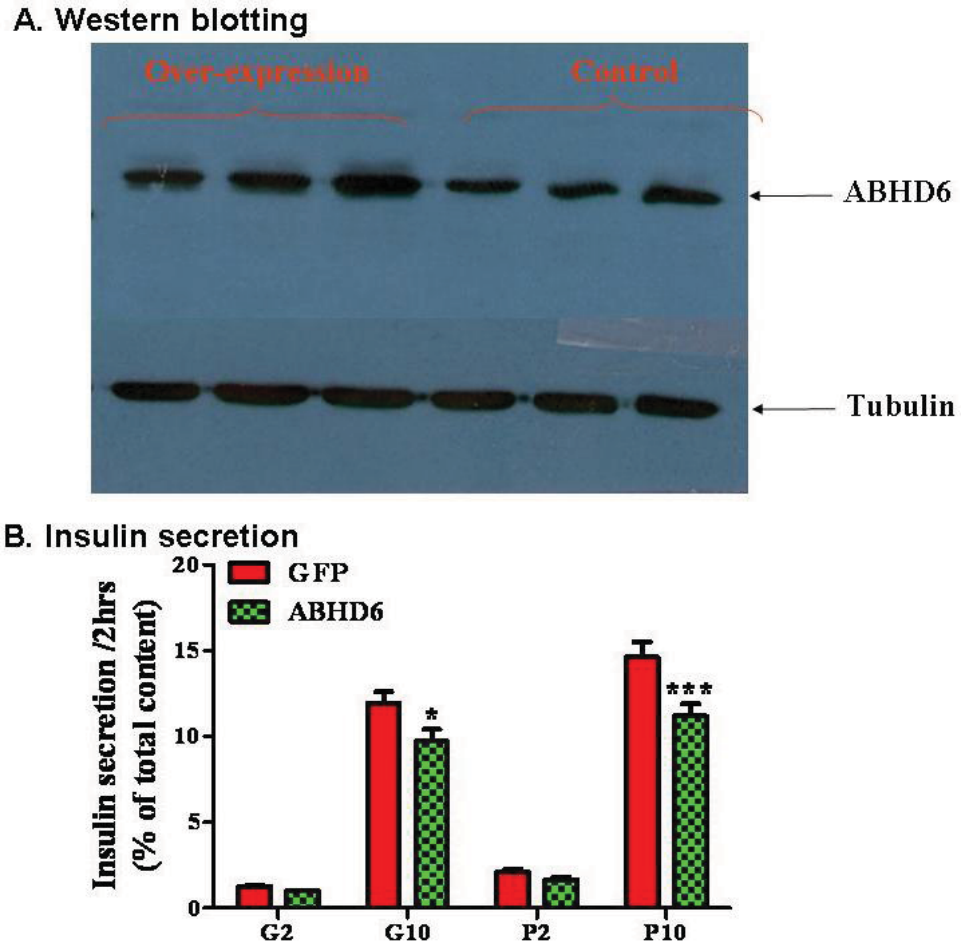


Figure 18 The effect of over-expression of ABHD6 on insulin secretion in INS832/13 cells. INS832/13 cells were transfected with Amaxa reagent. After 72h transfection, the cells were used for Western blotting and insulin secretion. Figure 18A shows the expression of ABHD6 by Western blotting; Figure 18B indicates the effect of over-expression of ABHD6 on insulin secretion. Results are means ± SE of 3 separate experiments, in triplicate wells. *p<0.05, **p<0.01, ***p<0.001 when compared to the corresponding "GFP" group.

3.8 TRPV1 is possiblly responsible for WWL70-enhanced insulin secretion.

The foregoing results showed that MAG, generated via GL/FFA cycle, plays a pivotal role in the lipid amplification of GSIS. However, the target of MAG in the □-cell, through

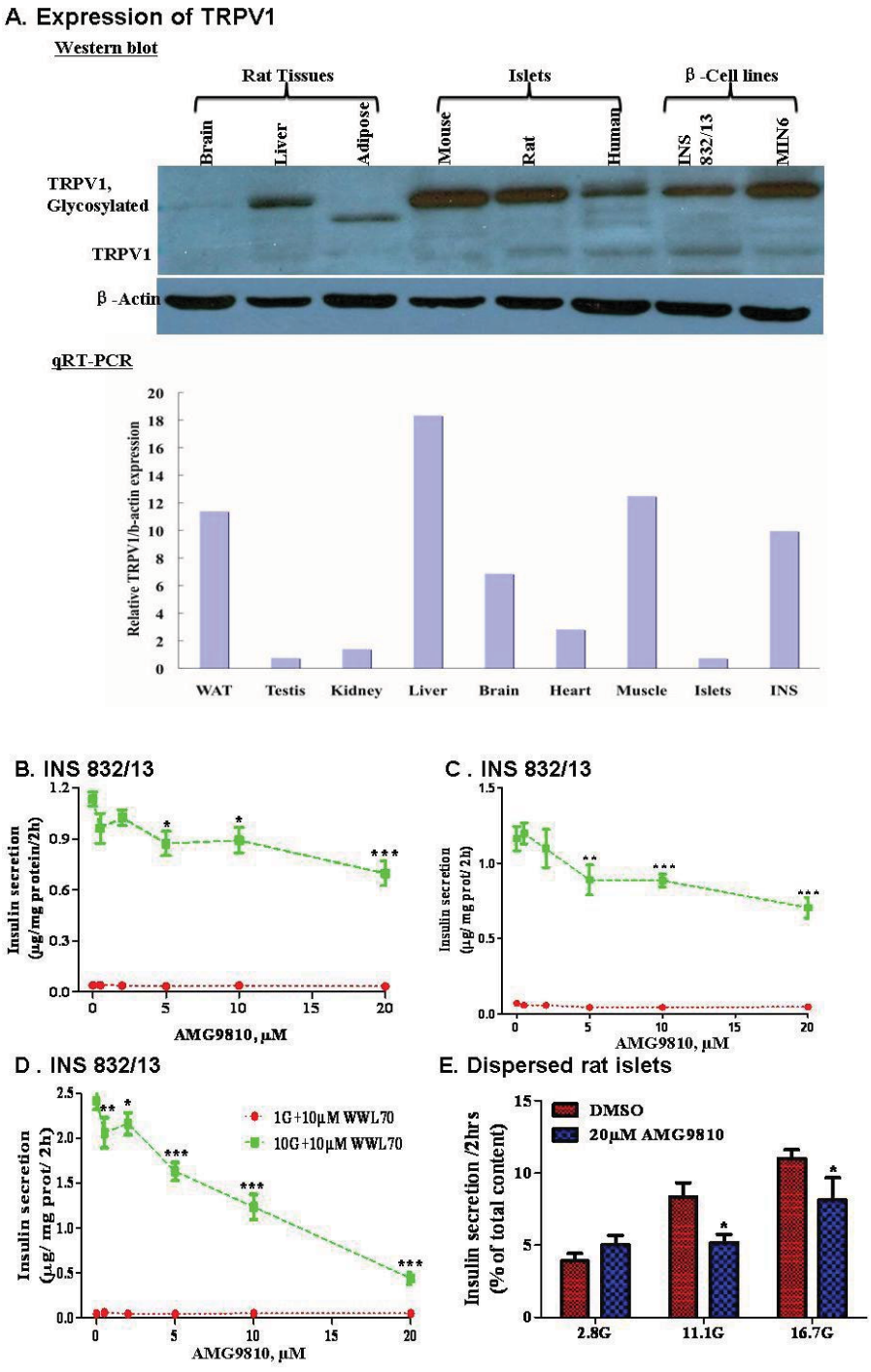
which insulin secretion is enhanced, is not known. Recently, it has been shown that MAGs could activate TRPV1 channel and cause calcium influx (Iwasaki et al., 2008). TRPV1 is a plasma membrane localized non-selective cation channel, which preferentially functions in the influx of Ca^{2+} ions, when stimulated (Venkatachalam and Montell, 2007). Besides, it is also known that capsaicin, which activates TRPV1, stimulates insulin secretion both in vitro in beta cells (Akiba et al., 2004) and in vivo in ZDF rats (Gram et al., 2007). The ligand-binding site of TRPV1 is known to be on the inner side of the plasma membrane. Considering this, we evaluated the possible role of TRPV1 in the elevated GSIS seen due to MAG accumulation. First, we assessed the expression of plasma membrane-bound TRPV1 in different rat tissues and mouse, rat and human islets by Western blotting and RT-PCR. As demonstrated in Figure 19A, liver expressed maximal amount of TRPV1, while its expression in beta cell line (MIN6 and INS832/13) and rat tissue is also quite significant.

As we noticed that □-cells do express TRPV1, we employed AMG9810 (Gavva et al., 2005), a specific antagonist of TRPV1, to check the role of TRPV1 and its activation by MAG in insulin secretion. As shown in Figure 19B and 19C, TRPV1 antagonist, AMG9810, could dose-dependently decrease GSIS, and this effect was more evident in the presence of 0.3mM palmitate, indicating that TRPV1 may contribute a significant part to the Ca^{2+} influx needed for insulin granule exocytosis. As MAG can activate TRPV1 and enhance Ca^{2+} influx and because the MAG levels increase in the presence of glucose and fatty acids, it is quite likely that the enhanced insulin secretion with glucose plus fatty acids may be mediated through the accumulation MAG, which in turn activates TRPV1 channel and thus insulin secretion. Thus the inhibitory effects of AMG9810 on insulin secretion are

more pronounced in the presence of 10μM WWL70, which causes significant accumulation of MAG by inhibiting ABHD6 (Fig. 19D).

In order to confirm the results seen with INS832/13 cells in primary beta cells, we also performed similar experiments on rat islets. As indicated in the Fig 19E & 19F, AMG9810 could significantly decrease GSIS and in the presence of WWL70 this inhibitory effect was more evident.

In order to check whether TRPV1 indeed plays a role in inducing calcium influx and thus insulin secretion, we performed calcium measurements in dispersed rat islets with or without WWL70 or AMG9810. There was a clear increase of calcium influx induced by WWL70, while AMG9810 decreased calcium influx (Fig 19G). This indicates that the enhanced insulin secretion by WWL70 is at least partially mediated by increased calcium influx conducted by TRPV1 channel. AMG9810, an antagonist of TRPV1, decreased the activity of TRPV1 channel, leading to reduced calcium influx and thus decreased insulin secretion.


A. Expression of TRPV1
Western blot
Rat Tissues
Islets
β -Cell lines
Brain
Liver
Adipose
Mouse
Rat
Human
INS 832/13
MIN6
TRPV1, Glycosylated
TRPV1
β -Actin
qRT-PCR
Relative TRPV1/b-actin expression
WAT
Testis
Kidney
Liver
Brain
Heart
Muscle
Islets
INS
B. INS 832/13
Insulin secretion (μg/mg protein/2h)
AMG9810, μM
C . INS 832/13
Insulin secretion (μg/ mg prot/ 2h)
AMG9810, μM
D . INS 832/13
1G+10μM WWL70
10G+10μM WWL70
Insulin secretion (μg/ mg prot/ 2h)
AMG9810, μM
E. Dispersed rat islets
DMSO
20μM AMG9810
Insulin secretion /2hrs (% of total content)
2.8G
11.1G
16.7G

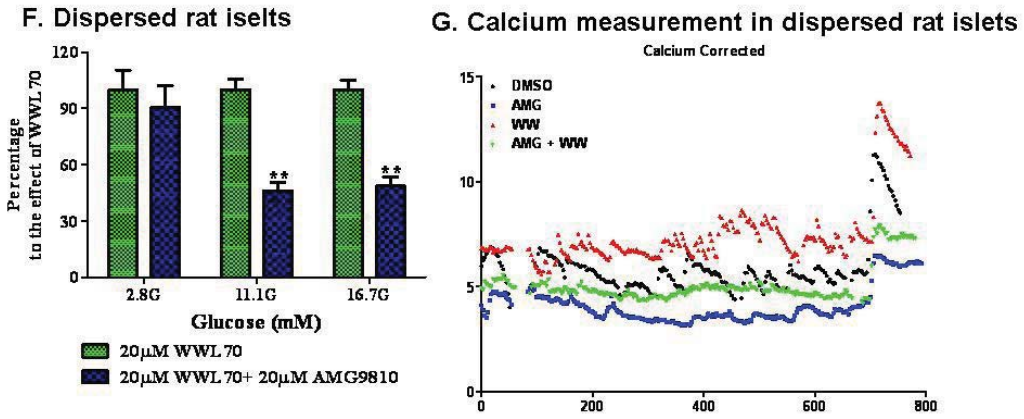


Figure 19 TRPV1 is responsible for WWL70-enhanced insulin secretion. The effect of the TRPV1 receptor in glucose-stimulated and WWL70-enhanced insulin secretion was examined using the specific antagonist AMG9810. Figure 19A indicates the expression level of TRPV1 in different rat tissues, islets and beta cell lines; Figure 19B indicates the effect of AMG9810 on GSIS (1mM glucose in red, 10 mM glucose in green); Figure 19C indicates the effect of AMG9810 on GSIS in the presence of exogenous palmitate (0.25mM); Figure 19D indicates the effect of AMG9810 on WWL70-enhanced insulin secretion at high glucose; Figure 19E indicates the effect of AMG9810 on insulin secretion in rat islets; Figure 19F shows the effect of AMG9810 on WWL70 enhanced insulin secretion in rat islets; Figure 19G indicates the effect of WWL and AMG9810 in inducing calcium influx in dispersed rat islets.Drugs were added at time 50 s and a depolarizing concentration of KCl (30mM) at time 700 s. Results are means ± SE of 3 separate experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ when compared to the corresponding glucose group.

4. Discussion

Insulin secretion by the pancreatic □-cell is an essential physiological process and this is governed by various cellular, nutritional and pathological factors. Pancreatic islets secrete insulin under normal conditions, in response to post-prandial increase in blood glucose levels. Other fuels including amino acids like leucine and glutamine and also fatty acids contribute to further augment insulin secretion. Even though the precise mechanism of insulin secretion is not known, the significance of the elevated Ca^{2+} influx and the role of K^{+}-ATP channels have been well documented. Prentki group and others (Prentki and Corkey, 1996) have provided ample evidence for the existence of a lipid amplification pathway for enhancing GSIS and as instrumental for the second phase insulin secretion. Recently, Prentki group has suggested that GL/FFA cycle as being an essential component in the lipid amplification pathway of insulin secretion (Nolan et al., 2006a; Nolan et al., 2006b; Prentki and Madiraju, 2008). Lipolysis has been shown to be important for insulin secretion as studies showed curtailed GSIS in islets upon inhibition of lipolysis (Masiello et al., 2002; Winzell et al., 2006). Prentki group and others provided evidence for the involvement of HSL, which prefers to hydrolyze DAG to MAG, in the regulation of GSIS both *in vivo* and *in vitro* (Fex et al., 2009; Peyot et al., 2004; Roduit et al., 2001). A recent study from Prentki lab showed that ATGL, which hydrolyzes TG to DAG, in the □-cells is also necessary for fuel and non-fuel stimulated insulin secretion, both in vitro and in vivo (Peyot et al., 2009b). Besides HSL, *sn1*-DAG lipase, a plasma membrane enzyme, was also suggested to play a role in the normal GSIS (Guenifi et al., 2001; Konrad et al., 1994). Orlistat, an inhibitor of lipolysis, is also known to antagonize fatty acid augmentation of

GSIS in isolated islets (Nolan et al., 2006a). The exact nature of the lipid-derived signal that is essential for lipid-amplification of GSIS is not clear, even though DAG was initially thought (Prentki and Matschinsky, 1987) to be the major lipolysis-derived signal for insulin secretion. However, since conditions that lead to DAG accumulation (eg., HSL ablation and DAG-lipase inhibition) also lead to lowered GSIS, it seems plausible that monoacylglycerol (MAG), generated from the lipolysis segment of GL/FFA cycling, likely functions as a metabolic coupling factor in GSIS.

As inhibition of lipolysis by orlistat almost completely obliterates GSIS in □-cells, we first investigated whether MAGs could reverse this inhibitory effect of orlistat on insulin secretion. Our initial experiments, revealed that orlistat-inhibited GSIS could be reversed with 2-AG, which is also effective in enhancing un-inhibited GSIS. As 2-AG is an endocannabinoid and also functions by activating CB1-endocannabinoid receptor, we ascertained if the 2-AG effects are related to its endocannabinoid-type activity. Earlier studies have demonstrated that 2-AG exerts both inhibitory (Juan-Pico et al., 2006) and stimulatory (Juan-Pico et al., 2006; Matias et al., 2006) effects on insulin secretion and that these effects are likely mediated via CB1/ CB2 receptors. Our results showed that 2-AG enhances insulin secretion at 2mM glucose, though this effect was not significant at high glucose concentration (10mM). We also found that unlike the earlier findings, CB1-receptors are not involved in the 2-AG mediated effects on GSIS and the restoration of GSIS after orlistat inhibition. Thus an antagonist of CB1 receptor, AM251 had no effect on 2-AG mediated increase in insulin secretion. Since 2-AG could reverse the inhibition of orlistat on insulin secretion, indicating MAG may be the putative metabolic coupling factor,

we tested whether other MAGs could also have similar effect. We noticed that two other MAGs, 1-SG and 1-PG could also completely restore insulin secretion after orlistat inhibition at intermediate glucose (5mM glucose) and partially at high glucose (10mM glucose). These results suggested that inhibition of lipolysis probably reduces the production of MAG, which likely acts as a lipid signal for augmenting GSIS, as exogenous supply of MAG restores the orlistat-inhibited insulin secretion.

To further test the hypothesis of MAG as a novel MCF, we decide to employ pharmacological and molecular biological methods to alter the MAG levels inside the cells. In order to achieve this, it was necessary to know the expression of different MAG lipases in the beta cells. Expression of MAG lipases in □-cells or islets was not reported before. The classical MAG lipase has been identified more than 40 years ago, and many reports have demonstrated that MAGL is highly expressed in adipocytes. Western blotting and RT-PCR analyses to detect the MAGL in different tissues and mouse, rat and human islets as well as two beta cell lines, MIN6 and INS832/13 revealed that adipocytes have high MAGL expression whereas, it is not expressed to any significant extent in beta cells (Fig. 14 A). Besides the classical MAGL, two other plasma membrane proteins with MAG lipase activity, viz., ABHD6 and ABHD12 were identified in the brain, and these enzymes were found to contribute to 15% of the total MAG hydrolytic activity in the brain (Blankman et al., 2007). Recent reports indicated that ABHD6 controls the accumulation of 2-AG and thus its availability to cannabinoid receptors in brain (Marrs et al., 2010). ABHD6 is an integral membrane protein with its active site facing the cytosolic side where as ABHD12

has its active site facing towards exterior of the cell (Blankman et al., 2007). As indicated in Fig 14B, ABHD6 is ubiquitously expressed in all sources of beta cells, including mouse, rat and human islets as well as beta cell lines, MIN6 and INS832/13. Expression of ABHD12 at protein level could not be ascertained with certainty due to the poor quality of antibody, even though its mRNA level could be easily detected (data not shown). These results indicated that ABHD6 may be the enzyme responsible for MAG hydrolysis and in controlling MAG levels in beta cells.

We then examined the effect of two specific inhibitors for MAGL and ABHD6, viz., JZL184 and WWL70, respectively, on insulin secretion. Inhibition of MAGL with JZL184 has no effect on insulin secretion (Fig. 15A) and this is in accordance with the poor expression of MAGL in □-cells. However, ABHD6 inhibitor- WWL70 caused a robust increase in insulin secretion (Fig. 15B), indicating that MAGs build-up inside the cells only upon inhibition of ABHD6, and thus leads to the enhanced insulin secretion. As a control we also included orlistat, which can reduce the production of MAGs by inhibiting lipases, showed strong inhibition of insulin secretion. WWL70 could dose-dependently increase insulin secretion (Fig. 15C), with the maximum effect being observed at 10μM. In order to confirm the results seen with INS cell line, we performed similar experiments in a more physiological beta cell model, i.e., isolated islets, from mouse, rat and human donors. In the islets from all the three species WWL70 could enhance GSIS (Fig. 15D, 15E and 15F), which confirmed the results obtained with INS832/13 cell line. These results provided

further support for the view that MAG, whose levels are regulated by the activity of ABHD6, likely mediates the lipid amplification of GSIS.

In order to ascertain that the effects of WWL70 are metabolism-dependent rather than direct membrane depolarization effects, we tested the effect of this drug on KCl induced insulin secretion. At low glucose concentration (1 mM), a large excess of KCl (35 mM) leads to plasma membrane depolarization and Ca^{2+} influx, which promotes the fusion of insulin granules that are already docked at the membrane. Thus this process bypasses the need for either K^{+}_{ATP} channels or other receptors for Ca^{2+} influx. WWL70 did not show any effect on KCl-stimulated insulin secretion in INS832/13 cells. This supports the notion that MAG that accumulates due to WWL70 inhibition of ABHD6 is needed for insulin secretion and high KCl bypasses this metabolism-based mechanism. Interestingly, stimulation of insulin secretion by amino acids, glutamine + leucine at low glucose, is also enhanced by WWL70, similar to GSIS. Thus, since the amino acid-stimulated insulin secretion also involves metabolism dependent signal generation, accumulation of MAG by WWL70 can further augment this process. We did not notice significant insulin secretion in response to arginine in INS832/13 cells.

In order to examine whether the effects of WWL70 and orlistat on insulin secretion are related to the altered levels of intracellular lipids, particularly MAG, we have tested the effect of these inhibitors on MAG accumulation and insulin secretion in INS832/13 □-cells. We noticed that under conditions where all the internal lipid carbons are eqilibrium-labeled

by [U-^{14}C]-glucose, incubation with 10 mM glucose led to increased synthesis of neutral lipid species including triglycerides, DAG and 1- and 2-MAG and also enhanced the amount of ^{14}C-FFA released into the medium (not shown). Results also showed that TG accumulated in the presence of orlistat, while ABHD6 inhibition by WWL70 only had marginal effect on TG accumulation (figure 16). Neither of the inhibitors had any effect on total DAG levels (including both 1,2- and 2,3-isomers). 2-MAG is formed mostly by the hydrolysis of DAG. Interestingly, while orlistat showed no effect on 2-MAG levels, inhibition of ABHD6 (WWL70) caused a modest elevation of this lipid (Fig. 16A). If DAG and MAG are in rapid equilibrium, it is possible that high concentration of orlistat (50 ¦ÌM) used in this experiment, could have maintained the steady-state level of 2-MAG, by inhibiting both its formation and degradation. On the other hand, orlistat significantly reduced the formation of 1-MAG, while inhibition of ABHD6 by WWL70 caused a significant increase. Considering that significant amount of the 1-MAG can also arise from the hydrolysis of LPA by lysophosphatidate phosphatase (LPP) isoenzymes, that are not known to be inhibited by orlistat, it is surprising that orlistat significantly lowered the production of 1-MAG. Whether there are any orlistat-sensitive pathways of 1-MAG production in □-cells, including the possible interconversion of 2-MAG to 1-MAG, needs to be examined. It is also important to consider that the intra-cellular location of MAG (either 1-MAG or 2-MAG) accumulation may vary depending upon the enzyme that is inhibited, i.e., MAGL or ABHD6. This may have relevance for the signaling functional ability of the accumulating MAG in a given cell. Thus, inhibition of the plasma membrane associated ABHD6 by WWL70 led to a significant increase in 1-MAG,

which is accompanied by a marked decline in the efflux of labeled FFA from INS832/13 □-cells (data not shown.

In order to know which specific MAG was increased after WWL70 treatment, we performed LC/MS/MS to separate different MAGs and DAGs. Total MAG and DAG levels increased in INS832/13 □-cells with increasing glucose concentration in the absence of FFA, indicating glucose could activate GL/FFA cycling, leading to elevated MAG production. In the presence of WWL70 with high glucose, the total MAG levels are increased significantly and there is only a modest change in total DAG levels (Fig. 16B), indicating that the inhibitor acts on the right target. The MAG profiles indicated that several MAG species increase at high glucose concentration, and with WWL70, there is a specific increase in 1-SG by several fold. Thus after treatment with WWL70, which blocks the ABHD6-mediated hydrolysis of MAG, there is a build-up of 1-SG in the □-cells, which perhaps triggers insulin granule exocytosis.

In order to further assess the role of MAG in insulin secretion, we checked whether exogenous MAG could induce insulin secretion. As shown in the Fig 17, various exogenous MAGs could increase insulin secretion in INS832/13 cells in a dose-dependent manner. Among the MAGs tested, 1-SG and 1-PG showed the strongest effect in inducing GSIS, which is highly consistent with the results of LC-MS/MS, which showed maximal accumulation of 1-SG after WWL70 treatment. We ruled-out the possibility that the added MAG is first converted to DAG in order to exert its effects on GSIS. Thus the inclusion of a

specific DAG-lipase inhibitor, RHC80267, which causes a build-up of 1,2-DAG in the cells, strongly inhibited GSIS and this inhibition is alleviated by the addition of 1-PG or 1-SG (data not shown).

Since the above-mentioned pharmacological approach clearly indicated the importance of ABHD6 in the regulation of GSIS, we attempted molecular biological methods to control the expression of this protein. First we tried to knockdown ABHD6 expression using specifc RNAi (Ambion). We noticed that even though the mRNA level of ABHD6, as measured by RT-PCR, at different time points (24h, 48h, 72h and 96h), showed ~80% decrease (data not shown); a corresponding decrease in the ABHD6 protein level was not seen, as assessed by Western blotting. In spite of many efforts, we were unable to see any significant decrease in the ABHD6 protein level, by RNAi-approach. We believe that this is due to the fact that the mRNA level of ABHD6, is quite high, about 1/5 of that of actin, because of which even if the ABHD6 mRNA decreases by 85% by RNAi, the remaining 15% is enough to make sufficient ABHD6 protein and hence no change in protein level. Another problem is related to ABHD6 being a plasma membrane protein, which generally has a longer half-life. It has been reported that the half-life of plasma membrane proteins can be longer than 4 days. This is a general problem for using RNAi to knockdown the membrane proteins. In our experiments, we did not measure the half-life of ABHD6, but at least this can be one of the reasons for the absence of a significant effect of RNAi on ABHD6 protein.

We next examined the effect of ABHD6 over-expression, using an expression plasmid (Origene) and a control protein (GFP) expression plasmid. As shown in Fig 18A, after 72h transfection, there is about 2 fold increase in the ABHD6 protein level. This is not a large increase compared to the over-expression of GFP in the controls and this may be because of the natural presence of relatively huge amount of ABHD6 mRNA in these cells. Thus, an additional increase in the mRNA content might have not led to a significant increase in the protein level. Interestingly, the 2-fold over-expression of ABHD6 decreased GSIS and the palmitate enhanced GSIS also compared with the GFP transfected control (Fig 18B). These results strongly implicated MAG, a substrate of ABHD6, as a MCF in the process of glucose-stimulated. Over-expresison of ABHD6 likely decreased the levels of MAG, thus lowered the insulin secretion.

One of the possible mechanisms, by which MAG induces insulin secretion, is probably via the activation of TRPV1 receptor. TRPV1 receptor, also known as vanilloid receptor is activated by various agents including H^+ ions, anandamide, capsaicin, heat etc. (Suri and Szallasi, 2008; Venkatachalam and Montell, 2007; Vriens et al., 2009). The presence of TRPV1-receptor in pancreatic □-cells has been investigated by different groups and while its presence and functionality has been shown in normal rat islet □-cells (Akiba et al., 2004; Barbasa and Fleig, 2008), studies done on ZDF rats (Gram et al., 2007) and in the Type-1 diabetes model, NOD mice (8 week old with insulinitis) (Razavi et al., 2006) were unable to show the TRPV1-R presence in the islets. It has been demonstrated that TRPV1-R has its

ligand/ agonist binding site intracellularly and its activation by capsaicin or other agonists leads to Ca^{2+} influx (Vriens et al., 2009), which is a pre-requisite for insulin granule fusion with plasma membrane for exocytosis. Because of the uncertainty regarding TRPV1-R localization in □-cells, we have conducted a systematic screening of islets and INS832/13 □-cells employing qRT-PCR and also Western blot analysis in the same samples. Our results demonstrated the expression of TRPV1-R at both mRNA as well as protein level (Fig 19A) in islets and in INS cells. Activation of TRPV1-R by capsaicin both *in vitro* (Akiba et al., 2004; Barbasa and Fleig, 2008) and also *in vivo* has been shown to elevate insulin secretion and Ca^{2+} influx (Alevizos et al., 2007; Gram et al., 2007; Moesgaard et al., 2005). Both 1- and 2-MAG have recently been shown to activate TRPV1-R *in vitro* leading to Ca^{2+} influx and also *in vivo* (Iwasaki et al., 2008). Since we found that TRPV1-R is expressed in islets and □-cells, we examined whether this receptor is involved in the augmentation of GSIS under conditions of MAG accumulation, such as ABHD6 inhibition by WWL70, by employing a highly specific TRPV1-R antagonist, AMG9810 (Gavva et al., 2005). We noticed that even though AMG9810 showed a dose-dependent inhibitory effect on GSIS, this effect is only marginal (though statistically significant) in INS cells at intermediate and high glucose concentrations (Fig. 19B and 19C). However, AMG9810 inhibition is more marked under conditions when GSIS is enhanced by WWL70 (Fig. 19D). Presence or absence of palmitate during GSIS incubations had no influence on AMG9810 inhibition. It is important to note that AMG9810 does not inhibit GSIS completely, even at high concentrations, which suggests that the TRPV1-R in □-cells only partially contributes to the total Ca^{2+} influx needed for exocytosis. It has been suggested that in islet □-cells,

while the 1st phase insulin secretion is coupled to L-type Ca^{2+} channels, the 2nd phase secretion is probably coupled to R-type Ca^{2+} channels (Jing et al., 2005; Pedersen and Sherman, 2009).

It has been reported that TRPV1-KO mice (C57Bl6J genetic background) are relatively resistant to high-fat diet induced obesity (Motter and Ahern, 2008) and also that these mice exhibit much better glucose tolerance and insulin sensitivity (Razavi et al., 2006). Also TRPV1-KO mice have reduced insulinemia unlike their controls, which have the tendency to show poor glucose tolerance. However, these effects were attributed primarily to the loss of TRPV1 in peripheral neurons in the islets (Razavi et al., 2006). Considering that activation of TRPV1-R by its ligands capsaicin and resiniferatoxin (Gram et al., 2005; Moesgaard et al., 2005) *in vivo* elevates plasma insulin levels and that deletion of TRPV1-R reduces plasma insulin along with the present observations that TRPV1-R is indeed expressed in islets and □-cells and has a role in insulin secretion *in vitro* and *ex vivo*, strongly indicate that this receptor in □-cells may have an important function *in vivo* to regulate insulin secretion.

Thus collectively our results support the concept that ABHD6 is an important regulator of GSIS and its selective inhibition causes accumulation of MAG, which activates TRPV1-R and thereby promotes insulin secretion. Therefore, compounds that selectively target ABHD6 have the potential to be developed as diabetes therapeutics, which increase circulating insulin levels. Such drugs may also have the potential to increase insulin

sensitivity in the peripheral tissues by TRPV1-R activation, as administration of capsaicin was shown to decrease insulin resistance.

5. Conclusion

5.1 Conclusion

Collectively our results support the concept (Fig. 20) that ABHD6 is an important regulator of GSIS and its selective inhibition causes accumulation of MAG, which activates TRPV1-R and thereby promoting Ca^{2+} influx and insulin secretion. Therefore, compounds that selectively target ABHD6 have the potential to be developed as diabetes therapeutics, which increase circulating insulin levels. Such drugs may also have the potential to increase insulin sensitivity in the peripheral tissues by TRPV1-R activation, as administration of capsaicin was shown to decrease insulin resistance.

5.2 Perspective

Our results demonstrate that MAG stimulates insulin secretion via TRPV-1 activation. But whether the effect of MAG is mediated only through this TRPV1 pathway or other possible mechanisms exist is still unknown. In some recent work, protein kinase-D (PKD) has been shown to associate with TRPV1 and also to phosphorylate TRPV1. Besides, PKD has been shown to contain the DAG-binding C1-domain and to be necessary for proper GSIS. We speculate that the C1-domain in PKD may also bind with MAG (Fig. 20), causing its activation and thus contributing to elevated insulin secretion. These studies are presently ongoing in our lab.

Furthermore, DAG was shown to bind with the C1-domain of MUNC13-1, an exocytosis-related protein, to stimulate insulin secretion. However, it has been reported that the C1-domain of MUNC13-1 has much lower affinity for DAG, owing to an overlapping Trp residue in the binding pocket, thus making this binding less efficient (Shen et al., 2005). We speculate that MAG may bind with the C1-domain of MUNC13-1 more effectively than DAG and thus able to activate this protein better to achieve insulin granule exocytosis with higher efficiency. We are currently conducting experiments related to MAG binding with C1-domain of MUNC13-1 with NMR (Fig. 20).

Because of the close relationship between MAG and ABHD6, we are presently generating ABHD6 whole-body and beta-cell-specific knockout mice, to further clarify the role of MAG as a new MCF and ABHD6 in insulin secretion and glucose homeostasis.

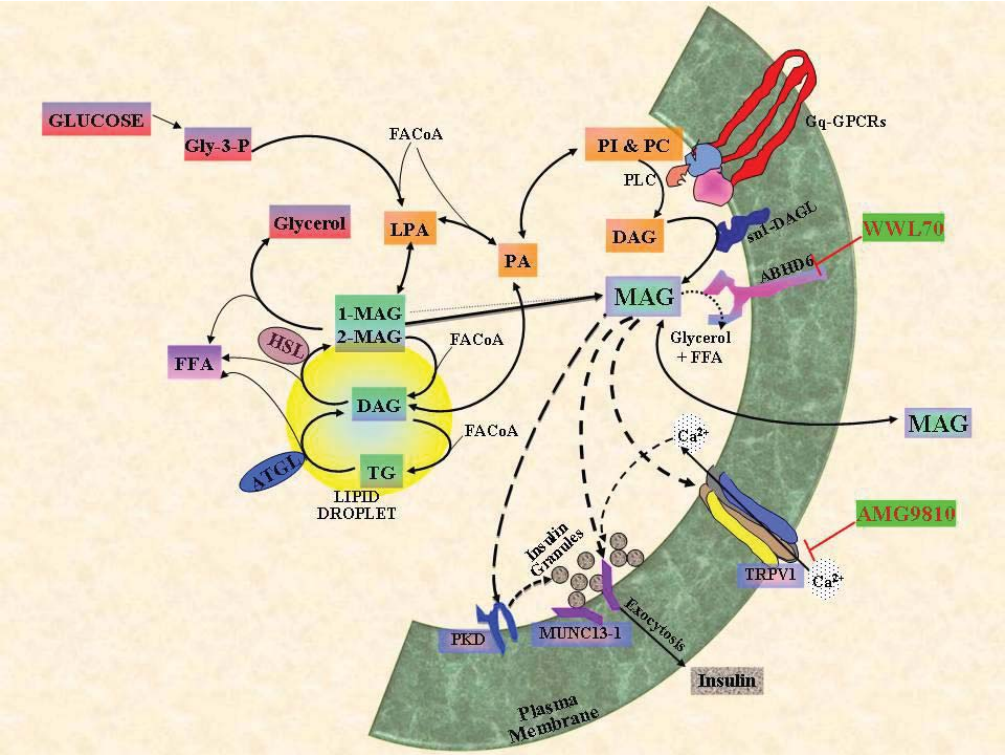


Figure 20 Lipolysis-derived MAG as a MCF for insulin secretion, targeting TRPV1 channels, PKD and Munc13-1. MAG, produced either by lipolysis on lipid droplets or due to signaling events on plasma membrane, can bind and activate on the cytosolic side: 1), TRPV1, which is a Ca2+ channel, 2), Munc13-1, which is an effector protein of exocytosis and 3), PKD, which is involved in the overall process of insulin granule exocytosis. Activation of these three essential components of exocytosis will lead to elevated insulin secretion. Thus, inhibition of ABHD6 by WWL70 can cause a build up of MAG near the plasma membrane and cause enhanced insulin secretion, by activating TRPV1 and promoting Ca^{2+} influx, which is blocked by TRPV1 antagonist, AMG9810. Besides TRPV1, Munc13-1 and PKD may also be the targets of MAG and contribute to insulin secretion.

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