Université de Montréal

Glucolipotoxicity and the Control of Pancreatic ß-Cell Apoptosis

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To be evaluated by the following members of the jury:

Dr. Richard Bertrand : President of jury
Dr. Marc Prentki : Research Director
Dr. Christine Des Rosiers: Member of jury

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SUMMARY

Type 2 diabetes is a heterogeneous condition characterized by elevated plasma glucose levels caused by a combination of insulin resistance and defective insulin secretion. As for type 1 diabetes, type 2 diabetes is increasingly being considered to be a disease of reduced β-cell mass. The pathogenic mechanisms causing β-cell loss in type 2 diabetes, however, are poorly understood. Many studies have indicated that elevated circulating free fatty acids (FFA) contribute to the underlying pathophysiology of the disease and that FFA represent a crucial link between insulin resistance and β-cell dysfunction. We previously proposed the "glucolipotoxicity" hypothesis in which elevated free fatty acids (FFA) together with hyperglycemia are synergistic in causing islet β-cell damage because high glucose inhibits fat oxidation and consequently lipid detoxification. The effects of 1-2 days culture of rat INS 832/13 β-cells was investigated in medium containing glucose (5,11, 20 mM) in the presence or absence of various FFA (0.25-0.4 mM) on pancreatic ß-cell death. A marked synergistic effect of elevated concentrations of glucose and saturated FFA (palmitate and stearate) on inducing β-cell death was found in INS 832/13 cells. In comparison, linoleate (polyunsaturated) synergized only modestly with high glucose, whereas oleate (monounsaturated) was not toxic. In INS 832/13 cells, the combination of elevated glucose and saturated FFA caused an increase in DNA condensation and the activity of caspase-3 indicating apoptosis. The pan-caspase inhibitor z-VAD-FMK reduced cell death induced by high glucose but not elevated glucose together with FFA, due to a switch from apoptosis to necrosis. Treating cells with the acyl-CoA synthase inhibitor triacsin C curtailed glucolipotoxicity. In contrast, the fat oxidation inhibitor etomoxir enhanced palmitate-induced cell death such that, at low glucose with palmitate, the same level of cell death occurred as in the condition of elevated glucose and palmitate without the inhibitor. The data indicate that FFA must be metabolized to LC-CoA to exert toxicity, the effect of which can be enhanced

by inhibiting fatty acid oxidation. Ceramide levels were increased after treatment with palmitate but only at elevated levels of glucose. Treating cells with fumonisin B1 or myriocin, both inhibitors of *de novo* ceramide synthesis, did not protect cells from palmitate-induced cell death suggesting that the action of FFA to promote ß-cell apoptosis does not occur via the *de novo* synthesis of ceramide. The results support the glucolipotoxicity hypothesis of ß-cell failure proposing that elevated FFA are particularly toxic in the context of hyperglycemia. In addition, the very divergent effects of saturated versus mononsaturated FFA on ß-cell apoptosis has potential dietary implications. Finally, strategies aimed at enhancing fat oxidation to counteract the inhibitory action of glucose might prove useful to protect ß-cells from glucolipotoxicity.

Key Words: Type 2 diabetes, glucolipotoxicity, apoptosis, long chain acyl-CoA, pancreatic β-cells, β-oxidation.

RESUME

Le diabète de type 2 est une pathologie multifactorielle caractérisée par une glycémie élevée résultant d'une résistance à l'insuline des tissus périphériques et d'une diminution de la sécrétion d'insuline en réponse au glucose des cellules-β pancréatiques. Comme pour le diabète de type 1, plusieurs études ont démontré une réduction du nombre de cellules-β lors des autopsies chez des personnes ayant souffert de diabète de type 2. Les mécanismes pathogéniques responsables de cette perte de cellules-β sont encore mal compris. Plusieurs études ont indiqué que le taux élevé en acides gras libres (AGL) circulants, souvent observé chez les diabétiques, contribue à la pathophysiologie de cette maladie et que les AGL représentent un lien primordial entre la résistance à l'insuline et la dysfonction des cellules-β. Nous avons proposé l'hypothèse de la «glucolipotoxicité» dans laquelle une synergie entre un haut taux d'AGL et une hyperglycémie induirait les cellules-β pancréatiques à entrer en apoptose. L'induction de l'apoptose serait initiée par l'action inhibitrice du glucose à haute concentration sur la β-oxydation des AGL et par conséquent empêcherait la détoxification lipidique.

Pour étudier cette hypothèse, nous avons utilisé le modèle de cellules-β de rat INS 832/13 et recherché les effets sur la mort cellulaire d'un traitement de 1 à 2 jours avec du glucose (5, 11 ou 20 mM) en présence ou absence de différents AGL (0,25-0,4 mM) couplés à la BSA Un effet synergique du glucose à concentration élevée et des AGL saturés (palmitate et stéarate) a été trouvé sur l'apoptose des cellules INS 832/13. Le linoléate (acide gras polyinsaturé) présentait une faible synergie avec le haut glucose alors que l'oléate (acide gras monoinsaturé) n'était pas toxique. Dans les cellules INS 832/13, la co-incubation des cellules en présence de concentrations élevées en glucose et AGL saturés a provoqué des augmentations de la condensation de l'ADN et de l'activité de la caspase-3, deux phénomènes caractéristiques du processus de mort cellulaire par apoptose. Le z-VAD-fmk, un inhibiteur non

spécifique des caspases, a réduit la mort cellulaire induite par le haut glucose mais pas celle provoquée par le co-traitement avec des taux élevés de glucose et d'AGL. Dans ce dernier cas, le z-VAD-fmk, a induit un changement de type de mort cellulaire, l'apoptose faisant place à la nécrose. Le traitement des cellules INS 832/13 avec la triacsin C, un inhibiteur de l'acyl-CoA synthase, a empêché la glucolipotoxicité. Par contre, l'etomoxir, un inhibiteur de la β-oxydation, a accru la mort cellulaire induite par le palmitate. En effet, nous avons observé les même taux de mort cellulaire à bas glucose en présence de palmitate et etomoxir qu'à haut glucose plus palmitate sans etomoxir. Ces résultats indiquent que les AGL doivent être métabolisés en acyl-CoA à longues chaînes pour exercer leur toxicité et que celle-ci peut être accrue suite à l'inhibition de la β-oxydation. Etant donné que plusieurs études ont démontré l'importance des céramides dans l'initiation de l'apoptose et que les céramides peuvent être produits à partir d'AGL (synthèse *de novo*), nous avons vérifié si les céramides contribuaient à l'initiation de l'apoptose induite par le palmitate et le haut glucose. Nous avons mesuré des taux élevés en céramides après incubation des cellules avec du palmitate à haut glucose seulement. Le traitement des cellules INS 832/13 avec la fumonisin B1 ou la myriocin, deux inhibiteurs de la synthèse *de novo* des céramides, n'a pas protégé les cellules de la mort cellulaire induite par le palmitate suggérant que l'effet pro-apoptotique des AGL n'a pas lieu via la synthèse *de novo* des céramides.

Dans leur ensemble, nos résultats appuient l'hypothèse de la glucolipotoxicité (toxicité de taux élevés d'AGL dans un contexte d'hyperglycémie) comme étant responsable de la perte en cellules-β pancréatiques. Finalement, les effets divergents des acides gras saturés *versus* les acides gras insaturés sur l'apoptose des cellules-β pourraient avoir des implications diététiques.

Finalement, des stratégies visant à accroire la β-oxydation pour compenser l'action inhibitrice du glucose pourraient se montrer utiles pour protéger les cellules-β de la glucolipotoxicité.

Mots clés: diabètes de type 2, glucolipotoxicité, apoptose, acyl-CoA à longues chaînes, cellules-β pancréatiques, β-oxydation.

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LIST OF ABBREVIATIONS

ACC	:	Acetyl-CoA carboxylase
Ac-CoA	:	Acetyl-CoA
Ac-DEVD-AFC	:	N-acetyl-Asp-Glu-Val-Asp-amino trifluoromethyl coumarin
ACO	:	Acyl-CoA oxidase
ACS	:	Acyl-CoA synthase
AGE	:	Advanced glycation end product
AICAR	:	5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMPK	:	AMP-activated protein kinase
BSA	:	Bovine serum albumin
CL	:	Citrate lyase
CPT I	:	Carnitine palmitoyltransferase-I
CS	:	Citrate synthase
DAG	:	Diacylglycerol
FAS	:	Fatty acid synthase
FB1	:	Fumonisin B1
FDP	:	Fructose 1,6-bisphosphate
FFA	:	Free fatty acid
F6P	:	Fructose 6-phosphate
GK	:	Glucokinase
GLP 1	:	Glucagon-like peptide-1
GLUT	:	Glucose transporter
GPAT	:	Glycerol-3-phosphate acyl transferase
G6P	:	Glucose 6-phosphate
G6Pase	:	Glucose 6-phosphatase
HSL	:	Hormone sensitive lipase
iNOS	:	Inducible nitric oxide synthase
K-ATP	:	Potassium ATP-dependent channel
KRBH	:	Krebs-Ringer bicarbonate hepes
LC-CoA	:	Long chain fatty acyl-CoA

LPA	:	Lysophosphatidic acid
MCD	:	Malonyl-CoA decarboxylase
MODY	:	Maturity-onset diabetes of the young
NEFA	:	Non-esterified fatty acid
NIDDM	:	Non-insulin-dependent diabetes mellitus
PA	:	Phosphatidic acid
PC	:	Pyruvate carboxylase
PARP	:	Poly (ADP-ribose) polymerase
PDH	:	Pyruvate dehydrogenase
PDX-1	:	«Pancreatic and duodenal homeobox protein-1»
PFK-1	:	6-Phosphofructose-1-kinase
PI	:	Propidium iodide
PI-3K	:	Phosphatidylinositol-3 kinase
PK	:	Pyruvate kinase
PKC	:	Protein kinase C
PL	:	Phospholipids
PPAR	:	Peroxisome proliferator activated receptor
SPT	:	Serine palmitoyltransferase
TG	:	Triglyceride
ZDF	:	Zucker diabetic fatty rat (fa/fa)
Z-VAD-FMK	:	Z-Val-Ala-Asp (OMe)-fluoromethylketone

The process of learning and the production of knowledge involve a large number of individuals and institutions. The development of every work is guided by the spirit and inspiration of all those who contributed in one way or another to every aspect of the work. The input of all those is substantially important.

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I am grateful to you all.

CHAPTER I: INTRODUCTION

1. HISTORICAL INFORMATION

It is eighty years after the discovery of insulin and we are still faced with many challenges concerning diabetes. Despite tremendous advances in our knowledge, we do not understand the accelerated rate of diabetes appearance in our present time. An improved understanding of the pathophysiological basis of diabetes is still required.

Diabetes Mellitus is a medical condition known to physicians for thousands of years. References to this disease can be found in many ancient writings. It was first described on an Egyptian papyrus which was discovered in Egypt in 1862, which is said to have been written between 3000 and 1500 BC. The first use of the term "Diabetes Mellitus" dates back to the second century AD. "Diabetes" stems from the Greek word "siphon" for 'pipe-like' or the passing through of water. 'Mellitus' is Latin for 'honey' or 'sweet' (Halliwell and Gutteridge, 1999).

In the middle of the 19th century, evidence from autopsies started to suggest a link between the pancreas and Diabetes Mellitus. In 1869, a German scientist called Paul Langerhans discovered the existence of two systems of cells in the pancreas: the acinar cells, secreting the pancreatic juice into the digestive system, and islets floating between the acini with, at that time, an unknown function. Several years later, these cells became known as the islets of Langerhans. In 1889 Minkowski and Von Mering gave the first direct evidence of the link between diabetes and the pancreas. They depancreatised a dog causing a state of polyuria indistinguishable from diabetes. This was followed by work by many researchers till the summer of 1920, when Banting and Best discovered insulin. On May 3, 1922, the discovery of insulin was officially announced to the medical community. It took another six years for

Steiner to establish that insulin is a protein, and until 1955 for the primary structure of insulin to be elucidated by Sanger and co-workers. (Historical information was obtained from: www.diabetesforum.net; www.med.uni-giessen.de; www.diabetes.ca).

2. CLASSIFICATION OF DIABETES

Diabetes mellitus is in reality, a group of disorders that have in common hyperglycemia resulting from defects in insulin production, insulin action or both. A hallmark in understanding the etiology of a disease lies in a proper classification and differentiating its various forms depending on various factors. Targeted research, treatment and prevention of diabetes mellitus depend on an appropriate classification. Thus, the growth of studies on the epidemiology and public health aspects was necessary for the field to move on. This required a new classification of the disease based on the huge scientific interest. The recent update of the classification of diabetes mellitus took place in 1997 by the World Health Organization (WHO) and the American Diabetes Association (ADA), which was a revision of the 1985 WHO Study Group (Zimmet 1999). According to this classification, the etiological types of diabetes are:

2.1 Type 1 Diabetes Mellitus

Type 1 (formerly called insulin-dependent diabetes mellitus, IDDM) is an autoimmune disease in which the β-cells of the pancreas are destroyed. In this type, insulin is required for survival to prevent ketoacidosis, coma and death. This is classically a disease of children and young adults, but recent studies indicate that type 1 diabetes can occur at any age. Genetic determinants such as HLA type seem important for the onset in many patients, with environmental factors, possibly viral infection a close second. Type 1 may account for 5 to 10 % of all diagnosed cases of diabetes.

2.2 Type 2 Diabetes Mellitus

Type 2, (formerly called non-insulin dependent diabetes mellitus NIDDM), is the most common form of diabetes. It is characterized by disorders of insulin resistance and insulin secretion, either of which can be the predominant feature. Both disorders are usually present at the time the disease is clinically manifested. It classically occurs in adults, especially the elderly, but recently it is being diagnosed with increasing frequency in children and adolescents. It is in most instances a polygenic disease, but also very life style dependent. Its prevalence, like obesity which is a major risk factor for its development, is increasing rapidly particularly in developing counties. Type 2 diabetes will be discussed in much greater depth later in the introduction.

2.3 MODY (Maturity Onset Diabetes of the Young)

MODY is a familial condition with autosomal dominant inheritance. It is a monogenic form of diabetes characterized by early age of onset (< 25 years), and pancreatic β-cell dysfunction. There are 6 types of MODY, four of which are caused by a mutation affecting a transcription factor. MODY 1, 3, 4 and 5 are caused by mutations affecting the transcription factors hepatocyte nuclear factor HNF-4α (MODY1), HNF-1α (MODY3), pancreatic and duodenal homeobox protein-1 (PDX-1) (MODY4), HNF-1β (MODY5) and NeuroD/β2 (MODY6). HNF regulates the expression of metabolic genes and glucose transporters whereas PDX-1 is implicated in the development of the pancreas and the regulation of the insulin gene. MODY2 is caused by a mutation affecting the glucokinase gene, the enzyme which catalyzes the rate limiting step of glycolysis (Froguel and Velho 1999; Wobser, Dussmann et al. 2002).

2.4 Statistics

Many surveys have confirmed that increasing urbanization and industrialization is associated with an increased prevalence of type 2 diabetes. The world Health Organization (WHO) estimates that, in 1997, 143 million people (6.2 % of the population) were afflicted with diabetes worldwide. It is predicted that these numbers will likely double by the year 2020 (O'Rahilly 1997). At home, diabetes is considered as a leading cause of death by disease in Canada and the number of Canadians afflicted with the disease has reached around 2 million people (www.diabetes.ca). Diabetes is one of the most common afflictions of the aged, but its prevalence, particularly of type 2 diabetes is rapidly increasing in younger individuals usually in association with obesity (Bramblett, Huang et al. 2000).

2.5 Burden of Diabetes for Affected Individuals

The long term complications of diabetes are divided into the microvascular complications, affecting the eye, kidney and nervous system, and the macrovascular complications with accelerated atherosclerosis and increased risk of amputation, myocardial infarction and stroke. Diabetes is considered to be the leading cause of all these complications, in addition to biochemical imbalances like ketoacidosis. The basic mechanisms of each of these complications is a combination of the adverse effects of hyperglycemia on tissues, stimulation of various growth factors, and the secondary effects of conditions frequently associated with diabetes such as hypertension and hyperlipidemia. People with diabetes are also more susceptible to many other illnesses. In 1999, diabetes was considered to be the sixth leading cause of death in the U.S., representing around 19 % of all deaths (www.ADA.com).
In addition to the health issues, diabetes represents another burden, an economic one, on the individual and on the government.

3. GLUCOSE HOMEOSTASIS

The blood glucose level is a balance between glucose absorption from the gut, glucose production by the liver and glucose utilization by insulin-independent tissues (such as the brain, kidney, and erythrocytes) and insulin-dependent tissues (such as fat and muscle). This balance is regulated by many neurohormonal factors including hormones of the endocrine pancreas (insulin from the β-cells and glucagon from the α-cells) which have an important central role (Kahn 1994). Blood glucose level is normally at 5 mM. Insulin secretion from β-cells and glucagon secretion from α-cells respond very precisely to small changes in glucose concentration in the physiologic range, thereby keeping glucose levels within the range of 3.7-7.0 mM in normal individuals (van Haeften 2002).

3.1 Post Prandial (Fed State)

Glucose, with other nutrients, is absorbed from the intestine into the portal circulation thus causing a rise in plasma glucose and other nutrient levels. The islet responds to this perturbation in nutrient levels by insulin secretion from the β-cells (Figure 1A). Glucose is the primary nutrient secretagogue for insulin, but its effectiveness as a secretagogue can be enhanced by other nutrients such as amino acids, fatty acids and non-nutrient stimuli (incretins) which are elevated after a mixed meal such as GLP-1 and gastric inhibitory peptide (GIP) (van Haeften 2002). The resultant increase in circulating insulin levels stimulate glucose uptake into insulin-sensitive tissues such as skeletal muscle, heart and adipose tissue and inhibits endogenous glucose production from the liver and kidney. Glucose utilization by insulin-insensitive tissues, such as the brain, change minimally after a meal. Thus, insulin causes an increase in glucose clearance from blood and reduced glucose entry into blood with the end effect of returning the post-prandial glucose rise back to pre-meal levels.

3.2 During Fasting

During fasting, the brain in particular requires a constant supply of glucose. This of course can not come from exogenous sources as glucose entry from the gut will be zero. It must come from an endogenous supply. Islet hormones are again instrumental in regulating glucose flux during fasting (Figure 1B).

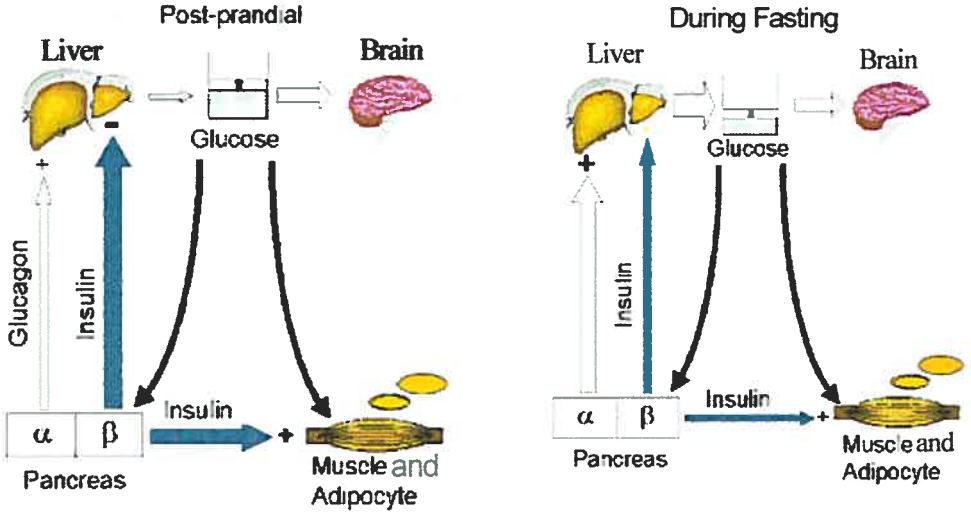


Figure 1. Glucose homeostasis (A) postprandially and (B) during fasting. A. After a meal, the intestine will absorb glucose and there will be an increase in plasma glucose levels (> 5.5 mM). The rise in glucose will stimulate the β-cells of the pancreas to secrete insulin. Glucose uptake by insulin-insensitive tissues such as brain will continue. Elevated insulin levels will inhibit endogenous glucose production from the liver and kidney and will promote storage of glucose in the liver, muscle and adipocytes. Thus normoglycemia is restored. B. During fasting, the brain requires a constant supply of glucose which has to come from endogenous sources. Glucose levels fall which results in reduced insulin secretion and increased glucagon secretion. Glucose uptake by insulin sensitive tissues is consequently reduced and endogenous glucose production by glucagon stimulation of glycogenolysis and gluconeogenesis is increased. Hepatic glycogenolysis is stimulated and glucose entry to the muscle and adipocytes is decreased. Glucose levels are maintained in the range of 4-5 mM (modified from Kahn, 1994).

With falling glucose levels, insulin secretion also falls, such that insulin effects on stimulating glucose entry into muscle and fat will be minimal and the inhibitory effect on endogenous glucose production will be abrogated.

Falling glucose levels also result in the secretion of glucagon from islet α-cells. Glucagon stimulates endogenous glucose production by promoting glycogenolysis and gluconeogenesis. Thus, endogenous glucose production will increase, and glucose entry into the muscle and the adipocytes will decrease (DeFronzo 1988). This state results in maintenance of normoglycemia (4-5 mM) with provision of the essential glucose supply to the brain.

Dysregulation of glucose homeostasis, whether resulting in elevated blood glucose (hyperglycemia) or low blood glucose levels (hypoglycemia), will have detrimental effects on the individual. Hyperglycemia is the hallmark of diabetes mellitus.

4. LIPID HOMEOSTASIS

Lipid, as for glucose homeostasis, is markedly altered between the fed and fasted states. Free fatty acids (FFA) constitute an important energy source in most body tissues, representing the primary oxidative fuel for liver, resting skeletal muscle, renal cortex and myocardium (Coppack, Jensen et al. 1994). FFA also have an important physiological role during pregnancy. During late pregnancy, lipolytic hormones stimulate fat breakdown, leading to elevations in plasma FFA concentrations, which induces peripheral insulin resistance and causes a switch in fuel metabolism from carbohydrate to fat oxidation, thus maximizing the availability of glucose for the developing foetus (Boden 1996).

4.1 Post-Prandial (Fed State)

In peripheral tissues, post-prandial rises in insulin promote the accumulation of triglyceride (TG) storage by the following: Inhibition of hormone sensitive lipase (HSL) with suppression of lipolysis of tissue TG stores; activation of

lipoprotein lipase with hydrolysis of TG within chylomicrons and VLDL and transfer of released FFA into cells for subsequent esterification and storage, and activation of lipogenesis pathways.

In the liver, fatty acid oxidation is inhibited whereas fatty acid estertification and synthesis of VLDL is promoted. Lipogenesis from glucose is also promoted (McGarry and Brown 1997).

4.2 During Fasting

In peripheral tissues, lipolysis of stored TG via HSL is promoted resulting in increased flux of FFA into the circulation and increased availability of FFA for oxidation particularly in heart and skeletal muscle.

In the liver, fatty acid oxidation is increased with the production of ketone bodies (McGarry and Brown 1997).

4.3 Role of Malonyl-CoA Regulation of Carnitine Palmitoyl Transferase I Activity in Lipid Homeostasis

The relationship between malonyl-CoA, an intermediate in lipogenesis, and carnitine palmitoyl transferase I (CPT I), an outer mitochondrial membrane enzyme which is the rate limiting enzyme in mitochondrial β-oxidation of fatty acids, was first reported in the liver. During states of high insulin/low glucagon ratio, i.e. with carbohydrate feeding the liver actively converts glucose carbons via malonyl-CoA to fatty acids and then to LC-CoA (McGarry and Brown 1997). If the newly formed LC-CoAs were to react with carnitine under the influence of CPT I, it would be converted back to acetyl-CoA through the process of β-oxidation, thus generating a futile cycle. This does not happen, however, as the elevated pool of malonyl-CoA produced from anaplerosis and the acetyl-CoA carboxylase (ACC) reaction during active lipogenesis inhibits CPT I. The LC-CoA, therefore can react with glycerol phosphate to form TG which in the liver can be exported in the form

of VLDL. However, in periods of low insulin/high glucagon, like starvation, the pathway of fatty acid synthesis comes to a halt, the malonyl-CoA concentration falls, CPT I becomes deinhibited and fatty acids reaching the liver readily enter the β-oxidation pathway to form ketone bodies.
This interaction between malonyl-CoA and CPT I has been observed to be at work in a variety of nonhepatic tissues, particularly the heart and skeletal muscle and the β-cell as well (Saddik, Gamble et al. 1993; Prentki and Corkey 1996; Saha, Vavvas et al. 1997; Merrill, Kurth et al. 1998). However, in the non-lipogenic tissues, the metabolic role of malonyl-CoA differs.

4.3.1 Malonyl-CoA

Malonyl-CoA is the product of ACC and in the liver as well as in other lipogenic tissues is the first committed intermediate in the pathway of fatty acid synthesis. In all tissues, lipogenic and non-lipogenic, it acquires its significant regulatory role by inhibiting CPT I, the first step specific to the opposing process of fatty acid oxidation (McGarry and Foster 1980; McGarry, Woeltje et al. 1989). Malonyl-CoA inhibits a class of carnitine acyltransferases that catalytically have access to the cytosolic pool(s) of long-chain acyl-CoA esters.

4.3.2 CPT I

The essential role of carnitine in the oxidation of long-chain fatty acids by mammalian tissues first emerged in the mid 1950. The formation of acylcarnitines enables acyl moieties to cross intracellular membranes, which are otherwise impermeable to acyl-CoA esters. This occurs through the action of specific carnitine/acylcarnitine carriers, highly expressed in both mitochondrial membranes and peroxisomal membranes. Acyl-CoA first reacts with carnitine under the influence of a CPT I on the outer aspect of the mitochondrial inner membrane, generating free CoA and acylcarnitine.

Acylcarnitine then permeates the inner membrane (possibly via a specific carrier mechanism) and reacts with a matrix pool of CoA in a reaction catalyzed by CPT II on the inner face of the inner membrane. The re-formed acyl-CoA then enters the pathway of β-oxidation, while the released carnitine returns to the extramitochondrial compartment (McGarry and Brown 1997). In the liver, CPT I plays a pivotal role in the regulation of fatty acid oxidation (McGarry, Mannaerts et al. 1977; McGarry, Leatherman et al. 1978).

This metabolic branch point (fatty acid traffic through esterification on oxidation) is controlled by malonyl-CoA (McGarry and Foster 1980; McGarry, Woeltje et al. 1989). Malonyl-CoA regulation of CPT I appears to play a crucially important physiological role in the pancreatic β-cell as is discussed below in the section on insulin secretion.

5. PANCREAS

5.1 Anatomy of the Pancreas

The pancreas is an elongated, tapered organ (about 22 cm long) located across the back of the abdominal cavity (Bramblett, Huang et al. 2000). It is in close proximity to the duodenum behind the stomach (Figure 2).

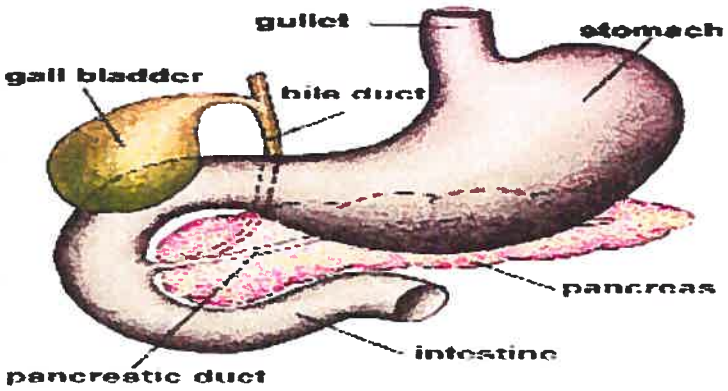


Figure 2. Anatomy of the pancreas. The right side of the organ (called the head) is the widest part of the organ and lies in the curve of the duodenum, the first division of the small intestine. The tapered left side extends slightly upward (called the body of the pancreas) and ends near the spleen (called the tail).

The structure of the pancreas is dominated by the fact that it is a dual function organ with both exocrine and endocrine cell types. The vast bulk of the pancreas is composed of exocrine tissue, and secretions from these cells flow into a series of ducts for ultimate delivery into the duodenum. Embedded within this exocrine tissue are roughly one million small clusters of cells called the Islets of Langerhans. Islets of Langerhans are the endocrine component of the pancreas. Islets contain several cell types (Figure 3) and are richly vascularized. They secrete insulin, glucagon and several other hormones (Bramblett, Huang et al. 2000).

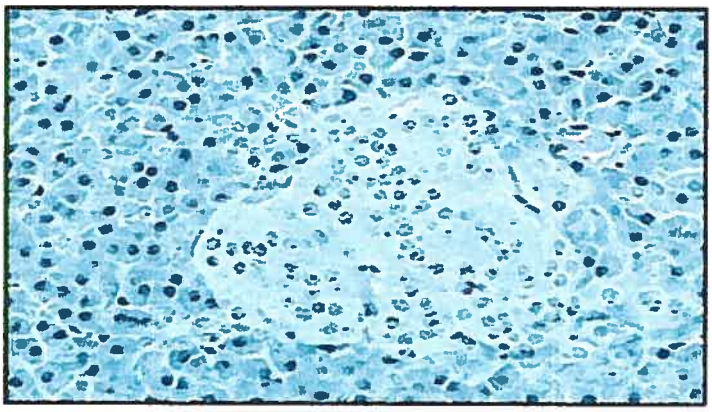


Figure 3. Islet histologic image.
A histologic image of an islet which is the large cluster of pale-staining cells in the middle. All of the surrounding tissue is exocrine tissue.

5.2 Exocrine Pancreas

The exocrine pancreas is composed of acini and ducts which comprises 90 % of the pancreas. The acini are composed of columnar to pyramidal epithelial cells with minimal stroma. The pancreas produces 2 liters/day of bicarbonate rich fluid containing digestive enzymes. The pancreatic enzymes are trypsin, chymotrypsin, aminopeptidases, elastases, amylases, lipases, phospholipases and nucleases.

5.3 Endocrine Pancreas

The endocrine pancreas consists of islets of Langerhans which are of endodermal origin and represents 1 % of the pancreas. The size of each islet is usually 0.1 to 0.2 mm. There are around one million islets per pancreas. The cellular composition of islets: beta (β) cells (68 %), alpha (α) cells (20 %), delta (δ) cells (10 %), PP cells (2 %) and serotonin cells (rare).

α-cells: secrete glucagon in response to low blood glucose levels which stimulates the release of glucose from stores thereby restoring blood glucose levels.
β-cells: secrete insulin in response to high levels of glucose in the blood.
δ-cells: secrete the hormone somatostatin (represses the release of insulin and glucagon).
PP cells: secrete pancreatic polypeptide (PP). The role of PP is not absolutely clear, but it appears to be involved in the regulation of other islet hormones and possibly food intake.

6. INSULIN

In the fall of 1920, working at the University of Toronto, Fred Banting and Charles Best were able to make a pancreatic extract which had anti-diabetic characteristics. This extract was called insulin which originates from the Latin word "island" (Best 2002).
At that time Sir Frederick Banting said:

"Insulin is not a cure, more work needs to be done".

Human insulin is a peptide hormone containing 51 amino acids. A single molecule consists of 2 polypeptide chains commonly labeled A (21 amino acids) and B (30 amino acids). The chains are linked by two disulfide bridges

(Kahn 1994). Insulin is secreted by the β-cells of the pancreas and has an important role in metabolism as discussed below.

6.1 Insulin Biosynthesis

The pancreatic β-cell normally maintains a stable balance among insulin secretion, insulin biosynthesis and insulin degradation to keep optimal intracellular stores of the hormone. Insulin biosynthesis follows the same pathway as other peptide hormones (Vander, Sherman et al. 1990). Its biosynthesis starts in the nucleus of the β-cell of the pancreas as a series of precursors beginning with pre-proinsulin, the protein encoded in the insulin gene. These precursors direct the prohormone into the secretory pathway ending finally in the secretory granules where it is converted into insulin and C-peptide. These products are stored and secreted together in a highly regulated manner in response to glucose and other stimuli (Steiner, Rouille et al. 1996).

6.2 Insulin Function

The actions of the anabolic hormone insulin are multifaceted affecting cellular metabolism (Kahn 1994; Bramblett, Huang et al. 2000). Insulin plays an important role in many aspects of cell physiology, however, its most prominent role is to stimulate glucose uptake in peripheral tissues after a meal. Insulin effects have been known for quite some time and these include:

- membrane transport of glucose, amino acids and certain ions;
- increased storage of glycogen;
- formation of triglycerides and other complex lipids;
- stimulation of DNA, RNA and protein synthesis.

These actions exert wide spectra of regulatory effects on the cell (Bramblett, Huang et al. 2000). They fall into two categories: metabolic and growth-

promoting effects. Metabolic effects are of short or long (with the involvement of genes) duration on the uptake, transport, intermediary metabolism and storage of small food molecules-hexoses, amino acids and fatty acids. Insulin reduces the levels of circulating fatty acids, amino acids and blood glucose levels by promoting conversion to their storage forms and also by inhibiting gluconeogenesis and lipolysis. The growth-promoting effects of insulin in other words mitogenic, are of long duration and are realized on the gene level. They induce expression of a number of specific genes, stimulation of the synthesis of DNA, RNA and specific proteins, and as a result the process of cell growth as a whole (Fantl, Johnson et al. 1993). Insulin has also been shown to suppress apoptosis (Rampalli and Zelenka 1995; Bertrand, Atfi et al. 1998; Diaz, Pimentel et al. 1999; Kang, Song et al. 2003).

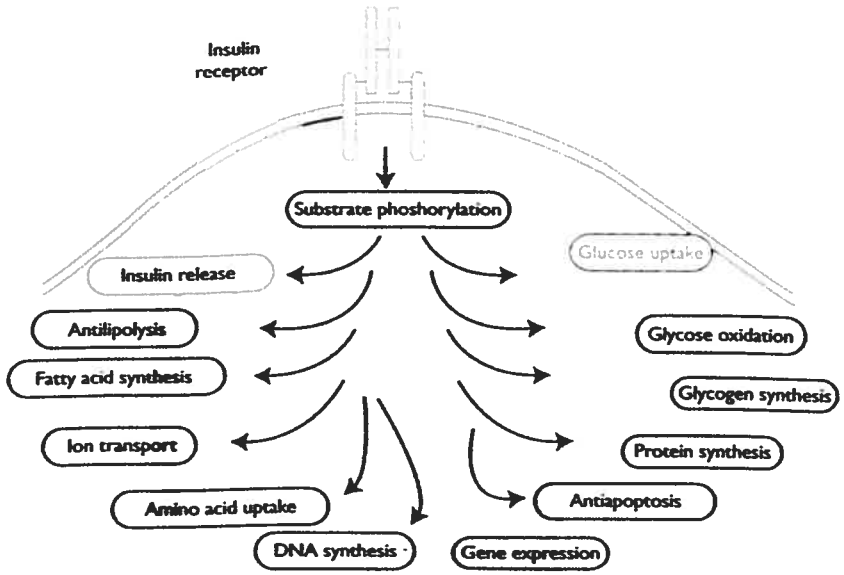


Figure 4: Protein phosphorylation is required to mediate insulin action. After receptor autophosphorylation, the β-subunit becomes active as a tyrosine-specific kinase and catalyzes phosphorylation of several intracellular proteins. This event provides the underpinning of the different actions of insulin which include: 1- stimulation of glucose turnover by favoring its transport across the plasma membrane; 2- promoting protein synthesis in almost all tissues by affecting gene transcription, messenger RNA translation and amino acid uptake; 3- increasing DNA synthesis and prevention of apoptosis via its mitogenic effect; 4- stimulation of ion transport across the plasma membrane; 5- stimulation of lipid synthesis; and 6- prevention of lipolysis by inhibiting hormone sensitive lipase (HSL) (Kahn 2000).

Once in the blood, insulin affects glucose homeostasis by stimulating the uptake of glucose into skeletal muscle and, to a lesser extent, into liver and adipose tissue and by inhibiting glucose production from liver or kidney. In muscle and adipocytes, glucose uptake is mediated by translocation of the insulin-sensitive glucose transporter GLUT-4 from intracellular glucose storage sites to the plasma membrane. Insulin also switches glycogen metabolism from glycogenolysis to glycogen synthesis in muscle. With respect to endogenous glucose production, insulin, either directly or as a consequence of suppressing FFA levels by inhibiting adipocyte lipolysis, inhibits glycogenolysis and gluconeogenesis. The enzymes involved in the insulin-regulated processes of glucose metabolism appear to be regulated by (de)phosphorylation of serine and/or threonine residues.

All known actions of insulin are initiated at the plasma membrane by insulin receptors responding to ligand binding. Ligand binding promotes ATP-binding and autophosphorylation of the cytoplasmic tyrosine kinase domain of the receptor.

The insulin receptor primarily regulates nutritional metabolic pathways, whereas all other receptor/tyrosine kinases mainly regulate cell growth and differentiation. The insulin receptor is a heterodimer consisting of two α and two β subunits linked by disulphide bridges. Transduction of the insulin signal includes the following events: (1) binding of hormone with the two extracellular α-subunits (135 KDa) of the receptor which performs a ligand binding function; and leads to (2) activation of receptor tyrosine kinase activity and autophosphorylation of the two transmembrane β-subunits (95 KDa); resulting in (3) induction of a downstream cascade of tyrosine phosphorylation of a wide spectrum of effector proteins (Pertseva, Shpakov et al. 2003). The insulin receptor signal transduction, as for other receptors in the receptor tyrosine kinase family, does not form direct complexes with substrates and/or effector molecules after autophosphorylation. Instead, the insulin receptor, upon autophosphorylation of at least the tri-tyrosine

subdomain, acquires exogenous kinase activity, phosphorylating its principal substrate: insulin receptor substrate 1 (IRS1). IRS1, in turn, binds effector molecules which are responsible for the actual processes of glucose transport, metabolism and cell growth (Kahn 1994; Pertseva, Shpakov et al. 2003).

7. INSULIN SECRETION

Insulin secretion from the pancreatic β-cell is a highly regulated process that maintains blood glucose levels within a very narrow range. Glucose homeostasis is achieved by a complex interplay between nutrients, hormones and the autonomic nervous system.

7.1 Pancreatic β-Cell Secretagogues

Insulin secretion is regulated by many factors such as nutrients and neuro-hormonal agents (Figure 5). The calorigenic agents which stimulate insulin secretion include glucose, free fatty acids and amino acids such as glutamine and leucine. Important examples of neurohormonal agents which stimulate insulin secretion include glucagon like peptide-1 (GLP-1), the 'gastric inhibitory peptide' (GIP), glucagon, cholecystokinin (CCK) and acetylcholine. (Hedeskov 1980; Wollheim and Sharp 1981; Prentki and Matschinsky 1987). Neurohormonal agents known to have an inhibitory effect on insulin secretion include somatostatin, galanine and β-sympatho-mimetic agents.

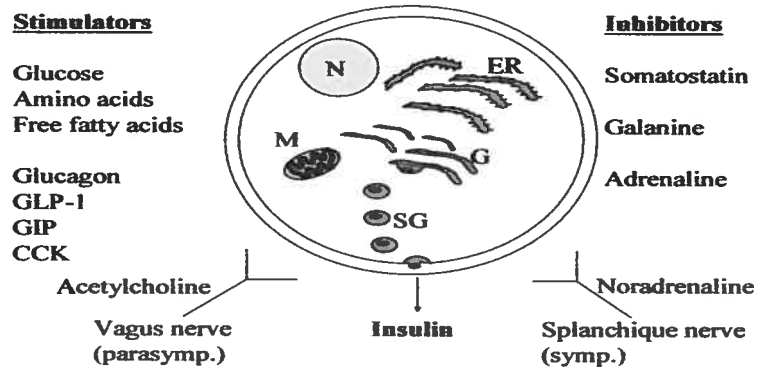


Figure 5. Stimulators and inhibitors of insulin secretion.
The calorigenic nutrients such as (glucose, amino acids and free fatty acids) and certain peptides like the incretin GLP-1, the gastrointestinal inhibitory polypeptide (GIP) and CCK stimulate the secretion of insulin, whereas somatostatin, galanine and adrenaline inhibit insulin secretion.

7.2 Mechanism of Insulin Secretion

Glucose, the primary stimulus for insulin secretion in the β-cell, elicits a biphasic insulin secretion. The first phase is sharp, reaches its maximum at 3-5 min, and lasts approximately 10 min. The second phase is more blunted and lasts as long as glucose levels remain elevated (Porte and Pupo 1969; Poitout and Robertson 1996). This biphasic response is believed to be due to two signaling pathways of glucose-stimulus-secretion coupling. One is the well-known K^+_{ATP} channel dependent pathway by which glucose depolarizes the β-cell, activates voltage-dependent-Ca^{2+} channels, and raises $[Ca^{2+}]_i$. The other pathway is the less known K^+_{ATP} channel independent pathway (Gembal, Gilon et al. 1992; Sato, Aizawa et al. 1992; Straub, James et al. 1998).

7.2.1 Calcium and K^+_{ATP} Channel Dependent Pathway

Glucose enters the β-cell through a facilitative glucose transporter, GLUT 2, which allows rapid equilibration between extra- and intracellular glucose

concentrations. Glucokinase (GK) phosphorylates glucose to produce glucose-6-phosphate (G6P). G6P then enters glycolysis producing pyruvate. In the mitochondria, pyruvate is decarboxylated to generate acetyl-CoA (Ac-CoA) by the action of pyruvate dehydrogenase. Ac-CoA then enters the Kreb's cycle and an increase in the ATP/ADP ratio is produced. The increase in ATP/ADP ratio inhibits the ATP-sensitive potassium channel which results in depolarization of the plasma membrane and opening of voltage-dependent calcium channels (VDCC). A major increase in cytosolic calcium ensues which triggers exocytosis of insulin (Prentki 1996) (Figure 6).

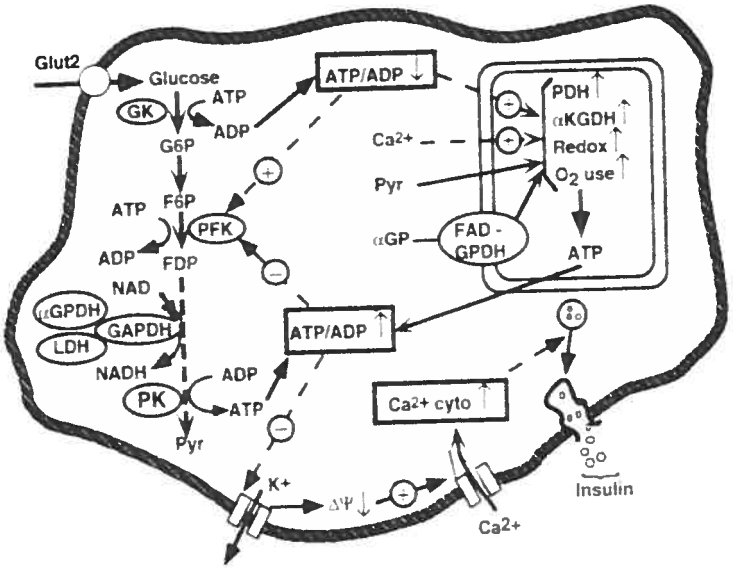


Figure 6. Model illustrating glucose induced insulin release in the pancreatic β-cell: Calcium and K^+_{ATP} channel dependent pathway.
Glucose enters the cell via the glucose transporter GLUT 2. It is metabolized and converted to pyruvate via glycolysis which is subsequently oxidized in the Kreb's cycle causing an increase in the ATP/ADP ratio. The increased ATP/ADP ratio closes the K^+ ATP-dependent channel (resulting in depolarization of the membrane) which causes opening of the Ca^{2+} voltage dependent channel and entry of Ca^{2+} into the cell. Ca^{2+} activates exocytosis of vesicles containing insulin granules. [GK, Glucokinase; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FDP, fructose 1,6-bisphosphate; PFK, phosphofructose-kinase; αGPDH, α-glycerol 3-phosphate dehydrogenase; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; LDH, lactate dehydro-genase; PK, pyruvate kinase; Pyr, pyruvate; PDH, pyruvate dehydrogenase; αKGDH, α-ketoglutarate dehydrogenase; αGP, α-glycerol 3-phosphate; FAD, flavin adenine dinucleotide] (Prentki 1996).

7.2.2 K^+_{ATP} Channel Independent Pathways of Insulin Secretion

It is clear however, that some additional components of glucose metabolism are needed for the full effect of glucose to be manifested. Prentki and Corkey Prentki, Corkey 1996) raised the intriguing possibility that one such component involves an element of glucose-fatty acid cross talk, in other words, that the malonyl-CoA/CPT I axis recognized in liver and later in the muscle and heart, may also be at work in the β-cell in relation to insulin secretion.

7.2.2.1 Anaplerotic /Malonyl-CoA/Lipid Signaling Pathway

Prentki and Corkey (Corkey, Glennon et al. 1989; Prentki, Vischer et al. 1992; Prentki, Corkey 1996) proposed a model of β-cell nutrient sensing and insulin secretion in which, in addition to the K^+_{ATP} dependent pathway, a parallel anaplerotic/lipid signaling pathway exists in which malonyl-CoA acts as a coupling factor. The cascade of events proposed in the model is as follows (Figure 7). Glucose is metabolised to pyruvate via glycolysis. Pyruvate is then metabolised by either pyruvate dehydrogenase (PDH) or pyruvate carboxylase (PC). PDH transforms pyruvate to Ac-CoA, while PC, on the other hand, is an anaplerotic enzyme which channels pyruvate into mitochondrial oxaloacetate. The latter anaplerotic mechanism results in efflux of citrate from the mitochondria to the cytosol. Cytosolic citrate is converted to Ac-CoA and oxaloacetate via the action of citrate lyase (CL). Cytosolic Ac-CoA is then carboxylated by acetyl-CoA carboxylase (ACC) to form malonyl-CoA. Malonyl-CoA can enter fatty acid synthesis via the action of the enzyme fatty acid synthase (FAS) which uses malonyl-CoA as a substrate. FAS, however, is not highly expressed in the β-cells (Brun, Roche et al. 1996) such that in response to elevated glucose and anaplerosis, cytosolic malonyl-CoA level rises. Malonyl-CoA is an inhibitor of CPT I, the enzyme which catalyzes the rate limiting step for fatty acid entry into the mitochondria to undergo β-

oxidation (Figure 7). Thus, CPT I inhibition by malonyl-CoA results in an increase in long chain acyl-CoA in the cytosol which potentiates insulin secretion either directly or indirectly by the production of complex lipid signaling molecules, activation of protein kinase C (PKC), or by protein acylation (Corkey, Deeney et al. 2000). In this model, malonyl CoA is believed to act as a regulatory molecule and long chain acyl-CoA and its metabolites as effector molecules in insulin secretion.

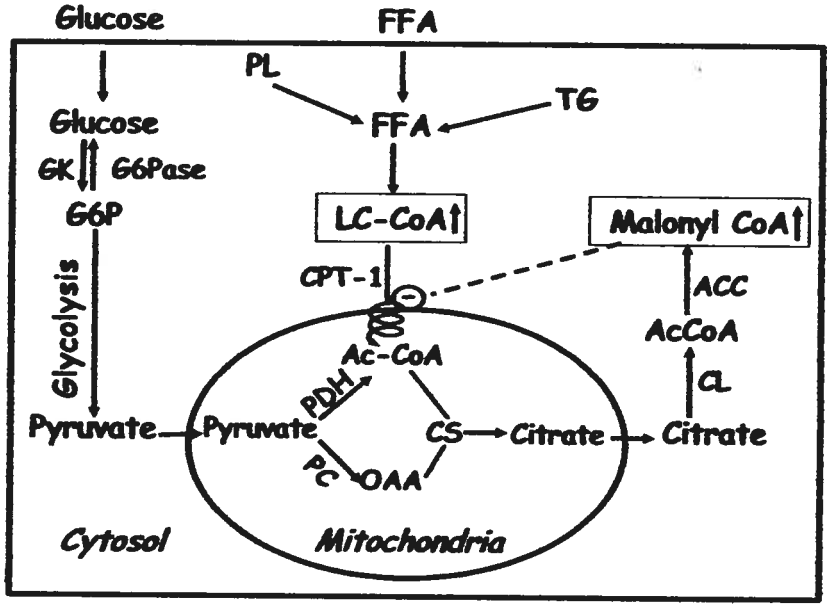


Figure 7. The regulation of cytosolic LC-CoA levels.
Cytosolic LC-CoA is derived from either circulating FFAs or endogenous complex lipids. The entry of LC-CoA into the mitochondria, where it is oxidized to acetyl-CoA, is controlled by malonyl-CoA derived from glucose or other fuels, which regulates CPT I. AcCoA, acetyl-CoA; CL, citrate lyase; CS, citrate synthase; G6P, glucose-6-phosphate; OAA, oxaloacetate; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PL, phospholipids; TG, triglyceride. *Adapted from Prentki M, Corkey B 1996.*

The evidence supporting the existence of an anaplerotic/malonyl-CoA/lipid signaling in the β-cell is summarized as follows: PC is abundant in the islet, yet the islet is neither a neoglucogenic nor a lipogenic tissue; citrate, an allosteric activator of ACC, rises markedly in response to glucose stimulation before secretion occurs (Corkey, Glennon et al. 1989; Roche, Farfari et al. 1998; Farfari, Schulz et al. 2000); only nutrients which cause an increase in

citrate levels cause insulin secretion (Brun, Roche et al. 1996); ACC is abundant in the β-cell; glucose increases ACC gene expression in INS cells, and basal insulin secretion correlates with the content of ACC protein (Brun, Roche et al. 1993); malonyl-CoA rises rapidly in response to glucose and only nutrients that elevate malonyl-CoA cause secretion (Corkey, Glennon et al. 1989; Liang and Matschinsky 1991; Prentki, Vischer et al. 1992). FAS is very low in the islets of Langerhans (Brun, Roche et al. 1996); potentiation of glucose-induced insulin secretion results after incubating the β-cells with fatty acids for a short period (Malaisse, Sener et al. 1979; Prentki, Vischer et al. 1992). Insulin release is promoted after inhibiting CPT I by pharmacological agents (Prentki, Vischer et al. 1992; Chen, Ogawa et al. 1994); the levels of fatty acyl-CoA in response to different nutrients correlate with insulin secretion (Prentki, Vischer et al. 1992). The above findings together provide a strong evidence for the concept that an anaplerotic/malonyl-CoA/lipid signaling pathway plays an instrumental role in β-cell nutrient signaling.

The following figure illustrates the two pathways of insulin secretion in the β-cell (Prentki, Joly et al. 2002).

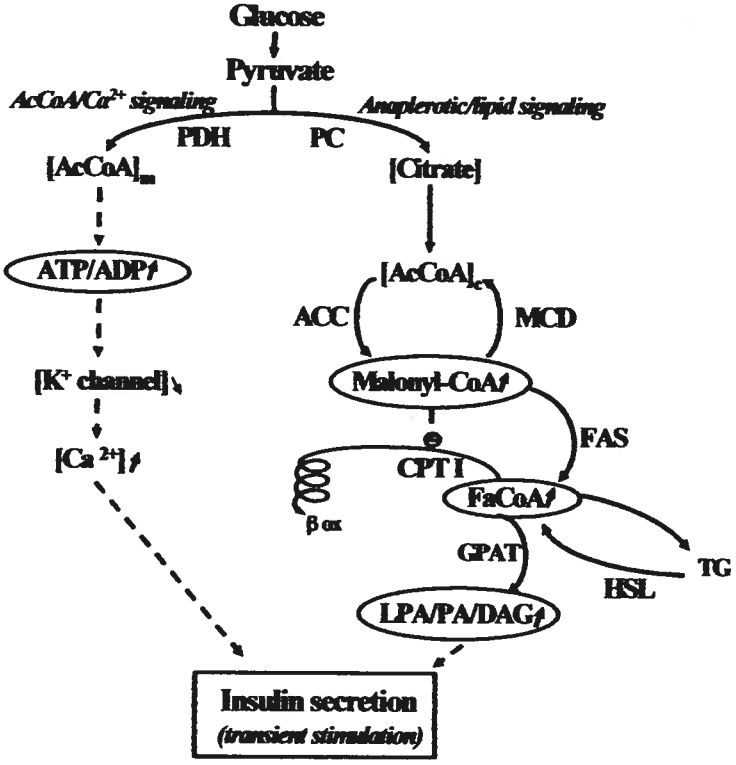


Figure 8. Model illustrating signal transduction pathway in the pancreatic β-cell. When transiently stimulated, the Ac-CoA/Ca^{2+} and anaplerotic/lipid signaling pathways synergize to promote insulin secretion. AcCoAc, cytosolic acetyl-CoA; AcCoAm, mitochondrial acetyl-CoA; DAG, diacylglycerol; LPA, lysophosphatidic acid; βox, β-oxidation of fatty acids; PA, phosphatidic acid; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; MCD, malonyl-CoA decarboxylase; TG, triglycerides; HSL, hormone sensitive lipase (Prentki 2002).

7.3 Role of FFA in Insulin Secretion

It is well documented that acute FFA administration leads to augmentation of glucose-stimulated insulin secretion (GSIS). FFA enhance both basal and GSIS and are essential for stimulus-secretion coupling in the pancreatic β-cell (Boden, Chen et al. 1998; Dobbins, Chester et al. 1998). Efforts to elucidate how fatty acids influence β-cell function led to a series of important findings which implicate an element of glucose-fatty acid cross-talk in stimulus-secretion coupling within the β-cell. Of these findings that are of importance and relevance to this thesis are: 1- exposure of Syrian-hamster insulinoma (HIT) cells or rat islets to glucose increases the cellular malonyl-CoA content which is proportional to the stimulation of insulin release (Corkey, Glennon et al. 1989; Liang and Matschinsky 1991; Prentki, Vischer et al. 1992); 2- glucose concentrations which stimulate insulin secretion also suppress the oxidation of long chain fatty acids (Tamarit-Rodriguez, Vara et al. 1984); 3- inhibitors of CPT I (like 2-bromopalmitate (2-BrP), 2- bromostearate (2-BrS) and etomoxir) which reduce fatty acid oxidation (McGarry, Woeltje et al. 1989; McGarry and Brown 1997) were found to stimulate insulin release from perifused islets (Bliss and Sharp 1992), perfused rat pancreas and in HIT cells (Prentki, Vischer et al. 1992); 4- exogenous long chain fatty acids appreciably potentiate GSIS from rat islets (Vara, Fernandez-Martin et al. 1988) and HIT cells (Prentki, Vischer et al. 1992) which is concomitant with the rise in LC-CoA esters.

All of these findings are consistent with the malonyl-CoA/LC-CoA hypothesis of Prentki and Corkey (Prentki and Corkey 1996). The increase in malonyl-CoA concentrations under these conditions suppresses CPT I activity and therefore fatty acid oxidation. This results in an increase in the concentration of cytosolic LC-CoAs which act as signaling molecules for insulin secretion in concert with the rise in $[Ca^{2+}]_i$ caused by the calcium and K^+_{ATP} channel dependent pathway. Glucose inhibition of LC-CoA oxidation will be associated with much greater increase in LC-CoA levels where the exogenous

FFA supply is simultaneously elevated, therefore amplifying the effect of elevated FFA.

This role of FFA in insulin secretion raises the question as to whether all FFA are equal in terms of their insulinotropic effects. To answer this question, studies were carried out in the perfused pancreas and it was found that the insulinotropic potency increased dramatically with increasing carbon chain and with a decrease in the number of double bonds (McGarry and Dobbins 1999). The reason for this difference between saturated and unsaturated fatty acids is not clear. Whether this is related to a difference in metabolism of the two types or to a difference in the physicochemical properties of the effector molecules still awaits an answer.

8. PATHOGENESIS OF TYPE 2 DIABETES

Type 2 diabetes is a heterogeneous condition, the etiology of which is polygenic and life style dependent (Kahn 1994; Zimmet, Alberti et al. 2001). It is invariably characterized by progressive deterioration in insulin secretory function, diminished hepatic glucoregulation and peripheral insulin resistance (DeFronzo 1988; Porte 1991; Kahn 1994; Poitout and Robertson 1996; Prentki and Corkey 1996; Prentki, Joly et al. 2002; Kahn 2003). Most often it is associated with, and usually preceeded by central obesity and dyslipidemia (Carey, Jenkins et al. 1996; Wei, Gaskill et al. 1997; Kahn 2003). The majority of obese subjects, however, despite varying degrees of insulin resistance, do not develop diabetes (Polonsky, Sturis et al. 1996; Mokdad, Ford et al. 2003). Insulin resistance, whether due to obesity or physiological conditions such as in pregnancy (Freinkel 1980; Catalano, Tyzbir et al. 1991), is usually compensated by islet ß-cells adaptation with both increased ß-cell number and/or volume (ß-cell mass) and augmented ß-cell function (Kloppel, Lohr et al. 1985; Sorenson and Brelje 1997; Lingohr, Buettner et al. 2002; Liu, Jetton et al. 2002; Butler, Janson et al. 2003; Kahn 2003). It is only when

insulin resistance is accompanied by failure of the islet ß-cell to compensate, that type 2 diabetes (Kahn 2003) and gestational diabetes (Buchanan 2001) develop. While the pathogenesis of the ß-cell failure is not well understood, it is becoming increasingly evident from animal models (Coleman and Hummel 1973; Pick, Clark et al. 1998) and human autopsy series (Stefan, Orci et al. 1982; Kloppel, Lohr et al. 1985; Clark, Wells et al. 1988; Butler, Janson et al. 2003) that, in addition to ß-cell dysfunction, reduction in ß-cell mass is involved.

The following figure illustrates the pathogenesis and the course of type 2 diabetes. (adapted from Kahn 2000).

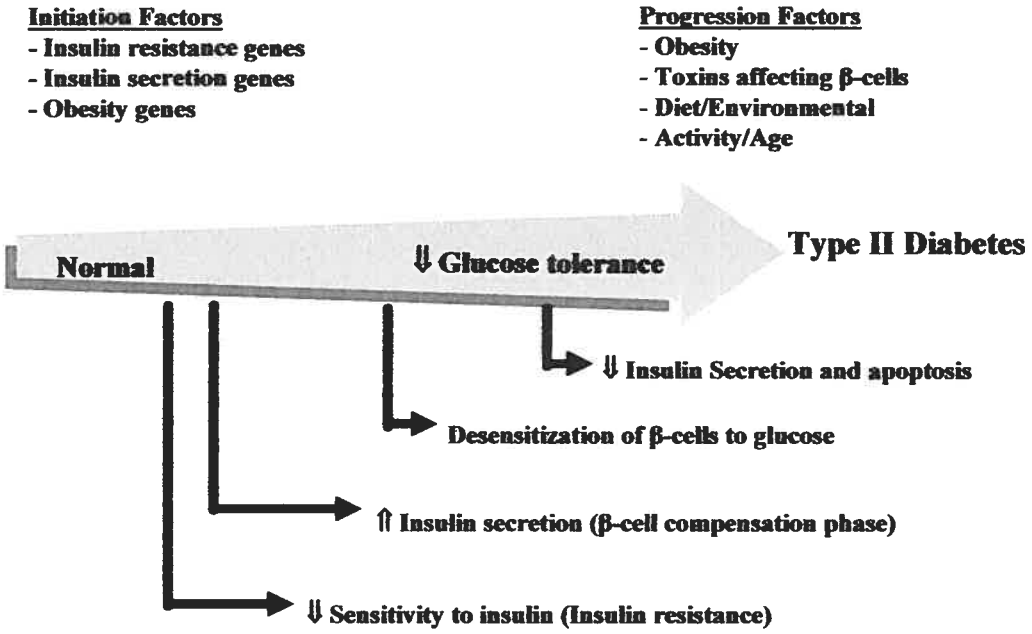


Figure 9. Pathogenesis of type 2 diabetes.

Many factors contribute to the etiology of type 2 diabetes including genetic and environmental factors. The genetic factors are likely to be multiple involving genes that affect insulin secretion, insulin sensitivity and obesity. A decrease in the sensitivity to insulin by the insulin target tissues results initially in augmentation of insulin secretion by the β-cells. This period is referred to as "β-cell compensation phase". With time, there is a gradual loss of β-cell compensation resulting in a decrease in glucose tolerance. Factors such as obesity, toxins and diet play a role in the progression of the disease and in desensitizing the β-cells to glucose. Eventually, there is a failure of glucose induced insulin secretion, β-cell death and thus the onset of the established disease of type 2 diabetes.

8.1 Peripheral Insulin Resistance

Type 2 diabetes is invariably characterized by peripheral insulin resistance which plays a major role in its development. Insulin resistance is defined as the impaired ability of insulin to promote peripheral glucose disposal. Insulin resistance is a hallmark of type 2 diabetes. Over 80 % of people with type 2 diabetes are obese, while virtually all are insulin resistant (Boden 1997). Thus, it is hypothesized that there is a tight correlation between obesity and insulin resistance. Human and animal studies support this notion indicating that weight loss/gain correlates closely with increasing/decreasing insulin sensitivity, respectively. Evidence indicates that insulin resistance is present years before the onset of the disease and it is considered as the best predictor for the disease (Shulman 2000). The peripheral action of insulin primarily includes the promotion of glucose uptake into skeletal muscle and inhibition of lipolysis in adipose tissue which are both impaired with insulin resistance. It has also been shown that muscle glycogen synthesis is decreased by 60 % in subjects with type 2 diabetes. Recent evidence have also indicated that insulin resistance is also present in the β-cell which might contribute to the abnormalities in insulin secretion observed in type 2 diabetes (Withers, Gutierrez et al. 1998; Kulkarni, Bruning et al. 1999). Many investigators have studied the mechanisms involved in insulin resistance at the level of the insulin receptor, the insulin-receptor signal transduction pathways, the insulin regulated intermediary metabolic pathways such as glycogen synthesis and lipolysis and the translocation of the insulin sensitive glucose transporter GLUT 4. Their findings are beyond the scope of this thesis other than to say that elevated FFA has been implicated in causing peripheral insulin resistance and this will be further discussed below.

8.2 Elevated Endogenous Glucose Production

Studies of hepatic glucose metabolism in type 2 diabetes invariably show increasing basal glucose production and a failure of insulin to suppress glucose production (DeFronzo 1988; Kahn 1994). Elevated glucose production is clearly important in the maintenance of hyperglycemia in both the basal (fasted) state and the post-prandial state.

8.3 Impaired Insulin Secretion

Impaired insulin secretion in type 2 diabetes is due to defects in β-cell function and β-cell mass. As discussed above, impaired insulin secretion is an essential component in the pathogenesis of type 2 diabetes.

8.3.1 Dysfunction

Islet dysfunction, characterized by loss of both first and second phase of glucose-induced insulin release, plays an important role in the pathogenesis of hyperglycemia (van Haeften 2002). Defects in the first phase insulin secretion are evident very early in the pathogenesis process with some studies showing this in subjects at risk of diabetes and those with impaired glucose tolerance. Defects in second phase secretion occur later and continue to deteriorate once type 2 diabetes has developed. Dysfunction is evident particularly with respect to the ability of glucose to induce insulin secretion, as secretion to arginine is preserved at least early in the pathogenesis process. There is considerable evidence indicating a desensitization of the islet function during persistent hyperglycemia *in vitro* (Grodsky 1989) and *in vivo* (Leahy, Cooper et al. 1986; Rossetti, Shulman et al. 1987; Leahy, Bonner-Weir et al. 1988).

8.3.2 Loss of β-Cell Mass

Another factor which plays an important role in failure of insulin secretion in the etiology of type 2 diabetes is the reduction in the number of β-cells. Regulation of β-cell mass appears to involve a balance between β-cell replication and apoptosis and islet neogenesis from exocrine pancreatic ducts (Finegood, Scaglia et al. 1995; Bonner-Weir 2000). Disruption of these processes causing either reduced β-cell formation and/or increased rates of β-cell death could cause a decrease in β-cell mass and, as a consequence, reduced insulin secretory capacity. There is controversy whether β-cell mass is decreased in type 2 diabetes (Stefan, Orci et al. 1982; Kloppel, Lohr et al. 1985; Clark, Wells et al. 1988; Guiot, Sempoux et al. 2001) and this is due to the scarcity of suitable pancreas specimens for histochemical analysis from individuals with type 2 diabetes. However, recent reports in which clinical information was better characterized concluded that β-cell mass is decreased in type 2 diabetes (Kloppel, Lohr et al. 1985; Clark, Wells et al. 1988; Sakuraba, Mizukami et al. 2002; Butler, Janson et al. 2003). Importantly, in a recently reported autopsy series (Butler, Janson et al. 2003), with adequate subject number and pre-death clinical data, islet ß-cell mass was shown to be reduced in both impaired glucose tolerance and type 2 diabetic subjects and was associated with evidence of increased apoptosis. Thus, both type 1 and 2 diabetes are increasingly viewed as ß-cell mass defects.

8.4 Role of Elevated FFA in the Pathogenesis of Type 2 Diabetes

Evidence is mounting to support a "lipocentric" approach to the understanding of the metabolic derangements of type 2 diabetes rather than the traditional "glucocentric" one (McGarry 1992). In support of this approach, it has long been known that, in addition to hyperglycemia, type 2 diabetic subjects almost invariably manifest a serious breakdown in lipid dynamics, reflected by elevated levels of circulating free fatty acids and triglycerides (TG) and

reduced levels of HDL cholesterol. Elevated plasma FFA are common in type 2 diabetes (Reaven, Hollenbeck et al. 1988) and in some studies have been shown to be predictive for the transition of patients from impaired glucose tolerance (IGT) to type 2 diabetes (Paolisso, Tataranni et al. 1995; Charles, Eschwege et al. 1997). High plasma FFA concentrations are also associated with a number of cardiovascular risk factors linked to insulin resistance including hypertension, dyslipidemia and abnormal fibrinolysis (Reaven 1988).

Moreover, evidence from *in vitro* studies involving long-term exposure of rat islets to high concentrations of fatty acids showed a pattern consistent with the characteristic features of β-cell dysfunction in human type 2 diabetes (Sako and Grill 1990; Unger 1995; McGarry and Dobbins 1999). These will be discussed later under the lipotoxicity section. In addition, observations drawn from experiments done on obese type 2 diabetics and on the Zucker diabetic fatty rat (ZDF) raised the possibility that in individuals genetically predisposed to develop type 2 diabetes, long exposure of the islets to elevated concentrations of circulating FFA and VLDL or both might have a deleterious effect on the β-cell as well as on the muscle and thus contribute to β-cell dysfunction. It is not clear, however, whether this breakdown in lipid homeostasis is a result of the disease or is instrumental in its development.

8.4.1 Insulin Resistance and Elevated FFA

Studies have indicated that elevated circulating FFA may contribute to the underlying pathophysiology of type 2 diabetes, in particular, the development of insulin resistance both in the periphery and the liver (Boden 1997; Shulman 2000). Central obesity is strongly linked to elevated FFA levels with central adipocytes possibly being first affected by insulin resistance as proposed by Bergman. (Bergman and Mittelman 1998). With the development of adipose tissue insulin resistance, insulin-mediated suppression of lipolysis is decreased

thus leading to increased circulating FFA and ultimately insulin resistance in skeletal muscle and liver (Boden 1997; Shulman 2000).

As mentioned earlier, skeletal muscle is also a major contributor to insulin resistance in type 2 diabetes. A strong correlation is observed between increased plasma FFA, intramyocellular lipid accumulation and insulin resistance (Krssak, Falk Petersen et al. 1999; Perseghin, Scifo et al. 1999; Boden, Lebed et al. 2001). FFA contribute to insulin resistance by having an inhibitory effect early on in glucose utilization in the muscle at the level of glucose transport (Cline, Petersen et al. 1999; Dresner, Laurent et al. 1999).

8.4.2 Endogenous Glucose Production and Elevated FFA

Endogenous glucose production, mainly contributed by the liver, is increased in type 2 diabetes (Boden and Shulman 2002). Plasma glucose levels have been closely correlated with the rate of hepatic glucose production. Little is known about the mechanism of increased endogenous glucose production, however, a role for elevated FFA has been postulated. High circulating FFA antagonize the effects of insulin to suppress endogenous glucose production. FFA are known to stimulate endogenous glucose production by promoting gluconeogenesis as shown by animal and human studies (healthy volunteers and type 2 diabetic subjects).

8.4.3 Insulin Secretion

The physiological nature of the glucose-fatty acid cross-talk in stimulus secretion coupling within the β-cell mentioned earlier, however, has a darker side. In order to maintain glucose homeostasis, the pancreatic β-cell constantly senses circulating nutrients and integrates these signals to secrete insulin accordingly. Abnormally elevated nutrient levels, however, may cause detrimental effects possibly via the same signaling pathways implicated in

insulin secretion. There is ample evidence that prolonged elevations of glucose "glucotoxicity" or FFA "lipotoxicity" are toxic to β-cells and cause both β-cell dysfunction and apoptosis. Prentki and Corkey (Prentki and Corkey 1996), however, hypothesized that elevated glucose would increase the toxicity of elevated FFA, a concept termed "glucolipotoxicity". Islet β-cell glucotoxicity, lipotoxicity and glucolipotoxicity will be discussed in detail below.

9. ISLET β-CELL FAILURE IN TYPE 2 DIABETES

9.1 Glucotoxicity

As mentioned earlier, the traditional approach to the understanding of type 2 diabetes pathogenesis was more "glucocentric". This was based on a considerable body of evidence suggesting that chronic hyperglycemia impairs glucose-induced insulin secretion and insulin gene expression (LeRoith 2002; Poitout and Robertson 2002). This is due to a diminished activity of two major β-cell transcription factors, pancreatic-duodenum homeobox-1 (PDX-1) (Olson, Redmon et al. 1993; Olson, Sharma et al. 1995) and the activator of the rat insulin promoter element 3b1 (Sharma, Olson et al. 1995; Poitout, Olson et al. 1996). The effects of prolonged hyperglycemia on β-cell function include distinct phenomena: glucose desensitization, β-cell exhaustion, and glucotoxicity. Glucose desensitization is a physiological adaptive mechanism and refers to the rapid and reversible refractoriness of the β-cell exocytotic machinery following a short exposure to elevated glucose. β-cell exhaustion, however, refers to the depletion of the readily releasable pool of intracellular insulin following prolonged exposure to a secretagogue (Sako and Grill 1990; Leahy, Bumbalo et al. 1994). The term glucotoxicity describes the slow and progressively "irreversible" effects of chronic hyperglycemia on pancreatic β-cell function. These defects of the β-cell are reversible up to a certain point in time after which they are irreversible. This suggests that β-cell exhaustion and

glucotoxicity is a continuum process, in which the latter becomes predominant after prolonged exposure to glucose (Moran, Zhang et al. 1997; Gleason, Gonzalez et al. 2000). *In vitro* and *in vivo* studies have shown that chronic hyperglycemia can decrease β-cell mass by inducing apoptosis (Efanova, Zaitsev et al. 1998; Pick, Clark et al. 1998).

Generation of oxidative stress has been reported as a biochemical basis of glucotoxicity (Matsuoka, Kajimoto et al. 1997; Tajiri, Moller et al. 1997; Ihara, Toyokuni et al. 1999; Tanaka, Gleason et al. 1999). Evidence from isolated islets chronically exposed to elevated glucose levels in vitro led to accumulation of advanced glycation end-products, (AGE), impaired β-cell function, and apoptosis, all of which can be prevented by antioxidant agents (Kaneto, Fujii et al. 1996; Tajiri, Moller et al. 1997). Normalization of plasma glucose levels and restoration of insulin secretion, insulin content, and insulin mRNA levels were achieved in the ZDF rats with antioxidants (Tanaka, Gleason et al. 1999). These findings support the hypothesis that glucotoxicity is mediated, at least in part, by chronic oxidative stress.

9.2 Lipotoxicity

A lipocentric approach is increasingly being taken in attempts to understand the pathogenesis of type 2 diabetes. Fatty acids (FA), which are essential β-cell fuels in the normal state, are toxic when present at elevated levels for long periods of time. This notion is supported by studies in isolated islets exposed to high concentrations of FFA for periods of 24-48h (Zhou and Grill 1995; Bollheimer, Skelly et al. 1998). This resulted in enhanced insulin secretion at low glucose concentrations, depressed proinsulin biosynthesis, depletion of insulin stores, and an impaired response of the β-cell to stimulatory concentrations of glucose, i.e., characteristics of type 2 diabetes. More evidence supporting this notion of "lipotoxicity" comes from studies with the ZDF rat, a model which shares many of the features of obesity-related type 2

diabetes in humans. A striking feature of this animal model is a pronounced increase in plasma NEFA and triglyceride concentrations in the perdiabetic phase and a sharp increase in islet triglyceride content immediately before the hyperglycemia appears, which is usually at about 9-11 weeks of age (Barnett, Collier et al. 1992; Lee, Hirose et al. 1994). Unger and colleagues (Barnett, Collier et al. 1992; Lee, Hirose et al. 1994) found that diet restriction of these animals greatly reduced their hyperlipidemia including FFA levels and, in association, islet function was largely restored and hyperglycemia did not develop. Under these conditions, there was no hyperglycemia and a substantial improvement of β-cell function was clearly evident, i.e. the entire phenotype of type II diabetes was prevented. The same relationship between increased TG content and deranged β-cell function was seen during long term exposure of rat islets to high fatty acid levels *in vitro* (Zhou, Ling et al. 1996).

Further studies from Unger and colleagues in the ZDF rat (Wang, Pan et al. 2001) focused on the mechanisms underlying islet lipotoxicity. Fatty acid-mediated upregulation of inducible nitric oxide synthase (iNOS) and ceramide synthesis (Figure 10) were both shown to be contributing to β-cell apoptosis, a documented event in the ZDF rat. Collectively, this body of work implicates fat accumulation in the islet as an important contributor to β-cell demise in the ZDF rat (and probably in other rodent models of obesity associated type 2 diabetes).

Further support for the 'lipotoxicity' hypothesis comes from experiments investigating the effect of troglitazone in islets from ZDF rats (Shimabukuro, Zhou et al. 1998). Incubation of ZDF rat islets with troglitazone resulted in improvement in insulin secretion in association with a reduction in the markedly increased islet triglyceride content as observed in untreated islets. These data support the hypothesis that increased circulating FFA and islet lipid accumulation are responsible for the suppression of insulin secretion seen in ZDF rats. One possible mechanism for the proposed lipotoxicity is that

surplus unoxidized FFA (characterized by elevated islet triglyceride content) increases the formation of nitric oxide (NO), which induces β-cell apoptosis (Unger, Zhou et al. 1999) (Figure 10).

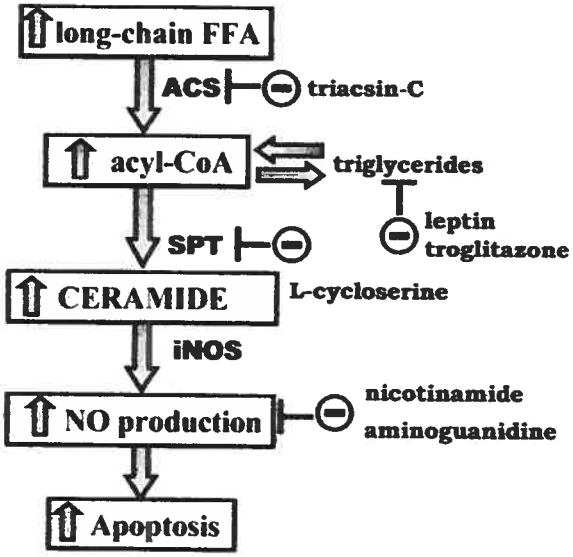


Figure 10: The proposed pathway of lipoapoptosis in the islets of the ZDF rat via the *de novo* pathway of ceramide. (Unger, Zhou et al. 1999). The formation of ceramide is catalyzed by the enzyme serine palmitoyl transferase (SPT) which is expressed at high levels in the ZDF rat islets.

Prolonged exposure of pancreatic β-cells to FFA increases basal insulin release but inhibits glucose-induced insulin secretion (McGarry and Dobbins 1999). In addition, FFA inhibit insulin gene expression in the presence of elevated glucose levels (Gremlich, Bonny et al. 1997; Ritz-Laser, Meda et al. 1999; Jacqueminet, Briaud et al. 2000), in part via negative regulation of the transcription factor PDX-1 (Gremlich, Bonny et al. 1997). Finally, excessive FFA induces β-cell death by apoptosis both *in vitro* (Cnop, Hannaert et al. 2001; Maedler, Spinas et al. 2001) and in ZDF rat islets (Pick, Clark et al. 1998; Shimabukuro, Higa et al. 1998).

9.3 Glucolipotoxicity: Glucose and FFA Synergize in Mediating β-Cell Toxicity

To reconcile and integrate the roles of elevated glucose and FFA in the pathogenesis of type 2 diabetes, Prentki and colleagues proposed the glucolipoxia concept (Prentki and Corkey 1996) which has subsequently been termed glucolipotoxicity (Prentki, Segall et al. 1998). The biochemical basis

of this hypothesis is underpinned by the β-cell anaplerosis/malonyl-CoA/lipid signaling model. This hypothesis states that the toxic actions of elevated FFA on various tissues will become particularly apparent in the context of hyperglycemia. Elevated glucose levels results in accumulation of cytosolic citrate, the precursor of malonyl-CoA, which inhibits CPT I. This curtails fat oxidation and consequently cellular detoxification of fatty acids. Under circumstances where both FFA and glucose are elevated, accumulation of metabolites derived from fatty acid esterification would inhibit glucose–induced insulin secretion and insulin gene expression and would also cause β-cell death. Thus, glucose induces and activates enzymes and transcription factors involved in fat synthesis and storage and simultaneously switches off fat oxidation via the accumulation of malonyl-CoA (McGarry and Brown 1997; Roche, Farfari et al. 1998; Jacqueminet, Briaud et al. 2000) and reduction in the expression level of peroxisomal proliferator activated receptor-α (PPARα) (Roduit, Morin et al. 2000). Sustained inhibition of fat oxidation results in an accumulation of long chain fatty acyl-CoAs (LC-CoA) which are thought to mediate the effects of chronically elevated FFA (Prentki and Corkey 1996). Whether LC-CoA accumulation affects the ß-cell (Kraegen, Cooney et al. 2001) directly or indirectly via triglyceride deposition or act as precursors for other lipid signaling metabolites such as phosphatidate, diacylglycerol and ceramide is unknown. Of note, the accumulation of LC-CoA in muscle, also via elevation of glucose and FFA together, has been implicated in the pathogenesis of insulin resistance via chronic activation of protein kinase C isoforms or ceramide (Ruderman, Saha et al. 1999; Kraegen, Cooney et al. 2001). For early ß-cell failure to occur, the hypothesis proposes that high normal postprandial glucose excursions, together with hyperlipidemia and elevated postprandial FFA levels, act in synergy to cause the initial ß-cell failure (Prentki, Joly et al. 2002) (Figure 11). The process is then likely to accelerate once hyperglycemia has developed.

Evidence to support this hypothesis has accumulated from many labs. First, in isolated islets differential effects of palmitate exposure on insulin gene expression are seen in the presence of low vs. high glucose concentrations (Jacqueminet, Briaud et al. 2000). A 72-h culture in the presence of palmitate does not affect insulin content or insulin mRNA levels at low glucose but does so at high glucose. Second, a prolonged culture of islets with palmitate is associated with glucose-dependent incorporation of FA into neutral lipids (Briaud, Harmon et al. 2001). It was found that glucose and palmitate have additive effects on FA metabolism into triglycerides (TG) only at elevated glucose levels.

These results clearly support the hypothesis that hyperglycemia is required for lipotoxicity to occur. They are consistent with the clinical observation that the majority of hyperlipidemic individuals have normal β-cell function and obesity and/or dyslipidemia are not sufficient to cause β-cell dysfunction.

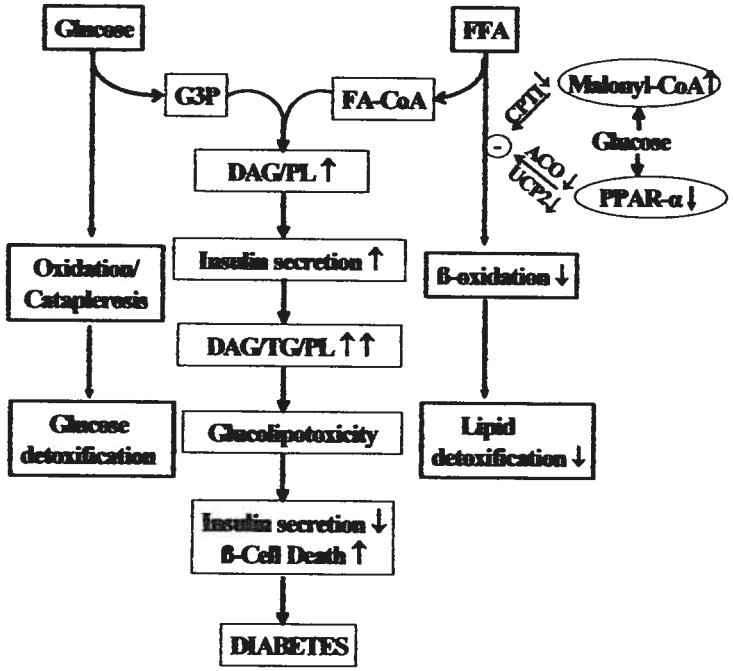


Figure 11: Possible mechanism of β-cell glucolipotoxicity implicating malonyl-CoA, PPAR-α and altered lipid partitioning. ACO, acyl CoA oxidase; DAG, diacylglycerol; G3P, glycerol 3-phosphate; PL, phospholipids; UCP2, uncoupling protein 2.

Thus, accordingly glucotoxicity and lipotoxicity are closely interrelated in the sense that lipotoxicity does not occur without chronic hyperglycemia and both are required to converge toward the generation of damaging effectors on β-cell function. Thus, lipotoxicity can be viewed as one mechanism of glucotoxicity.

9. AIMS OF THE THESIS

The Role of Glucose and Fatty Acid Synergy in ß-Cell Toxicity in the Pathogenesis of Type 2 Diabetes

To date the actions of glucose and fatty acids on ß-cell growth and death have been studied mostly independently. As explained in the introduction above, we hypothesized that the toxicity of elevated FFA would be much enhanced in the context of hyperglycemia; a concept which we termed "glucolipotoxicity". The main aim of this thesis, therefore, was to investigate the role of synergy between elevated glucose and FFA on ß-cell toxicity.

Specific Objectives

1. To determine the effects of elevated glucose alone and various FFA (saturated, monounsaturated and polyunsaturated) at both low glucose and high glucose on ß-cell death.

2. To determine the mode of ß-cell death (apoptosis *vs* necrosis) induced by elevated glucose, elevated FFA or the combination of elevated glucose and FFA.

3. To determine, as proposed by the model of glucolipotoxicity, whether the inhibition of fatty acid oxidation by elevated glucose is involved in the

mechanism of synergy between elevated glucose and FFA in causing ß-cell death.

4. To determine whether the *de novo* pathway of ceramide synthesis is involved in glucolipotoxicity.

Perspectives:

The ß-cell glucolipotoxicity concept, if successfully verified by the planned studies, will be of major importance to the fields of diabetes reasearch and practice. It will be a major advance in the understanding of the pathogenesis of type 2 diabetes which will guide the development of both preventive and treatment strategies for this disease. This concept may also be of importance in the understanding of the pathogenesis of type 1 diabetes. Whereas the major etiologic factor for this disease is undoubtedly immunologic, glucolipotoxicity could be involved in markedly increasing the rate of decline in ß-cell mass once glucose levels start to rise. Glucolipotoxicity may also be involved in loss of the replaced ß-cell mass which occurs in the early phases of islet transplantation in the treatment of type 1 diabetes. Thus, dietary and pharmacologic strategies aimed at limiting glucolipotoxicity, thereby enhancing ß-cell survival, may be of importance in the management of all forms of diabetes.

CHAPTER 2: Materials and Methods

Materials. Regular and glucose-free RPMI medium and cell culture supplements were purchased from Invitrogen (Burlington, Ont, Canada). The caspase-3 substrate Ac-DEVD-AFC, the pan-caspase inhibitor z-VAD-fmk, the ceramide inhibitors fumonisin B1 (FB1) and myriocin and the acyl-CoA synthase inhibitor triacsin C were purchased from Biomol (Plymouth Meeting, PA). Fatty acids were purchased from Nu-Check Prep (Elysian, MN). Fatty acid-free bovine serum albumin (BSA) (fraction V), etomoxir, AICAR, metformin, Hoechst 33342 (bis-benzimide) and propidium iodide (PI) were obtained from Sigma (St Louis, MO). n-Octyl-ß-D-glucopyranoside and diacylglycerol kinase were purchased from Calbiochem (San Diego, CA), and dioleoyl-phosphatidyl-glycerol from Avanti Polar Lipids (Alabaster, AL). [γ-^{32}P]ATP was from Amersham Pharmacia Biotech (QC, Canada). Silica Gel 60 thin-layer chromatography plates were from Whatman (New Jersey, USA).

Cell Culture. INS 832/13 cells (kindly provided by Dr. C.B. Newgard, Duke University) (Hohmeier, Mulder et al. 2000) between passages 36-70 were grown as previously described (Asfari, Janjic et al. 1992) in monolayer at 37°C with 5 % CO_2 in regular RPMI-1640 medium at 11 mM glucose supplemented with 10 % heat-inactivated fetal bovine serum (FBS), 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate and 50 µM ß-mercaptoethanol (complete medium). To measure glucose and FFA induced cell death, cells were seeded at a density of $4x10^4$ cells/cm^2 and grown for two days in complete RPMI. After the culture establishment period, medium was removed and cells were then incubated in RPMI medium supplemented as above with 1 % FBS (described thereafter as RPMI incubation medium), at either 5, 11 or 20 mM glucose in the presence of 0.5 % BSA alone (control) or with various FFA (0.25, 0.3 or 0.4 mM) bound to 0.5 % BSA as indicated. In some experiments, following culture establishment, cells were pre-treated with the inhibitors, zVAD-fmk (50 µM), FB1 (50 µM), myriocin (20 µM),

etomoxir (0.2 mM), triacscin C (5 μM) for 1-2 h as indicated in complete RPMI containing 10 % FBS, followed by FFA treatment at 5 or 20 mM glucose in complete RPMI containing 1 % FBS (incubation medium) for 24 h, also, in the presence of the inhibitor. The antidiabetic agent metformin (0.5 mM) and the AMPK activator AICAR (1 mM) were added together with the FFA for 24 h in complete RPMI containing 1 % FBS.

Preparation of BSA-Bound Fatty Acids. Stock solutions of fatty acids bound to BSA were prepared as described by Roche and coll. (Roche, Buteau et al. 1999). Briefly, a saturating quantity of the sodium salt fatty acid was dissolved at 37°C for 16 h under a nitrogen atmosphere in Krebs-Ringer bicarbonate buffer containing 10 mM Hepes (pH 7.4) buffer (KRBH) and 5 % (wt/vol) fatty acid-free BSA. Solutions were then filtered through a 0.2 μm filter. BSA-bound FFA was quantitated using the NEFA C kit (WAKO chemicals GmbH, Germany) and stock solutions were finally adjusted to 4 mM FFA using 5 % fatty acid free BSA in KRBH and stored at -20°C.

Quantification of Cell Death. INS 832/13 cell viability was determined by the ability of cells to exclude trypan blue. To discriminate between necrosis and apoptosis, cells were double stained with the fluorescent DNA-staining dyes Hoechst 33342 and propidium iodide (PI). INS 832/13 cells ($8x10^4$ cells) were seeded on 12 mm glass coverslips placed in 24-well plate in complete RPMI medium for two days. Medium was changed to supplemented RPMI containing 1 % FBS, 0.5 % BSA, at either 5, 11 or 20 mM glucose with or without the various FFA (0.25, 0.3, 0.4 mM) as indicated for an additional 24 h. At the end of the treatment period, cells were stained with Hoechst 33342 (10 μg/ml) and PI (1 μg/ml) in media simultaneously for 30 min at 37°C. Coverslips were then placed on slides and the stained nuclei were immediately visualized by fluorescence microscopy with a Zeiss Axioskop microscope using Hoechst and PI filter sets. Cells were defined as apoptotic when they exhibited a condensed nuclear chromatin or a fragmented nuclear membrane

when visualized with Hoechst 33342. Necrotic cells were characterized by nuclear PI staining but without condensed chromatin or fragmented nuclear membranes. Cells without apoptotic or necrotic features were considered viable. Quantitative analysis of each sample was performed by randomly choosing 5 fields and by counting at least 200 cells per assay condition.

Annexin staining: Annexin-V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS). Detection of cell-surface PS with annexin V serves as a marker for apoptotic cells. Annexin V will not bind intact normal cells. It will detect apoptotic cells with membrane alterations (PS translocation). To differentiate apoptosis from necrosis, cells are simultaneously stained with Annexin V and PI. INS 832/13 cells were grown on coverslips as mentioned earlier for cell death quantification. After the treatment period, coverslips were rinsed with 1X binding buffer and then stained with Annexin-V FITC and PI according to the manufacturer's protocol (Clonetech) for 15 minutes at 37°C at room temperature in the dark. Coverslips were then washed, placed on slides and visualized under fluorescent microscope using a dual filter set for FITC and rhodamine. Cells having an intense green fluorescence surrounding the plasma membrane are considered apoptotic cells. Cells with green staining and red cytoplasm staining are considered late apoptotic cells which have lost their membrane integrity.

Western Blotting: For experiments analyzing poly (ADP-ribose) polymerase (PARP) cleavage, cells were seeded and treated as indicated earlier. At 24 h following treatment, cells were scraped and washed with ice-cold PBS, centrifuged at 1000 rpm for 10 minutes at 4°C. The cell pellet was then resuspended in a reducing loading buffer according to the manufacturer's instructions (Enzyme Systems Products, Livermore, CA) (62.5 mM Tris, pH 6.8; 6 M urea; 10 % glycerol; 2 % SDS; 0.003 % bromophenol blue; 5 % β-mercaptoethanol), and sonicated for 20 sec on ice. Protein concentrations were

determined using the Pierce assay. Equal amounts of protein, 50 µg, were incubated for 15 min at 65°C before loading on SDS-PAGE (8 %) and then transferred to nitrocellulose membrane. Both the native (116 Kd) and the cleaved (86 Kd) bands of PARP were identified by the polyclonal antibody (419) (Enzyme Systems Products) used at a dilution of 1:15000 and an anti-rabbit conjugated to peroxidase (Jackson laboratories) secondary antibody at a dilution of 1:10000. The signal was visualized by enhanced chemiluminescence (Amersham-Pharmacia biotech).

***In vitro* Caspase-3 Activity Assay.** Caspase-3 activity was measured using the substrate Ac-DEVD-AFC and was assayed according to the manufacturer's protocol. Briefly, INS 832/13 cells established in culture were incubated for 24 h in RPMI containing 1 % FBS and 0.5 % BSA at 5 or 20 mM glucose with or without various fatty acids (0.25, 0.3, 0.4 mM) as indicated in figure legends. Both adherent and unattached cells were then harvested and combined. After sedimentation at 500 g for 10 min, the cells were washed twice with ice cold PBS, lysed for 10 min on ice with a cell lysis buffer (Invitrogen), and centrifuged (10 min, 15,000 g, 4°C) to remove debris. Fifty micrograms of proteins determined by the BCA protein quantification kit (Pierce) were incubated for 30 min with 50 µM Ac-DEVD-AFC at 30°C. Fluorescence was analyzed using a FluoStar-Optima microplate reader (BMG lab Technologies, Offenburg, Germany) in fluorescence mode using an excitation filter of 380 nm (10 nm bandpass) and an emission filter of 505 nm (bandpass 10 nm). The reaction was allowed to proceed for 30 min with a reading every minute. Caspase-3 activities were obtained by calculating the slope of the reaction over 30 min and by reporting the slope for each condition.

Ceramide Measurement. INS 832/13 cells were seeded in complete RPMI medium at 11 mM glucose containing 10 % FBS in 6-well plates and grown for 2 days. They were then incubated for 24 h in supplemented RPMI

containing 1 % FBS at 5 or 20 mM glucose with or without the various fatty acids bound to 0.5 % BSA. Lipids were extracted according to the method of Bligh and Dyer (Bligh and Dyer 1959). Briefly, cells were scraped, washed with PBS and the lipids were extracted with 3 ml chloroform/methanol (1:2, v/v) in 13 X 100 mm screw-top glass tubes. The monophase was supplemented with 0.7 ml distilled water, and after vigorous mixing the phases were left to settle for 10 min. Following the further addition of 1 ml chloroform and 1 ml water, shaking and centrifugation at 500 g for 5 min at room temperature, the organic and aqueous phases were separated. The organic phase was transferred to new tubes and dried under nitrogen. Lipids were then resuspended in 1 ml chloroform and stored at -80°C. Ceramide levels were measured using a modified Escherichia coli diacylglycerol kinase assay (Preiss, Loomis et al. 1986; Okazaki, Bielawska et al. 1990) using external standards. Sixty percent of each of the lipid samples was dried under N_2 and then solubilized by sonication in a bath sonicator in 20 µl octyl-ß-D-glucoside (7.5 %)/dioleoyl-phosphatidylglycerol (25 mM) micellar solution. The reaction buffer was prepared using a 2x solution [containing 10 mM imidazole/HCl buffer (pH 6.6), 100 mM LiCl, 25 mM $MgCl_2$ and 2 mM EGTA], mixed with an equal volume of dilution buffer [10 mM imidazole/1 mM diethylenetriamine-penta-acetic acid (pH 7.0)]. Ninety µl of reaction buffer was added to the lipid micelles together with 5 µg of diacylglycerol kinase. The reaction was started by adding 10 µl of 2.5 mM γ^{32}P-ATP solution (specific activity of 6,000 Ci/mmol) and allowed to proceed for 30 min at room temperature. Lipids were re-extracted as described above and the organic phase was dried under nitrogen. Lipids were then resuspended in 50 µl methanol/chloroform (1:10, v/v), and 25 µl were spotted on silica gel TLC plates along with ceramide standards (Sigma). Plates were then run with chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, by vol) mobile phase, and subjected to autoradiography. The radioactive spots corresponding to ceramide phosphate were scraped and counted on a scintillation counter. Linear curves of ceramide phosphorylation were produced with external

standards. Ceramide levels were normalized to total lipid phosphate. Briefly, 40 % of the lipid sample was dried down under nitrogen and oxidized with 0.15 ml of 70 % perchloric acid at 160°C for 45 min. The tubes were allowed to cool before adding 0.83 ml water, followed by 0.17 ml of 2.5 % ammonium molybdate and 0.17 ml of 10 % ascorbic acid. The tubes were then incubated at 50°C for 15 min, and the absorbance was read at 820 nm and compared with standards.

Statistical analysis. Data were analyzed by ANOVA followed by Bonferroni post-hoc test. $P<0.05$ was considered significant. Data are expressed as mean ± standard error of the mean (S.E.).

CHAPTER 3: RESULTS

1. Optimization of Culture Conditions for ß-Cell Toxicity Experiments.

Culture conditions for INS 832/13 cells were established considering several factors; firstly, we wanted to work in conditions that mimic islets *in vivo* in the sense that there is minimal proliferation. Secondly, INS 832/13 cells proliferate rapidly when cultured at high compared to low glucose concentrations in 10 % FBS. For these reasons, in order to select the lowest % of FBS at which both cell death and proliferation is minimal, INS 832/13 cells were cultured in complete RPMI medium at 11 mM glucose with 10, 5, 2, 1, 0.5, 0.2 and 0 % FBS for 96 hours and cell viability was assessed by the ability of cells to exclude trypan blue. At 96 h in 1 % FBS, the percent cell death was around 15 percent in comparison to the five percent with 10 % FBS (Figure 12). Thus, 1 % FBS concentration was chosen to run all assays.

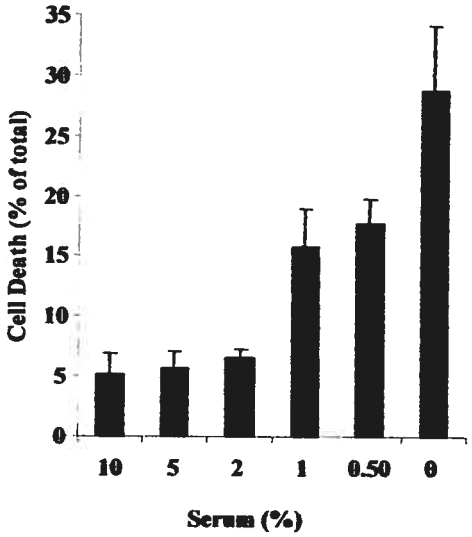


Figure 12: Optimization of culture conditions. INS 832/13 cells were cultured in complete RPMI media at 11 mM glucose in 24-well plate at a density of $0.08x10^6$ cells/well. The medium was then changed containing different percentages of serum as indicated for another 96 hours. At the end of the incubation period, cells were harvested by trypsinization and both cell death and cell density were calculated using the trypan blue exclusion method.

2. Various Fatty Acids have Differential Effects on ß-cell Death which, for Some are Highly Glucose Dependent.

INS 832/13 cells were treated for 24 h with different concentrations of FFA (palmitate, oleate and linoleate) at 5 and 20 mM glucose. At 5 mM glucose, palmitate and linoleate, even at high physiological concentrations (0.4 mM), showed minimal toxicity (Figure 13 A, C).

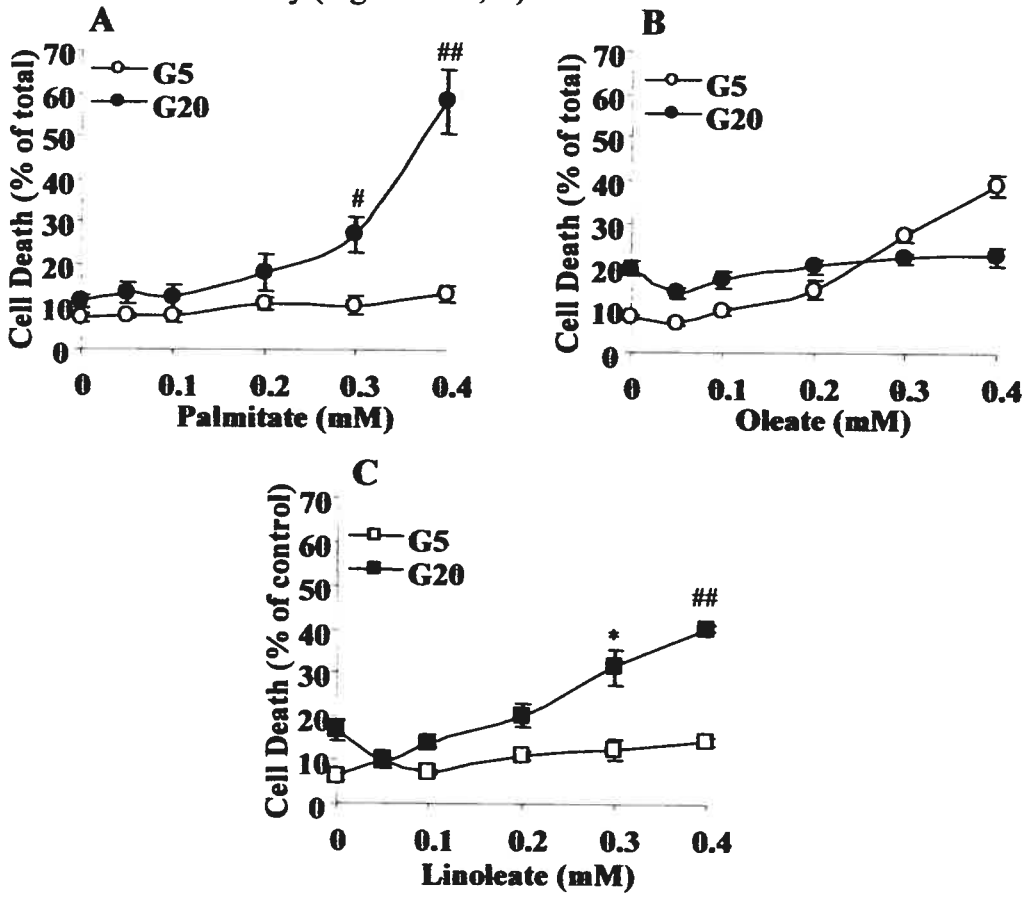


Figure 13. Dose response curve of FFA.
After establishing the cells in culture, the INS 832/13 cells were cultured for 24 h in RPMI incubation medium containing 0.5 % BSA at 5 (G5) and 20 (G20) mM glucose in the absence (control) or presence of 0.05, 0.1, 0.2, 0.3, 0.4 mM FFAs. After treatment, both adherent and floating cells were collected, combined and the cells were stained with trypan blue. Positive (dead) and negative (live) cells were counted. Results are expressed as percent of total cell number. #, P=0.01 compared to palmitate 5 mM glucose. *, P<0.05 compared to linoleate at 5 mM glucose. ##, P<0.01 compared to palmitate at 5 mM glucose and linoleate at 5 mM glucose.

At this low concentration of glucose, oleate at 0.3 and 0.4 mM was moderately toxic which has been recently observed by another group (Figure 13B) (Wrede, Dickson et al. 2002). At 20 mM glucose, however, palmitate and linoleate caused cell death at concentrations greater than 0.2 mM (Figure 13A,C). In contrast, at 20 mM glucose, oleate was not toxic at its highest concentration (0.4 mM) (Figure 13B). Thus 0.3 or 0.4 mM of palmitate and 0.4 mM of linoleate and oleate were used to run subsequent experiments with all FFAs used except for stearate which was used at 0.25 mM because of its high toxicity at elevated glucose levels since toxicity of FFA increases with chain length.

INS 832/13 cells were then exposed for 24 h to 5, 11 or 20 mM glucose in the absence or presence of the three most abundant FFA found in the blood i.e. saturated palmitate (C 16:0), monounsaturated oleate (C 18:1) and polyunsaturated linoleate (C 18:2) (Figure 14). In the absence of exogenous FFA, cells exposed to 5 and 11 mM glucose showed minimal cell death (less than 5 %) as measured by trypan blue exclusion. Elevated glucose (20 mM) caused a modest increase in cell death that reached 7 % of total cell number.

At 5 mM glucose, all tested FFA increased cell death 1.8-2.0-fold. Higher glucose concentrations markedly increased the lipotoxicity of palmitate, had an intermediate effect on the lipotoxicity of linoleate, but had no effect on the toxicity of oleate. Thus, a dramatic synergistic action of palmitate and glucose on cell death was observed at both 11 and 20 mM glucose (Figure 14). Linoleate did synergize with elevated glucose but in a reduced manner. In marked contrast, oleate showed low cytotoxicity at the elevated glucose concentrations examined. Various fatty acids, therefore, are not equivalent with respect to their action on cell death and the cytotoxicity of some fatty acids is markedly glucose dependent.

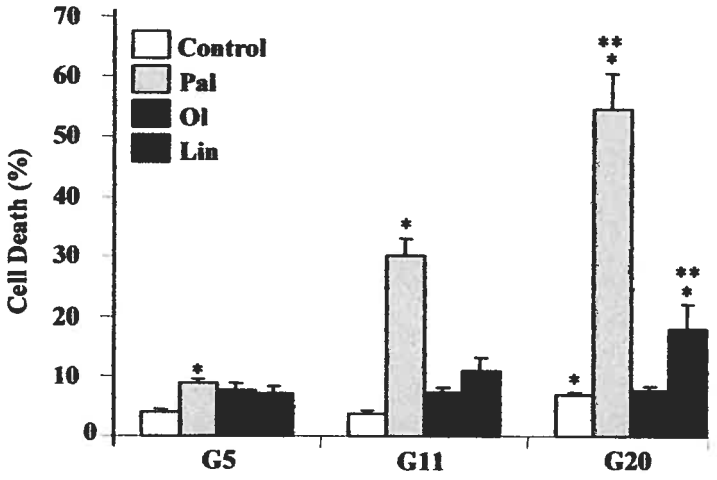
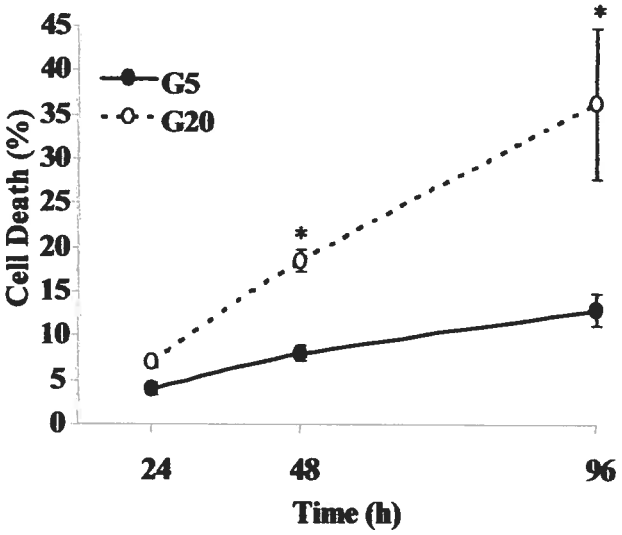


Figure 14: Effect of various free fatty acids at different glucose concentrations on ß-cell death. INS 832/13 cells were cultured for 24 h in RPMI incubation medium as described in methods containing 0.5 % BSA at 5 (G5), 11 (G11) or 20 (G20) mM glucose in the absence (control) or presence of 0.4 mM palmitate (Pal), oleate (Ol) or linoleate (Lin). After treatment, both adherent and floating cells were collected and combined and the cells were stained with trypan blue. Positive (dead) and negative (live) cells were counted. Results are expressed as percent of total cell number. Data are mean ± S.E. of three different experiments. One-way ANOVA with Bonferroni post-hoc test; *, $P<0.001$ compared to control at same glucose concentration. **, $P<0.001$ compared to corresponding palmitate at 5 and 11 mM glucose.

The cytotoxicity induced by high glucose alone however was further increased at 48 and 96 h to 18.5±1.31 and 36.3±8.5 %, respectively (Figure 15).

Figure 15: Time dependence of the apoptotic action of elevated glucose on the β-cell. INS 832/13 cells were cultured as in figure 14 in the presence of glucose 5 and 20 mM for 24, 48 and 96 h as indicated in the figure. *P<0.05* for 20 mM glucose at 48 and 96h as compared to 5 mM glucose for the same time point.



3. Saturated Fatty Acids are Particularly Efficient in Synergizing with Glucose to Induce ß-Cell Apoptosis.

To determine the mode of cell death induced by the various FFA at elevated glucose, INS 832/13 cells were stained with the dye Hoechst 33342 (Figure 16). Elevated levels of glucose (20 mM) caused minimal apoptosis at 48 hours. Saturated fatty acids (palmitate and stearate) and the polyunsaturated FFA linoleate also caused considerable apoptosis as indicated by DNA condensation at 20 mM glucose (Figure 16 shown by the arrows). Oleate was not toxic at 20 mM glucose as the amount of apoptosis with oleate was equivalent to that of 20 mM glucose without fatty acid. Examples of apoptotic nuclei are shown in Figure 16, and the quantification of condensed nuclei at 5 and 20 mM glucose at 24 h is shown in Figure 17.

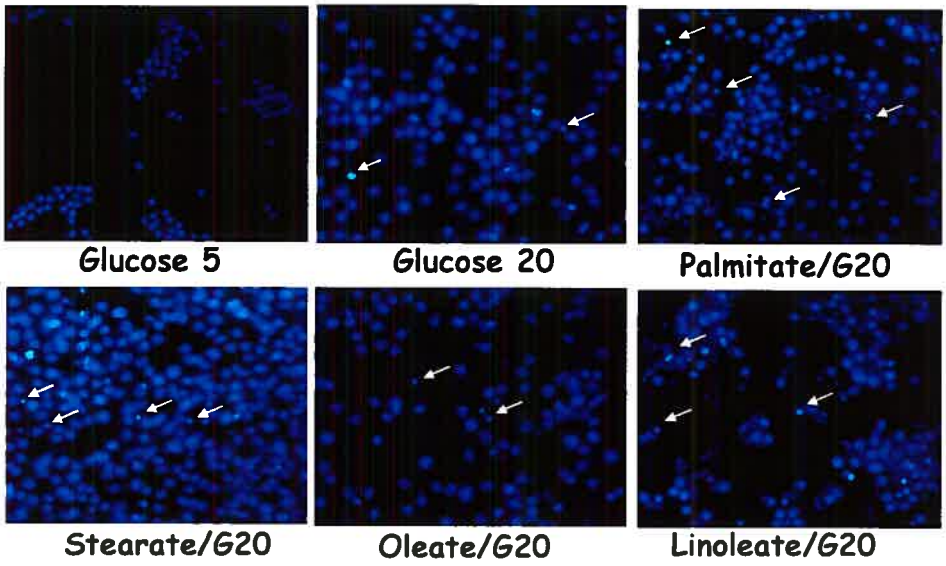


Figure 16: High glucose and different FFA caused DNA condensation to various extents. INS 832/13 cells were seeded on glass coverslips and then treated at 5 or 20 mM glucose in the absence (control) or presence of (0.4 mM) palmitate, oleate, linoleate or (0.25 mM) stearate for 48 h. Cells were stained with Hoechst 33342 and then visualized using fluorescence microscopy. Examples of condensed nuclei, a hallmark of apoptosis, are indicated by arrows.

To examine whether cell death induced by various FFA at elevated glucose occurs via necrosis as well as apoptosis, INS 832/13 cells were stained with

the dyes Hoechst 33342 and PI to distinguish both types of cell death (Figure 17). Under all tested experimental conditions (various fatty acids at different glucose concentrations) the percentage of necrotic cells was modest (less than 7 %), except for palmitate at 20 mM glucose (18 %) (Figure 17).

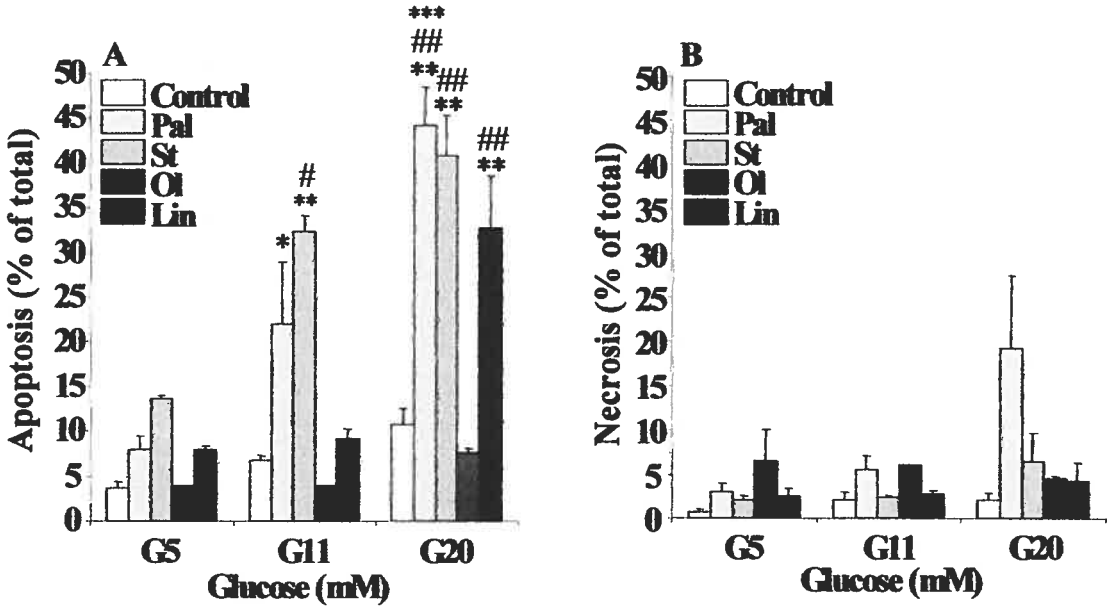


Figure 17: Quantification of the effect of various free fatty acids at different glucose concentrations on ß-cell apoptosis and necrosis. INS 832/13 cells were cultured for 24 h as described in Figure 1 at 5 (G5), 11 (G11) or 20 mM (G20) glucose in the absence (control) or presence of 0.4 mM palmitate (Pal), oleate (Ol), linoleate (Lin) or (0.25 mM) stearate (St). Cells were then stained with the dyes Hoechst 33342 and PI, analyzed by fluorescence microscopy and scored according to the morphology and type of the fluorescence of the nucleus, as described in the methods section. Results are expressed as percent of total cell number for A. Apoptotic cells and B. Necrotic cells. Data represents means ± S.E. of four different experiments. **, $P<0.001$ compared to control at same glucose concentration. *, $P<0.05$ and ***, $P<0.001$ compared to control at 5 mM glucose. #, $P<0.05$ compared to stearate at 5 mM glucose. ##, $P<0.001$ compared to corresponding FFA at 5 mM glucose. (One-way ANOVA with Bonferroni post-hoc test).

As concluded from the cell toxicity data, elevated glucose moderately increased apoptosis in the absence of fatty acids. Palmitate, stearate and linoleate induced cell death primarily by apoptosis and, as for cell death

examined by trypan blue (Figure 14), FFA-induced apoptosis was strongly glucose concentration dependent. The saturated FFA, palmitate and stearate, were more potent compared to linoleate at high glucose and unlike linoleate, were able to induce more apoptosis at intermediate (11 mM) compared to low (5 mM) glucose. Linoleate caused significant apoptosis (30 %) only at elevated glucose (20 mM). Oleate was minimally toxic at 5 mM glucose and its toxicity in terms of apoptosis and necrosis was not increased at elevated glucose.

The apoptosis induced by FFA at elevated glucose was further examined and confirmed when cells were analyzed with AnnexinV binding under fluorescence microscopy (Figure 18).

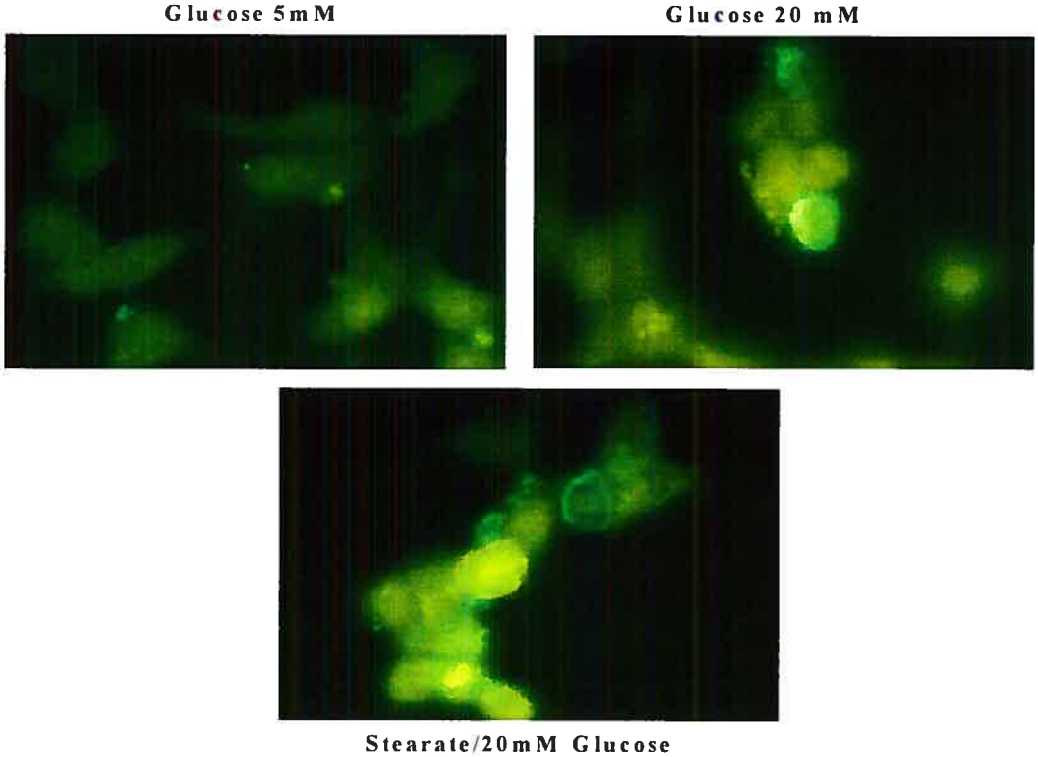


Figure 18: Stearate at elevated glucose levels cause phosphatidylserine flip. INS 832/13 cells were grown on glass coverslips placed in 6-well plate for 48 hours, then cells were treated at 5 or 20 mM glucose in the absence (control) or presence of 0.25 mM stearate for 24 hours. Cells were then stained with Annexin-V and PI as described under Materials and Methods. Annexin-V staining was visualized under fluorescence microscopy.

In cells undergoing apoptosis, phosphatidylserine (PS) which is usually present on the inner layer of the plasma membrane flips to the outer layer and is bound to annexin-V. Annexin-V is a phospholipid-binding protein with a high affinity for PS. PS flip serves as a marker for apoptotic cells. Annexin V will not bind intact normal cells but cells undergoing either apoptosis or necrosis. In necrotic cells, leakage can occur, thereby, Annexin V binds to PS on the inner leaflet. Thus, to differeniate apoptosis from necrosis, cells are simultaneously stained with annexin V and PI. Cells undergoing apoptosis therefore, will be observed with this technique to have green fluorescence staining of the plasma membrane. The effect of fatty acid stearate was examined using this technique at both 5 and 20 mM glucose (Figure 18).

As shown in figure 18, at 20 mM glucose few cells exhibited annexin V staining. At 20 mM glucose together with stearate however, more cells exhibited annexin V staining indicative of apoptosis.

4. Palmitate at Elevated Glucose Induces Caspase-3 Activation and PARP cleavage

To confirm that these morphological changes were part of an apoptotic process, we evaluated the effect of the various FFA on the activation of caspase-3, a hallmark of apoptosis (Figure 19).

None of the tested FFA significantly increased caspase-3 activity at low glucose. Palmitate and stearate synergized with high glucose to increase caspase-3 activity by 8- and 11-fold, respectively. Oleate did not cause activation of caspase-3, and linoleate, together with high glucose, increased caspase 3 activity by about 4-fold.

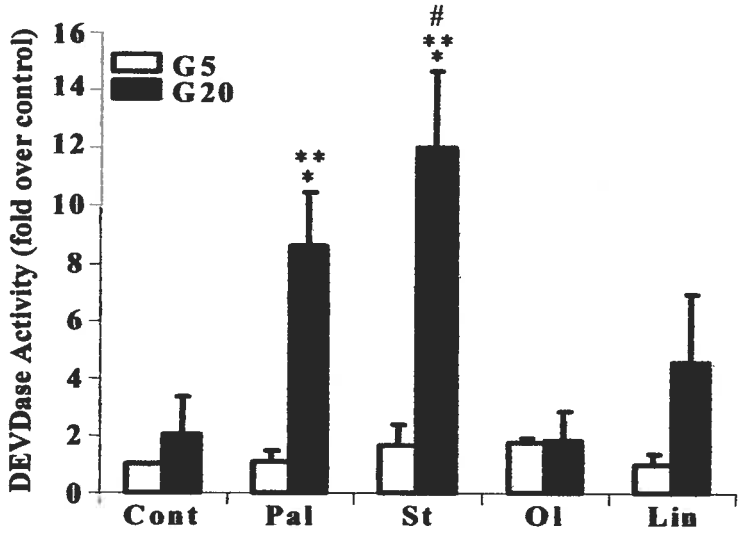
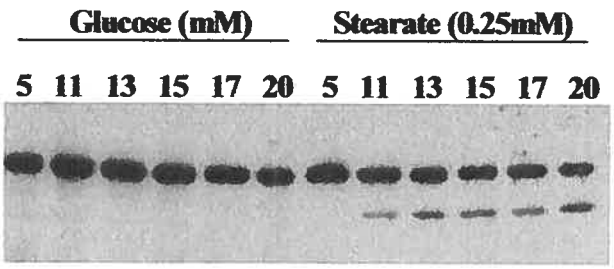


Figure 19: The action of various free fatty acids on caspase-3 activation is glucose-dependent. INS 832/13 cells were cultured for 24 h as described earlier in RPMI incubation medium at 5 or 20 mM glucose in the absence (control) or presence of 0.4 mM palmitate (Pal), oleate (Ol), linoleate (Lin) or 0.25 mM stearate (St). Cell lysates were incubated with the caspase-3 substrate Ac-DEVD-AFC. Fluorescence of released AFC was measured as described in Methods. Results are presented as fold increase over control and are means ± S.E. of four separate experiments. *, $P<0.05$ compared to control at 5 mM glucose. **, $P<0.05$ compared to corresponding FFA at 5 mM glucose. #, $P<0.001$ compared to control at 20 mM glucose. (One-way ANOVA with Bonferroni post-hoc test).

Saturated fatty acids at elevated glucose concentrations also caused cleavage of poly ADP-ribose polymerase (PARP) with the formation of the 85 Kd fragment, characteristic of apoptosis as shown in figure 20.

Figure 20: PARP cleavage induced by stearate is glucose dependent. INS 832/13 cells were grown as discussed earlier in Figure 19 treated with stearate (0.25 mM) at different glucose concentrations for 48 hours. Cells were harvested, lysed and proteins were quantitated as discussed under Materials and Methods.



To determine whether inhibiting caspases could provide a means by which ß-cell death induced by elevated glucose and fatty acids, singly or combined could be reduced, apoptotic and necrotic events were quantitated in the absence and presence of the pancaspase inhibitor zVAD-FMK. As expected, z-VAD-FMK was highly effective at both inhibiting caspase-3 activation (Figure 21) and reducing apoptosis induced by elevated glucose alone and elevated glucose with palmitate (Figure 22A). This translated into an inhibition of cell death induced by elevated glucose singly, but surprisingly, had no effect on the very high rate of cell death induced by high glucose and palmitate together due to a dramatic shift from apoptosis to necrosis (Figure 22A).

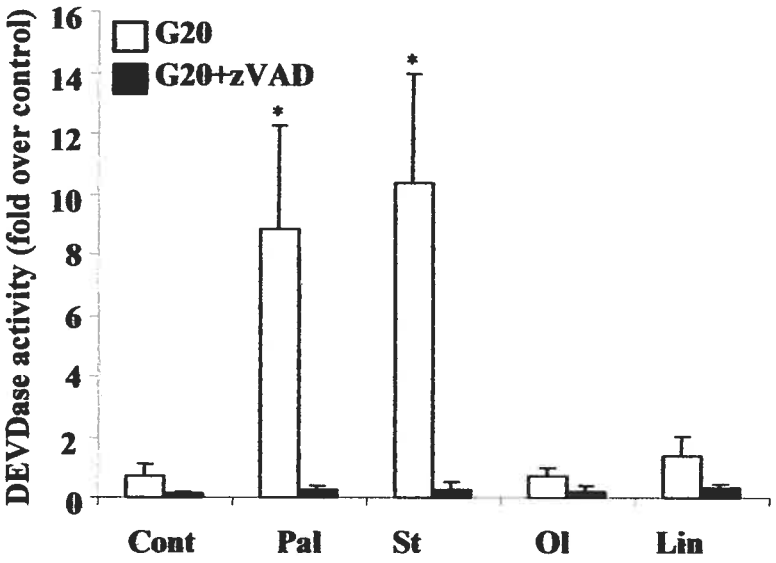


Figure 21: z-VAD-FMK blocks activation of caspase-3 induced by FFA at elevated glucose levels. INS 832/13 cells were cultured for 24 h as described in Figure 19 at 20 mM glucose in the absence (control) or presence of 0.4 mM palmitate (Pal), oleate (Ol), linoleate (Lin) or 0.25 mM stearate (St) and 50 µM z-VAD-FMK. Cell lysates were incubated with the caspase-3 substrate Ac-DEVD-AFC. Fluorescence of released AFC was measured as described in the Materials and Methods section. Data represent three different duplicate experiments and are expressed as mean ± S.E. *, $P<0.01$ compared to palmitate or stearate at 20 mM glucose without z-VAD-fmk. (One-way ANOVA with Bonferroni post-hoc test).

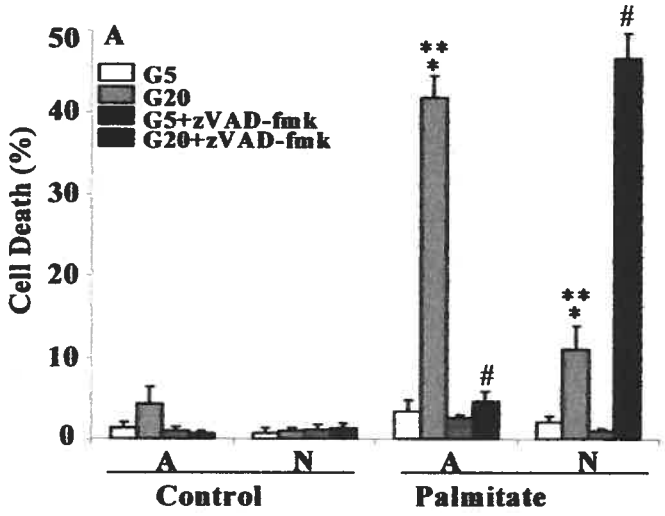
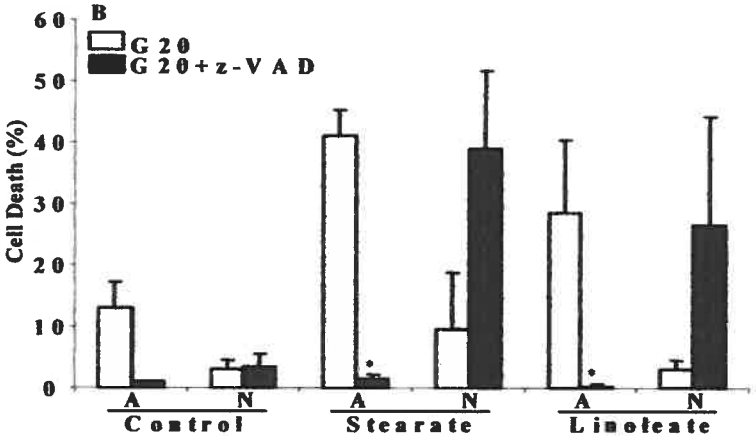


Figure 22: Inhibition of palmitate-induced caspase activation is coupled with a switch to necrosis. A. INS 832/13 cells were grown in complete RPMI medium containing 10 % FBS at 11 mM glucose on coverslips for two days. They were then pre-treated with the caspase inhibitor z-VAD (50 μM) for two hours in complete RPMI medium. Medium was changed to RPMI incubation medium containing 0.5 % BSA at 5 (G5) or 20 mM (G20) glucose in the absence (control) or presence of 0.4 mM palmitate (Pal) and z-VAD-fmk for 24 h. Cells were then stained with Hoechst 33342 and PI and viable, apoptotic (A) and necrotic (N) cells were counted using fluorescent microscopy from five fields chosen randomly. Results are expressed as percent of total cell number. B. Results are expressed as total cell death (sum of apoptotic and necrotic cells). Data represent four different duplicate experiments and are expressed as mean ± S.E. *, $P<0.01$ compared to palmitate at 5 mM glucose. **, $P<0.01$ compared to control at 20 mM glucose. #, $P<0.001$ compared to same condition without z-VAD-fmk. (One-way ANOVA with Bonferroni post-hoc test).



The same observation was made with stearate and linoleate at elevated glucose concentrations (Figure 22B).

Altogether, this series of experiments establishes that an apoptotic pathway is induced in INS 832/13 cells by either high glucose or some fatty acids. Together, however, elevated glucose and, in particular, saturated fatty acids are markedly synergistic in this effect. In addition, glucotoxicity but not glucolipotoxicity, can be prevented by a caspase inhibitor (Figures 22).

5. Metabolism of Palmitate but not its Mitochondrial ß-Oxidation is Required for Glucolipotoxicity.

Treating INS 832/13 cells with triacsin-C, an inhibitor of acyl-CoA synthase (Noel, Antinozzi et al. 1997), blocked cell death induced by palmitate at 20 mM glucose (Figure 23). For reasons that are unclear, triacsin C exhibited cytotoxicity in the absence of exogenous FFA but not in their presence. Perhaps in the absence of exogenous FFA, triacsin-C competes with the small amount of endogenously released FFA for reactions that are limiting for various essential cell function; these processes would not be limited by the drug when exogenous FFA are provided. In any event, the data indicate that CoA-esterification of fatty acids, the first step of FFA metabolism, is required for the toxic action of fatty acids at elevated glucose.

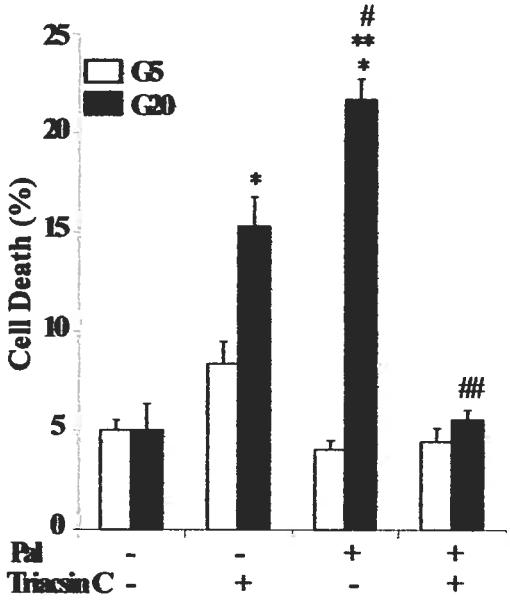


Figure 23: Palmitate must be metabolized to synergize with elevated glucose to induce ß-cell death. INS 832/13 cells were preincubated (as described in Figure 22 for 1 h) with or without the acyl-CoA synthase inhibitor triacsin C (5 µM) and subsequently cultured for 24 h in RPMI incubation medium with or without triacsin C at 5 (G5) or 20 mM (G20) glucose in the absence or presence of 0.3 mM palmitate (Pal). Cell death was evaluated with trypan blue staining. Mean ± S.E. of four determinations (two separate duplicate experiments). *, $P<0.001$ compared to Pal(-), Triacsin(-) at 5 mM and 20 mM glucose. #, $P<0.01$ compared to Pal(-), Triacsin (+) at 20 mM glucose. **, $P<0.001$ compared to Pal(+), Triacsin(-) at 5 mM glucose. ##, $P<0.001$ compared to Pal(+), Triacsin(-) at 20 mM glucose. (One-way ANOVA with Bonferroni post-hoc test).

In addition the results show that glucolipotoxicity is not the result of an unspecific toxic effect of FFA themselves.

Treating INS 832/13 cells with etomoxir, an inhibitor of CPT I that catalyzes the rate-limiting step of the ß-oxidation of FFA, markedly amplified cell death (Figure 24A) and caspase-3 activation (Figure 24B) induced by palmitate (0.3 mM) at elevated glucose. Interestingly, etomoxir alone at low glucose was not toxic in the absence of exogenous palmitate but allowed lipotoxicity of palmitate at low glucose that was similar in its extent to the glucolipotoxicity caused by the combined presence of high glucose and palmitate (Figure 24A).

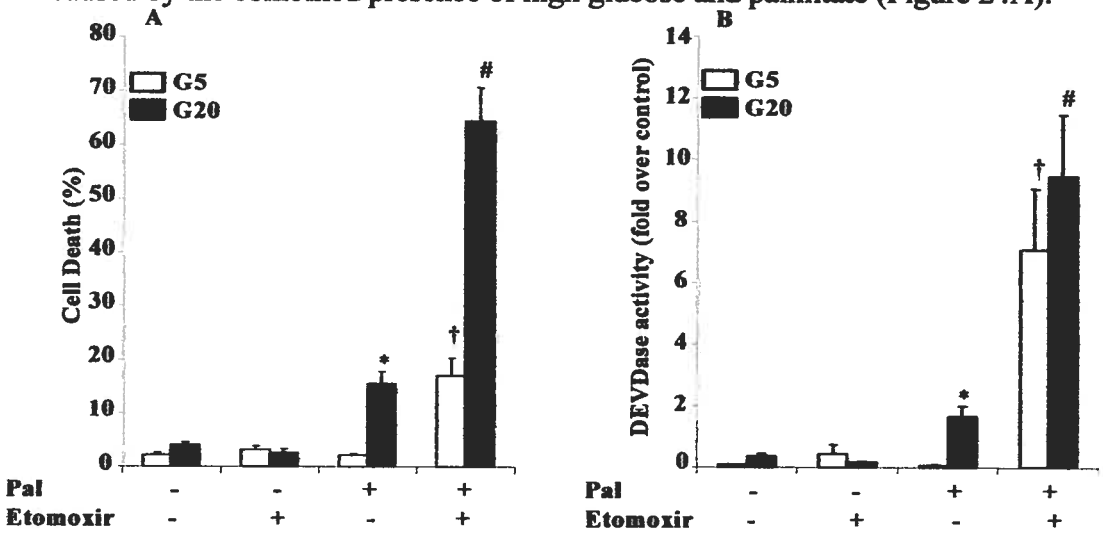


Figure 24: The fatty acid ß-oxidation inhibitor etomoxir induces palmitate toxicity at low glucose and enhances palmitate toxicity at high glucose. A. INS 832/13 cells were preincubated as described in Figure 22 for 1 h with or without etomoxir (0.2 mM) and subsequently cultured for 24 h in RPMI incubation medium at 5 (G5) or 20 mM (G20) glucose in the absence or presence of 0.3 mM palmitate (Pal) with etomoxir. Dead (trypan blue positive) cells were counted and reported as percentage of total cells counted. Mean ± S.E. of three separate duplicate experiments. B. Cells were treated as in panel A and caspase-3 activity was measured as described in Figure 19. . Mean ± S.E. of three separate duplicate experiments. B. Cells were treated as in panel A and caspase-3 activity was measured as described in Figure 3. Mean ± S.E. of three separate experiments. *, *P<0.05* compared to Pal(-), Etomoxir(-) at 5 mM glucose control; †, *P<0.01* compared to Pal(+), etomoxir(-) at 5mM glucose; #, $P<0.001$ compared to Pal(+), Etomoxir(-) at 20 mM glucose.

This suggests that the ß-oxidation of fatty acids is not involved in the mechanism by which palmitate induces apoptosis at elevated glucose, but rather that glucose inhibition of fat oxidation (Prentki and Corkey 1996; Prentki, Joly et al. 2002) is involved in this process.

6. Accumulation of Ceramide after Saturated Fatty Acid Treatment

Ceramide is a lipid signaling molecule involved in regulating key biological processes such as cell senescence, stress responses, cell cycle arrest and apoptosis (Hannun 1996; Hannun and Luberto 2000). Some evidence for a role of the *de novo* synthesis pathway of ceramide in ß-cell lipotoxicity has been provided in the ZDF diabetic rat model (Shimabukuro, Higa et al. 1998). Saturated fatty acids such as palmitate and stearate enter directly into the *de novo* synthesis of ceramide. To gain insight into the mechanism of ß-cell glucolipotoxicity, the ceramide content of INS 832/13 cells treated with elevated glucose and fatty acids was measured (Figure 25). Elevated glucose or the various FFA (palmitate, stearate, oleate and linoleate) alone did not alter the ceramide content. The ceramide level of INS 832/13 cells, however, increased 2-3 times in palmitate- and stearate-treated cells at high glucose compared to low glucose (Figure 25).

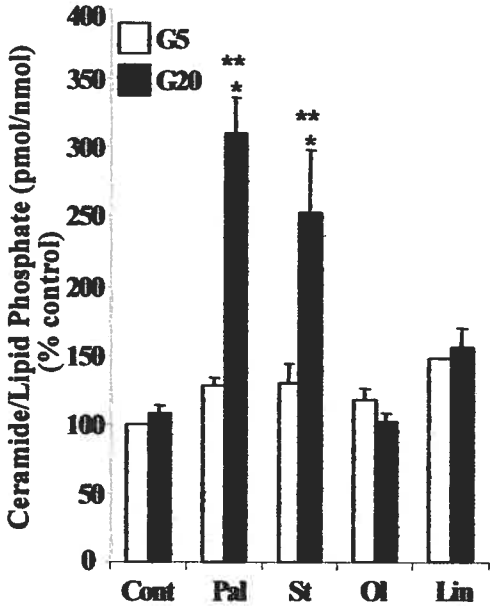


Figure 25: Elevated glucose plus saturated fatty acids cause ceramide accumulation in INS 832/13 cells. Cells were cultured for 24 h as described earlier in the absence or presence of 0.4 mM palmitate (Pal), oleate (Ol), linoleate (Lin) or 0.25 mM stearate (St) at 5 (G5) and 20 mM (G20) glucose. The cellular ceramide content was determined using the diacylglycerol kinase assay and normalized to total lipid phosphate content (as described under Materials and Methods). Means $\pm$ S.E. of five different duplicate experiments. *, $P<0.001$ compared to control at 5 and 20 mM glucose **, $P<0.001$ compared to Pal or St at 5mM glucose. (One-way ANOVA with Bonferroni post-hoc test).

To further investigate the mechanism of ceramide accumulation, we treated INS 832/13 cells with two inhibitors (myriocin and fumonisin FB1) of the *de novo* pathway of ceramide which inhibit serine palmitoyltransferase (SPT) and ceramide synthase, respectively. Both myriocin and fumonisin B1 did not block ceramide accumulation (Figure 26) or cell death (Figure 27) induced by the combined presence of elevated glucose and palmitate.

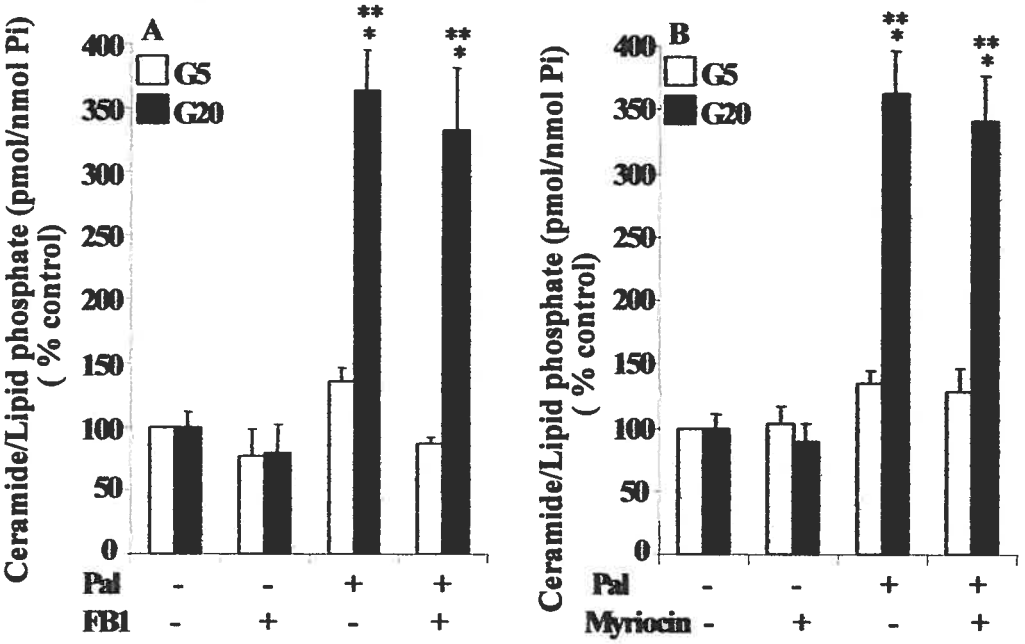


Figure 26: The ceramide synthase inhibitors fumonisin B1 (FB1) and myriocin do not block the rise in cellular ceramide caused by the combined presence of elevated glucose and palmitate. Cells were pre-incubated with (A) FB1 (50 μM) or (B) myriocin (20 μM) for 2 h as described earlier followed by another incubation in RPMI incubation medium and 0.5 % BSA at 5 (G5) or 20 mM (G20) glucose in the absence (control) or presence of 0.4 mM palmitate (Pal) and FB1 or myriocin for 24 h. Cellular ceramide content was measured as in Figure 25. Means ± S.E. of three different duplicate experiments. *, $P<0.001$ compared to Pal(-), FB1(-) or myriocin (-) at 5 and 20 mM glucose. **, $P<0.001$ compared to Pal at 5mM glucose. (One-way ANOVA with Bonferroni post-hoc test).

The data are compatible with the possibility that ceramide might be involved in signaling apoptosis induced by saturated fatty acids at elevated glucose. However, in INS 832/13 cells, the *de novo* pathway of ceramide synthesis

does not appear to be involved either in the mechanism promoting ceramide accumulation or in cell death induced by glucolipotoxicity.

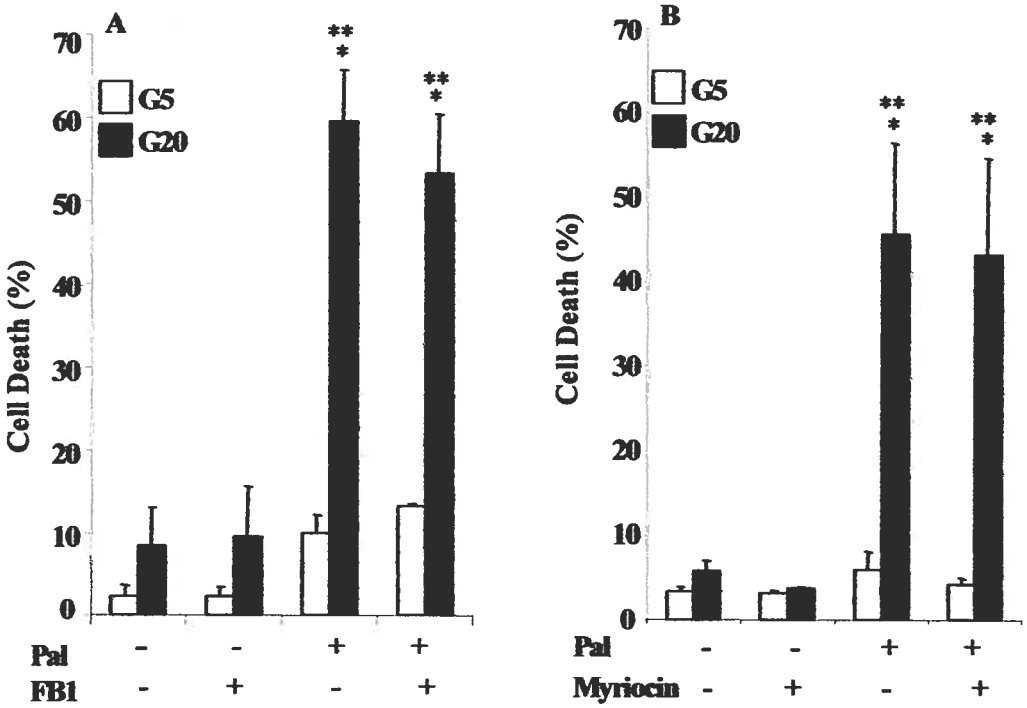


Figure 27: The ceramide synthase inhibitors fumonisin B1 (FB1) and myriocin do not block palmitate induced cell death at elevated and palmitate. Cells were pre-incubated with (A) FB1 (50 μM) or (B) myriocin (20 μM) for 2 h (as described in Figure 26) followed by another incubation in RPMI incubation medium with 0.5 % BSA at 5 (G5) or 20 mM (G20) glucose in the absence (control) or presence of 0.4 mM palmitate (Pal), FB1 or myriocin for 24 h. Adherent and floating cells were then collected, combined and stained with trypan blue to measure cell death. Results are expressed as percent of total cell number. Means ± S.E. of three different duplicate experiments. *, $P<0.001$ compared to Pal(-), FB1(-) or myriocin (-) at 5 and 20 mM glucose. **, $P<0.001$ compared to Pal at 5mM glucose. (One-way ANOVA with Bonferroni post-hoc test).

CHAPTER 4: DISCUSSION

In this study, we demonstrated for the first time a marked synergistic effect of high glucose and saturated FFA on inducing cell death by apoptosis in INS 832/13 ß-cells (Figures 13, 14 and 17). Elevated glucose and FFA together were much more toxic than either of these factors alone. In addition, we found differential effects of the various fatty acids tested at high glucose, with palmitate (saturated FFA) dramatically having the most toxicity, linoleate (polyunsaturated FFA) having modest toxicity and oleate (monounsaturated FFA) having poor or no toxicity (Figures 13 and 14). Thus, for saturated fatty acids in particular, the results are consistent with the glucolipotoxicity hypothesis (Prentki and Corkey 1996; Prentki, Roduit et al. 2001; Prentki, Joly et al. 2002), and provide strong support for the concept that this process is implicated in the progressive loss of ß-cell mass by apoptosis which is involved in the pathogenesis of type 2 diabetes (Butler, Janson et al. 2003). As reviewed recently (Prentki, Joly et al. 2002), glucolipotoxicity may also contribute to the etiology of type 1 diabetes and has implications for transplanted type 1 diabetic patients. Another component of the glucolipotoxicity hypothesis not addressed in this study is that which causes ß-cell dysfunction. In support of this component, Poitout and coll. (Jacqueminet, Briaud et al. 2000) showed in rat islets that 72 h of culture in the presence of palmitate at low glucose did not affect insulin mRNA and insulin biosynthesis, but at high glucose these parameters were reduced. Similarly, the palmitate effect on decreasing IDX-1 expression, a ß-cell differentiation factor, was shown to be glucose dependent (Gremlich, Bonny et al. 1997).

After correcting for the difference in BSA concentration used in the media of our experiments (0.5%), and the much higher BSA concentration of human plasma (~3.5 %), the unbound FFA concentrations we used were in the order of 5-6 fold higher than is usually present in the plasma of subjects at high risk

of diabetes (Stefan, Stumvoll et al. 2003). However, our media did not contain triglyceride in VLDL particles, the fatty acids of which are available to islets *in vivo* via lipoprotein lipase (LPL) which has been shown to be present and active in islet tissue (Cruz, Kwon et al. 2001). Furthermore, triglyceride levels in diabetic plasma and in subjects at risk of type 2 diabetes are commonly elevated above 2 mM. Hence, the actual supply of FFA to the INS cells in our experiments (from media FFA only) compared to islets of subjects at risk of type 2 diabetes (from plasma FFA and triglyceride) may not be very different.

Hoechst and PI staining, Annexin-V binding, PARP cleavage and caspase-3 experiments showed that both apoptosis and necrosis are implicated in FFA-induced cytotoxicity, however apoptosis is the major pathway leading to ß-cell death caused by elevated glucose and FFA singly or combined (Figures 16-20). When caspase-3 activation was inhibited using the pan-caspase inhibitor z-VAD-fmk (Figure 21), apoptosis induced by glucose was blocked; however, in the presence of palmitate at high glucose levels, z-VAD-fmk caused a switch from apoptosis to necrosis (Figure 22). This has been observed earlier in other systems (Lemaire, Andreau et al. 1998; Sane and Bertrand 1999). This suggests that glucolipotoxicity exerts a very severe insult to the ß-cell in comparison to glucotoxicity that can be halted by an antiapoptotic agent.

The mechanism of FFA-induced apoptosis is not well understood. However, according to the glucolipotoxicity hypothesis (Prentki, Joly et al. 2002) it is predicted that, at elevated glucose, LC-CoA derived from simultaneously elevated FFA will accumulate due to the inhibitory effect of the glucose on lipid detoxification via ß-oxidation. This results in increased LC-CoA partitioning towards toxic cellular processes either directly or indirectly via acylation and/or esterification products. In support of this concept, treating cells with the fatty acyl-CoA synthetase (ACS) inhibitor triacsin C demonstrated, in accordance with previous results in ZDF rat islets

(Shimabukuro, Higa et al. 1998), that apoptosis induced by saturated fatty acids is not caused by the FFA themselves, but by LC-CoA and/or metabolites derived from them (Figure 23). Furthermore, etomoxir, an inhibitor of CPT I the rate-limiting enzyme in the ß-oxidation of FFA, enhanced cell death and caspase-3 activation at both low and elevated glucose levels suggesting that the toxicity of fatty acids at elevated glucose is not mediated via their oxidation (Figure 24). Interestingly, etomoxir allowed lipotoxicity of palmitate at low glucose at a level equivalent to the glucolipotoxicity caused by the combination of high glucose and palmitate. Also consistent with the view that glucose-induced inhibition of fat oxidation is important in the mechanism whereby glucose and FFA synergize to cause ß-cell death, long term exposure of ß-cells to glucose causes sustained accumulation of the CPT I inhibitor malonyl-CoA (Prentki, Vischer et al. 1992), chronically suppresses fat oxidation (Roche, Farfari et al. 1998) and stimulates fat esterification processes (Roche, Farfari et al. 1998). In the ß-cell, we (Prentki, Vischer et al. 1992) and others (Jacqueminet, Briaud et al. 2000) have also reported that elevated glucose and palmitate or oleate synergize in promoting lipid esterification with increases particularly in triglyceride esterification and deposition.

With respect to the triacsin C results, there are at least five isoforms of ACS with different tissue distribution, subcellular localization, substrate preference and regulatory mechanisms. Only two of these five ACS are inhibited by triacsin C (Kim, Lewin et al. 2001; Lewin, Kim et al. 2001; Coleman, Lewin et al. 2002). The mitochondrial membrane bound ACS-5, an isoform thought to channel LC-CoA to CPT I, is not sensitive to triacsin C (Coleman, Lewin et al. 2002). Thus, in agreement with this finding, it is possible that triacsin C inhibited only the LC-CoA pool which is channeled for esterification.

It has been proposed, using ZDF rat islets as a model (Shimabukuro, Higa et al. 1998), that FFA mediate apoptosis of ß-cells via the *de novo* synthesis of

ceramide. Our results clearly show that saturated FFA caused an increase in ceramide accumulation only at elevated glucose (20 mM) (Figure 25). This suggests that ceramide accumulation could be implicated in FFA-induced apoptosis at high glucose. Two inhibitors of the *de novo* pathway of ceramide synthesis, however, neither blocked cell death nor ceramide accumulation which suggest that *de novo* synthesis of ceramide is not involved in FFA-induced apoptosis at elevated glucose levels in INS 832/13 cells. This is partly in contrast with other studies in which ceramide synthesis inhibitors reduced apoptosis of ß-cell death (Shimabukuro, Higa et al. 1998; Maedler, Spinas et al. 2001; Lupi, Dotta et al. 2002). The difference between the other studies and our results could be attributed to differences in the concentration and composition of fatty acids used and to differences in experimental systems. However, we cannot exclude the possibility that the inhibitors did not work in our system. We feel this is unlikely since the same inhibitor preparations gave positive results in another cell system in our hands (Hardy S, Prentki M, JBC in Press). Nevertheless, we will further study these inhibitors and these experiments will include a positive control in INS 832/13 cells. Our results do not underscore the importance of ceramide in FFA induced apoptosis, however other pathways of ceramide generation should be examined such as the sphingomyelin pathway (Liu, Obeid et al. 1997).

The minimal toxicity of oleate, an abundant FFA in serum, is particularly interesting. Unsaturated fatty acids such as oleate and palmitoleate have been shown in some cellular systems to have either no effect on apoptosis (de Vries, Vork et al. 1997; Maedler, Spinas et al. 2001), or even to protect from palmitate-induced apoptosis (de Vries, Vork et al. 1997; Hardy, Langelier et al. 2000; Maedler, Spinas et al. 2001). The difference between palmitate and oleate on ß-cell cytotoxicity could be attributed to differences in metabolism of the fatty acids, in particular the make up of the phospholipid pool. Thus, the enrichment of phospholipids by saturated FFA lowers membrane fluidity, impairing various membrane functions (Innis and Clandinin 1981; Stubbs and

Smith 1990). Reduced cardiolipin synthesis is another possibility as palmitate has been shown to decrease the synthesis of cardiolipin in cardiomyocytes (Ostrander, Sparagna et al. 2001) and the breast cancer cells MDA-MB231 (S. Hardy and M.P., JBC in press). The phospholipid cardiolipin binds cytochrome C in the mitochondrion which prevents the release of cytochrome C into the cytoplasm, this release being an important event in the apoptotic process (Zou, Li et al. 1999). We previously showed that oleate activates PI-3 kinase in MDA-MB231 cells and promotes cell proliferation, whereas palmitate had opposite effects (Hardy, Langelier et al. 2000). Thus, it would be of interest to determine whether palmitate also reduces PI-3 kinase activity in the ß-cell considering that this enzyme plays a critical role in the control of cell growth and apoptosis (Cantley 2002). In any event, the differential effects of saturated *versus* unsaturated FFA on ß-cell apoptosis is of interest from a nutritional stand point and provides a plausible explanation for various epidemiological and dietary intervention studies which have consistently led to the recommendation of a low saturated/high monounsaturated fat diet for type 2 diabetes prevention (Thanopoulou, Karamanos et al. 2003).

The experiments described in this study were all performed in an immortalized pancreatic ß-cell line and we clearly understand that there are great limitations in extrapolating the findings to human islets. In this respect, we have already started performing experiments on dissociated human islet cells *ex-vivo* and on rat islet tissue in order to verify the cell line results. The initial islet results are clearly consistent with the findings of the INS cell experiments.

The clinical importance of the islet ß-cell glucolipotoxicity concept deserves further consideration taking into account the results of the current study. Continual loss of islet ß-cell mass is almost certainly involved in the progressive deterioration in insulin secretion which occurs through both the early (e.g. conversion from impaired glucose tolerance (IGT) to diabetes) and

late stages of type 2 diabetes (e.g. secondary failure of oral hypoglycemic agents) (Butler, Janson et al. 2003; Kahn 2003). Islet ß-cell loss is also a major problem both pre- and post-transplantation in the islet replacement programs for type 1 diabetes (Contreras, Bilbao et al. 2001). Aggressive strategies to limit ß-cell loss, therefore, need to be developed and, it follows from this study, that these strategies should include the avoidance of glucolipotoxicity. Whereas reduction in glucolipotoxicity may have been a mechanism involved in the success of lifestyle modification with weight loss in some studies of type 2 diabetes prevention (Pan, Li et al. 1997; Tuomilehto, Lindstrom et al. 2001; Knowler, Barrett-Connor et al. 2002), the successful STOP-NIDDM trial (Chiasson, Josse et al. 2002) is more directly supportive of using approaches to limit glucolipotoxicity. Thus, in this study (Chiasson, Josse et al. 2002), acarbose, which primarily acts by reduction of postprandial glucose but can also reduce postprandial lipid levels (Kado, Murakami et al. 1998), was used and reduced conversion to diabetes. Our findings also point to two further approaches that show potential for reducing glucolipotoxicity. The first is dietary substitution of saturated with monounsaturated fatty acids. The second is using pharmacological tools to activate β-cell fatty acid oxidation such as activators of AMP-activated protein kinase (AMPK). Preliminary experiments performed in our laboratory using metformin and 5-amino-imidazole-4-carboxamide-1-ß-D-ribofuranoside (AICAR), both pharmacological activators of AMPK, showed protection from glucolipotoxicity. Of relevance to this latter approach, metformin has also been shown to reduce progression from IGT to diabetes (Knowler, Barrett-Connor et al. 2002).

Future Directions

To further test the hypothesis that glucose-derived malonyl-CoA and elevated cytosolic long chain acyl-CoA esters act as proapoptotic metabolic signals mediating the actions of elevated glucose and FFA on β-cell death, experiments targeting the malonyl-CoA/CPT I interaction are potentially very useful. Pharmacologic and molecular biology tools provide two approaches which could help in establishing whether increased fat esterification and reduced β-oxidation are implicated in β-cell glucolipotoxicity. The correlation of malonyl-CoA and LC-CoA determinations with apoptosis measurements will also be instrumental in determining whether malonyl-CoA and FACoA are proapoptotic metabolic signals.

To this end, we plan to alter the expression level of enzymes involved in the regulation of malonyl-CoA levels and the partitioning of lipids in the β-cell by using both a recombinant adenovirus approach and post transcriptional gene silencing (PTGS) by small interference RNAi (siRNA). In support of this approach, a recent report showed that inhibitors of FAS which caused a rise in malonyl-CoA, induced apoptosis of human breast cancer cells. Apoptosis in these cells was rescued by inhibitors of ACC which lowered malonyl-CoA levels (Pizer, Thupari et al. 2000).

1. Two enzymes tightly regulate the levels of malonyl-CoA, acetyl-CoA carboxylase (ACC) and malonyl-CoA decarboxylase (MCD).

a) ACC plays a unique role in the production of malonyl-CoA, since it is the only enzyme which produces malonyl-CoA. Glucose induced increases in ACC activity, together with increased substrate supply for glucose driven anaplerosis result in production of malonyl-CoA, a powerful inhibitor of CPT I. Glucose is involved in both short and long term activation of ACC. Short term activation is achieved by dephosphorylation of ACC (Louis and Witters

1992; Zhang and Kim 1995) and long term activation is accomplished by means of the activation of gene expression via PII promoter of ACC (Brun, Roche et al. 1993).

ACC will be targeted using siRNA. In this model, malonyl-CoA level should decrease and thus CPT I should be de-inhibited at elevated glucose levels. We expect that apoptosis induced by FFA at elevated glucose levels will thus decrease.

b) MCD has been proposed to regulate the levels of malonyl-CoA at least in non-lipogenic tissues where FAS level is low (Voilley, Roduit et al. 1999). MCD catalyzes the decarboxylation of malonyl-CoA back into acetyl-CoA. Our laboratory cloned the rat MCD cDNA from an insulin-secreting pancreatic β-cell line (INS-1) (Voilley, Roduit et al. 1999). Evidence indicating the importance of MCD in regulating malonyl-CoA levels and in supporting the glucolipotoxicity concept came from experiments done by our group (Roduit R, Prentki M, submitted). The enzyme was expressed in a tetracycline regulatable manner in INS 832/13 cells and in rat islets. MCD activity was increased more than five fold resulting in markedly reduced malonyl-CoA content under both low and high glucose conditions. This was associated with altered lipid partitioning in terms of glucose induced lipid esterification processes. The esterfication products phospholipids, diacylglycerol, and triacylglycerol were all significantly decreased under MCD overexpression at conditions of elevated glucose levels. The data from this study, confirm the presence of important links between the nutrient coupling factor malonyl-CoA and the partitioning of fatty acids to esterification products.

This model of MCD overexpression will be extended to include the role for glucose-derived malonyl-CoA and exogenous FFA via LC-CoA on β-cell toxicity. In this respect, MCD will be targeted by two approaches.

i- MCD will be overexpressed using an adenovirus in a tetracycline regulatable manner and FFA induced β-cell toxicity at elevated glucose levels will be tested as well as lipid partitioning in terms of fatty acid esterification and oxidation. We expect that under these conditions, toxicity induced by FFA at elevated glucose levels will decrease since glucose derived malonyl-CoA level will be lowered by MCD overexpression such that CPT I will be de-inhibited. Thus, we expect that FFA toxicity will be decreased because these are channeled towards oxidation away from esterification.

ii- Another way to target MCD is via PTGS using sRNAi. In this method, the MCD gene will be silenced post transcriptionally such that malonyl-CoA should accumulate to higher levels at elevated glucose levels. This will cause inhibition of CPT I such that LC-CoA will accumulate and will be channeled to esterifcation. In this model, we expect that toxicity induced by FFA at elevated glucose concentrations will further increase.

In both models, malonyl-CoA levels will be measured in order to correlate the levels of toxicity with malonyl-CoA levels.

2- **CPT I**, is a third enzyme we believe has a key role in glucolipotoxicity which we wish to target. CPT I is the rate limiting enzyme in mitochondrial β-oxidation of fatty acids. A mutant enzyme which is active but insensitive to malonyl-CoA inhibition will be overexpressed using an adenoviral construct. We expect that overexpressing this mutant CPT I will prevent malonyl-CoA inhibition of CPT I activity thus altering the partitioning of LC-CoA from esterification to mitochondrial β-oxidation particularly in situations of high glucose.

We hypothesize that increased CPT I activity at high glucose will cause increased oxidation of FFA. Thus, this detoxification step might rescue the β-cell from glucolipotoxicity.

3- *In vivo* studies are important to confirm the data obtained *in vitro*. Studies on two different animal models could be carried out:

a) The Zucker fatty (ZF) rats. These rats have elevated levels of FFA but normal glucose levels. Infusing these animals with glucose to elevate plasma concentrations (saline for control) for a period of time will be useful to monitor islet β-cell toxicity. Apoptotic assays could be performed on islets of these animals to measure β-cell toxicity and fatty acid-glucose synergy.

b) A second model would utilize high fat feeding (diets high in saturated *vs* monounsaturated fat) to rats in addition to infusing glucose to elevate plasma concentrations (saline for control) with heparin (which stimulates lipoprotein lipase (LPL) thus increasing the levels of saturated or monounsaturated FFA).

We believe that these experiments will confirm the process of glucolipotoxicity *in vivo*. Recently, our data and the glucolipotoxicity hypothesis were supported by a clinical study carried out on Pima Indians (Stefan, Stumvoll et al. 2003). This study showed deterioration in insulin secretion in subjects with the combination of elevated glucose and fatty acids but no deterioration if only glucose alone or fatty acids alone were elevated.

Other Potential targets in the control of glucolipotoxicity include PPAR-α, AMPK and GLP I.

4- PPAR-α and AMPK are other potential targets in the control of glucolipotoxicity. As mentioned earlier, glucose at high concentrations downregulates PPAR-α expression, which inturn downregulates ACO and UCP-2 expression. Thus, with hyperglycemia (post prandial or chronic), the 3 pathways of fat oxidation are reduced because of:

a) a sustained elevation in malonyl-CoA which inhibits CPT I, the key enzyme in the mitochondrial β-oxidation of FA,

b) a reduction in PPAR-α expression which causes a downregulation in acyl-CoA oxidase (ACO) which is important for peroxisomal β-oxidation and downregulation of uncoupler protein 2 (UCP2), which is important for mitochondrial uncoupled oxidation.

Experiments targeting PPAR-α and AMPK expression and activity, therefore are important in exploring the importance of lipid partitioning in glucolipotoxicity. Some such experiments are currently being carried out in our laboratory which include the use of:

i- AICAR which activates AMPK;

ii- Metformin which also activates AMPK, has in addition antioxidant properties;

iii- AMPK dominant negative and AMPK constitutively active adenoviruses.

5- The peptide glucagon like peptide 1 (GLP 1) is an incretin secreted from intestinal cells which has also been demonstrated to have an antiapoptotic activity in glucolipotoxicity models in our laboratory. This is another area which warrants further investigation.

The following scheme illustrates potential strategies to further understand the glucolipotoxicity concept (Figure 28).

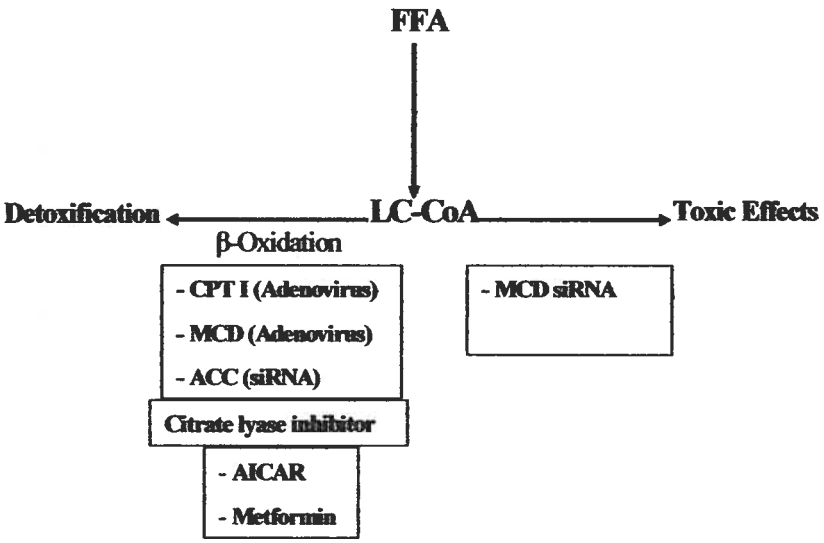


Figure 28: Planned experimental approaches to further investigate the role of malonyl-CoA and lipid partitioning in glucolipotoxicity. Molecular biological and pharmacological tools will be utilized to either increase or decrease malonyl-CoA. In addition, tools will be used to either increase fatty acid β-oxidation to detoxify LC-CoA or increase the availability of LC-CoA for toxicity (see text for discussion).

We feel that the concept of altered lipid partitioning together, with the experiments mentioned above are, instrumental in addressing glucolipotoxicity (Figure 28). This is because of the data we and others have accumulated showing that elevated levels of glucose and FFA synergize in causing TG deposition and apoptosis in β-cell. We believe that the data already obtained and presented here in addition to the proposed experiments will have important implications for prevention and treatment of type 2 diabetes.

CHAPTER 5: BIBLIOGRAPHY

Asfari, M., D. Janjic, et al. (1992). "Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines." Endocrinology **130**(1): 167-78.

Barnett, M., G. R. Collier, et al. (1992). "The longitudinal effect of inhibiting fatty acid oxidation in diabetic rats fed a high fat diet." Horm Metab Res **24**(8): 360-2.

Bergman, R. N. and S. D. Mittelman (1998). "Central role of the adipocyte in insulin resistance." J Basic Clin Physiol Pharmacol **9**(2-4): 205-21.

Bertrand, F., A. Atfi, et al. (1998). "A role for nuclear factor kappaB in the antiapoptotic function of insulin." J Biol Chem **273**(5): 2931-8.

Best, C. (2002). "The discovery of insulin: the work of Frederick Banting and Charles Best." JBC **277**(June): e15.

Bligh, E. G. and W. J. Dyer (1959). "A rapid method of total lipid extraction and purification." Can J Biochem Physiol **37**: 911-917.

Bliss, C. R. and G. W. Sharp (1992). "Glucose-induced insulin release in islets of young rats: time-dependent potentiation and effects of 2-bromostearate." Am J Physiol **263**(5 Pt 1): E890-6.

Boden, G. (1996). "Fuel metabolism in pregnancy and in gestational diabetes mellitus." Obstet Gynecol Clin North Am **23**(1): 1-10.

Boden, G. (1997). "Role of fatty acids in the pathogenesis of insulin resistance and NIDDM." Diabetes **46**: 3-10.

Boden, G., X. Chen, et al. (1998). "Acute lowering of plasma fatty acids lowers basal insulin secretion in diabetic and nondiabetic subjects." Diabetes **47**(10): 1609-12.

Boden, G., B. Lebed, et al. (2001). "Effects of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects." Diabetes **50**(7): 1612-7.

Boden, G. and G. I. Shulman (2002). "Free fatty acids in obesity and type 2 diabetes: defining their role in the development of insulin resistance and beta-cell dysfunction." Eur J Clin Invest **32 Suppl 3**: 14-23.

Bollheimer, L. C., R. H. Skelly, et al. (1998). "Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation." J Clin Invest **101**(5): 1094-101.

Bonner-Weir, S. (2000). "Islet growth and development in the adult." J Mol Endocrinol **24**(3): 297-302.

Bramblett, D. E., H. P. Huang, et al. (2000). "Pancreatic islet development." Adv Pharmacol **47**: 255-315.

Briaud, I., J. S. Harmon, et al. (2001). "Lipotoxicity of the pancreatic beta-cell is associated with glucose-dependent esterification of fatty acids into neutral lipids." Diabetes **50**(2): 315-21.

Brun, T., E. Roche, et al. (1996). "Evidence for an anaplerotic/malonyl-CoA pathway in pancreatic beta-cell nutrient signaling." Diabetes **45**(2): 190-8.

Brun, T., E. Roche, et al. (1993). "Glucose regulates acetyl-CoA carboxylase gene expression in a pancreatic beta-cell line (INS-1)." J Biol Chem **268**(25): 18905-11.

Buchanan, T. A. (2001). "Pancreatic B-cell defects in gestational diabetes: implications for the pathogenesis and prevention of type 2 diabetes." J Clin Endocrinol Metab **86**(3): 989-93.

Butler, A. E., J. Janson, et al. (2003). "Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes." Diabetes **52**(1): 102-10.

Cantley, L. C. (2002). "The phosphoinositide 3-kinase pathway." Science **296**(5573): 1655-7.

Carey, D. G., A. B. Jenkins, et al. (1996). "Abdominal fat and insulin resistance in normal and overweight women: Direct measurements reveal a strong relationship in subjects at both low and high risk of NIDDM." Diabetes **45**(5): 633-8.

Catalano, P. M., E. D. Tyzbir, et al. (1991). "Longitudinal changes in insulin release and insulin resistance in nonobese pregnant women." Am J Obstet Gynecol **165**(6 Pt 1): 1667-72.

Charles, M. A., E. Eschwege, et al. (1997). "The role of non-esterified fatty acids in the deterioration of glucose tolerance in Caucasian subjects: results of the Paris Prospective Study." Diabetologia **40**(9): 1101-6.

Chen, S., A. Ogawa, et al. (1994). "More direct evidence for a malonyl-CoA-carnitine palmitoyltransferase I interaction as a key event in pancreatic beta-cell signaling." Diabetes **43**(7): 878-83.

Chiasson, J. L., R. G. Josse, et al. (2002). "Acarbose for prevention of type 2 diabetes mellitus: the STOP-NIDDM randomised trial." Lancet **359**(9323): 2072-7.

Clark, A., C. A. Wells, et al. (1988). "Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes." Diabetes Res **9**(4): 151-9.

Cline, G. W., K. F. Petersen, et al. (1999). "Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes." N Engl J Med **341**(4): 240-6.

Cnop, M., J. C. Hannaert, et al. (2001). "Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation." Diabetes **50**(8): 1771-7.

Coleman, D. L. and K. P. Hummel (1973). "The influence of genetic background on the expression of the obese (Ob) gene in the mouse." Diabetologia **9**(4): 287-93.

Coleman, R. A., T. M. Lewin, et al. (2002). "Do long-chain acyl-CoA synthetases regulate fatty acid entry into synthetic versus degradative pathways?" J Nutr **132**(8): 2123-6.

Contreras, J. L., G. Bilbao, et al. (2001). "Cytoprotection of pancreatic islets before and soon after transplantation by gene transfer of the anti-apoptotic Bcl-2 gene." Transplantation **71**(8): 1015-23.

Coppack, S. W., M. D. Jensen, et al. (1994). "In vivo regulation of lipolysis in humans." J Lipid Res **35**(2): 177-93.

Corkey, B. E., J. T. Deeney, et al. (2000). "The role of long-chain fatty acyl-CoA esters in beta-cell signal transduction." J Nutr **130**(2S Suppl): 299S-304S.

Corkey, B. E., M. C. Glennon, et al. (1989). "A role for malonyl-CoA in glucose-stimulated insulin secretion from clonal pancreatic beta-cells." J Biol Chem **264**(36): 21608-12.

Cruz, W.S., G. Kwon, et al. (2001). "Glucose and insulin stimulate heparin-releasable lipoprotein lipase activity in mouse islets and INS-1 cells." J Biol Chem **276**(15): 12162-68

de Vries, J. E., M. M. Vork, et al. (1997). "Saturated but not mono-unsaturated fatty acids induce apoptotic cell death in neonatal rat ventricular myocytes." J Lipid Res **38**(7): 1384-94.

DeFronzo, R. A. (1988). "Lilly lecture 1987. The triumvirate: beta-cell, muscle, liver. A collusion responsible for NIDDM." Diabetes **37**(6): 667-87.

Diaz, B., B. Pimentel, et al. (1999). "Apoptotic cell death of proliferating neuroepithelial cells in the embryonic retina is prevented by insulin." Eur J Neurosci **11**(5): 1624-32.

Dobbins, R. L., M. W. Chester, et al. (1998). "Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans." Diabetes **47**(10): 1613-8.
Dresner, A., D. Laurent, et al. (1999). "Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity." J Clin Invest **103**(2): 253-9.

Efanova, I. B., S. V. Zaitsev, et al. (1998). "Glucose and tolbutamide induce apoptosis in pancreatic beta-cells. A process dependent on intracellular Ca2+ concentration." J Biol Chem **273**(50): 33501-7.

Fantl, W. J., D. E. Johnson, et al. (1993). "Signalling by receptor tyrosine kinases." Annu Rev Biochem **62**: 453-81.

Farfari, S., V. Schulz, et al. (2000). "Glucose-regulated anaplerosis and cataplerosis in pancreatic beta-cells: possible implication of a pyruvate/citrate shuttle in insulin secretion." Diabetes **49**(5): 718-26.

Finegood, D. T., L. Scaglia, et al. (1995). "Dynamics of beta-cell mass in the growing rat pancreas. Estimation with a simple mathematical model." Diabetes **44**(3): 249-56.

Freinkel, N. (1980). "Banting Lecture 1980. Of pregnancy and progeny." Diabetes **29**(12): 1023-35.

Froguel, P. and G. Velho (1999). "Molecular Genetics of Maturity-onset Diabetes of the Young." Trends Endocrinol Metab **10**(4): 142-146.

Gembal, M., P. Gilon, et al. (1992). "Evidence that glucose can control insulin release independently from its action on ATP-sensitive K+ channels in mouse B cells." J Clin Invest **89**(4): 1288-95.

Gleason, C. E., M. Gonzalez, et al. (2000). "Determinants of glucose toxicity and its reversibility in the pancreatic islet beta-cell line, HIT-T15." Am J Physiol Endocrinol Metab **279**(5): E997-1002.

Gremlich, S., C. Bonny, et al. (1997). "Fatty acids decrease IDX-1 expression in rat pancreatic islets and reduce GLUT2, glucokinase, insulin, and somatostatin levels." J Biol Chem **272**(48): 30261-9.

Grodsky, G. M. (1989). "A new phase of insulin secretion. How will it contribute to our understanding of beta-cell function?" Diabetes **38**(6): 673-8.

Guiot, Y., C. Sempoux, et al. (2001). "No decrease of the beta-cell mass in type 2 diabetic patients." Diabetes **50 Suppl 1**: S188.

Halliwell, B. and J. Gutteridge (1999). "Free radicals in biology and medicine." **3rd edition**(Oxford science publications).

Hannun, Y. A. (1996). "Functions of ceramide in coordinating cellular responses to stress." Science **274**(5294): 1855-9.
Hannun, Y. A. and C. Luberto (2000). "Ceramide in the eukaryotic stress response." Trends Cell Biol **10**(2): 73-80.

Hardy, S., Y. Langelier, et al. (2000). "Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects." Cancer Res **60**(22): 6353-8.

Hedeskov, C. J. (1980). "Mechanism of glucose-induced insulin secretion." Physiol Rev **60**(2): 442-509.

Hohmeier, H. E., H. Mulder, et al. (2000). "Isolation of INS-1-derived cell lines with robust ATP-sensitive K+ channel-dependent and -independent glucose-stimulated insulin secretion." Diabetes **49**(3): 424-30.

Ihara, Y., S. Toyokuni, et al. (1999). "Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes." Diabetes **48**(4): 927-32.

Innis, S. M. and M. T. Clandinin (1981). "Dynamic modulation of mitochondrial membrane physical properties and ATPase activity by diet lipid." Biochem J **198**(1): 167-75.

Jacqueminet, S., I. Briaud, et al. (2000). "Inhibition of insulin gene expression by long-term exposure of pancreatic beta cells to palmitate is dependent on the presence of a stimulatory glucose concentration." Metabolism **49**(4): 532-6.

Kado, S., T. Murakami, et al. (1998). "Effect of acarbose on postprandial lipid metabolism in type 2 diabetes mellitus." Diabetes Res Clin Pract **41**(1): 49-55.

Kahn, C. (2000). "Atlas of Diabetes."

Kahn, C. R. (1994). "Banting Lecture. Insulin action, diabetogenes, and the cause of type II diabetes." Diabetes **43**(8): 1066-84.

Kahn, S. E. (2003). "The relative contributions of insulin resistance and beta-cell dysfunction to the pathohysiology of type 2 diabetes." Diabetologia.

Kaneto, H., J. Fujii, et al. (1996). "Reducing sugars trigger oxidative modification and apoptosis in pancreatic beta-cells by provoking oxidative stress through the glycation reaction." Biochem J **320 (Pt 3)**: 855-63.

Kang, S., J. Song, et al. (2003). "Insulin can block apoptosis by decreasing oxidative stress via phosphatidylinositol 3-kinase- and extracellular signal-regulated protein kinase-dependent signaling pathways in HepG2 cells." Eur J Endocrinol **148**(1): 147-55.

Kim, J. H., T. M. Lewin, et al. (2001). "Expression and characterization of recombinant rat Acyl-CoA synthetases 1, 4, and 5. Selective inhibition by triacsin C and thiazolidinediones." J Biol Chem **276**(27): 24667-73.

Kloppel, G., M. Lohr, et al. (1985). "Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited." Surv Synth Pathol Res **4**(2): 110-25.

Knowler, W. C., E. Barrett-Connor, et al. (2002). "Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin." N Engl J Med **346**(6): 393-403.

Kraegen, E. W., G. J. Cooney, et al. (2001). "The role of lipids in the pathogenesis of muscle insulin resistance and beta cell failure in type II diabetes and obesity." Exp Clin Endocrinol Diabetes **109**(Suppl 2): S189-S201.

Krssak, M., K. Falk Petersen, et al. (1999). "Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study." Diabetologia **42**(1): 113-6.

Kulkarni, R. N., J. C. Bruning, et al. (1999). "Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes." Cell **96**(3): 329-39.

Leahy, J. L., S. Bonner-Weir, et al. (1988). "Minimal chronic hyperglycemia is a critical determinant of impaired insulin secretion after an incomplete pancreatectomy." J Clin Invest **81**(5): 1407-14.

Leahy, J. L., L. M. Bumbalo, et al. (1994). "Diazoxide causes recovery of beta-cell glucose responsiveness in 90% pancreatectomized diabetic rats." Diabetes **43**(2): 173-9.

Leahy, J. L., H. E. Cooper, et al. (1986). "Chronic hyperglycemia is associated with impaired glucose influence on insulin secretion. A study in normal rats using chronic in vivo glucose infusions." J Clin Invest **77**(3): 908-15.

Lee, Y., H. Hirose, et al. (1994). "Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships." Proc Natl Acad Sci U S A **91**(23): 10878-82.

Lemaire, C., K. Andreau, et al. (1998). "Inhibition of caspase activity induces a switch from apoptosis to necrosis." FEBS Lett **425**(2): 266-70.

LeRoith, D. (2002). "Beta-cell dysfunction and insulin resistance in type 2 diabetes: role of metabolic and genetic abnormalities." Am J Med **113 Suppl 6A**: 3S-11S.

Lewin, T. M., J. H. Kim, et al. (2001). "Acyl-CoA synthetase isoforms 1, 4, and 5 are present in different subcellular membranes in rat liver and can be inhibited independently." J Biol Chem **276**(27): 24674-9.
Liang, Y. and F. M. Matschinsky (1991). "Content of CoA-esters in perifused rat islets stimulated by glucose and other fuels." Diabetes **40**(3): 327-33.

Lingohr, M. K., R. Buettner, et al. (2002). "Pancreatic beta-cell growth and survival--a role in obesity-linked type 2 diabetes?" Trends Mol Med **8**(8): 375-84.

Liu, B., L. M. Obeid, et al. (1997). "Sphingomyelinases in cell regulation." Semin Cell Dev Biol **8**(3): 311-322.

Liu, Y. Q., T. L. Jetton, et al. (2002). "beta-Cell adaptation to insulin resistance. Increased pyruvate carboxylase and malate-pyruvate shuttle activity in islets of nondiabetic Zucker fatty rats." J Biol Chem **277**(42): 39163-8.

Louis, N. A. and L. A. Witters (1992). "Glucose regulation of acetyl-CoA carboxylase in hepatoma and islet cells." J Biol Chem **267**(4): 2287-93.

Lupi, R., F. Dotta, et al. (2002). "Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated." Diabetes **51**(5): 1437-42.

Maedler, K., G. A. Spinas, et al. (2001). "Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function." Diabetes **50**(1): 69-76.

Malaisse, W. J., A. Sener, et al. (1979). "Insulin release: the fuel hypothesis." Metabolism **28**(4): 373-86.

Matsuoka, T., Y. Kajimoto, et al. (1997). "Glycation-dependent, reactive oxygen species-mediated suppression of the insulin gene promoter activity in HIT cells." J Clin Invest **99**(1): 144-50.

McGarry, J. D. (1992). "What if Minkowski had been ageusic? An alternative angle on diabetes." Science **258**(5083): 766-70.

McGarry, J. D. and N. F. Brown (1997). "The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis." Eur J Biochem **244**(1): 1-14.

McGarry, J. D. and R. L. Dobbins (1999). "Fatty acids, lipotoxicity and insulin secretion." Diabetologia **42**(2): 128-38.

McGarry, J. D. and D. W. Foster (1980). "Regulation of hepatic fatty acid oxidation and ketone body production." Annu Rev Biochem **49**: 395-420.

McGarry, J. D., G. F. Leatherman, et al. (1978). "Carnitine palmitoyltransferase I. The site of inhibition of hepatic fatty acid oxidation by malonyl-CoA." J Biol Chem **253**(12): 4128-36.

McGarry, J. D., G. P. Mannaerts, et al. (1977). "A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis." J Clin Invest **60**(1): 265-70.

McGarry, J. D., K. F. Woeltje, et al. (1989). "Regulation of ketogenesis and the renaissance of carnitine palmitoyltransferase." Diabetes Metab Rev **5**(3): 271-84.

Merrill, G. F., E. J. Kurth, et al. (1998). "Influence of malonyl-CoA and palmitate concentration on rate of palmitate oxidation in rat muscle." J Appl Physiol **85**(5): 1909-14.

Mokdad, A. H., E. S. Ford, et al. (2003). "Prevalence of obesity, diabetes, and obesity-related health risk factors, 2001." Jama **289**(1): 76-9.

Moran, A., H. J. Zhang, et al. (1997). "Differentiation of glucose toxicity from beta cell exhaustion during the evolution of defective insulin gene expression in the pancreatic islet cell line, HIT-T15." J Clin Invest **99**(3): 534-9.

Noel, R. J., P. A. Antinozzi, et al. (1997). "Engineering of glycerol-stimulated insulin secretion in islet beta cells. Differential metabolic fates of glucose and glycerol provide insight into mechanisms of stimulus-secretion coupling." J Biol Chem **272**(30): 18621-7.

Okazaki, T., A. Bielawska, et al. (1990). "Role of ceramide as a lipid mediator of 1 alpha,25-dihydroxyvitamin D3-induced HL-60 cell differentiation." J Biol Chem **265**(26): 15823-31.

Olson, L. K., J. B. Redmon, et al. (1993). "Chronic exposure of HIT cells to high glucose concentrations paradoxically decreases insulin gene transcription and alters binding of insulin gene regulatory protein." J Clin Invest **92**(1): 514-9.

Olson, L. K., A. Sharma, et al. (1995). "Reduction of insulin gene transcription in HIT-T15 beta cells chronically exposed to a supraphysiologic glucose concentration is associated with loss of STF-1 transcription factor expression." Proc Natl Acad Sci U S A **92**(20): 9127-31.

O'Rahilly, S. (1997). "Science, medicine, and the future. Non-insulin dependent diabetes mellitus: the gathering storm." Bmj **314**(7085): 955-9.

Ostrander, D. B., G. C. Sparagna, et al. (2001). "Decreased cardiolipin synthesis corresponds with cytochrome c release in palmitate-induced cardiomyocyte apoptosis." J Biol Chem **276**(41): 38061-7.

Pan, X. R., G. W. Li, et al. (1997). "Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study." Diabetes Care **20**(4): 537-44.

Paolisso, G., P. A. Tataranni, et al. (1995). "A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM." Diabetologia **38**(10): 1213-7.

Perseghin, G., P. Scifo, et al. (1999). "Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents." Diabetes **48**(8): 1600-6.

Pertseva, M. N., A. O. Shpakov, et al. (2003). "A novel view on the mechanisms of action of insulin and other insulin superfamily peptides: involvement of adenylyl cyclase signaling system." Comp Biochem Physiol B Biochem Mol Biol **134**(1): 11-36.

Pick, A., J. Clark, et al. (1998). "Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat." Diabetes **47**(3): 358-64.

Pizer, E. S., J. Thupari, et al. (2000). "Malonyl-coenzyme-A is a potential mediator of cytotoxicity induced by fatty-acid synthase inhibition in human breast cancer cells and xenografts." Cancer Res **60**(2): 213-8.

Poitout, V., L. K. Olson, et al. (1996). "Chronic exposure of betaTC-6 cells to supraphysiologic concentrations of glucose decreases binding of the RIPE3b1 insulin gene transcription activator." J Clin Invest **97**(4): 1041-6.

Poitout, V. and R. P. Robertson (1996). "An integrated view of beta-cell dysfunction in type-II diabetes." Annu Rev Med **47**: 69-83.

Poitout, V. and R. P. Robertson (2002). "Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity." Endocrinology **143**(2): 339-42.

Polonsky, K. S., J. Sturis, et al. (1996). "Seminars in Medicine of the Beth Israel Hospital, Boston. Non-insulin-dependent diabetes mellitus - a genetically programmed failure of the beta cell to compensate for insulin resistance." N Engl J Med **334**(12): 777-83.

Porte, D., Jr. (1991). "Banting lecture 1990. Beta-cells in type II diabetes mellitus." Diabetes **40**(2): 166-80.

Porte, D., Jr. and A. A. Pupo (1969). "Insulin responses to glucose: evidence for a two pool system in man." J Clin Invest **48**(12): 2309-19.

Preiss, J., C. R. Loomis, et al. (1986). "Quantitative measurement of sn-1,2-diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells." J Biol Chem **261**(19): 8597-600.

Prentki, M. (1996). "New insights into pancreatic beta-cell metabolic signaling in insulin secretion." Eur J Endocrinol **134**(3): 272-86.

Prentki, M. and B. E. Corkey (1996). "Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM?" Diabetes **45**(3): 273-83.

Prentki, M., E. Joly, et al. (2002). "Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes." Diabetes **51 Suppl 3**: S405-13.

Prentki, M. and F. M. Matschinsky (1987). "Ca2+, cAMP, and phospholipid-derived messengers in coupling mechanisms of insulin secretion." Physiol Rev **67**(4): 1185-248.

Prentki, M., R. Roduit, et al. (2001). "Glucotoxicity, lipotoxicity and pancreatic beta cell failure: a role for malonyl-CoA, PPAR alpha and altered lipid partitioning." Can J Diabetes Care **25**(36-46).

Prentki, M., L. Segall, et al. (1998). "[Gluco-lipotoxicity and gene expression in the pancreatic beta cell]." Journ Annu Diabetol Hotel Dieu: 17-27.

Prentki, M., S. Vischer, et al. (1992). "Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion." J Biol Chem **267**(9): 5802-10.

Rampalli, A. M. and P. S. Zelenka (1995). "Insulin regulates expression of c-fos and c-jun and suppresses apoptosis of lens epithelial cells." Cell Growth Differ **6**(8): 945-53.

Reaven, G. M. (1988). "Banting lecture 1988. Role of insulin resistance in human disease." Diabetes **37**(12): 1595-607.

Reaven, G. M., C. Hollenbeck, et al. (1988). "Measurement of plasma glucose, free fatty acid, lactate, and insulin for 24 h in patients with NIDDM." Diabetes **37**(8): 1020-4.

Ritz-Laser, B., P. Meda, et al. (1999). "Glucose-induced preproinsulin gene expression is inhibited by the free fatty acid palmitate." Endocrinology **140**(9): 4005-14.

Roche, E., J. Buteau, et al. (1999). "Palmitate and oleate induce the immediate-early response genes c-fos and nur-77 in the pancreatic beta-cell line INS-1." Diabetes **48**(10): 2007-14.

Roche, E., S. Farfari, et al. (1998). "Long-term exposure of beta-INS cells to high glucose concentrations increases anaplerosis, lipogenesis, and lipogenic gene expression." Diabetes **47**(7): 1086-94.

Roduit, R., J. Morin, et al. (2000). "Glucose down-regulates the expression of the peroxisome proliferator-activated receptor-alpha gene in the pancreatic beta -cell." J Biol Chem **275**(46): 35799-806.

Rossetti, L., G. I. Shulman, et al. (1987). "Effect of chronic hyperglycemia on in vivo insulin secretion in partially pancreatectomized rats." J Clin Invest **80**(4): 1037-44.

Ruderman, N. B., A. K. Saha, et al. (1999). "Malonyl-CoA, fuel sensing, and insulin resistance." Am J Physiol **276**(1 Pt 1): E1-E18.

Saddik, M., J. Gamble, et al. (1993). "Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart." J Biol Chem **268**(34): 25836-45.

Saha, A. K., D. Vavvas, et al. (1997). "Malonyl-CoA regulation in skeletal muscle: its link to cell citrate and the glucose-fatty acid cycle." Am J Physiol **272**(4 Pt 1): E641-8.

Sako, Y. and V. E. Grill (1990). "A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation." Endocrinology **127**(4): 1580-9.

Sako, Y. and V. E. Grill (1990). "Coupling of beta-cell desensitization by hyperglycemia to excessive stimulation and circulating insulin in glucose-infused rats." Diabetes **39**(12): 1580-3.

Sakuraba, H., H. Mizukami, et al. (2002). "Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients." Diabetologia **45**(1): 85-96.

Sane, A. T. and R. Bertrand (1999). "Caspase inhibition in camptothecin-treated U-937 cells is coupled with a shift from apoptosis to transient G1 arrest followed by necrotic cell death." Cancer Res **59**(15): 3565-9.

Sato, Y., T. Aizawa, et al. (1992). "Dual functional role of membrane depolarization/Ca2+ influx in rat pancreatic B-cell." Diabetes **41**(4): 438-43.

Sharma, A., L. K. Olson, et al. (1995). "The reduction of insulin gene transcription in HIT-T15 beta cells chronically exposed to high glucose concentration is associated with the loss of RIPE3b1 and STF-1 transcription factor expression." Mol Endocrinol **9**(9): 1127-34.

Shimabukuro, M., M. Higa, et al. (1998). "Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression." J Biol Chem **273**(49): 32487-90.

Shimabukuro, M., Y. T. Zhou, et al. (1998). "Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes." Proc Natl Acad Sci U S A **95**(5): 2498-502.

Shulman, G. I. (2000). "Cellular mechanisms of insulin resistance." J Clin Invest **106**(2): 171-6.

Sorenson, R. L. and T. C. Brelje (1997). "Adaptation of islets of Langerhans to pregnancy: beta-cell growth, enhanced insulin secretion and the role of lactogenic hormones." Horm Metab Res **29**(6): 301-7.

Stefan, N., M. Stumvoll, et al. (2003). "Elevated Plasma Non-Esterified Fatty Acids are Associated with Deterioration of Acute Insulin Response in IGT but not NGT." Am J Physiol Endocrinol Metab.

Stefan, Y., L. Orci, et al. (1982). "Quantitation of endocrine cell content in the pancreas of nondiabetic and diabetic humans." Diabetes **31**(8 Pt 1): 694-700.

Steiner, D. F., Y. Rouille, et al. (1996). "The role of prohormone convertases in insulin biosynthesis: evidence for inherited defects in their action in man and experimental animals." Diabetes Metab **22**(2): 94-104.

Straub, S. G., R. F. James, et al. (1998). "Glucose activates both K(ATP) channel-dependent and K(ATP) channel-independent signaling pathways in human islets." Diabetes **47**(5): 758-63.

Stubbs, C. D. and A. D. Smith (1990). "Essential fatty acids in membrane: physical properties and function." Biochem Soc Trans **18**(5): 779-81.

Tajiri, Y., C. Moller, et al. (1997). "Long-term effects of aminoguanidine on insulin release and biosynthesis: evidence that the formation of advanced glycosylation end products inhibits B cell function." Endocrinology **138**(1): 273-80.

Tamarit-Rodriguez, J., E. Vara, et al. (1984). "Starvation-induced changes of palmitate metabolism and insulin secretion in isolated rat islets stimulated by glucose." Biochem J **221**(2): 317-24.

Tanaka, Y., C. E. Gleason, et al. (1999). "Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants." Proc Natl Acad Sci U S A **96**(19): 10857-62.

Thanopoulou, A. C., B. G. Karamanos, et al. (2003). "Dietary Fat Intake as Risk Factor for the Development of Diabetes: Multinational, multicenter study of the Mediterranean Group for the Study of Diabetes (MGSD)." Diabetes Care **26**(2): 302-307.

Tuomilehto, J., J. Lindstrom, et al. (2001). "Prevention of type 2 diabetes mellitus by changes in lifestyle among subjects with impaired glucose tolerance." N Engl J Med **344**(18): 1343-50.

Unger, R. H. (1995). "Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications." Diabetes **44**(8): 863-70.

Unger, R. H., Y. T. Zhou, et al. (1999). "Regulation of fatty acid homeostasis in cells: novel role of leptin." Proc Natl Acad Sci U S A **96**(5): 2327-32.

van Haeften, T. W. (2002). "Early disturbances in insulin secretion in the development of type 2 diabetes mellitus." Mol Cell Endocrinol **197**(1-2): 197-204.

Vander, A., J. Sherman, et al. (1990). "Human physiology text book." **5th edition**.

Vara, E., O. Fernandez-Martin, et al. (1988). "Palmitate dependence of insulin secretion, "de novo" phospholipid synthesis and 45Ca2+-turnover in glucose stimulated rat islets." Diabetologia **31**(9): 687-93.

Voilley, N., R. Roduit, et al. (1999). "Cloning and expression of rat pancreatic beta-cell malonyl-CoA decarboxylase." Biochem J **340 (Pt 1)**: 213-7.

Wang, Z. W., W. T. Pan, et al. (2001). "The role of leptin resistance in the lipid abnormalities of aging." Faseb J **15**(1): 108-114.

Wei, M., S. P. Gaskill, et al. (1997). "Waist circumference as the best predictor of noninsulin dependent diabetes mellitus (NIDDM) compared to body mass index, waist/hip ratio and other anthropometric measurements in Mexican Americans--a 7-year prospective study." Obes Res **5**(1): 16-23.

Withers, D. J., J. S. Gutierrez, et al. (1998). "Disruption of IRS-2 causes type 2 diabetes in mice." Nature **391**(6670): 900-4.

Wobser, H., H. Dussmann, et al. (2002). "Dominant-negative suppression of HNF-1 alpha results in mitochondrial dysfunction, INS-1 cell apoptosis, and increased sensitivity to ceramide-, but not to high glucose-induced cell death." J Biol Chem **277**(8): 6413-21.

Wollheim, C. B. and G. W. Sharp (1981). "Regulation of insulin release by calcium." Physiol Rev **61**(4): 914-73.

Wrede, C. E., L. M. Dickson, et al. (2002). "Protein Kinase B/Akt Prevents Fatty Acid-induced Apoptosis in Pancreatic beta -Cells (INS-1)." J Biol Chem **277**(51): 49676-84.

Zhang, S. and K. H. Kim (1995). "Glucose activation of acetyl-CoA carboxylase in association with insulin secretion in a pancreatic beta-cell line." J Endocrinol **147**(1): 33-41.

Zhou, Y. P. and V. Grill (1995). "Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans." J Clin Endocrinol Metab **80**(5): 1584-90.

Zhou, Y. P., Z. C. Ling, et al. (1996). "Inhibitory effects of fatty acids on glucose-regulated B-cell function: association with increased islet triglyceride stores and altered effect of fatty acid oxidation on glucose metabolism." Metabolism **45**(8): 981-6.

Zimmet, P., K. G. Alberti, et al. (2001). "Global and societal implications of the diabetes epidemic." Nature **414**(6865): 782-7.

Zimmet, P. Z. (1999). "Diabetes epidemiology as a tool to trigger diabetes research and care." Diabetologia **42**(5): 499-518.

Zou, H., Y. Li, et al. (1999). "An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9." J Biol Chem **274**(17): 11549-56.