MECHANISMS OF FATTY ACID INDUCED DECREASE IN \( \beta \)-CELL FUNCTION

By

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science

University of Toronto

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General Abstract

An important mechanism involved in the pathogenesis of type 2 diabetes is elevation of plasma free fatty acids which induce insulin resistance and may impair both β-cell function and mass (β-cell lipotoxicity). The objective of my thesis was to investigate the role of oxidative stress in β-cell lipotoxicity, using in vivo, ex vivo, and in vitro models.

I used in vivo models of 48h i.v. oleate or olive oil infusion in Wistar rats followed by hyperglycemic clamps, or islet secretion studies ex vivo, and in vitro models of 48h exposure to oleate in isolated islets.

My first study showed that 48h oleate infusion decreased the insulin response to a hyperglycemic clamp, an effect prevented by coinfusion of the antioxidants N-acetylcysteine and taurine. Similar to the findings in vivo, 48h infusion of oleate decreased glucose stimulated insulin secretion (GSIS) ex vivo, and induced oxidative stress in isolated islets, effects prevented by coinfusion of the antioxidants N-acetylcysteine, taurine, or tempol. Islets exposed to oleate or palmitate showed a decreased insulin response to high glucose and increased levels of oxidative stress, effects prevented by taurine. Therefore, my data are the first demonstration that oxidative
stress plays a role in the decrease in β-cell secretory function induced by prolonged exposure to FFA, in vitro and in vivo.

My second study addressed downstream effects of oxidative stress involving inflammation. A 48h infusion of oleate or olive oil decreased β-cell function during a hyperglycemic clamp, an effect prevented by coinfusion of the IKKβ inhibitor salicylate. GSIS in isolated islets was impaired by olive oil or oleate and restored by salicylate. These results suggest a potential role for both oxidative stress and inflammation in lipid-induced β-cell dysfunction.

My third study addressed downstream effects of oxidative stress involving β-cell insulin signalling. A 48h infusion of oleate or olive oil decreased β-cell function during a hyperglycemic clamp, an effect prevented by coinfusion of the tyrosine phosphatase inhibitor bisperoxovanadate. GSIS in isolated islets was impaired by olive oil or oleate and restored by bisperoxovanadate, suggesting a role of FFA in decreasing β-cell function by induction of β-cell insulin resistance.
The amount of energy, both physical and intellectual, that one puts into a PhD project, is enormous, and rather hidden behind the final product, which is the thesis. This effort is most of the time only understood by those close to the author, throughout his struggle. And it is for this very special reason that I would like to acknowledge all those involved with my project!

For sure, I will start by saying a great “Thank you!” to my supervisor, Dr. Adria Giacca, without your care and patience, none of this would have ever happened! You are a true Professor, and mentor!

Second, I need to give a lot of recognition to my former mentor, Professor Nurit Kaiser, who was too kind and understanding, to help me find the way toward my desired career! A big Thank you! to my former co-supervisor and friend, Dr. Gil Leibowitz. You both shaped my first steps in research, and this is something so great, that will never be forgotten!

To the members of my supervisory committee, Dr. Gary Lewis, Dr. George Fantus, Dr. Michael Wheeler, as well as to my examining committee members, Dr. Allan Volchuk, Dr. Mladen Vranic, Dr. Minna Woo, Dr. Carol Greenwood and Professor Vincent Poitout, I would like to say Thank you!, for your time and patience during my research years here in Toronto, and for a great number of advises regarding my small steps in this career! Your help and kindness are greatly appreciated!

I would like to bring my recognition to my beloved wife, Sînziana Oprescu, who is now only at the beginning of this great battle, called Medicine. I want to say thank you,
for everything you mean to me, for everything you did, as part of the lab, or outside, to support a science-obsessed full-time graduate student, and sometimes, part-time husband!

Obviously, my parents would come next, Stelian and Elena Oprescu, as well as my grandparents, Gheorghe and Maria Costescu, you all wanted me so much to be a doctor, and I have always promised you, that I will try to become more than that! I hope I am now, one more step toward that ideal!

I will not forget the wonderful people in the Institute of Medical Science, who were amazingly helpful with all the matters required by my PhD examinations throughout the years, Thank you, Ms. Hazel Pollard, Ms. Josie Chapman, and a big, big Thank you! to Ms. Dianne Fukunaga!

This few pages should become another manuscript, if I am to say thank you to all those who helped and contributed to my success, but it is very important for me, to say the names of my co-workers and more important, friends, Loretta Lam, there would be no thesis without your support and knowledge! Ed Park, Jiwan Dhaliwall, Christine Tang, Khajag Koulaian, Danna Breen, Sandra Pereira, thank you to all of you, for the wonderful part of the time spent in the lab! And for sure, I will not forget the help from my project students throughout the years, such as Tsveta Klimentova, Alexandra Mardimae, Kattie Chum, Sicong Li, to name only a few.

To all of you, one more time, a great, big, Thank you!, and all my best wishes for your life, and career!
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<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
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<tr>
<td>BPV</td>
<td>Bisperoxovanadium</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CON</td>
<td>Control</td>
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<td>CPT-1</td>
<td>Carnitine Palmitoyl Transferase-1</td>
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<td>Cyclooxygenase-1</td>
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<td>COX2</td>
<td>Cyclooxygenase-2</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DCFH-DA</td>
<td>Dichlorofluorescein Diacetate</td>
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<td>DI</td>
<td>Disposition Index</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>eIF2</td>
<td>Eukaryotic initiation factor 2</td>
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<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FFA</td>
<td>Free fatty acids</td>
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<td>FoxO-1</td>
<td>Forkhead transcription factor 1</td>
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<td>GABA</td>
<td>Gamma Aminobutyric Acid</td>
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<td>GINF</td>
<td>Glucose infusion</td>
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<td>GLP-1</td>
<td>Glucagon-like peptide</td>
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<td>GLUT</td>
<td>Glucose transporter</td>
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<td>GPR</td>
<td>G protein-coupled receptor</td>
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<td>GPx-1</td>
<td>Glucagon Like Peptide</td>
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<td>GSIS</td>
<td>Glucose-stimulated insulin secretion</td>
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<td>H-NAC</td>
<td>High concentration of N-acetyl-L-cysteine</td>
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<td>HO-1</td>
<td>Hemoxygenase 1</td>
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<td>HRP</td>
<td>Horseradish Peroxidase</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>IGT</td>
<td>Impaired glucose tolerance</td>
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<td>IKK</td>
<td>IkBα kinase</td>
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<td>IL-6</td>
<td>Interleukin 6</td>
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<td>iNOS</td>
<td>Inducible Macrophage Type Nitric-oxide Synthase</td>
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<td>IkBα</td>
<td>Inhibitor of κBα</td>
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<td>IRS</td>
<td>Insulin receptor substrate</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<td>JNK</td>
<td>c-Jun NH₂-terminal kinase</td>
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<tr>
<td>KRB</td>
<td>Krebs Ringer buffer</td>
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<tr>
<td>KRB-H</td>
<td>Krebs Ringer buffer-Heppes</td>
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<tr>
<td>LC-CoA</td>
<td>Long-chain coenzyme A</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>L-NAC</td>
<td>Low concentration of N-acetyl-L-cysteine</td>
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<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>MEHA</td>
<td>4-Methyl-Histamine</td>
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<td>M/I</td>
<td>Sensitivity index</td>
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<td>Mito-SOX</td>
<td>Mitochondrial superoxide dye</td>
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<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acids</td>
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<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<td>NAD</td>
<td>Beta-Nicotinamide Adenine Dinucleotide</td>
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<td>NADH</td>
<td>1,4-Dihydronicotinamide Adenine Dinucleotide</td>
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<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate Hydrogen</td>
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<td>NFK-B</td>
<td>Nuclear factor κB</td>
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<td>OB-R</td>
<td>Leptin receptor</td>
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<td>OLE</td>
<td>Oleate</td>
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<td>OLO</td>
<td>Olive Oil</td>
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<td>PDX-1</td>
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<td>PI3K</td>
<td>Phosphatidylinositol 3 kinase</td>
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<td>PKB</td>
<td>Protein kinase B</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>POD</td>
<td>Peroxidase</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyl serine</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein tyrosine phosphatase</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
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<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute Medium</td>
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<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>SAL</td>
<td>Saline</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
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<td>SFA</td>
<td>Saturated Fatty Acid</td>
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<td>SH2</td>
<td>Src homology 2</td>
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<td>Suppressor of cytokine signaling 3</td>
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<td>SOD</td>
<td>Superoxide dismutase</td>
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<td>SREBP-1</td>
<td>Sterol regulatory element-binding protein 1</td>
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<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
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<tr>
<td>TAU</td>
<td>Taurine</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<td>TEMPOL</td>
<td>4-Hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl</td>
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<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>TPO</td>
<td>Thermoxidized Palm Oil</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinediones</td>
</tr>
<tr>
<td>UCP</td>
<td>Uncoupling protein</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker Diabetic Fatty</td>
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Introduction

Diabetes mellitus is an *important* public health concern that is said to be “one of the main threats to human health in the 21st century” (1). Many have referred to the increase in the prevalence of diabetes as an epidemic. A survey of the U.S. Center for Disease Control and Prevention found that in 2002 6.3% of U.S. population had diagnosed diabetes (all types) with the greatest prevalence in individuals older than the age of 65 (2). The prevalence of diabetes appears to be similar in other Western industrialized nations, including Canada (3). It has been predicted that over the next 25 years, the global prevalence of diabetes will increase by 35%, with the greatest increase being in the developing world (3).

Diabetes mellitus describes a heterogeneous group of metabolic disorders characterized clinically and metabolically by elevated blood glucose levels (hyperglycemia). These features result from the low effect of insulin, either because of
insulin deficiency alone (type 1 diabetes mellitus), or because of defective insulin secretion in relation to a decrease in insulin sensitivity (type 2 diabetes mellitus). Hyperglycemia arises from a defect in glucose homeostasis. Plasma glucose levels are regulated by: i) acute and sustained insulin secretion; ii) inhibitory effect of insulin on hepatic glucose production and stimulatory effect of insulin on hepatic glucose utilization (hepatic insulin sensitivity); iii) stimulatory effect of insulin on glucose uptake by muscle and fat (peripheral insulin sensitivity). Alterations in insulin secretion, or alterations in hepatic and/or peripheral insulin sensitivity when uncompensated by a corresponding increase in insulin secretion will result in abnormal glucose homeostasis. In diabetes, there are concomitant defects in both amino acid and lipid metabolism that also result from the low effect of insulin.

Diabetes leads to premature morbidity and mortality. The overall age-adjusted mortality rate in diabetic adults is approximately twice that in non-diabetic individuals (4). Acidosis and coma are acute complications that are the result of uncontrolled diabetes mellitus. Nowadays, the greatest morbidity and mortality in diabetic individuals stems from chronic complications such as macrovascular, microvascular, and neuropathic diseases. Aggressive treatment of diabetes has been shown to delay the onset of these complications (5).

The most common forms of diabetes mellitus fall into two broad categories, each characterized by a different etiology and pathology. The first, known as type 1 diabetes mellitus, typically develops early in life and is characterized by a tendency to ketoacidosis and dependence on insulin for survival. The second form, type 2 diabetes, is a much more prevalent form of diabetes and is characterized by the absence of an
absolute dependence on insulin for survival and a lesser likelihood of developing ketoacidosis.
1.1. Type 2 Diabetes Mellitus

Type 2 diabetes mellitus is the most common form of diabetes, accounting for approximately 90% of all cases of this disease. This form of the disease affects between 5 and 20 % of the population in Western industrialized countries (6). Type 2 diabetes is characterized by defects both in insulin sensitivity and in insulin secretion. The exact cause and pathogenesis of type 2 diabetes is not well understood, but its development has been linked both to genetic and environmental factors. Studies conducted in monozygotic twins demonstrate a concordance rate of nearly 100%, suggesting a strong genetic background for this disease (7). Type 2 diabetes is known to be a polygenic disease with complex inheritance patterns. Despite longstanding investigations, the precise genes that lead to the development of the disease have yet to be identified, although a number of candidates were shown by recent studies to be associated with a high risk of disease (8).

Available epidemiological evidence suggests a strong relationship between type 2 diabetes and obesity. It has been estimated that 85% of type 2 diabetic individuals are also obese (9). In particular, upper body (central) obesity is closely linked to insulin resistance and type 2 diabetes mellitus (10).

1.1.1. Type 2 Diabetes, Obesity and Insulin Resistance

Insulin mediates its cellular effects through a series of well-defined steps. Stimulation of insulin action requires that the hormone must first bind to specific
receptors present on the cell surface of all insulin-target tissues. The insulin receptor is a heterotetrameric receptor tyrosine kinase. Upon binding insulin, the receptor undergoes autophosphorylation on specific tyrosine residues located on the intracellular domain of the β subunit, which activates the kinase activity of the receptor. Once activated, the insulin receptor phosphorylates the tyrosine moiety on a number of intracellular substrates, including members of the insulin receptor substrate family (IRS-1/2/3/4), the Shc adaptor protein, Gab-1, and Cbl.

It has been well established that obesity is associated with insulin resistance. One mechanism that explains this association is that fatty acids, which are often elevated in obesity, mainly because of release from an expanded adipose tissue mass, induce intracellular alterations that impair insulin sensitivity. In the 1960’s, Randle and his colleagues proposed that an increase in free fatty acids (FFA) induce a state of insulin resistance in cardiac and skeletal muscle cells by causing a decrease in glucose metabolism via a fatty acid/glucose inhibitory cycle, by inhibiting glycolytic usage of glucose, with subsequent derangements of glucose transport and glycogen synthesis. More recently, fatty acids have been suggested to impair insulin action by activating intracellular protein kinase C (PKC) enzymes (11;12), which phosphorylate serine residues on the insulin receptor and IRS, thereby inhibiting their tyrosine phosphorylation. These changes in phosphorylation disrupt the insulin signaling cascade. Furthermore, it has been suggested that FFA may impair insulin sensitivity by stimulating the production of ceramides and also of free radicals (13;14). These act in part via activation of proinflammatory stress-activated kinases with similar effects on the insulin signaling cascade as PKC. Findings from a recent study suggest that a predisposition to
develop type 2 diabetes mellitus, such as a strong family history, may be important in determining whether FFA alter insulin sensitivity (15). Experimental data from this paper showed that a 4-day physiological increase in plasma FFA to levels seen in obesity and patients with type 2 diabetes impairs insulin action/insulin signaling in control subjects, but does not worsen preexisting insulin resistance in normal glucose tolerant subjects with a strong family history of type 2 diabetes. This may be due to preexisting lipotoxicity or to other mechanisms of insulin resistance.

In addition to being an energy storehouse, adipose tissue has been shown to be a secretory organ. Adipocytes secrete a number of cytokines, including TNFα. The levels of this particular cytokine correlate with the adipose tissue mass. TNFα has been found to reduce insulin sensitivity (16), and therefore this cytokine may serve as a mediator of obesity induced insulin resistance. TNF-α induces insulin resistance by promoting serine phosphorylation of the insulin receptor and IRS-1, which inhibits the tyrosine phosphorylation (and therefore activation) of these key molecules in the insulin signalling cascade (17). TNF-α induces serine phosphorylation of IRS-1 through the activation of the inhibitor kappa B kinase (IKK) complex (18) and through the activation of JNK (19).

The cytokine interleukin-6 (IL-6) is also secreted by adipocytes and has been associated with obesity induced insulin resistance (20). A recent in vivo study in mice showed that chronic (5 day) IL-6 treatment selectively impaired hepatic insulin signaling through a mechanism that may involve the induction of suppressor of cytokine signalling-3 (SOCS-3) (21;22). SOCS-3 can suppress insulin-stimulated receptor autophosphorylation and IRS-1 tyrosine phosphorylation in the liver (22), possibly by binding to the tyrosine (960) residue of insulin receptor and interfering with the association of IRS with the insulin
receptor (23). SOCS can also mediate specific degradation of IRS-1 and 2 proteins via the elongin BC ubiquitin-ligase complex in multiple cell types including hepatocytes (24).

Several other factors secreted by adipocytes have also been found to impair insulin sensitivity. A peptide secreted by adipocytes known as resistin has also been suggested to play a role in linking obesity, insulin resistance, and diabetes. In a study published in 2001, resistin was shown to induce insulin resistance both in vivo in mice, and in vitro in 3T3-L1 adipocytes (25). The precise mechanisms by which resistin induces insulin resistance have yet to be determined, but appear to include decreased AMP-activated kinase (AMPK) activity (26).

In addition to secreting molecules that impair insulin sensitivity, adipose tissue has also been shown to synthesize and secrete molecules that enhance insulin action. The fat-derived hormones leptin (27) and adiponectin (28) are known to enhance insulin sensitivity. Leptin, a product of the \textit{ob} gene, is a hormone secreted in proportion to the degree of obesity (29) and is strongly correlated with insulin sensitivity. Leptin binds to OB-R receptors, which belong to the class 1 cytokine receptor family and are ubiquitously distributed. Binding of leptin to its receptor activates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signal transduction pathway, leading to its numerous functions. There is considerable amount of crosstalk between the leptin signaling and other signaling pathways, including insulin-stimulated phosphatidylinositol 3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling (30). Leptin has been shown to enhance insulin sensitivity by depleting the intracellular lipid stores, which are known to impair insulin action (31), by activating AMPK (32). Humans with rare leptin deficiency or leptin receptor mutations are severely obese (33). Similarly, two of the most studied animal models of obesity, \textit{ob/ob} and \textit{db/db}
mice, have a genetic defect in leptin hormone and leptin receptor, respectively. These mice become obese due to excessive food intake and reduced energy expenditure. As a result, they develop severe insulin resistance and eventually diabetes (34). In addition to leptin’s role as a satiety hormone that regulates food intake and energy balance via its actions in ventromedial hypothalamus (35;36), leptin also has direct insulin-sensitizing effects on peripheral tissues (37-39).

Adiponectin (Acrp 30) is a 30kDa hormone secreted mainly by adipocytes. Two receptor forms have been cloned for adiponectin: AdipoR1, which is a high affinity receptor for a putative proteolytic fragment of adiponectin containing globular terminal domain but with very low affinity for full-length adiponectin and AdipoR2, which has intermediate affinity for both forms of adiponectin (40). AdipoR1 is abundantly expressed in skeletal muscle and at moderate levels in other tissues, whereas AdipoR2 is predominantly expressed in the liver. The complete signaling pathway for adiponectin remains to be elucidated but a docking protein called adaptor protein containing PH domain 1 mediates adiponectin-induced activation of AMPK (41;42).

Expression and circulating levels of adiponectin are decreased in obese humans and mice, and correlate strongly with insulin sensitivity (43). Mice deficient in adiponectin are insulin resistant (44) and, conversely, an adiponectin administration to obese, insulin resistant mice improves insulin sensitivity (45-47). Adiponectin increases glucose utilization by fat and muscle, where AdipoR1 is predominantly expressed (48). In the liver where AdipoR2 is mainly expressed, adiponectin acts to enhance suppression of hepatic glucose production (45). Adiponectin also appears to have central effects on the
regulation of body weight, as intracerebroventricular administration of the hormone increases energy expenditure and decreases body weight of mice (49).

1.1.2. Pathophysiology of Type 2 Diabetes Mellitus

As previously described, the pathophysiology of type 2 diabetes is multi-factorial, featuring insulin resistance (both peripheral and hepatic), and impaired β-cell function. Prospective studies, which follow patients from normal glucose tolerance, through impaired glucose tolerance (IGT), to the onset of type 2 diabetes mellitus, have shown that insulin resistance may be the earliest defect associated with the disease (50). However, IGT already features β-cell dysfunction (51;52). Insulin secretion is increased in an attempt to maintain normal glucose homeostasis. Typically in a normal individual, insulin secretion increases to perfectly compensate for a defect in insulin sensitivity. However, in individuals predisposed to develop type 2 diabetes, insulin secretion fails to compensate for insulin resistance, leading to impaired glucose tolerance and hyperglycemia.

1.1.2.1. Free Fatty Acids and Insulin Secretion

Insulin is normally secreted by the pancreatic β-cell in response to a variety of different secretagogues. Plasma glucose is the major regulator of insulin secretion.
Glucose enters the β-cell via a glucose transporter (GLUT2), which is present in large numbers in the plasma membrane, and does not require insulin for activation. Once inside the cell, glucose is metabolized to glucose-6-phosphate by the rate-limiting enzyme glucokinase. In the β-cell, glucose-6-phosphate is further metabolized through glycolysis and the tricarboxylic acid cycle. Glucose metabolism is also coupled to mitochondrial oxidative phosphorylation. The end result of this process is a net increase in intracellular ATP concentration. An increase in the ATP/ADP ratio stimulates the closure of the ATP sensitive K⁺ channels (K⁺<sub>ATP</sub> channels), resulting in a depolarization of the cell. Voltage-gated Ca²⁺ channels (Ca²⁺<sub>v</sub> channels) open in response to the change in membrane potential, resulting in an influx of calcium into the cell. The increase in intracellular calcium triggers the exocytosis of the insulin containing granules. Some oral hypoglycemic agents, such as sulfonylurea derivatives and nateglinide, increase insulin release by closing the K⁺<sub>ATP</sub> channels (direct effect) in the β-cells.

Glucose has also been found to induce insulin secretion through a mechanism that does not depend on the closure of K⁺<sub>ATP</sub> channels. This K⁺<sub>ATP</sub> independent mechanism does not require changes in intracellular Ca²⁺ and is likely accounted for by direct effects on exocytosis of the increase in the ATP/ADP ratio and/or putative ‘coupling factors’ derived from glucose or increased by glucose such as long-chain acyl CoA (LC-CoA) (53). Glucose is metabolized through the glycolytic pathway to pyruvate, which is then converted into citrate in the mitochondria. In addition to being oxidized by the TCA cycle, some citrate may also be exported into the cytoplasm where it can be converted to malonyl-CoA via the sequential action of ATP-citrate lyase and acetyl-CoA carboxylase. Malonyl-CoA is a potent allosteric inhibitor of the mitochondrial membrane enzyme
carnitine palmitoyltransferase-1 (CPT-1), which controls the transport of LC-CoA into the mitochondria to be oxidized. Thus, malonyl-CoA acts to switch β-cell metabolism from fatty acid oxidation to glucose oxidation. An important consequence of this switch is an increase in cytosolic LC-CoA, which is proposed to act as an effector molecule in the β-cell. It has been demonstrated that LC-CoA can directly stimulate the insulin exocytotic machinery, independently of known modulators of this process (54). This direct effect may result from the fact that LC-CoA facilitates the fusion of secretory granules with the β-cell plasma membrane, promoting insulin release. It has been suggested that this effect is mediated by protein acylation (55). In addition to the direct effects of LC-CoAs on insulin secretion, signaling molecules have also been shown to affect intracellular signaling pathways. It has been suggested that LC-CoAs could directly modulate the activity of enzymes such as PKC. LC-CoAs have been shown to mainly stimulate the activity of PKC isoforms in β-cells (56). Activation of PKC stimulates glucose-induced insulin secretion in these insulin producing cells (54). It has been suggested that the acute stimulatory effect of LC-CoA on insulin secretion is the result of the direct activation of PKC isoforms in the pancreatic β-cell. Finally, LC-CoAs have been found to modulate the activity of certain ion channels. There exists evidence to indicate that LC-CoA can also inhibit insulin secretion by preventing closure of the K+ATP channels (57), thereby preventing the membrane depolarization required to trigger insulin exocytosis.

In addition to glucose, FFAs are also insulin secretagogues. FFAs can increase insulin secretion by promoting islet ATP generation. However, it is now accepted that
most of the FFA induced insulin secretion is mediated through LC-CoA. As previously described, LC-CoA play an important role in modulating insulin secretion.

In both humans (58;59) and animals (60), insulin secretion normally occurs in a biphasic pattern. The first phase insulin response (early insulin response), characterized by a spike in circulating plasma insulin levels, is followed by the more sustained release of insulin during the second-phase insulin secretory response. It is believed that the initial first-phase response is due to release of readily-releasable pool of granules at the cell surface, whereas the sustained release or second phase of insulin is due to mobilization of granules from inside the cell of newly synthesized insulin (61). Another interesting characteristic of insulin secretion is that it occurs in an oscillatory manner (62). It is thought that oscillations in glucose metabolism, which lead to oscillations in intracellular calcium levels, are at least in part responsible for this phenomenon (63). However, the physiological benefit of such a secretion pattern has yet to be determined (62).

1.1.2.2. Free Fatty Acids and Diabetes

The association between obesity and diabetes mellitus has been well established. More than 85% of individuals diagnosed with type 2 diabetes are obese (9). Plasma free fatty acids (FFA), which are often elevated in obesity, play an important role in the development of diabetes. Plasma free fatty acids are derived from lipolysis of adipose tissue triglyceride stores. In general, obesity results in higher circulating plasma FFA levels. This is primarily due to the fact that FFA turnover (lipolysis) is directly
proportional to body fat mass (64). However, studies have suggested that not all obese states are equivalent. Body adipose mass distribution plays an important role in determining the circulating plasma FFA levels. Higher circulating plasma FFA concentrations have been associated with upper body obesity (also known as abdominal or central obesity), rather than lower body obesity (also known as peripheral obesity) (65). Upper body obesity, which is associated with higher circulating FFA levels, is more closely associated to the development of diabetes (10), strengthening the hypothesis that FFA play an important role in the development of this disease. A great number of experimental studies have also provided evidence that artificially elevated plasma FFA levels lead to defects that are characteristic of impaired glucose tolerance and type 2 diabetes, such as insulin resistance and impaired insulin secretion (66).

As discussed above, several different mechanisms have been suggested to mediate the impairing effects of FFA on insulin sensitivity. These mechanisms include the Randle cycle, activation of PKC isoforms, and oxidative stress. However, in subjects with a family history of diabetes, the effect of FFA to impair insulin sensitivity appears to be reduced (15), whereas these subjects appeared to be particularly susceptible to an FFA-induced impairment in β-cell function (67).

1.1.2.3. Free Fatty Acids and the β-cell

Acutely, FFA stimulate insulin secretion (68-73), but chronically can decrease β-cell function and mass, an effect referred to as β-cell lipotoxicity (66). Although the
mechanisms behind β-cell lipotoxicity are unclear, one possibility points toward oxidative stress. Pancreatic β-cells have low antioxidant defenses (74) and are thus susceptible to reactive oxygen species (ROS)-induced decrease in function and viability (75;76). Oxidative stress has been implicated in the insulin secretory impairment of glucotoxicity (77;78), which is in many respects similar to that of lipotoxicity (53;79).

FFAs have been shown to have a time-dependent effect on β-cells. As mentioned above, acutely FFA stimulate insulin secretion in vitro (68-70;72;73). This effect is more on GSIS than on basal insulin secretion, as shown by these in vitro studies (80;81). Early studies conducted by Crespin et al. showed that acute exposure to elevated plasma FFA levels enhanced insulin secretion also in vivo, in fasted animals (69). The effect was found to be proportional to the length and degree of saturation of the fatty acid (82). In addition to directly stimulating insulin secretion, acute exposure to FFA was found to potentiate both glucose and non-glucose stimulated insulin secretion in fasted animals (83). These findings have been confirmed by other in vitro studies (80;81). In addition, acute lowering of plasma FFA with nicotinic acid resulted in reduction in basal plasma insulin in both non-obese and obese healthy fasted individuals and in patients with type 2 diabetes (84).

In contrast, prolonged exposure to elevated FFA levels has been shown to have a different effect on β-cell function. Prolonged (> 24-h) exposure to FFA increases insulin secretion at basal and moderately elevated glucose levels in rat islets (85;86), but not human (86) or mouse (87) islets. However, prolonged (> 24 h) exposure to FFA has been shown to desensitize the β-cell secretory response to high physiological and pathophysiological concentrations of glucose. Both in vitro and in situ studies in isolated
islets (86;87), perfused rat pancreas (85), and in β-cell lines (88;89) have shown that long-term fatty acid exposure impairs GSIS.

The effects of a prolonged elevation of FFA on GSIS in vivo are more controversial than those found in vitro. Magnan et al. infused rats with Intralipid and heparin for 48 h and GSIS was measured in vivo in the fed state and in vitro in isolated islets (90). In this study, the authors found that a prolonged lipid infusion suppressed insulin secretion in the isolated islets, however, the 48 h fatty acid elevation appeared to enhance GSIS in vivo. In another study conducted by Boden et al. in healthy humans, a 48 h Intralipid and heparin infusion increased GSIS in vivo, under conditions of a 48 h hyperglycemic clamp (91). These authors found that elevated FFA levels coupled with hyperglycemia caused insulin hypersecretion throughout the infusion period. In contrast, other studies have shown that a prolonged FFA elevation impairs GSIS in vivo. In a study conducted by Mason et al, a 48 h elevation of plasma FFAs levels was shown to impair GSIS in fasted rats (92). In this study, the impairing effect of a prolonged infusion of Intralipid and Heparin (triglyceride emulsion containing mostly polyunsaturated fatty acids) on GSIS was less than that of oleate (monounsaturated fatty acid). Two independent studies demonstrated that FFAs chronically desensitize the insulin secretory response to glucose in humans. In a study conducted by Paolisso et al. in humans, the acute insulin response to a standard glucose load was decreased after 24 h of Intralipid and Heparin infusion, and this decrease was reversed 24 h after cessation of the lipid infusion (71). In a separate study, Carpentier et al. found that a 48-h infusion of Intralipid and Heparin did not affect the absolute insulin secretory response to a glucose challenge. However, when insulin secretion was corrected for the fatty acid induced defect in insulin
action, a prolonged FFA infusion was shown to actually impair β-cell function, i.e. the ability of the β-cell to compensate for insulin resistance (93).

Several reasons may account for the discordant results discussed above. First, differences in experimental protocols (i.e. glucose levels, fasting vs. fed) may influence the results presented in the various studies. Second, the effect of FFA on insulin sensitivity was not considered in every study discussed. In contrast to isolated islets, GSIS in vivo depends not only on glucose levels, but also on insulin sensitivity. Therefore, for an accurate assessment of in vivo GSIS, the results must be interpreted in the context of insulin sensitivity, since in vivo the normal β-cell increases its secretion to compensate for reduced insulin sensitivity. Both Bergman et al (94), and Kahn et al (95) have introduced the concept that the relationship between insulin sensitivity and insulin secretion is hyperbolic so that the disposition index (DI), which is the product of insulin sensitivity and insulin secretion, is constant. That is, when insulin sensitivity is low (insulin resistance), insulin secretion is high and vice versa in normal subjects. Since it is well established that FFA induce insulin resistance, which would be expected to result in increased insulin secretion, even an unchanged or mildly elevated absolute GSIS may indicate β-cell dysfunction.

There exists evidence to indicate that in addition to affecting β-cell function, FFA can also affect β-cell mass. In the short to “medium” term, the main effect on mass is stimulatory, meant to compensate for a certain degree of insulin resistance (96;97). Accordingly, proliferation was found to increase (96;97). However, in several studies, FFA induced β-cell apoptosis in vitro (98-100) and perhaps also in vivo (97;101). Furthermore, there exists in vitro evidence that prolonged FFA exposure can also
decrease β-cell proliferation (99;102). Increased apoptosis imbalanced by a corresponding increase in proliferation is thought to promote the long term failure of the β-cell mass to compensate for insulin resistance in type 2 diabetes (103). The mechanisms responsible for the observed changes in both β-cell function and mass will be discussed in the following section.
1.2. Cellular Processes and Mechanisms Involved in the Effect of Acute Increase of Free Fatty Acids Levels on β-cell

The acute effect of FFA to enhance insulin secretion does not appear to be specific for a glucose stimulus (83), which suggests that final common events in stimulus secretion coupling may be involved. Some of the mechanisms involved have been discussed above, such as protein acylation and PKC activation. These mechanisms may be operational in both β-cells and peripheral tissues and thus link insulin resistance and hyperinsulinemia at the cellular level (104;105).

PKCs comprise a family of isoenzymes that play a key role in cell functions (106). PKCs are classified into three major subgroups: 1) ‘conventional’ PKCs (α, βI, βII, and γ), which require phosphatidylserine (PS) and are activated by calcium and DAG; 2) ‘novel’ PKCs (δ, ε, μ, η, θ) which require PS and DAG but not calcium for activation; and 3) ‘atypical’ PKCs (ζ, λ), which are calcium- and DAG-independent but PS-dependent (107). When PKC is activated, the enzyme translocates from the cytosol to the plasma membrane.

PKC isoforms are ubiquitously expressed. In the major insulin-responsive tissues, i.e. liver, skeletal muscle, and adipose tissue, PKC isoforms from each of the three categories are expressed to varying degrees (108). For instance, PKC-θ is expressed predominantly in the skeletal muscle (109) but is not highly expressed in the liver. In contrast, PKC-δ is expressed robustly in all major insulin-responsive tissues (110).

Selective activation of individual PKC isoforms by physiological stimuli provides one explanation for isoform-selective responses (111). Novel PKCs are selectively
activated by DAG produced from phospholipase D pathway, which does not produce a
calcium transient (112) whereas the calcium from phosphoinositide hydrolysis may be
required for full activation of conventional PKC isoforms (113). The subcellular
localization of PKCs also determines isoform-dependent functions (111).

PKC, in particular novel isoforms, are known to phosphorylate a number of
serine/threonine residues of the insulin receptor and IRS (114-116). This diminishes
insulin receptor tyrosine kinase activity and interferes with tyrosine phosphorylation of
the IRS, thereby impairing insulin signaling. For instance, Greene et al. have shown that
activated PKC-δ can directly phosphorylate specific serine residues of human IRS-1,
including serine 307, 323, 573, that diminishes the ability of insulin receptor to tyrosine
phosphorylate IRS-1 in vitro (115). Moreover, PKC-θ decreases tyrosine phosphorylation
of IRS-1 by phosphorylating serine 1101 of IRS-1 in cultured murine C2C12 myocytes
and 3T3 L1 fibroblasts (117). These results point to a potentially direct role of PKC in
impairment of insulin signaling.

Activation of PKC isoforms, both conventional and novel, is associated with
various animal models of insulin resistance. In addition, elevated PKC activity has been
observed in muscle tissue from individuals with type 2 diabetes and in liver of humans
with type 2 diabetes (118). Therefore, PKC activation is likely a key mediator of insulin
resistance, particularly in the context of elevated plasma FFA and tissue fat accumulation.

Regarding the β-cell, it is well known that PKC isoforms are present (119). β-cell
expresses the classical α-form as well as the β2-form, but not the γ-form (120). They also
express the novel ε-form whereas the φ and θ isoforms seem to be present in small
amounts (120). PKC-ζ was also reported to be present in islets (121) as well as PKC-λ.
PKC are generally thought to be stimulatory for insulin secretion (120) and FFA activation of novel and/or atypical PKC isoforms has been found to increase insulin secretion acutely (56;122). However, a recent paper has implicated the novel isoform PKC ε in the decrease in β-cell function induced by FFA chronically (123). In this paper, β-cell function was enhanced in PKC-ε null mice under conditions of high fat feeding but not control feeding. Similarly, insulin secretion was greater in PKC-ε null islets compared to control islets when islets were exposed to 48h palmitate. These results suggest that inhibition of PKC-ε may prevent β-cell lipotoxicity. The mechanism is unclear, but might include changes in lipid metabolism, insulin content and insulin gene transcription (123). The relation to oxidative stress has not been investigated. In other cell types, activation of PKC can induce oxidative stress (124) and can be also activated by oxidative stress (125).

**1.2.1. β-cell and GPR Receptors**

Studies have shown that FFA do not need to be metabolized to act upon the secretory mechanism of the β-cell. Interestingly, exogenous FFA can act directly on the β-cell as ligands for the G-protein coupled receptor GPR40 (126) present in β-cells. This receptor was shown to be responsive to saturated 12-16 carbon FFA and unsaturated 18-20 carbon FFA (127). Different types of FFA, such as medium- and long-chain saturated and unsaturated fatty acids can activate GPR40 in a dose-dependent manner (128). Short-chain fatty acids activate receptors GPR41 and GPR43 (129). The mechanism involved in
GPR signaling was hypothesized to involve activation of PLC-mediated hydrolysis of PIP$_2$ into DAG and PIP$_3$, which further activate PKC and mobilize calcium from the endoplasmic reticulum to stimulate insulin secretion (126). Recently, it was observed that a large part of the acute enhancing effect of FFA on insulin secretion is also mediated through the GPR40 receptor (126;130). However, the role of this receptor in FFA induced β-cell dysfunction is still controversial, as prolonged islets exposure to FFA decreased GSIS in islets of GPR40-null mice in one study (131) but not in another (132).

1.2.2. β-Cell and Toll-Like Receptors

FFA can bind to LPS or toll-like receptors (TLR), and TLR-null mice were found to be protected from FFA-induced insulin resistance (133). Toll-like receptors belong to another family of newly described receptors, of which some members are also present on the β-cell. Among these, CD14 is a myeloid differentiation antigen described first in monocytes but also expressed in neutrophils and B lymphocytes. CD14 is membrane glycoprotein receptor of the complex formed by lipopolysaccharides with lipopolysaccharides binding protein, an acute phase response protein normally present in trace amounts in human serum (134). CD14 was also found to be expressed in islet cells (135) and also to function as LPS receptor on β-cells. Other receptors such as TLR4 and TLR2 and the leucine repeat rich MD-2 proteins are also present in β-cells (135). Whereas these receptors which may play a role in the development of autoimmune type 1
diabetes are also important for lipotoxicity and Type 2 diabetes, remains to be investigated.
1.3. Cellular Processes and Mechanisms Involved in the Effect of Chronic Increase of Free Fatty Acids Levels on β-Cell

The mechanisms that mediate the impairing effect of a prolonged FFA elevation on insulin secretion have been the focus of intense research. Experimental data has shown that this effect is only present and specific for glucose, and not other secretagogues, suggesting interference with glucose metabolism, or any other downstream mechanism induced by glucose (136;137). It has been suggested that the observed lipid induced defects in insulin secretion may be the consequence of an impairment in islet glucose metabolism. An enhanced rate of fatty acid oxidation might reduce glucose metabolism, and consequently glucose-induced insulin secretion, if Randle’s cycle (also known as the glucose-fatty acid cycle) were operative in β-cells exposed to fat. This relationship between fatty acid oxidation and glucose metabolism has been well documented in tissues such as heart muscle and liver (138). According to this concept, increased fatty acid oxidation enhances the generation of both NADH and acetyl-CoA, which inhibit pyruvate dehydrogenase, and thus glucose oxidation. Furthermore, the Randle cycle also predicts an elevation in the intracellular concentration of citrate. This metabolite is known to negatively regulate the key rate limiting enzymes involved in glucose metabolism known as phosphofructokinase, resulting in a lower glucose utilization rate. Studies have also shown that the FFA-induced impairment in insulin secretion is associated with an enhanced capacity of the β-cell for fatty acid oxidation (89;139), and thus an enhanced Randle’s cycle, due to alterations in the expression of key metabolic enzymes. The levels of the messenger RNA (mRNA) for acetyl-coenzyme A (CoA)
carboxylase (ACC), which catalyzes the formation of malonyl-CoA, decreases in response to prolonged exposure to fatty acids (89). Simultaneously, the transcription of the gene encoding carnitine palmitoyltransferase I (CPT-I), the rate-limiting enzyme for β-oxidation of FFAs, is upregulated by fatty acids (88). There is some in vitro evidence suggesting that Randle’s cycle may in fact be established in pancreatic islets exposed to fat. Results from an in vitro study demonstrate that inhibiting fatty acid oxidation with etomoxir (an inhibitor of CPT-1) prevents the FFA-induced defect in glucose-induced insulin secretion (140). Furthermore, prolonged exposure to the fatty acid palmitate has been shown to reduce the activity of the PDH enzyme in isolated rodent islets, also indicating that the glucose-fatty acid cycle may be operative in β-cells after fat exposure (141). In other studies fatty acids did not affect the β-cell glucose utilization rate (142), or the glucose-6-phosphate and citrate levels (139), suggesting that a reduction in glucose metabolism, as predicted by the glucose-fatty acid cycle, is not the major cause of blunted insulin release in response to glucose stimulation.

It is well established that the production of ATP by the mitochondria is essential for GSIS. Therefore, any dissociation of phosphorylation from oxidation (uncoupling), which will decrease the efficiency of ATP synthesis, will impair glucose-induced insulin secretion. Proteins of the uncoupling protein (UCP) family are located in the inner mitochondrial membrane and uncouple the electrochemical gradient produced by the respiratory chain from ATP synthesis. Exposure to FFA has been shown to increase the expression of UCP2 (143), the only member of the UCP family located in the pancreatic islet. The findings obtained from overexpression (144) and knockout (145) studies are consistent with the hypothesis that alterations in the protein levels of UCP2 modulate
GSIS. FFA also impair oxidative phosphorylation in β-cells via acting as cofactors for the proton transport function of UCP2 (146). This uncoupling action of FFA is even accentuated in β-cells after prolonged FFA exposure, most likely due to activation of UCP2 by intramitochondrially produced superoxide (146).

Effects of FFA on expression of metabolic genes (CPT-1 and UCP-2) are thought to be mediated by PPAR and SREBP-1. FFA can directly influence lipogenic gene expression by modulating the activity and expression of transcription factors, including PPAR and sterol regulatory element-binding protein (SREBP)-1 (147). PUFA are strong activators of PPARα and a diet high in unsaturated fat increases PPARα gene expression in the liver (148). PPARα increases gene expression of enzymes involved in peroxisomal and mitochondrial FFA oxidation (149,150). PUFA suppress the expression of SREBP-1 and thus the expression of lipogenic enzymes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (151-155). The consequence of suppression of ACC is a decrease in malonyl-coA, which not only results in a reduction in TG accumulation but also an increase in FFA oxidation as malonyl-coA inhibits carnitine palmitoyltransferase (CPT)-1. PUFA suppress the expression of SREBP-1 by decreasing mRNA stability (150) and via post-translational modification (156).

SREBP-1c, a member of the SREBP family, is expressed in β-cells (157). Studies using β-cell-specific SREBP-1c transgenic mice and SREBP-1c knockout mice demonstrated that SREBP-1c induces lipogenesis and thus exaggerates lipotoxicity, resulting in decreased β-cell function and mass (157).

Activation of the nuclear receptor PPAR γ leads to changes in the expression of genes involved in both glucose and lipid homeostasis (158). Systemic activation of PPAR
γ increases GLUT4 expression, regulates release of adipocyte-derived signaling factors that affect insulin sensitivity in muscle, and contributes to a turnover in adipose tissue, inducing the production of smaller, more insulin sensitive adipocytes, thus reducing plasma FFA. Activation of PPAR γ with TZDs affects islet lipotoxicity, improving β-cell function (158), however it is not clear whether this is only an indirect effect, or is also due to a direct effect of PPAR γ on β-cells. Similarly, PPAR α decrease plasma TG and thereby also indirectly affects islet lipotoxicity. As PPAR α stimulates FFA oxidation, a direct effect to increase β-cell function is also likely (159).

Long-term exposure to free fatty acids has been found to also alter gene expression and/or activity of transcription factors which are important for glucose-induced insulin gene transcription. Exposure of isolated rat pancreatic islets to palmitate induced a significant decrease in mRNA and protein expression of PDX-1 (160), a transcription factor expressed in the pancreatic β-cells. This resulted in an expected decrease in the expression of β-cell genes transactivated by PDX-1 in addition to the insulin gene, such as those for GLUT2 and glucokinase (160). Both GLUT2 and glucokinase play an important role in the β-cell’s glucose sensing mechanism, and thus alterations in the expression of these genes would predictably alter the glucose-induced insulin secretory response. Other studies found that palmitate did not affect the expression, but reduced the nuclear localization of PDX-1 (161). Palmitate did however block the transcriptional induction of MaFa by glucose (161). These effects appeared to be specific for saturated fats (161) perhaps through ceramide synthesis (162).

Furthermore, prolonged exposure to fatty acids may impair insulin production by inhibiting proinsulin biosynthesis (136;140). Finally, there exists evidence to suggest that
FFAs may also delay processing of proinsulin in β-cells. A study conducted in the MIN6 pancreatic β-cell line found that long-term fatty acid exposure impaired the function of the prohormone convertase enzymes (PC2 and PC3), which catalyze the conversion of proinsulin into insulin (163).

A recent paper (164) has shown that 2h exposure to palmitate decreased levels of carboxypeptidase E (CPE, the enzyme which is involved in the final conversion of proinsulin to insulin) through a mechanisms involving translocation of CPE to the Golgi system followed by CPE lysosomal degradation. Decreased CPE caused ER stress, presumably because of a backlog of unprocessed proinsulin in the secretory pathway and ER stress further led to cell death.
1.4. Mechanisms Involved in the Chronic Effect of Free Fatty Acids on β-Cell Function

As referred above in section 1.3, FFA can decrease β-cell function by direct effects of FFA on: 1) enzymes (i.e. Randle cycle); 2) channels (i.e. effect of long-chain FFA on ATP-sensitive K channel); 3) transcription factors (i.e. PPAR mediated increase in UCP-2 expression), and 4) possible direct effects of PKCɛ and GPR40 on chronically decreasing β-cell function. In addition to these mechanisms, oxidative stress and inflammation may play a role in the FFA-induced impairment in insulin secretion.

1.4.1. Role of Oxidative Stress in Decreasing β-cell Function

The term oxidative stress is used to describe an environment where there is an excess concentration of free reactive oxygen species (ROS). ROS are free radicals and other oxygen-containing molecules that react with other tissue molecules changing their structure. ROS can damage cellular proteins, lipids, and DNA. As described in more detail in the chapter Glucotoxicity, Lipotoxicity and Oxidative Stress chronic FFA exposure results in generation of ROS. It has been demonstrated that exposure to FFAs enhance ROS production in islets (165). The pancreatic β-cells, which have a low antioxidant capacity, are especially vulnerable to oxidative stress (74). This sensitivity has been found to affect both β-cell function and viability. Exposure to ROS such as hydrogen peroxide (75) or to lipid peroxidation products (i.e. reactive aldehydes such as
4-hydroxy-2-noneal) (166) results in a marked impairment in GSIS. Taken together, these findings suggest that FFA may impair GSIS by enhancing oxidative stress in islets. The precise mechanism by which oxidative stress impairs β-cell function and the site of impairment involved, remains to be elucidated. However, several possible mechanisms have been proposed. One possibility is that oxidative stress impairs insulin secretion by reducing ATP production. As previously described, an increase in the ATP/ADP ratio is essential for normal insulin secretory function. Therefore, any alteration in ATP synthesis will undoubtedly affect insulin secretion. In addition to being an important source of ROS, the mitochondrion is paradoxically very sensitive to oxidative stress. Treatment with hydrogen peroxide, a free radical donor, was found to reduce the generation of ATP through a mechanism involving a change in mitochondrial membrane permeability and mitochondrial membrane potential. Furthermore, studies have shown that mitochondrial enzymes, such as aconitase (167), and mitochondrial DNA (168) are susceptible to oxidative modification. Oxidative stress inhibits glucose metabolism in the β-cell. Miwa et al have shown that both glucose utilization and glucose oxidation are significantly reduced in isolated islets exposed to lipid peroxidation products, which are the end product of a reaction between free radicals and membrane lipids (166). Oxidative stress may also reduce the mitochondrial ATP production by inducing the expression (169;170) and/or activity (146;171) of a mitochondrial uncoupling protein (UCP-2) in the pancreatic β-cell. Finally, oxidative stress may also affect insulin gene expression directly, or via inflammatory pathways, as described in Oxidative Stress and Inflammatory Pathways. In a study conducted in a model of glucotoxicity, which is
also associated to oxidative stress, antioxidants were shown to prevent the ROS-mediated decrease in insulin gene expression and insulin gene promoter activity (78).

Glucose-induced oxidative stress can impair β-cell function at the level of insulin gene transcription (77;78), because of decreased binding of the transcription factors PDX-1 (172;173) and MafA (174;175) to the insulin promoter. Oxidative stress increases FoxO-1 nuclear retention (176;177) which results in nuclear exclusion of PDX-1 (178) and consequent inhibition of insulin gene transcription. At least part of the effect of oxidative stress on FoxO-1 is currently thought to be indirect and mediated by JNK (179;180).

β-cell lines chronically exposed to high glucose levels showed an impairment in insulin gene expression associated with a decrease in PDX-1 and MafA binding (181). In studies performed in vivo in partially pancreatectomized hyperglycemic rats (182) and in the diabetic gerbil Psammomys obesus (183) PDX-1 expression is also reduced. Its binding activity is decreased in islets from Zucker Diabetic Fatty rats (184). MafA expression was reduced in mouse diabetic models (185) and in the glucotoxic insulin-secreting HIT-T15 cell the reduction in MafA binding activity was shown to be due to a loss of protein expression without changes in mRNA expression (174). The decrease in insulin gene transcription and MaFA expression induced by glucotoxicity is prevented by antioxidants (78;174). As described in (186), impairment of insulin gene transcription also occurs after prolonged islet exposure to palmitate (187-191), but not oleate (136;161), or a physiologic mixture of oleate + palmitate (163). The palmitate effect has been attributed to ceramides (162), which can induce oxidative stress (192), and activate JNK in many cell types (79). However, the role of oxidative stress in this effect has not
been investigated. In a recent study, palmitate was found to decrease insulin gene transcription via JNK activation in islets (193). Again, the role of oxidative stress in palmitate induced JNK activation has not been explored.

Prolonged exposure of human islets to glucose decreases (pro)insulin biosynthesis (194), however it is unclear whether this is secondary to decreased insulin gene transcription. In in vitro models of lipotoxicity, insulin biosynthesis is reduced (86;136;163) also in the absence of decreased insulin gene transcription (136;163). This is due to the effect of fatty acids to impair translation (86;136;195;196). Studies have shown unchanged insulin gene expression, but decreased insulin content in oleate-treated MIN6 cells, an effect partially reversed by NAC (81). These results raise the possibility that FFA-induced oxidative stress affects insulin biosynthesis directly at the level of translation.

1.4.2. The Effect of Chronic Increase in Free Fatty Acids Levels on β-Cell Mass

Obese normoglycemic subjects have an increased relative β-cell volume compared with lean normoglycemic subjects (66;68). Although it cannot be excluded that obese subjects have a larger β-cell mass regardless of the presence of obesity, most data from animal studies show that obesity induces an increase in β-cell mass. In Caucasians, relative β-cell mass is reduced by 40% in obese subjects with impaired fasting glucose, and by 63% in obese subjects with type 2 diabetes compared with obese normoglycemic
subjects (68). A reduction in relative β-cell mass is also observed in Asian people with type 2 diabetes (76;77). Subjects prone to developing type 2 diabetes might have an inherent marginal β-cell mass because of environmental, possibly intrauterine, and genetic factors; alternatively, they might have an impaired capacity for β-cell mass adaptation to metabolic stimuli. However, an increased number of apoptotic β-cells in the islets of diabetic subjects compared with those of normoglycemic subjects, with no difference in replicating cells, provides support for a progressive decline in β-cell mass during the course of the disease (68). In addition, diabetes is associated with increased β-cell apoptosis in Zucker diabetic fatty (ZDF) rats (78).

In addition to impairing the glucose stimulated insulin secretory response, a prolonged FFA elevation has been shown to affect β-cell mass dynamics. Fatty acids affect proliferation and apoptosis of the insulin producing cells of the pancreatic islet. The effect of FFA on proliferation remains controversial. There exists evidence to suggest that prolonged exposure to fatty acids can both enhance and impair β-cell proliferation. Prolonged exposure to FFAs has been shown to stimulate β-cell proliferation in vitro (197) and also in vivo (96;97) with consequent increase in β-cell mass. The signaling mechanisms that may be involved in increasing β-cell proliferation have yet to be completely elucidated. However, there exists evidence to indicate that fatty acids upregulate the expression of the c-fos and nur-77 transcription factors (198), which are known to induce the expression of other genes encoding for proteins necessary for cells to progress through the cell cycle. In contrast, prolonged exposure to FFAs has also been shown to inhibit β-cell proliferation. In a study by Cousin et al, a prolonged exposure to elevated plasma FFA levels was shown to inhibit both glucose and insulin-
like growth factor I (IGF-1) induced β-cell proliferation, through a mechanism that may involve the inhibition of protein kinase B (PKB) activity and / or activation of an atypical PKC isoform (PKC ζ) (102).

Long-term FFA exposure has also been found to promote β-cell apoptosis (98;98;101). Several mechanisms have been proposed to explain this finding. Studies have found that fatty acid-induced β-cell apoptosis depends in part on the generation of ceramides. These lipid second messengers are involved in the apoptotic response induced by a variety of different triggers including cytokines, ionizing radiation, and heat shock (199). Studies have noted that ceramide levels are elevated in fat rich islets (101;200), presumably due to the abundance of substrate for de novo ceramide synthesis. Treatment with ceramide synthase inhibitors prevented the FFA-induced β-cell apoptosis in both rodent (101) and human (98) islets. Ceramides, which are known to enhance the generation of free radicals, may trigger apoptosis by promoting oxidative stress. Ceramides have been shown to increase the production of ROS in the mitochondrion (192), and they have also been found to upregulate the expression of the iNOS gene (201). However, fatty acids have also been shown to enhance the production of ROS, independent of ceramides (202). Free radicals have been implicated as important regulators of the apoptotic pathway (203). Therefore, it is not surprising that there exists evidence indicating that FFA-induced β-cell apoptosis is ROS-dependent. Studies have found that prolonged exposure to FFA stimulates the generation of ROS in β-cells (165;204). Treatment with either antioxidants or inhibitors of inducible nitric oxide synthase (iNOS, as NFκB-dependent gene) has been found to prevent FFA-induced β-cell apoptosis (100;101;204). This suggests that fatty acids depend on oxidative stress to
induce β-cell apoptosis. A recent study linked reduced β-cell mass, caused by FFA-induced apoptosis through NFκB activation (205) with the activation of the ER stress response (194). Finally, activation of PKCδ has been implicated in the fatty acid induced loss of β-cell mass through a mechanism that may involve inhibition of glucose/IGF-I mediated mitogenesis (206). PKCδ has also been implicated in FFA induced β-cell apoptosis (207).
1.5. **Glucotoxicity, Lipotoxicity and Related Signaling Pathways**

The development of impaired glucose tolerance in obese individuals is characterized by progressive elevation of FFA and progressive hyperglycemia. Chronic hyperglycemia can exert deleterious effects on insulin sensitivity, β-cell function and mass, referred to as glucotoxicity (208). Over time, both glucotoxicity and lipotoxicity contribute to the progressive deterioration of glucose homeostasis characteristic of diabetes. Recently, the concept of glucolipotoxicity has been proposed. This concept attempts to explain the synergistic effects of a combined chronic increase of glucose and FFA mostly on the impairment of pancreatic β-cell function and mass (79). This synergism may be explained by the fact that glucose alters the partition between fat oxidation and esterification via its stimulatory effect on acetyl-CoA carboxylase which increases malonyl-CoA, the physiologic inhibitor of FFA oxidation. Thus, long-chain fatty acyl-CoAs accumulate in the cytosol rather than being oxidized, and are esterified to diacylglycerol (DAG, also derived from glucose) and ceramides (209). LCFA-CoA and diacylglycerol activate PKC. The effect of FFA to decrease glucose stimulated insulin secretion has been attributed to DAG (161) and the effect of FFA to decrease insulin gene transcription in the presence of glucose has been attributed to ceramides (162). Notably, PKC and ceramides are known inducers of oxidative stress (210;211).

That glucotoxicity and lipotoxicity may be both mediated by oxidative stress, is supported by the following lines of evidence: 1) both glucose (212) and FFA (165) can induce oxidative stress in islets; 2) β-cells have low antioxidant defenses (74); 3)
oxidative stress can impair GSIS (213); 4) many proposed pathways of β-cell gluco- and lipotoxicity are upstream, downstream or more often both upstream and downstream of oxidative stress. These include hexosamines, PKC, ceramides pathways, which can induce oxidative stress (124;192;214) and are also activated by oxidative stress (125;215;216), and JNK (stress-activated kinases) and IKK/NFκB pathways which are proinflammatory downstream effectors of oxidative stress (213;217).
1.6. Reactive Oxygen Species and Oxidative Stress

The process of ATP generation to sustain the ongoing needs of living cells is accompanied by ROS production in minute amounts by the mitochondrial electron transport chain, which is usually buffered by native antioxidant defense mechanisms such as superoxide dismutase, catalase, and glutathione. However, in states of energy excess (increased glucose and FFA) this balance is tipped towards increased mitochondrial production of ROS by FFA and glucose. FFA may also amplify mitochondrial ROS production by changes in mitochondrial membrane composition. Other intracellular sources of ROS induced by FFA are those triggered by the plasma membrane NADPH oxidase, activated by PKC (124), ceramides, peroxisomal oxidases, endoplasmic reticulum oxidases, cyclooxygenases and lipoxygenases or hexosamines (reviewed in (218)). Other potential pathways of ROS generation by glucose include glycosylation, the glucosamine pathways, PKC activation and glucose auto-oxidation. The uncontrolled production of ROS or a reduction in antioxidant species is regarded as oxidative stress and is thought to be deleterious by altering gene expression and protein structure and function. In the presence of oxygen, superoxide is generated, which in turn forms hydrogen peroxide via superoxide dismutase. In the presence of heavy metals, hydrogen peroxide generates highly toxic hydroxyl radicals. There is evidence that this sequence of events might occur specifically in β-cells (81;218;219) with deleterious effect on several key steps in insulin secretion and/or β-cell viability.
1.6.1. Glucotoxicity, lipotoxicity and oxidative stress

There is experimental evidence that the mechanisms of glucotoxicity involve oxidative stress in β-cells. In β-cell lines (78), as well as in islets (214;220) generation of reactive oxygen species by chronic exposure to elevated glucose leads to decreased transcription of the insulin gene, an effect prevented by antioxidants (78;213;214). Glucotoxicity induced oxidative stress decreases insulin gene expression through JNK activation (213). Also, the impairing effect of FFA on β-cell insulin secretion was found by in vitro studies in islets to involve the generation of oxidative stress (165), although in some studies no increase in ROS was found (161) and antioxidants were not effective. Our previous study in MIN6 cells showed that the antioxidant NAC prevented the decrease in insulin content induced by oleate (81), however, insulin secretion was not affected. In healthy subjects, infusion of Intralipid, a source of FFA, caused systemic oxidative stress, as judged by increased malondialdehyde levels and a decline in the plasma reduced/oxidized glutathione ratio (13). Another recent paper from our group showed that oral treatment with the antioxidant taurine ameliorates Intralipid-induced impairment in insulin sensitivity and β-cell function in obese and overweight, non-diabetic men (221).

1.6.2. Type 2 diabetes and oxidative stress

Many studies suggest that patients with type 2 diabetes are subjected to chronic oxidative stress (222). Pro-oxidants and markers for oxidative tissue damage, such as
hydroperoxides, and oxidated DNA bases, are elevated in plasma of patients with type 2 diabetes (223-225) and erythrocytes from diabetic patients contain low levels of reduced glutathione (GSH), the primary intracellular antioxidant (226), a defect ameliorated by improved metabolic control (227).

Antidiabetic effects of the antioxidant N-Acetylcysteine were demonstrated in db/db mice (77). As these mice developed hyperglycemia, their insulin content and insulin gene expression decreased. N-Acetylcysteine treatment was associated with preserved insulin content and insulin mRNA as well as increased amounts of PDX-1, an important insulin gene transcription factor. N-Acetylcysteine also reversed the progressive worsening of hyperglycemia, the decrease in glucose tolerance and insulin secretion, the decrease in PDX-1 binding to the insulin promoter, and the decrease in insulin gene expression in ZDF rats (78). Moreover, treatment with the antioxidant vitamin E had beneficial effects on glycemic control in GK rats, which was accompanied by improvement in insulin secretion and lower levels of HbA₁c (228). Daily supplementation with 1.5 grams of the antioxidant taurine for 8 weeks had no effect on insulin secretion or sensitivity, or on blood lipid levels (229) in patients at high risk for type 2 diabetes; however, intravenous infusion of GSH in type 2 diabetic patients improved insulin secretion and glucose tolerance during an oral glucose tolerance test (230).
1.6.3. Oxidative Stress and Inflammatory Pathways

PKC, ceramides, hexosamines (which can be increased by both glucose and fat (231)) and ROS are mediators of the insulin resistance induced by gluco- and lipotoxicity (209;232;233). Ceramides inhibit the insulin-induced activation of Protein Kinase B (PKB) which is downstream of the insulin receptor substrate (IRS) in the insulin signalling cascade (234). PKC, ceramides and ROS (235) can stimulate IKKβ, which in addition to activating the inflammatory transcription factor NFκB, induces serine/threonine phosphorylation of IRS, which decreases their insulin-induced tyrosine phosphorylation (236). Other inflammatory pathways activated by PKC, ceramides and ROS are the JNK/p38 pathways which also phosphorylate IRS at serine-threonine sites. In previous studies in rats in Dr. Giacca’s laboratory, antioxidants prevented insulin resistance induced by FFA (237), glucose (238) and glucosamine (239). The effect of antioxidants to prevent the FFA-induced hepatic insulin resistance appeared to be due to the inhibition of activation of IKKβ (237) and JNK. In the β-cell, JNK activation has been linked to the decrease in insulin gene expression induced by glucotoxicity (213) and lipotoxicity (162). Inflammatory pathways could directly influence insulin secretion and can influence insulin secretion and β-cell growth indirectly by impairing insulin signalling in islets.
1.6.3.1. ROS and the JNK pathway

Oxidative stress activates inflammatory pathways such as JNK. When β-cell lines or isolated rat islets were exposed to oxidative stress, JNK, p38 MAPK (a stress activated kinase), and PKCβ were activated, and a decrease of insulin gene expression was observed (213). However, only the JNK inhibitor prevented this decrease. Oxidative stress induced activation of JNK was shown to cause nucleocytoplasmic translocation of PDX-1, which lead to suppression of insulin gene expression (180). Prolonged exposure to both glucose (213) and palmitate (193) decreased insulin gene expression through the JNK pathway.

1.6.3.2. ROS and the NFκB pathway

*In vitro* studies have reported FFA-mediated activation of transcription factor NFκB (205) as a likely consequence of the ability of FFAs to increase ROS (217;218); however, the role of NFκB remains controversial, as other studies have excluded it as a potential candidate for lipotoxicity (240). Although oxidative stress is a known activator of the IKK/NFκB pathway, the role of NFκB in β-cells remains controversial. There are reports that NFκB may actually be beneficial for GSIS (241;242), unless stimulated by cytokines (243). However, IKKβ inhibition that, in addition to NFκB, also inhibits serine phosphorylation of IRS, increases β-cell function (244;245).

NFκB is a transcription factor for iNOS and COX2 genes. An important role of iNOS induction in β-cell lipotoxicity has been postulated by Unger (66;200;204), but
has been disputed by others (78). It has been reported that high-dose salicylate (inhibitor of IKK), partially restores β-cell glucose sensitivity in type 2 diabetes (246). This effect has been attributed to inhibition of COX2 (247;248), due to a decrease in insulin secretion induced by PGE, although the inhibition of iNOS as well as enhancement of insulin signalling in β-cells by salicylate may also have played a role.

1.6.4. Oxidative Stress and β-Cell Insulin Resistance

Inflammatory and stress-associated kinases could affect β-cell function via inducing insulin resistance directly at the β-cell level. Insulin receptor and insulin signalling molecules are expressed in the β-cell, and it has recently become evident that insulin may affect β-cell function and growth. Short-term effect on GSIS can be either inhibitory or stimulatory (249-252), whereas effects on proinsulin biosynthesis and insulin gene transcription appear to be stimulatory (253-255). Although there is still debate about the importance of insulin for β-cell function, there is consensus about the concept that insulin signalling molecules, however activated (i.e., by insulin, directly by glucose or other hormones (256), play an important and mainly stimulatory role in β-cell function and growth (257;258). This raises the possibility that known inducers of insulin resistance at peripheral sites, such as oxidative stress, may also induce “insulin resistance” at the β-cell level. However, there are only a few reports (206;259) on the effect of prolonged exposure to FFA and glucose on the insulin
signalling cascade in the β-cell. These reports from in vitro studies suggest a possible role for β-cell insulin resistance in both glucotoxicity (259) and lipotoxicity (206).

1.6.5. ROS and endoplasmic reticulum stress

It is known that oxidative stress can induce ER stress and vice versa (240). Oxidative stress can induce ER stress via depletion of ER Ca\(^{2+}\) (260;261). In turn, ER stress can induce oxidative stress because the formation of disulphide bonds by ERO1p oxidase results in the production of ROS (262). Studies with mice involving mutations in ER stress-activated PKR-like ER kinase (PERK) and its downstream effector, the translation initiation complex eukaryotic initiation factor 2 (eIF2) showed that islets from these mice have a marked decrease not only in β-cell development and survival, but also in function (262). ER stress is also implicated in the activation of JNK (263) and can also activate IKKβ/NFκB (264).

Treatment of INS-1 cells with high glucose resulted in induction of the ER stress marker genes, BIP and Chop10 and SREBP-1c mediated decrease in IRS2 (265) (SREBP is processed by the same pathway involved in the processing of the ATF-6 during ER stress). Palmitate was shown to activate PERK and other ER stress markers in β-cell lines (240;266), whereas oleate has less effect (266).
1.6.6. ROS and the hexosamine pathway

Hexosamine pathway activation results in significant deterioration of GSIS along with reduction in expression of insulin gene, coincident with an increase in ROS (214). In turn, ROS can increase flux through the hexosamine pathway through hyperglycemia-induced decrease in glyceraldehyde-3-phosphate dehydrogenase activity in β-cells (211). PKC, ceramides and hexosamines (which can be increased by both glucose and fat (231)) are mediators of the insulin resistance induced by gluco- and lipotoxicity (209;232), and may play a role in the β-cell, because recently, FFA-induced JNK-mediated decrease in insulin gene expression has been linked to impairment of β-cell insulin signaling via serine phosphorylation of IRS-1 (193).
1.7. Summary

An important mechanism which may be involved in the pathogenesis of type 2 diabetes in obese individuals is elevation of plasma free fatty acids (FFA) which induce insulin resistance and may impair both β-cell function and mass (β-cell lipotoxicity). Oxidative stress has been shown to play an important role in β-cell glucotoxicity, i.e. the impairment in β-cell function and mass induced by glucose, which is in many aspects similar to that induced by FFA. There is considerable evidence from *in vitro* and *in vivo* studies that elevated FFA levels (both alone and in combination with glucose) result in the generation of ROS and consequently increased oxidative stress. Mechanisms of FFA-induced oxidative stress and possible sites of impairment of β-cell function are summarized in Figure 1.1 (figures are at the end of this chapter). Possible sources of FFA-induced reactive oxygen species which may affect β-cell function are summarized in Figure 1.2.
1.8. Rationale and Significance of the Studies

Despite the *in vitro* evidence that links oxidative stress to the FFA-induced impairment in β-cell function, proof that oxidative stress is causal had not been provided prior to the studies presented in this thesis. There were only 2 *in vitro* studies; in one of them, by our group, NAC restored insulin content in MIN6 cells exposed to oleate, whereas secretion was not affected (81). In the other study in rat islets, antioxidant treatment was ineffective (161). *In vivo* models have not been studied so far. Furthermore, downstream mechanisms of FFA-induced oxidative stress affecting β-cell function have not been investigated, especially in the *in vivo* model.

The significance of the studies performed and presented in my thesis is: 1) To provide new insights into the physiology and pathophysiology of insulin secretion and into the mechanisms of the β-cell dysfunction caused by lipotoxicity; 2) To establish the rationale for FFA lowering therapies in preventing deterioration of β-cell function in type 2 diabetes and 3) To identify novel targets susceptible to nutritional and/or pharmacological intervention to prevent and treat β-cell dysfunction in obesity associated type 2 diabetes.
1.9. General Hypothesis

The general hypothesis of this thesis is that oxidative stress plays an important role in FFA-induced decrease in β-cell function \textit{in vivo} via pathways that involve inflammation and/or β-cell insulin resistance.
1.10. Specific Aims

**Aim 1.** To investigate the causal role of oxidative stress in FFA-induced decrease in β-cell function.

**Aim 2.** To investigate the causal role of activation of inflammatory pathways, such as IKKβ/NFκB in the FFA-induced decrease in β-cell function.

**Aim 3.** To start investigating the causal role of β-cell “insulin resistance” in the FFA-induced decrease in β-cell function.
1.11. Studies

This thesis consists of three studies that examined the role of oxidative stress (Study 1), inflammatory pathways (Study 2) and β-cell insulin signaling (Study 3) in lipotoxicity and allowed us to draw preliminary conclusions regarding how these factors are linked in the pathway of β-cell lipotoxicity, at the same time suggesting potential targets in its prevention.
Figure 1.1. Mechanisms of free fatty acid (FFA)-induced oxidative stress and sites of impairment of β-cell function.

This figure is not intended to be all-inclusive.
Figure 1.2. Possible sources of free fatty acid (FFA)-induced reactive oxygen species (ROS).

This figure is not intended to be all-inclusive.
2 General Methods

2.1. Procedures

2.1.1. Experimental Animal Model and Surgical Procedures

2.1.1.1. Animals

For all studies, normal Female Wistar rats (Charles River, Quebec, Canada) aged 11-13 weeks and weighing 250-300g were used for experiments, as in our previous studies when we have established the β-cell lipotoxicity model (92). The rats were housed in the University of Toronto’s Department of Comparative Medicine. They were exposed to a 12h light/dark cycle. The rats were fed a Teklad Global diet containing 21% Protein with 64% carbohydrate and 14% fat, Harland Teklad Global Diets, Madison, WI.
2.1.1.2. Surgery

After a week of adaptation to the facility, rats were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg·ml⁻¹, 1 μl·g⁻¹ of body weight; Study 1) or with isofluorane (Studies 2 and 3), and indwelling catheters were inserted into the right internal jugular vein for infusions and the left carotid artery for sampling. Polyethylene catheters (PE-50; Cay Adams, Boston, MA), each extended with a segment of silastic tubing (length of 3 cm, internal diameter of 0.02 inches; Dow Corning, Midland, MI), were used. The venous catheter was extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch. Both catheters were tunneled subcutaneously and exteriorized. The catheters were filled with a mixture of 60% polyvinylpyrrolidone and heparin (1,000 U·ml⁻¹) to maintain patency and were closed at the end with a metal pin. The rats were allowed a minimum 3-4 days period of post-surgery recovery before experiments, after which they were connected to the infusion apparatus. The infusion lines ran inside a tether that was fitted to the subcutaneous implant. Each rat was placed in a circular cage, and the infusion lines were protected by a tether and run through a swivel, which was suspended on top of the cage to give complete freedom of movement to the rat. 48 h infusions were started through the jugular vein, whereas a slow infusion of heparinized saline was used to keep the carotid artery patent for sampling. Throughout the infusion period, rats had free access to water and to their standard pelleted food. All procedures were in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto.
2.1.2. Preparation of Infused Solutions

We have established an *in vivo* model of β-cell lipotoxicity in rats (92), for which we have used a 48 h iv infusion of oleate, a monounsaturated fatty acid, bound to bovine serum albumin (BSA). The BSA is used to prevent the detergent action of the fatty acid, and in this way, the oleate infusion can be given to animals through a central iv line (267). We found that a 48 h two fold elevation of plasma FFA obtained with oleate infusion, which did not induce significant insulin resistance, decreased GSIS during 2 step hyperglycemic clamps in rats (92). The fat emulsions were freshly prepared (72) as in our previous studies (268) and protected from light. The infused olive oil was prepared as in (268). Taurine, NAC, TEMPO (Study 1), Salicylate, BMS (Study 2) and BPV (Study 3) were dissolved in saline at 7.4 pH. During the 48 h infusions the rats had free access to food and water. Samples for FFAs, glucose, and insulin were taken at 18, 24, and 46 hours after the onset of the Saline or fat infusion, i.e. –30, -24, and –2 h before the onset of the hyperglycemic clamp (time = 0). Food was removed at 19:00 the day before the two-step hyperglycemic clamp. Clamp studies or islet isolation were performed in overnight fasted, conscious rats at the end of the 48 h infusions.
2.1.3. Hyperglycemic Clamp Studies

After the 48 h infusion period, basal samples were taken over 20 min (basal period). The two-step hyperglycemic clamp was performed in conscious rats fasted overnight. Thereafter, a two-step hyperglycemic clamp was performed to evaluate GSIS from the plasma insulin and C-peptide response to the rise in plasma glucose. Both C-peptide and insulin were measured because they are co-secreted but cleared by different mechanisms (kidney and liver). Therefore, a change in both, indicates a change in secretion. Furthermore, insulin clearance may be decreased by FFA, whereas there is no effect of FFA on C-peptide clearance (269). At –20 min, the continuous arterial infusion of heparinized saline was stopped in all rats, since the same total amount of heparinized saline was used to dilute the erythrocytes that were reinfused into the rats after plasma separation from blood samples. The venous infusion saline/treatment was continued throughout the experiment. The glucose infusion was given through the jugular catheter. Both the glucose and the saline/treatment infusion lines were connected to the jugular line through a Y shaped connector. Using an infusion of 37.5% glucose, at time = 0 min, glucose level was gradually raised and then maintained at 13 mmol·l⁻¹ (upper physiological glucose level for rats) for 120 min. The gradual rise in glucose avoids any oleate bolus from the dead space of the infusion line which may cause heart arrhythmia and sudden death of the rat. At time = 120 min the glucose level was again raised and then maintained at 22 mmol·l⁻¹ (maximum stimulatory levels) for another 120 min. Plasma glucose was 'clamped' at either 13 mmol·l⁻¹ or 22 mmol·l⁻¹ by a variable glucose infusion adjusted according to
frequent (every 5-10 min) glycemic readings obtained on a Beckman 2 glucose analyzer. Samples for insulin, C-peptide and FFA were taken at regular intervals. The sample volume was minimized to avoid anemia. A total of 2.5ml of blood was withdrawn from the rats. After removal of plasma from centrifuged whole blood samples, erythrocytes were suspended in heparinized saline (4U/ml) and reinfused into the rats. FFA levels were measured by colorimetric kits (Wako and Boehringer Chemicals, resp.), rat insulin and C-peptide by RIA kits (Linco Research Inc, MO). In each experiment, an index of insulin sensitivity (93) was obtained from the hyperglycemic clamp data, i.e. during the determination of GSIS, as described in the following chapter and in the methods section of Study 2. At the end of the experiment, the rats were deeply anesthetized and the pancreas was excised and fixed for histochemical analysis.

2.1.4. Evaluation of GSIS and β-Cell Function In Vivo

C-peptide levels were taken as indices of absolute insulin secretion, as calculations of insulin secretion rate by C-peptide deconvolution, which is commonly performed in humans (93;270;271) cannot be performed in the rat. This is because the parameters of C-peptide kinetics cannot be derived in the rat, as rat C-peptide (species specific) is not available in amounts sufficient for in vivo injections. However, studies in humans have shown that the kinetics of C-peptide are not influenced by glucose or fat (269;271;272). In vivo, absolute insulin secretion is influenced by insulin
sensitivity, as the β-cell adapts to insulin resistance (reciprocal of insulin sensitivity). However, it is possible to take into account the effect of insulin sensitivity on absolute insulin secretion and thereby assess β-cell function based on the hyperbolic relationship between insulin secretion and insulin sensitivity described by (273). β-cell function in vivo (insulin secretion corrected for insulin sensitivity) is assessed by calculating the content of the hyperbola, termed as Disposition Index (DI = C-peptide multiplied by an index of insulin sensitivity). Insulin sensitivity during hyperglycemic clamps is calculated as M/I index, that is the glucose infusion rate required to maintain the glycemic target divided by the insulin concentration. In our oleate infusion model, absolute insulin secretion and β-cell function tend to vary in parallel as there are minimal effects of oleate on insulin sensitivity. We have previously shown that M/I and C-peptide are inversely related, as implied by the DI concept, in our model (274).

2.1.5. *Ex vivo* Islet Studies

The islet secretion studies were performed to investigate β-cell function in an open loop system, i.e. where insulin secretion cannot be acutely influenced by the prevailing insulin resistance or by changes in insulin clearance. These studies did therefore complement the studies in vivo where the contribution of insulin resistance to insulin secretion can only be 'eliminated' by calculation (i.e., DI method), and where the contribution of changes in insulin clearance to β-cell function is still a
factor. Islets were also used for biochemical and molecular studies that allowed us to gain insights into the mechanisms of lipotoxicity. At the end of the 48 h infusions, islets were isolated and used for insulin secretion studies and for determination of ROS using the dichlorofluorescein diacetate (DCFH-DA) method.

2.1.5.1. Islet Isolation

Pancreatic islets were isolated in overnight fasted rats, after the 48h infusion period. Rats were anesthetized in a similar manner as described above. The visceral contents were exposed and rats were exanguinated through an incision in the abdominal aorta. The common bile duct was quickly isolated and a collagenase solution was infused into the pancreas (~15 ml). The pancreas was then removed and placed in a sterile 50 ml Falcon tube. The pancreas was then incubated in a water bath (37°C). Following the incubation, the contents of the Falcon tube were subjected to vigorous shaking. The mixture was then centrifuged, and the pellet resuspended and passed through a 300 µm filter, followed by another centrifugation. The pellet was resuspended and the islets were isolated by a Histopaque 1077 density gradient (275;276). Islet recovery was monitored as possible indication of substantial changes in β-cell mass. During isolation, glucose was kept at 2.8 mmol·l⁻¹.
2.1.6. Evaluation of GSIS and β-Cell Function Ex Vivo

After 60 min preincubation in KRB, islets of approximately the same size were handpicked and used for static incubation at various glucose concentrations (5 islets/tube in triplicate). Incubations were performed at low glucose (2.8 mmol·l\(^{-1}\)) to obtain a measure of the glucose-independent component of insulin secretion, 6.5 mmol·l\(^{-1}\) which corresponds to basal glucose level in rats, and at the same glucose concentrations as studied in vivo (13 and 22 mmol·l\(^{-1}\)) for an equal length of time (120 min). Insulin release was evaluated from insulin concentrations in the medium.

2.1.7. In vitro Studies in Islets

*In vitro Islet Studies* were performed in order to totally eliminate the indirect effect of insulin resistance on insulin secretion, which is still present in the 48h infusion period in the *ex vivo* studies, and also to perform mechanistic studies, as the amount of islets is greater than obtained through *ex vivo* studies (when large quantities were required, islets from few animals were pooled). The same Wistar rats were used as in chronic infusion models. Islets were isolated using the same protocol as in the *ex vivo* studies. Batches of 200–300 islets of similar size were collected and maintained at 37°C in a 5% CO\(_2\) atmosphere in suspension in 15 ml RPMI medium without antioxidants, containing 10% fetal bovine serum, different glucose concentrations, and different agents according to the experimental protocols, for 48 h. In
the end of the 48 h period, islet insulin secretion was determined through static incubation. The same determinations were performed as described above for my *ex vivo* studies, including measurements of ROS with the DCF method.

### 2.1.8. ROS Determination

Pancreatic islets were incubated with 10μM dichlorofluorescein diacetate (DCFH-DA) (Molecular probes, Invitrogen, Burlington, ON, Canada) in KRBH buffer containing no glucose for 30 minutes at 37 °C (277). DCFH-DA is a cell permeable compound that diffuses across the cell membrane, whereafter it is hydrolyzed by intracellular esterases in living cells to yield dichlorofluorescein (DCFH), a nonfluorescent compound. Subsequently, DCFH can be oxidized by ROS to the fluorescent compound dichlorofluorescein (DCF). After loading with DCFH-DA, the medium was removed and replaced with fresh KRB buffer containing no glucose. Thereafter, the islets were imaged and fluorescence was recorded in an Olympus fluorescent BX51W1 microscope fitted with a 10x water immersion objective and cooled CCD camera equipped with a magnification changer, allowing for an additional increase in the magnification by 4 times. Measurements were performed at 480nm excitation and 510nm emission using a xenon lamp-based DeltaRam high speed monochromator (Photon technology international) and videocamera. The collected data was analyzed using the ImageMaster 3 software.
2.2. Laboratory Methods

2.2.1. Plasma Glucose

Plasma glucose concentrations were measured by the glucose oxidase method using a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). A 10 µl sample of plasma containing D-glucose is pipetted into a solution containing oxygen and glucose oxidase. The glucose reacts with oxygen in the following reaction catalyzed by glucose oxidase:

\[
\text{D-Glucose} + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconic Acid} + \text{H}_2\text{O}_2
\]

In the reaction, oxygen is used at the same rate as glucose to form gluconic acid. A polarographic oxygen sensor is used to detect oxygen consumption, which is directly proportional to the glucose concentration in the sample. Results are obtainable within 30 sec following sample addition. Plasma samples were reanalyzed until repeated measurements were within a difference of 3 mg·dL^{-1}. The analyzer was calibrated before use and frequently during the experiment with the 150/50 glucose/urea nitrogen standard (Beckman Instruments Inc., Naguabo, Puerto Rico, USA) that accompanied each kit.
2.2.2 Plasma Free Fatty Acids Assay

Plasma levels of FFA were analyzed using a colorimetric kit under enzymatic reaction from Wako Industrials (Neuss, Germany). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA produced is oxidized by adding acyl-CoA oxidase (ACOD), which generates $\text{H}_2\text{O}_2$. $\text{H}_2\text{O}_2$, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(B-hydroxyethyl)-aniline (MEHA) with 4-aminophenazone to form the final reaction product, which is a purple colored adduct. This can be measured colorimetrically at 550 nm. The results are correct to within 1.1%. The reactions of this assay is listed below:

\[\text{ACS}\]
\[\text{FFA} + \text{ATP} + \text{CoA} \longrightarrow \text{Acyl-CoA} + \text{AMP} + \text{PPi}\]

\[\text{ACOD}\]
\[\text{Acyl-CoA} + \text{O}_2 \longrightarrow 2,3\text{-trans-Enoly-CoA} + \text{H}_2\text{O}_2\]

\[\text{POD}\]
\[2 \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{MEHA} \longrightarrow \text{Final Reaction Product} + 3 \text{H}_2\text{O}\]
2.2.3. Plasma Insulin Assay

Radioimmunoassay (RIA) kit, double antibody RIA, specific for rat insulin from Linco Research Inc. (St. Charles, MO, USA) were used to determine plasma insulin concentrations. Insulin in the plasma sample competes with a fixed amount of $^{125}$I-labelled insulin for the binding sites on the specific antibodies. A standard curve was determined using insulin standards at 0, 3, 10, 30, 100, 240 µU·ml$^{-1}$ in duplicate. An addition of a second antibody immunoadsorbent followed by centrifugation and aspiration of the supernatant separated the bound and free insulin. The radioactivity of the pellet was then measured and was inversely proportional to the quantity of insulin in the sample. $^{125}$I-insulin (50 µl to all tubes) and rat insulin antibody (50 µl to all tubes) were mixed with plasma sample (100 µl to all tubes). The tubes were then vortexed and incubated overnight at 4 degrees. 1000 µl of precipitating reagent was added to all tubes followed by vortexing and incubating for 20 min at 4°C. The tubes were then spunned at 1500 g for 40 min. The supernatant was aspirated and the radioactivity in the pellet was counted for 4 min in a gamma counter (Beckman Instruments, Fullerton CA, USA). The counts (B) for each of the standards and unknowns were expressed as a percentage of the mean counts of the “0 = standard” (Bo):

$$\text{% activity bound} = \frac{B \; (\text{Standard or sample})}{B_O} \times 100 \%$$

The % activity bound for each standard was plotted against the known concentration in order to construct the standard curve. The unknown sample was determined by the
interpolation of the standard curve. The coefficient of interassay variation determined on
reference plasma was less than 10 \%.

2.2.4. Plasma C-Peptide assay

Linco’s Rat C-peptide RIA kit was used to determine the plasma C-peptide levels. The
kit uses an antibody specific for rat C-peptide (Linco Research, Inc, St. Charles, MO
USA). The principle is the same as insulin RIA as described above. The procedures are
the same as insulin RIA with the exception of one extra day. On the first day, only rat C-
peptide antibody was added followed by an overnight incubation at 4°C. On the second
day, \( ^{125}I \)-rat C-peptide was added followed by vortexing and overnight incubation at 4°C.
On the last day, 1000 \( \mu l \) of precipitating reagent was added to all tubes followed by
vortexing and incubation for 20 min at 4°C. Then, the tubes were centrifuged at 1500 g
for 40 min. The supernatant was the aspirated, and the radioactivity in the pellet was
counted for 4 min in a gamma counter. The % activity bound was calculated the same as
insulin RIA kit. The % activity bound for each standard was plotted against the known
concentration to obtain standard curve. The concentration of the unknown samples were
determined by interpolation with a coefficient of inter assay variation determined on
reference plasma less than 10.5\%.
2.3. Calculations

2.3.1. Insulin clearance

The C-Peptide/Insulin ratio was used as an index of insulin clearance. The C-peptide level was divided by the insulin level at each time point in the last 40 minutes of each step for the two-step hyperglycemic clamp.

2.3.2. Insulin sensitivity index

The insulin sensitivity index (M/I) was calculated at individual time points during the last 40 minutes of each step of the two-step hyperglycemic clamp according to the following formula:

\[
\frac{M}{I} = \frac{G_{\text{INF}}}{\text{Insulin}}
\]

where \( G_{\text{INF}} \) is the rate of glucose infusion, Insulin is the insulin concentration, and Glucose is the plasma glucose level at individual time points during the last 40 minutes of each step of the hyperglycemic clamp. This equation assumes that the change in glucose uptake and production induced by a change in insulin concentration is proportional to the ambient glucose and insulin concentration. M/I is reported in units of deciliter per kilogram per minute per microunit per milliliter. Unfortunately, there are limitations to
using this method to assess insulin sensitivity at elevated insulin levels. It has been reported that the relationship between circulating insulin levels and insulin action is not linear at insulin concentrations higher than 180 μU·ml⁻¹ (53).

2.3.3. Disposition Index

The disposition index (DI), which was used as an index of insulin secretion corrected for the ambient degree of insulin resistance, was calculated for each time point as DI=M/IxC-peptide; the rationale for using this index was provided in the chapter Evaluation of GSIS and β-cell Function In Vivo.
2.4. Statistical Analysis

Presented data are means ± SE. One way non-parametric ANOVA for repeated measurements, followed by Tukey’s t-test was used to compare treatments. Calculations were performed using SAS (Cary, NC).
Study 1

Free Fatty Acid-Induced Reduction in Glucose Stimulated Insulin Secretion—Evidence for a Role of Oxidative Stress *In Vitro* and *In Vivo*

* The results of this study are incorporated in a manuscript published in Diabetes, 56(12):2927-37, 2007.
3.1. Abstract

Objective An important mechanism in the pathogenesis of type 2 diabetes in obese individuals is elevation of plasma FFA, which induce insulin resistance and chronically decrease β-cell function and mass. Our objective was to investigate the role of oxidative stress in FFA-induced decrease in β-cell function.

Research Design and Methods We used an in vivo model of 48h i.v. oleate infusion in Wistar rats followed by hyperglycemic clamps, or islet secretion studies ex vivo, and in vitro models of 48h exposure to oleate in islets and MIN6 cells.

Results 48h Infusion of oleate decreased the insulin and C-peptide responses to a hyperglycemic clamp (p<0.01), an effect prevented by coinfusion of the antioxidants N-acetylcysteine and taurine. Similar to the findings in vivo, 48h infusion of oleate decreased glucose stimulated insulin secretion ex vivo (p<0.01), and induced oxidative stress (p<0.001) in isolated islets, effects prevented by coinfusion of the antioxidants N-acetylcysteine, taurine, or tempol. 48h infusion of olive oil induced oxidative stress (p<0.001) and decreased the insulin response of isolated islets similar to oleate (p<0.01). Islets exposed to oleate or palmitate and MIN6 cells exposed to oleate showed a decreased insulin response to high glucose and increased levels of oxidative stress (both p<0.001), effects prevented by taurine. Real-time RT-PCR showed increased mRNA levels of antioxidant genes in MIN6 cells following oleate exposure, an effect partially prevented by taurine.
Conclusions Our data are the first demonstration that oxidative stress plays a role in the decrease in β-cell secretory function induced by prolonged exposure to FFA, *in vitro* and *in vivo*. 
3.2. Introduction

Type 2 diabetes is characterized by both insulin resistance and defective insulin secretion (50). Obesity is the major predisposing factor for type 2 diabetes and is associated with excessive release of fatty acids from the expanded adipose tissue mass, leading to elevated plasma FFA, known to induce insulin resistance (9;232). Acute FFA exposure stimulates insulin secretion (53), but studies in vitro and in situ have shown that prolonged FFA exposure decreases glucose stimulated insulin secretion (GSIS) (278). The effect of prolonged FFA elevation on β-cell function in vivo has been more controversial, as absolute GSIS was found to be increased (90;91;279), unchanged (93;280), or decreased (71;92;281) by FFA. However, in most of these studies β-cell function was inadequate to compensate for FFA-induced insulin resistance (71;92;93;280;281), at least in predisposed individuals (67). Although the mechanisms behind FFA-induced decrease in β-cell function are unclear, one possibility points toward oxidative stress. Pancreatic β-cells have low antioxidant defenses (74) and are thus susceptible to reactive oxygen species (ROS)-induced decrease in function and viability (75;76). Oxidative stress has been implicated in the decrease in GSIS induced by prolonged exposure to glucose (77;78), which is in many respects similar to that induced by prolonged exposure to FFA (53;79). However, whether oxidative stress plays a role in FFA-induced decrease in β-cell function is still unclear. The majority of studies have shown that FFA generate ROS in islets (165) and β-cell lines (81;282), although in a recent paper ROS were not increased by FFA in cultured islets. In the same paper, antioxidants did not prevent the FFA-induced defect in GSIS (161). By contrast, in our
previous study, although the antioxidant N-acetylcysteine did not restore secretion in MIN6 cells, it did prevent the decrease in insulin content induced by oleate (81). There are no other studies linking oxidative stress to FFA-induced decrease in β-cell function, although insulin sensitizers with anti-oxidant activity restored GSIS \textit{in vitro} (210;283).

In this study we investigated the effects of prolonged exposure to oleate, alone and in combination with antioxidants, on GSIS during hyperglycemic clamps \textit{in vivo, ex vivo} in islets of oleate-treated rats and \textit{in vitro} in islets and MIN6 β-cells. Both hyperglycemic clamps and \textit{ex vivo} studies in islets were performed in rats infused i.v. for 48h with oleate/saline + antioxidants. We used three antioxidants, N-acetylcysteine (NAC), taurine, and tempol (4-Hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl). Oleate is the most prevalent circulating fatty acid, followed by palmitate. For \textit{in vitro} studies in islets, both oleate and palmitate were used. Unfortunately, palmitate has low solubility and thus is not suitable for i.v. infusion. Oleate was used instead of the standard infusion of Intralipid and heparin because we have previously shown that a prolonged infusion of oleate impairs GSIS to a greater extent than Intralipid + heparin (92). For \textit{in vitro} studies in MIN6 cells, oleate was used, and we measured ROS and expression of genes induced by oxidative stress.
3.3. Materials and Methods

3.3.1. Studies in rats

3.3.1.1. Animals and surgery

Female Wistar rats (250-300g, Charles River, Canada) were cannulated as previously described in the General Methods section and in (92).

3.3.1.2. Intravenous infusions

Rats were infused for 48h with either: 1) saline (SAL); 2) oleate (OLE); 3) oleate and lower dose of N-acetylcysteine (OLE+L-NAC); 4) oleate and higher dose of N-acetylcysteine (OLE+H-NAC); 5) oleate and taurine (OLE+TAU); 6) lower dose of N-acetylcysteine (NAC); 7) higher dose of N-acetylcysteine (NAC); 8) taurine (TAU), followed by a two-step hyperglycemic clamp. For ex vivo GSIS determinations in islets, we added three more treatments: 9) olive oil/heparin alone (OLO); 10) oleate and tempol (OLE+TPO) or 11) tempol alone (TPO). We used the same infusion protocol as in (92). Oleate (Sigma, St. Louis, MO) was prepared in fatty acid free BSA (Sigma, St. Louis, MO) according to the Bezman-Tarcher method (267) as modified by Miles et al. (284), and given at 1.3 µmol·min⁻¹. The 20% olive oil emulsion was prepared by mixing...
chemical grade (Sigma) olive oil (unrefined olive oil contains antioxidants) with phosphatidylcholine, glycerol and penicillin-streptomycin, as described in (268). Heparin was added to a final concentration of 50 U·ml$^{-1}$, and the emulsion was infused at 5 µl·min$^{-1}$. Saline was given as control (equi-volume) as we have shown no difference between saline and BSA infusion, using the same experimental protocol (92). NAC was given at 2.14 µmol·kg$^{-1}$·min$^{-1}$, dose that reversed insulin resistance induced by glucose in our previous study (238), and at 2.76 µmol·kg$^{-1}$·min$^{-1}$; taurine was given at this equimolar dose. Tempol was given at 2.41 µmol·kg$^{-1}$·min$^{-1}$, the dose that showed protection against ROS toxicity in experimental pancreatitis (285). All antioxidants (Sigma) were dissolved in saline at pH=7.4. After 48h infusion and ~12h fasting, we performed either in vivo studies, by using two-step hyperglycemic clamps, or ex vivo studies in isolated islets.

### 3.3.1.3. Hyperglycemic clamps

GSIS in vivo was determined by measuring insulin and C-peptide during two-step hyperglycemic clamps. An infusion of 37.5% glucose was started at time=0min. We did not use a glucose prime because we wished to avoid possible arrhythmias caused by an oleate bolus from the dead space of the infusion line. Plasma glucose was maintained at 13 mmol·l$^{-1}$ by adjusting the rate of the glucose infusion according to frequent (every 5-10min) glucose determinations. At 120min, the glucose infusion was raised to achieve and maintain plasma glucose levels of 22 mmol·l$^{-1}$ until the end of experiments (time=240min), as described in the General Methods section.
3.3.1.4. Plasma assays

Plasma glucose, insulin and C-peptide levels were measured as described in the General Methods section.

3.3.1.5. Islet isolation and secretion studies

Islets of in vivo infused rats were isolated as in (277), and preincubated for 1h at 37°C in Krebs Ringer buffer containing 10 mmol·l⁻¹ HEPES (KRBH), and 2.8 mmol·l⁻¹ glucose. Thereafter, 5 islets of approximately the same size were incubated in triplicate at 2.8, 6.5, 13 and 22 mmol·l⁻¹ glucose for 2h, 37°C. Insulin was measured in the supernatant with Linco’s kit. For in vitro studies, before preincubation, islets of untreated rats were cultured for 48h in RPMI 1640 without antioxidants, containing 0.4 mmol·l⁻¹ oleate or 0.4 mmol·l⁻¹ palmitate in 0.5% BSA, or 25 µmol·l⁻¹ H₂O₂, with or without 1 mmol·l⁻¹ taurine.

3.3.2. Studies in MIN6 Cells (performed by G. Bikopoulous)

3.3.2.1. Cell culture
Oleate, BSA, taurine and KRBH were purchased from Sigma. Oleate bound to fatty acid free BSA was prepared as in (81). MIN6 β-cells (286), gift from Dr. S. Seino, Chiba University (passage number 35–45) were cultured in DMEM containing 25 mmol·l⁻¹ glucose and supplemented with 10% fetal bovine serum, 100 units·ml⁻¹ penicillin and 100 μg·ml⁻¹ streptomycin. Cells (3.5x10⁵) were then incubated overnight in DMEM containing 10 mmol·l⁻¹ glucose, followed by additional exposure to: 1) 0.5 % (wt/vol) BSA, 2) 0.4 mmol·l⁻¹ oleate in BSA, 3) 0.4 mmol·l⁻¹ oleate in BSA plus 1 mmol·l⁻¹ taurine, or 4) 1 mmol·l⁻¹ taurine in BSA for 48h.

3.3.2.2. Insulin secretion

MIN6 cells were preincubated for two sequential periods of 30 min in KRBH with no glucose and incubated in KRBH containing 0 or 16.7 mmol·l⁻¹ glucose for 1h. Insulin was determined in the supernatant with Linco’s kit.

3.3.2.3. ROS measurements

Islets or MIN6 cells were incubated with 10μM dihydro-dichlorofluorescein-diacetate (H₂DCF-DA, D6883 Sigma) in KRBH containing 2.8 mmol·l⁻¹ (islets) or 0 mmol·l⁻¹ (MIN6 cells) glucose for 30 min (277). Then, the medium was replaced with
fresh KRBH containing no glucose and fluorescence measured at 480 nm excitation and 510 nm emission with an Olympus microscope. Data were analyzed using ImageMaster3.

3.3.2.4. RT-PCR

Primers were designed using Primer Express (PE Applied Biosystem, Perkin Elmer, Foster City, CA). Total RNA was extracted using the RNeasy kit (Qiagen, Burlington, Canada). Total RNA was reverse transcribed using oligo-dT primers and Superscript II (Invitrogen, Burlington, Canada). The real-time RT-PCR was monitored and analyzed by the Sequence Detection System (PE Applied Biosystem, Perkin Elmer). All genes were normalized to β-actin. Primers sequences are available upon request.

3.3.3. Statistics

Data are means±SE. One way non-parametric ANOVA for repeated measurements, followed by Tukey’s t-test was used to compare treatments. Calculations were performed using SAS (Cary, NC).
3.4. Results

3.4.1. In vivo clamp studies

During the 48h infusions, as expected, the oleate treated rats had higher plasma FFA than the rats treated with saline or antioxidant alone (Table 3.1, tables and figures are at the end of this chapter). The infusion of oleate or antioxidants did not affect plasma glucose or insulin (data not shown).

Following the 48h infusions, we evaluated insulin secretion in vivo during hyperglycemic clamps. As shown in Figures 3.1.A and 3.1.D, basal plasma FFA prior to the clamps were higher in oleate-treated than control rats, or rats treated with antioxidant alone. FFA levels declined during the clamp because of hyperglycemia and hyperinsulinemia. However, FFA remained higher in oleate-treated rats.

Basal plasma glucose was similar in all groups. During the first step of the clamp, glucose levels rose to 13 mmol·l⁻¹ (upper physiological in rats), and during the second step to 22 mmol·l⁻¹ (pathological but maximally stimulatory) with no differences among groups (Figures 3.1.B and 3.1.E). The glucose infusion rate (GINF) necessary to maintain the target glucose level was lower in the oleate- than saline-treated group, consistent with decreased insulin secretion, decreased insulin sensitivity, or both (Figures 3.1.C and 3.1.F). With the low NAC dose in combination with oleate, GINF was only partially restored (p=NS, OLE+L-NAC vs. OLE or SAL, Figure 3.1.C). When the dose of NAC was increased by 25%, GINF was completely restored (Figure 3.1.C). Taurine,
when coinfused with oleate at a dose equimolar to the higher NAC dose, also restored GINF to control levels (Figure 3.1.F). Antioxidants alone had no effect on GINF.

Basal insulin and C-peptide levels were similar in all groups. As expected, plasma insulin rose in response to increasing glucose levels (Figures 3.2.A and 3.2C). Plasma C-peptide also rose, indicating that the rise in insulin was due to increased secretion (Figures 3.2.B and 3.2.D). Plasma insulin and C-peptide were lower in oleate- than saline-treated rats. In the group infused with oleate and low-dose NAC, plasma insulin and C-peptide were restored to control levels only at 13 mmol l⁻¹ glucose (p=NS, OLE+L-NAC vs SAL; p<0.05, OLE+L-NAC vs. OLE), whereas at 22 mmol l⁻¹ glucose the effect of oleate was only partially prevented (p=NS, OLE+L-NAC vs. SAL or OLE). When the higher dose of NAC, or an equimolar dose of taurine was added to oleate, plasma insulin and C-peptide were completely restored (Figures 3.2.A-B and 3.2.C-D). Antioxidants alone had no effect on insulin or C-peptide levels.

As evidenced by Figures 3.1.C-1F and 3.2.A-3.2.C, GINF and insulin levels were proportionally decreased in OLE vs. SAL groups, therefore the sensitivity index M/I=GINF/Insulin (287) was not significantly different during both the first step (0.404±0.047 μmol·kg⁻¹·min⁻¹/pmol·l⁻¹ for SAL, and 0.323±0.023 μmol·kg⁻¹·min⁻¹/pmol·l⁻¹ for OLE, p=NS) and the second step (0.260±0.033 μmol·kg⁻¹·min⁻¹/pmol·l⁻¹ for SAL, and 0.269±0.030 μmol·kg⁻¹·min⁻¹/pmol·l⁻¹ for OLE, p=NS) of the clamp. Both antioxidants had no effect on M/I.
3.4.2. *Ex vivo* studies in islets

Rats were infused for 48h with oleate/saline at the same rate as for our *in vivo* studies. At the end of the 48h infusions, islets were isolated and incubated at the following glucose concentrations: 2.8 mmol·l⁻¹ (non-stimulatory); 6.5 mmol·l⁻¹ (basal glucose levels in rats); 13 mmol·l⁻¹ and 22 mmol·l⁻¹ (as in hyperglycemic clamps). The insulin secretory response of islets isolated from oleate-infused rats was markedly decreased compared with control, both at 13 and 22 mmol·l⁻¹ glucose (*Figure 3.3.B*). To exclude any possible non-specific effect on insulin secretion due to the surfactant activity of direct infusion of FFA, we infused for 48h an emulsion of 20% olive oil (OLO), a triglyceride mixture containing 71% oleate. Heparin was added to the olive oil emulsion to activate lipoprotein lipase, which releases FFA from the triglycerides of olive oil. At the end of olive oil infusion, the increase in FFA levels was similar to that observed following the oleate infusion (*Figure 3.3.A*). Olive oil infusion decreased GSIS (*Figure 3.3.B*), and increased ROS (*Figure 3.3.C, 3.3.D*) of isolated islets, similar to the oleate infusion. We also performed *in vivo* infusion of NAC and TAU alone, and in combination with oleate, followed by evaluation of insulin secretion in isolated islets. Similar to our results *in vivo*, the lower NAC dose prevented the oleate-induced GSIS decrease at 13 mmol·l⁻¹ glucose (p=NS, OLE+L-NAC vs. SAL, and p<0.05, OLE+L-NAC vs. OLE), whereas its protective effect at 22 mmol·l⁻¹ glucose was only partial (p=NS, OLE+L-NAC vs. OLE or SAL, *Figure 3.4.A*). The higher NAC dose (*Figure 3.4.A*) and taurine (*Figure 3.4.B*) prevented the oleate-induced GSIS decrease at both 13 and 22 mmol·l⁻¹ glucose. Since NAC can be converted to taurine, which might affect insulin secretion.
independent of its antioxidant effect (288-290), we used another antioxidant, tempol, a superoxide dismutase (SOD)-mimetic, unrelated to NAC or taurine. Similar to the other antioxidants, when given alone, tempol did not affect plasma FFA during the 48h infusion (OLE+TPO: 1058±108 µmol·l⁻¹ at 48h, p=NS vs. OLE: 1093±86 µmol·l⁻¹; TPO: 713±49 µmol·l⁻¹ at 48h, p=NS vs. SAL: 663±59 µmol·l⁻¹). When tempol was added to the oleate infusion, it prevented the oleate-induced GSIS decrease in isolated islets, similar to NAC and taurine (Figure 3.4.C). No significant effect of any antioxidant alone was observed at any glucose concentration. Addition of high dose NAC (Figure 3.4.D), taurine (Figure 3.4.E) or tempol (Figure 3.4.F) completely prevented the oleate-induced increase in ROS, whereas no significant change in ROS was observed with any antioxidant alone.

3.4.3. In Vitro studies in islets

We performed in vitro studies in islets, to investigate whether 48h exposure to oleate had the same effect as in our in vivo or ex vivo studies; moreover, we also used palmitate, as it is commonly used in vitro, and hydrogen peroxide as a positive control for ROS induction (213). 0.4 mmol·l⁻¹ oleate or palmitate in 0.5% BSA decreased GSIS (Figure 3.5.A and 3.5.C) to approximately the same degree, as did 25 µmol·l⁻¹ hydrogen peroxide (Figure 3.5.D), whereas coincubation with 1 mmol·l⁻¹ taurine restored GSIS. Taurine alone had no effect on insulin secretion. Oleate, palmitate or hydrogen peroxide also increased ROS, which was prevented by the addition of taurine (Figure 3.6.A-C).
Taurine alone did not affect ROS. Presence of 0.4 mmol·l⁻¹ oleate during the 2h of GSIS increased the level of insulin in all groups, however, the inhibitory effect of 48h oleate exposure was still present, and prevented by addition of taurine (Figure 3.5.B).

3.4.4. In Vitro studies in MIN6 cells

We also performed studies in MIN6 cells, to investigate whether oleate upregulates genes of antioxidant enzymes, and whether this effect is reversed by antioxidants. 0.4 mmol·l⁻¹ oleate in 0.5% BSA for 48h increased insulin secretion at 0 glucose, but decreased GSIS (Figure 3.7.A). 2.8 mmol·l⁻¹ glucose concentrations were not studied here because already stimulatory in MIN6 cells (80;81), however, our previous data suggest no effect of oleate at 2.8 mmol·l⁻¹ glucose (81). Coincubation with taurine restored GSIS and tended to normalize insulin secretion at 0 glucose. Taurine alone had no effect on insulin secretion. Oleate also increased ROS, which was prevented by the addition of taurine (Figure 3.7.B). Taurine alone did not affect ROS. To confirm the increase in ROS independent of the DCF method, we performed real-time RT-PCR for the following genes induced by oxidative stress: inducible heme oxygenase-1 (HO-1, cytoplasmic or nuclear), glutathione peroxidase-1 (GPx-1, mitochondrial and cytoplasmic), catalase (cytoplasmic), Cu/Zn-superoxide dismutase (Cu/Zn-SOD, SOD3, extracellular), Mn-superoxide dismutase (Mn-SOD, SOD2, mitochondrial). We also evaluated the effect of oleate on the metabolic genes CPT-1 and UCP-2 as a control. Oleate increased the mRNA of CPT-1, UCP-2, Cu/Zn-SOD, GPx-1 and catalase (Figure
3.8), but not SOD2 (data not shown). Addition of taurine did not affect the upregulation of CPT-1 as expected, UCP-2 or GPx-1 mRNA, but prevented the upregulation of catalase (p<0.05, OLE+TAU vs. OLE), whereas the effect on Cu/Zn-SOD was only partial. HO-1 mRNA showed a tendency to increase in response to oleate, which appeared to be reversed by taurine.
3.5. Discussion

We have examined the effects of prolonged FFA exposure with or without antioxidants, on β-cell function in vivo, ex vivo, and in vitro. In our in vivo clamp studies both insulin and C-peptide responses to glucose were lower in rats treated with oleate. These results indicate reduced insulin secretion, an effect consistent with that observed ex vivo and in vitro in isolated islets and in vitro in MIN6 cells. Insulin secretion during hyperglycemic clamps should be interpreted in the context of insulin sensitivity, since in vivo normal β-cells compensate for insulin resistance. Although FFA induce insulin resistance, this effect was not observed in the present study. This was not surprising, as we noticed only a trend for insulin sensitivity to decrease (92) in our previous studies performed using the same oleate infusion protocol in the same animal model. The lack of detectable insulin resistance may be related to a number of factors, including only moderate elevation of FFA, their monounsaturation, the female sex of the rats, and the use of the hyperglycemic clamp rather than the gold-standard hyperinsulinemic-euglycemic clamp. Since there was no change in insulin sensitivity, and therefore the β-cell did not have to compensate for insulin resistance, our results are similar in vivo and ex vivo.

The mechanism whereby prolonged exposure to fatty acids decreases β-cell function is not completely understood, and is probably due to oxidative stress-dependent and independent pathways (81). Studies have shown that fatty acids promote the generation of ROS in islets (165) and β-cell lines (81;282). Although ROS in minimal amounts can increase basal insulin secretion (291), both lipid peroxides (166) and
hydrogen peroxide (75) decreased GSIS. Cytokine exposure (292) and chronic hyperglycemia (77;78) are two other conditions where reduced β-cell function was found to be associated with oxidative stress and antioxidants improved GSIS (77;78;293). Decreased GSIS in the presence of oxidative stress may be an adaptive response of the β-cell to limit further ROS generation and reduce endoplasmic reticulum stress, thereby allowing the β-cell to resist apoptosis. Thus, in terms of β-cell survival, decreased function can be interpreted as a beneficial adapting phenomenon (liподadaptation as in (53)). In terms of whole body physiology, however, decreased β-cell function can also be considered as maladaptive, and therefore lipotoxic, because it results in decreased glucose tolerance (as evidenced by the reduced GINF during our clamps) which can lead to hyperglycemia (93).

We here show that the FFA-induced decrease in GSIS was prevented by antioxidants. Both NAC and taurine were effective in vivo, and according to our results with NAC, the effect was dose-dependent. To assess β-cell function independent of systemic factors influencing the β-cell response to glucose in vivo (e.g. not only the prevailing insulin sensitivity but also islet autonomic innervation), we examined GSIS ex vivo in isolated islets. We also measured islet ROS, as direct proof of the presence of oxidative stress. Furthermore, to eliminate any indirect effect of in vivo treatments, we exposed islets and MIN6 cells directly to fat in vitro. 48h infusion of oleate, or olive oil, which contains mostly oleate, increased ROS and impaired GSIS ex vivo in isolated islets, consistent with our in vivo findings. Treatment with NAC, taurine or tempol protected the islets from the FFA-mediated impairment in GSIS. Direct exposure of islets for 48h to oleate or palmitate decreased the insulin response at high glucose, and taurine
restored GSIS, similar to our *ex vivo* model. The same results were obtained with oleate in MIN6 cells, where the oleate-induced increase in expression of antioxidant genes catalase and Cu/Zn-SOD was partially prevented by taurine. The reason for the only partial prevention by taurine is unclear, but may be related to cell line variability.

Taurine and NAC are sulfur-containing amino acids that scavenge aldehydes and can directly or indirectly increase the content of glutathione. Besides its antioxidant properties, taurine has other effects that could influence insulin secretion. At high concentrations (>10-fold those used here) taurine can alter calcium flux (290) and interact with GABA receptors (288), however whether this occurs in the β-cell is not known. At high concentrations taurine can also close $K^+_{ATP}$ channels in β-cells (289). In support of taurine’s action as an antioxidant, the other antioxidant used, NAC, proved to be equally effective to taurine in preventing the FFA-induced decrease in GSIS; however, since NAC can be converted to taurine, we cannot totally exclude that the NAC effect may also be independent of its antioxidant properties. To address this possibility, we performed *ex vivo* studies with the antioxidant tempol, a SOD-mimetic, unrelated to NAC or taurine, which also prevented the oleate-induced decrease in GSIS and the increase in ROS.

Taken together, our findings demonstrate that oxidative stress mediates FFA-induced decrease in GSIS. Consistent with these findings, our previous study in MIN6 cells showed that NAC prevented the decrease in insulin content induced by oleate (81). In the above study, NAC had no effect on GSIS, however, a model of extreme FFA-mediated β-cell dysfunction was used, as the cells were exposed for 72 hours to oleate, which abolished GSIS. In another study in rat islets (161), NAC also failed to prevent the oleate-induced decrease in GSIS. However, the *in vitro* model used was also extreme
(72h oleate exposure), and much higher concentrations of NAC were used, raising the possibility of NAC toxicity. Furthermore, the islets were cultured in RPMI 1640, which contains antioxidants. The effect of NAC on insulin content was not examined in that study (161). The latter study of Moore et al. also did not show an increase in ROS. However, the probe used for determination of ROS, carboxy-H$_2$DCF-DA, is a slightly different probe than ours (H$_2$DCF-DA), which raises the possibility of different detection levels. In this study, ROS were measured in dispersed islets. We analyzed ROS in situ, and islets or MIN6 cells did not go through any latency or mechanical stress which may allow clearance of the dye. Independent of the DCF method, we also showed induction of antioxidant genes, the functional consequence of which is uncertain, but which is evidence for the presence of oxidative stress in living cells.

The mechanisms whereby fat-induced oxidative stress affects β-cell function have yet to be clarified. Oxidative stress can inhibit glucose oxidation (166) and/or decrease ATP generation by inducing UCP-2 in β-cells (277). In our MIN6 cells UCP2 mRNA was induced by oleate, but taurine did not prevent this effect, suggesting that ROS were not implicated. However, ROS could have increased the activity of UCP-2 (294). Oxidative stress can also decrease insulin gene expression (78) by inducing nuclear retention of FoxO1, with decreased PDX-1 but increased NeuroD expression (185). MaFA expression was shown to be increased by one group (185), but decreased by glucotoxicity, an oxidative stress–mediated event, by another group (174). In our study (81) in MIN6 cells, oleate did not affect insulin gene expression, but reduced the insulin content which was partially restored by NAC. This raises the possibility that antioxidants prevent the impairment in insulin biosynthesis induced by fat that was shown by previous
studies (136;140;163). Another explanation for the decreased GSIS observed with oleate, and its prevention by antioxidants may be a change in β-cell mass. However, our unpublished results show that 48h exposure to fat is too short a period to induce a decrease in β-cell mass, which is in accordance with other authors’ findings (90;96).

In summary, our study demonstrates that prolonged exposure to oleate which induces oxidative stress in islets, decreases glucose-stimulated insulin secretion both in vitro and in vivo. These findings are the first direct demonstration that oxidative stress is involved in the FFA-induced decrease in β-cell secretory function, and that antioxidants may be useful in its prevention.
Table 3.1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>FFA ($\mu$mol·l$^{-1}$)</th>
<th>Significance (vs. SAL)</th>
</tr>
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<td>SAL</td>
<td>0</td>
<td>634±67</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>489±73</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>407±70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>693±26</td>
<td>-</td>
</tr>
<tr>
<td>OLE</td>
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<td>646±67</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>773±92</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>864±103</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1050±118</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>OLE+L-NAC</td>
<td>0</td>
<td>682±79</td>
<td>NS</td>
</tr>
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<td>18</td>
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<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1128±110</td>
<td>p&lt;0.001</td>
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<tr>
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<td></td>
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<td>1003±92</td>
<td>p&lt;0.001</td>
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<tr>
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<td>NS</td>
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</tr>
<tr>
<td></td>
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<td>1158±84</td>
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</tr>
<tr>
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<td>NS</td>
</tr>
<tr>
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<td>NS</td>
</tr>
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</tr>
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</tr>
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<td>811±60</td>
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<td>638±71</td>
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<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>805±28</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 3.1. Plasma FFA levels during the 48h infusion period. Rats were treated with:

1) Saline alone (SAL, n=11), 2) Oleate alone (OLE, 1.3µmol·min⁻¹, n=10), 3) Oleate+low dose of N-Acetylcysteine (OLE+L-NAC, oleate-1.3µmol·min⁻¹, L-NAC-2.14µmol·kg⁻¹·min⁻¹, n=8), 4) Oleate+high dose of NAC (OLE+H-NAC, oleate-1.3µmol·min⁻¹, H-NAC-2.76µmol·kg⁻¹·min⁻¹, n=7), 5) Oleate+Taurine, (OLE+TAU, oleate-1.3µmol·min⁻¹, taurine-2.76µmol·kg⁻¹·min⁻¹, n=14, equimolar dose with H-NAC), 6) Low dose of NAC alone, (L-NAC, n=8), 7) High dose of NAC alone, (H-NAC, n=9) or 8) Taurine alone (TAU, n=13) Data are means±SE. For groups infused with oleate alone (OLE) or oleate in combination with the antioxidant NAC at low/high dose, or taurine (OLE+L-NAC, OLE+H-NAC, OLE+TAU) the plasma FFA concentrations were raised to levels higher than control rats (SAL) or rats treated with antioxidants alone (L-NAC, H-NAC or TAU).
Figure 3.1

A

B

C

D

E

F

# oleate infused groups vs. non-oleate infused groups, p<0.01 throughout the hyperglycemic clamp
† OLE vs. all, except OLE+L-NAC, p<0.001 throughout the hyperglycemic clamp
**Figure 3.1.** Effects of low and high dose N-acetylcysteine (A-C) and taurine (D-F) on plasma FFA, glucose and GINF during two-step hyperglycemic clamps with/without 48h oleate infusion. Rats were treated with: 1) Saline alone (SAL, n=11, black circles), 2) Oleate alone (OLE, 1.3µmol·min⁻¹, n=10, white circles), 3) Oleate+low dose of N-Acetylcysteine (OLE+L-NAC, oleate-1.3µmol·min⁻¹, L-NAC-2.14µmol·kg⁻¹·min⁻¹, n=8, white diamonds), 4) Oleate+high dose of NAC (OLE+H-NAC, oleate-1.3µmol·min⁻¹, H-NAC-2.76µmol·kg⁻¹·min⁻¹, n=7, white triangles), 5) Oleate+Taurine, (OLE+TAU, oleate-1.3µmol·min⁻¹, taurine-2.76µmol·kg⁻¹·min⁻¹, n=14, equimolar dose with H-NAC, white squares), 6) Low dose of NAC alone, (L-NAC, n=8, black diamonds), 7) High dose of NAC alone, (H-NAC, n=9, black triangles) or 8) Taurine alone (TAU, n=13, black squares) Data are means±SE. Oleate increased plasma FFA, as expected. Plasma glucose levels were superimposable among groups. Oleate impaired the GINF during the two-step hyperglycemic clamp; the low dose of NAC partially prevented the effect of oleate, whereas the high dose of NAC or taurine completely prevented the impairment induced by oleate.
Figure 3.2

# OLE vs all, except OLE+L-NAC, p<0.01 during the first and p<0.001 during the second step of the hyperglycemic clamp
Figure 3.2. Effects of low and high dose N-acetylcysteine (A-B) and taurine (D-E) on insulin and C-peptide during two-step hyperglycemic clamps with/without 48h oleate infusion. Groups are described in the legend of Figure 1. Oleate impaired both the insulin and C-peptide levels during the two-step hyperglycemic clamp; the low dose of NAC partially prevented the effect of oleate, whereas the high dose of NAC or taurine completely prevented the impairment induced by oleate.
Figure 3.3

A

Plasma FFA (μmol·L⁻¹)

B

Insulin Secretion (pmol·Islet⁻¹·h⁻¹)

Glucose Concentration (mmol·L⁻¹)

C

ROS Levels (% of Control)

D

SAL

OLE

OLO

† p < 0.001, OLE and OLO vs. SAL

# p < 0.01, OLE and OLO vs. SAL
Figure 3.3. Effects of 48h oleate or olive oil infusion on plasma FFA (A) and insulin secretion (B) in islets. Islets were isolated from rats treated with: Saline (SAL, n=16, black); Oleate (OLE, 1.3µmol·min⁻¹, n=13, white); Olive oil (OLO, 5µl·min⁻¹ of 20% olive oil and 50 U·ml⁻¹ heparin, n=10, light vertical). A. Both oleate and olive oil increased the plasma FFA levels to the same extent after 48h, compared with saline controls; B. Both oleate and olive oil infusions impaired the insulin response from isolated islets at 13 and 22 mmol·l⁻¹ glucose, compared with control. C. Islets ROS (SAL, n=7; OLE, n=6; OLO, n=4). D. Representative fluorescent images for ROS (200X). Light images are available upon request.
Figure 3.4

A

Insulin secretion (pmol/islet·h⁻¹)

<table>
<thead>
<tr>
<th>Glucose Concentration (mmol·l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
</tr>
<tr>
<td>SAL</td>
</tr>
<tr>
<td>1.0</td>
</tr>
</tbody>
</table>

B

Insulin secretion (pmol/islet·h⁻¹)

<table>
<thead>
<tr>
<th>Glucose Concentration (mmol·l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
</tr>
<tr>
<td>SAL</td>
</tr>
<tr>
<td>1.0</td>
</tr>
</tbody>
</table>

C

Glucose Concentration (mmol·l⁻¹)

| 5  | 7  | 19 | 22 |
| SAL | OLE | OLE+TPO | TPO |
| 1.0 | 1.5 | 2.0 | 2.5 |

# p < 0.01, OLE vs. SAL

† p < 0.001, OLE vs. SAL

D

ROS Levels (% of Control)

| SAL | OLE | OLE+H-NAC | H-NAC |
| 100 | 100 | 100 | 100 |

E

ROS Levels (% of Control)

| SAL | OLE | OLE+TAU | TAU |
| 100 | 100 | 100 | 100 |

F

ROS Levels (% of Control)

| SAL | OLE | OLE+TPO | TPO |
| 100 | 100 | 100 | 100 |

† p < 0.001, OLE vs. all

G

SAL | H-NAC | TAU | TPO

OLE | OLE+H-NAC | OLE+TAU | OLE+TPO
Figure 3.4. Effects of oleate and low and high dose N-acetylcysteine (A), taurine (B) and tempol (C) on insulin secretion (A-C) and ROS (D-F) in islets. A. Rats were treated for 48h with: SAL, n=16, black; OLE, n=13, white; OLE+L-NAC, n=5, narrow horizontal; OLE+H-NAC, n=12, dark horizontal; L-NAC, n=6, dashed horizontal; H-NAC, n=6, light horizontal; Oleate impaired the insulin secretion at both 13 and 22 mmol·l⁻¹ glucose; the low dose of NAC completely prevented the effect of oleate at 13 mmol·l⁻¹ glucose, but had only a partial effect at 22 mmol·l⁻¹ glucose; the high dose of NAC completely prevented the impairment induced by oleate at both 13 mmol·l⁻¹ glucose and 22 mmol·l⁻¹ glucose. B. SAL as in A.; OLE as in A.; OLE+TAU, n=17, large checker; TAU, n=12, large grid; Taurine completely prevented the impairment induced by oleate at both 13 mmol·l⁻¹ glucose and 22 mmol·l⁻¹ glucose. C. SAL as in A.; OLE as in A.; Oleate+Tempol (OLE+TPO, 2.41μmol·kg⁻¹min⁻¹, n=12, wide upward diagonal); Tempol (TPO, n=12, narrow upward diagonal). Tempol completely prevented the impairment induced by oleate at both 13 mmol·l⁻¹ glucose and 22 mmol·l⁻¹ glucose. D-F: ROS levels in cells exposed to treatments in A-C. (SAL, n=7; OLE, n=6; OLE+H-NAC, n=6; OLE+TAU, n=4; OLE+TPO, n=7; H-NAC, n=5; TAU, n=5; TPO, n=6). Data are means±SE, calculated as fold change from control. Infusion of oleate increased ROS levels as compared to saline control, whereas coinfusion of each antioxidant completely prevented the increase in ROS induced by oleate. Antioxidants alone had no significant effect. G. Representative fluorescent images for ROS (200X). Light images are available upon request.
**Figure 3.5. Effects of oleate, palmitate and hydrogen peroxide on insulin secretion in cultured islets.** Islets were exposed for 48h to: **A.** BSA (CON, n=7, black); Oleate (OLE, 0.4 mmol·l⁻¹ in BSA, n=14, white); Oleate + Taurine, (OLE+TAU, oleate-0.4 mmol·l⁻¹ in BSA, Taurine-1 mmol·l⁻¹, n=10, checker); Taurine (TAU, 1 mmol·l⁻¹, n=7, large grid); **B.** Oleate 0.4 mmol·l⁻¹ in BSA was added to KRB during the 2h static incubation; BSA, (CON+OLE in KRB, n=5, black); Oleate (OLE+OLE in KRB, 0.4 mmol·l⁻¹ in BSA, n=5, white); Oleate + Taurine, (OLE+TAU+OLE in KRB, oleate-0.4 mmol·l⁻¹ in BSA, Taurine-1 mmol·l⁻¹, n=4, checker); Taurine (TAU+OLE in KRB, 1 mmol·l⁻¹, n=4, large grid); **C.** BSA as in **A.**, Palmitate (PAL, 0.4 mmol·l⁻¹ in BSA, n=12, spheres); Palmitate + Taurine, (PAL+TAU, palmitate-0.4 mmol·l⁻¹ in BSA, Taurine-1 mmol·l⁻¹, n=10, white dots on black); Taurine as in **A.**; **D.** BSA as in **A.**, Hydrogen peroxide, (H₂O₂, 25 mmol·l⁻¹ in BSA, n=5, horizontal brick); Hydrogen peroxide + Taurine, (H₂O₂+TAU, H₂O₂-25 mmol·l⁻¹ in BSA, Taurine-1 mmol·l⁻¹, n=6, trellis); Taurine as in **A.** Oleate, palmitate or hydrogen peroxide impaired the insulin secretion at both 13 and 22 mmol·l⁻¹ glucose, an effect completely prevented by taurine. Our results demonstrate that although the levels of insulin were increased in all the groups exposed to oleate in KRB, the previous exposure to oleate for 48h still impaired GSIS, an effect prevented by taurine.
Figure 3.6

A

B

C

D

SAL

OLE

PAL

H2O2

TAU

OLE+TAU

PAL+TAU

H2O2+TAU

* p<0.05, vs. CON

# p<0.01, vs. CON

† p<0.001, vs. CON
Figure 3.6. Effects of oleate (A), palmitate (B) and hydrogen peroxide (C) on islet ROS. The groups are described in the legend of Figure 5. (CON, n=4; OLE, n=6; PAL, n=5; H2O2, n=6; OLE+TAU, n=5; PAL+TAU, n=4; H2O2+TPO, n=6; TAU, n=5). Data are means±SE, calculated as fold change from control. E. Representative fluorescent images for ROS (200X). Light images are available upon request. Oleate, palmitate and hydrogen peroxide increased ROS levels as compared to BSA control, whereas taurine reversed this effect.
Figure 3.7

A

Insulin Secretion (pg/3.5.10^6 Cells.h^-1)

- CON
- OLE
- OLE+TAU
- TAU

Glucose Concentration (mmol.l^-1)

B

ROS Levels (% of Control)

- CON
- OLE
- OLE+TAU
- TAU

C

- CON
- OLE
- OLE+TAU
- TAU

# p < 0.01 vs. CON
† p = 0.001 vs. all
Figure 3.7. Effects of oleate and taurine on insulin secretion (A) and ROS (B) in MIN6 cells. Cells were exposed for 48h to: BSA (CON, n=18, black); Oleate (OLE, 0.4 mmol·l⁻¹ in BSA, n=18, white); Oleate + Taurine, (OLE+TAU, oleate-0.4 mmol·l⁻¹ in BSA, Taurine-1 mmol·l⁻¹, n=16, checker); Taurine (TAU, 1 mmol·l⁻¹, n=18, large grid). 0 glucose concentration was used to evaluate the glucose stimulated insulin secretion because at 2.8 mmol·l⁻¹, which are non-stimulatory for islets, MIN6 cells show a moderate to maximal glucose stimulation. Oleate impaired the insulin secretion at 16.7 mmol·l⁻¹ glucose; taurine completely prevented the impairment induced by oleate at 16.7 mmol·l⁻¹ glucose. Oleate increased insulin secretion at 0 glucose, an effect partially prevented by taurine. B. ROS levels in cells exposed to treatments in A. (CON, n=9; OLE, n=9; OLE+TAU, n=8; TAU, n=8). Data are means±SE, calculated as fold change from control. Oleate increased ROS levels as compared to BSA control, whereas taurine completely prevented the increase in ROS induced by oleate. C. Representative fluorescent images for ROS of MIN6 cells (200X). Light images are available upon request.
Figure 3.8

A  Heme Oxygenase-1

B  Glutathione Peroxidase-1

C  Catalase

D  Cu/Zn-Superoxide Dismutase

E  Carnitine-Palmitoyl Transferase-1

F  Uncoupling Protein-2

* p<0.05 vs. CON
# p<0.01 vs. CON
† p<0.001 vs. CON
**Figure 3.8.** Real Time RT-PCR from mRNA of MIN6 cells. Cells were exposed for 48h to: 1) BSA (CON, n=18), 2) Oleate alone (OLE, 0.4 mmol·l⁻¹ in BSA, n=18), 3) Oleate + Taurine, (OLE+TAU, oleate-0.4 mmol·l⁻¹ in BSA, taurine-1 mmol·l⁻¹, n=16) and 4) Taurine alone (TAU 1 mmol·l⁻¹, n=18). The level of expression was measured for the following genes: (A) Heme-oxygenase 1 (HO-1, cytoplasmic or nuclear) (B) Glutathione peroxidase-1 (GPx-1, mitochondrial and cytoplasmic), (C) Catalase (CAT, cytoplasmic), (D) Superoxide-dismutase (Cu/Zn-SOD, SOD3, Extracellular SOD), (E) Carnitine Palmitoyl Transferase-1 (CPT-1), (F) Uncoupling Protein 2 (UCP-2). Data are means±SE, calculated as fold change from control. Real Time-PCR of MIN6 cells treated with oleate showed induction of metabolic genes CPT-1 (E) and UCP-2 (F), and of oxidative stress response genes GPx-1 (B) and Catalase (C), but not Mn-SOD (mitochondrial, not shown). Addition of taurine did not affect the up-regulation of CPT-1 as expected (E), UCP-2 (F) or GPx-1 (B), but partially prevented the upregulation of catalase (C) and Cu/Zn-SOD (D). HO-1 (A) and Cu/Zn-SOD (D) showed a tendency to increase in response to oleate, which appeared to be reversed by taurine.
Study 2

High Dose Salicylate Prevents Fat-Induced $\beta$-Cell Dysfunction \textit{In Vitro and In Vivo}*  

* The results of this study are incorporated in a manuscript to be submitted to Diabetes by January 2009.
4.1. Abstract

Objective We have previously shown that antioxidants prevented the impairment in β-cell function induced by prolonged exposure to FFA. The objective of this study is to address potential downstream effects of oxidative stress involving inflammation in pancreatic islets. More specifically, we wished to determine whether IKKβ is involved in lipid-induced decrease in GSIS.

Research Design and Methods 48h oleate (1.3 µmol·min⁻¹) or 20% olive oil+heparin (40 U·ml⁻¹) at 5 µl·min⁻¹ were infused in Wistar rats with or without the IKKβ inhibitor salicylate (0.117 mg·kg⁻¹min⁻¹, the dose that prevented lipid-induced insulin resistance). After the 48h infusion, 2-step hyperglycemic clamps were performed to evaluate insulin secretion in vivo, or islets were isolated to evaluate GSIS ex vivo. I also exposed the islets in culture conditions to oleate±salicylate or BMS-345541 (a specific IKKβ inhibitor, BMS) for 48h to evaluate GSIS in vitro.

Results 48h infusion of oleate or olive oil resulting in ~2-fold elevation of plasma FFA impaired β-cell function in vivo as measured with the disposition index during a two-step hyperglycemic clamp (13 and 22 mmol·l⁻¹ glucose), whereas coinfusion of salicylate prevented the effect of oleate or olive oil. GSIS ex vivo in isolated islets of oleate or olive oil-treated rats was impaired, but was restored by coinfusion of salicylate, while salicylate alone had no effect. Similar results were obtained in cultured islets, where 48h exposure to 0.4 mmol·l⁻¹ oleate impaired the GSIS at 13 mmol·l⁻¹ and 22 mmol·l⁻¹, an effect prevented by salicylate or BMS-345541. BMS-345541 also prevented fat-induced β-cell dysfunction in vivo during hyperglycemic clamp in mice.
Conclusion Our results suggest a potential role for the activation of inflammatory pathways involving IKKβ in lipid-induced β-cell dysfunction.
4.2. Introduction

A number of studies has established a link between oxidative stress and type 2 diabetes. Oxidative stress is present in diabetic patients (222) and has been implicated in diabetic complications (295) and insulin resistance (296). Studies in rats in our laboratory have shown that lipotoxicity-induced insulin resistance is mediated by oxidative stress (297) and the role of oxidative stress in fat-induced insulin resistance was also recently demonstrated in humans (221). In our previous study, we demonstrated that prolonged exposure to oleate which induces oxidative stress in islets, decreases glucose stimulated insulin secretion both *in vitro* and *in vivo*. Oxidative stress is a known activator of IKKβ, which, by phosphorylating the inhibitor IκBα, activates NFκB. The effect of IKKβ/NFκB on β-cell function is currently controversial. There are reports that NFκB may be beneficial for GSIS (241;242), unless stimulated by cytokines (243). However, IKKβ which can induce serine phosphorylation of IRS (18;297) decreases β-cell function (244;245). It is also controversial whether fat activates the IKKβ/NFκB pathway in β-cells (205;240). Although fatty acids did not activate NFκB in INS-1 or primary rat β-cells in one study (240), lipotoxicity was associated with NFκB activation and inhibited by an IKKβ inhibitor in INS-1 β-cells in another study (205). The effect of fat on IKKβ/NFκB *in vivo* has not been investigated.

To address the role of IKKβ in fat-induced β-cell dysfunction *in vivo* we used the IKKβ inhibitors salicylate and BMS-345541 (298). Normal Wistar rats were intravenously infused with either saline or fat to elevate plasma FFA levels 2 fold with or without salicylate for 48 hours. We used two types of fat infusion: direct oleate infusion
in BSA, and olive oil plus heparin infusion which avoids the surfactant activity of direct infusion of FFA. After the 48h infusion period, insulin secretion was evaluated in vivo through a 2-step hyperglycemic clamp, or ex vivo, in isolated islets. We also used the specific IKKβ inhibitor BMS-345541 (BMS) in studies performed with 48h oleate infusion in mice. In addition, we also performed in vitro studies, in which rat islets were exposed for 48h to oleate, with or without salicylate or BMS. To address the mechanism of the effect of IKKβ inhibition on β-cell function we measured the levels of serine-phosphorylated IRS-1 in islets from rats exposed for 48h to fat, with or without salicylate. We found that IKKβ inhibitors prevent fat-induced β-cell dysfunction in association with prevention of fat-induced IRS-1 serine phosphorylation in islets.
4.3. Materials and Methods

4.3.1. Studies in rats

4.3.1.1. Animals and surgery

Female Wistar rats (250-300g, Charles River, Canada) were cannulated as previously described in the General Methods section and in (92).

4.3.1.2. Intravenous infusions

The rats were fasted overnight and randomized to the following infusion groups (n=6-12/group): 1) Saline control (SAL, 5 μl·min⁻¹); 2) Fat, either oleate at the dose used in the study presented in the Chapter 3 of this thesis (OLE) or olive oil plus heparin (OLO, 50 U·ml⁻¹ heparin; 5.5 μl·min⁻¹); Olive oil is a triglyceride mixture containing 75% oleate, and 16% saturated fat. Heparin was added to the olive oil emulsion to activate lipoprotein lipase, which releases FFA from the triglycerides of olive oil. 3) Oleate plus salicylate (0.0117 mg·kg⁻¹·min⁻¹, the dose that reversed insulin resistance in (236)) or Olive oil plus salicylate (0.0117 mg·kg⁻¹·min⁻¹) and 4) Salicylate alone. A 2-step hyperglycemic clamp was performed after the 48h infusion period, using the same protocol described in the General Methods.
4.3.1.3. Islet isolation and secretion studies

Islets of in vivo infused rats were isolated as in (277), and preincubated for 1h at 37°C in Krebs Ringer buffer containing 10 mmol·l⁻¹ HEPES (KRBH), and 2.8 mmol·l⁻¹ glucose. Thereafter, 5 islets of approximately the same size were incubated in triplicate at 2.8, 6.5, 13 and 22 mmol·l⁻¹ glucose for 2h, 37°C. Insulin was measured in the supernatant with Linco’s kit. For in vitro studies, before preincubation, islets of untreated rats were cultured for 48h in RPMI 1640 without antioxidants, containing 0.4 mmol·l⁻¹ oleate in 0.5% BSA with or without 0.25 mmol·l⁻¹ salicylate (the same concentration as was used in (245)) or 3 µmol·l⁻¹ BMS-345541 (BMS), a dose that was calculated based on our pilot dose-response curve studies results. Whereas salicylate is also a weak inhibitor of both COX1 and COX2, (299), BMS is a highly specific inhibitor of IKKβ (300) with no reported direct COX inhibiting activity.

4.3.1.4. ROS measurements

ROS measurements were performed following the same protocol as described in the General Methods section, as well as in the Methods section of Chapter 3.
4.3.2. Western Blot Analysis

Serine 307 phosphorylated IRS1 (Antibody purchased from Cedarlane Lab. Ltd., 1:500 dilution) was measured by Western blotting using islet lysates of fat/control infused rats. Islet pellets (100~150 islets per sample) were lysed in RIPA buffer (50 mmol·l⁻¹ Tris–HCl, pH 7.4, 150 mmol·l⁻¹ NaCl, 1 mmol·l⁻¹ EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 1 mmol·l⁻¹ PMSF, 10 µg·ml⁻¹ Aprotinin, 10 µg·ml⁻¹ Leupeptin) and incubated on ice for 30 min. The samples were then centrifugation at 13000 rpm, 4°C for 10 min. The supernatant was removed and the protein concentration was measured using the BCA protein assay (Pierce). 30 µg of protein from each islet lysate was resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-phospho-IRS1 (Ser307) antibody. Secondary antibody conjugated to HRP and enhanced chemiluminescence system (Amersham Biosciences) were used for detection. The bands obtained from immunoblotting were quantified by scanning densitometry.

4.3.3. Phosphorylated IκBα Assay

Whole islets-extract was obtained according to manufacturer’s protocol. Protein concentration in all islet samples was determined as described above, in the Western Blot Analysis section. Islet-lysates were tested for Phosphorylated IκBα by an ELISA kit (Active Motif, Carlsbad, CA).
4.3.4. NFκB p65 Activity Assay

Nuclear proteins were extracted according to the manufacturer’s instructions (Active Motif nuclear extraction kit, Carlsbad, CA). The supernatant (nuclear fraction) was kept at -80°C until analysis. The active NFκB contained in the nuclear extracts was measured by its DNA binding activity on immobilized oligonucleotides encoding a specific consensus site using a NFκB p65 transcription factor ELISA kit (Active Motif, Carlsbad, CA).

4.3.5. Studies in Mice

4.3.5.1. Animals and Surgery

For all studies, normal Female C57/Bl6 mice (Charles River, Quebec, Canada) aged 4-8 weeks and weighing 22-25 g were used for experiments. The mice were housed in the University of Toronto’s Department of Comparative Medicine. They were exposed to a 12h light/dark cycle. The mice were fed mouse chow containing 18% protein, 64% carbohydrate, and 14% fat, Teklad Global 2018 (Madison, WI).

After a week of adaptation to the facility, mice were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg·ml⁻¹, 1 µl·g⁻¹ of body weight) and an indwelling catheter was inserted into the right internal jugular vein for infusion. The tail vein was used for sampling. Polyethylene catheters (PE-10; Cay Adams, Boston, MA),
each extended with a segment of silastic tubing (length of 1.5 cm, Dow Corning, Midland, MI), were used. The catheter was extended to the level of the right atrium, and tunneled subcutaneously and exteriorized. The catheter was filled with heparin (1,000 U·ml⁻¹) in normal saline to maintain patency and was closed at the end. The mice were allowed a minimum 3-4 days period of post-surgery recovery before experiments, after which they were connected to the infusion apparatus. The infusion lines ran inside a tether that was fitted to the subcutaneous implant. Each mouse was placed in a cage, and the infusion lines were protected by a tether and run through a swivel, which was suspended on top of the cage to give complete freedom of movement to the mouse. 48 h infusions were started through the jugular vein. Throughout the infusion period, mice had free access to water and to their standard pelleted food. All procedures were in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto.

4.3.5.2. Preparation of Infused Solutions

We have transferred our in vivo model of β-cell lipotoxicity from rats to mice, for which we have used the same 48 h iv infusion of oleate given at 3.9 µmol·min⁻¹ bound to bovine serum albumin (BSA), prepared as described in the General Methods section. BMS-345541 (30 mg·kg⁻¹·day⁻¹) was dissolved in saline at 7.4 pH. Samples for FFAs, glucose, and insulin were taken at -20 and -10 minutes before the onset of the
hyperglycemic clamp (time = 0). Food was removed 4 h before the two-step hyperglycemic clamp.

4.3.5.3. Hyperglycemic Clamp Studies

After the 48 h infusion period, basal samples were taken over 20 min (basal period). Thereafter, the hyperglycemic clamp was performed to evaluate GSIS from the plasma insulin and C-peptide response to the rise in plasma glucose. The venous infusion saline/treatment was continued throughout the experiment. The glucose infusion was given through the jugular catheter. Both the glucose and the saline/treatment infusion lines were connected to the jugular line through a Y shaped connector. Using an infusion of 37.5% glucose, at time = 0 min, glucose level was gradually raised and then maintained at 22 mmol·l⁻¹ (maximum stimulatory levels) for 120 min. Plasma glucose was 'clamped' at 22 mmol·l⁻¹ by a variable glucose infusion adjusted according to frequent (every 5-10 min) glycemic readings obtained on a Hemocue glucose analyzer. Samples for insulin, C-peptide and FFA were taken at regular intervals. The sample volume was minimized to avoid anemia. A total of 0.3 ml of blood was withdrawn from the mice. FFA levels were measured by colorimetric kits (Wako and Boehringer Chemicals, resp.), mouse insulin and C-peptide by RIA kits (Linco Research Inc, MO). In each experiment, an index of insulin sensitivity (93) and a Disposition Index was obtained during the last 30 minutes of the hyperglycemic clamp data, i.e. during the determination of GSIS, as described in the General
Methods section. At the end of the experiment, the mice were deeply anesthetized. The protocol is summarized in Figure 4.1.

4.3.5.4. Plasma assays

Plasma glucose, insulin and C-peptide levels were measured as described in the General Methods section.

4.3.6. Calculations

Please refer to the General Methods section for calculations used in this study.

4.3.6.1. Insulin clearance

The C-Peptide/Insulin ratio was used as an index of insulin clearance. The C-peptide level was divided by the insulin level at each time point in the last 40 minutes of each step for the two-step hyperglycemic clamp.
4.3.7. Statistical Analysis

Data are means±SE. One way non-parametric ANOVA for repeated measurements, followed by Tukey’s t-test was used to compare treatments. Calculations were performed using SAS (Cary, NC).
4.4. Results

4.4.1. In vivo clamp studies

During the 48h infusions the groups treated with oleate or olive oil had higher plasma FFA than the rats treated with saline or salicylate alone (Table 4.1.A-B, tables and figures are at the end of this chapter). The infusion of oleate, olive oil or salicylate did not affect plasma glucose or insulin (data not shown).

Following the 48h infusions, we evaluated insulin secretion in vivo using hyperglycemic clamps. As shown in Figures 4.2.A and 4.2.B, basal plasma FFA prior to the clamps were higher in oleate or olive oil-treated than control rats, or rats treated with salicylate alone. FFA levels decreased during the clamp because of hyperglycemia and hyperinsulinemia.

Basal plasma glucose was similar in all groups. During the first step of the clamp, glucose levels rose to 13 mmol·l⁻¹ (upper physiological in rats), and during the second step to 22 mmol·l⁻¹ (pathological but maximally stimulatory) with no differences among groups (Figures 4.3.A and 4.3.B). The glucose infusion rate (GINF) necessary to maintain the target glucose level was lower in both oleate and olive oil groups as compared with saline or salicylate treated groups, consistent with decreased insulin secretion, decreased insulin sensitivity, or both (Figures 4.4.A and 4.4.B). When the IKKβ inhibitor salicylate was used in combination with oleate or olive oil, GINF was completely restored to control levels (Figures 4.4.A and 4.4.B). Salicylate alone or added to olive oil had no effect on GINF.
Basal insulin and C-peptide levels were similar in all groups (Figures 4.5.A-B and 4.6.A-B) As expected, plasma insulin and C-peptide rose in response to glucose in all groups, indicating increased secretion. Plasma insulin and C-peptide were lower in the oleate-treated rats, but not in the groups treated with olive oil. In the group infused with oleate and salicylate, plasma insulin and C-peptide were restored to control levels both at 13 mmol\(^{-1}\) glucose (p=NS, OLE+SLY vs SAL) and at 22 mmol\(^{-1}\) glucose (p=NS, OLE+SLY vs. SAL, Figures 4.5.A-B and 4.6.A-B). Salicylate alone or salicylate+olive oil had no effect on insulin or C-peptide levels.

As evidenced by Figures 4.4.A-B and 4.5.A-B, GINF and insulin levels were proportionally decreased in OLE vs. SAL groups, therefore the sensitivity index calculated as M/I=GINF/Insulin (287) was not significantly different (Figure 4.7.A). Salicylate had no significant effect on M/I, either alone, or in combination with oleate. In the group treated with olive oil GINF was reduced as compared to the control group, whereas insulin levels were similar, therefore the M/I was reduced indicating the presence of insulin resistance (Figure 4.7.B). The decrease in M/I was prevented by the addition of salicylate, whereas salicylate alone had no effect on M/I.

The disposition index (DI) was used as an index of \(\beta\)-cell function (or insulin secretion corrected for the ambient degree of insulin resistance), and was calculated as C-peptide levels multiplied by M/I. When DI was used to evaluate \(\beta\)-cell function, we found this to be impaired with both oleate and olive oil infusions (Figure 4.8.A-B), but completely restored with the addition of salicylate. Salicylate alone had no effect on DI.

The C-Peptide/Insulin ratio was taken as an index of insulin clearance. There was no significant difference in this ratio among the groups (Table 4.2).
4.4.2. *Ex vivo* studies in islets

Rats were infused for 48h with fat/saline at the same rate as for our *in vivo* studies. At the end of the 48h infusion period, islets were isolated and static stimulation studies were performed at the following glucose concentrations: 2.8 mmol·l\(^{-1}\), representing non-stimulatory levels for the \(\beta\)-cell; 6.5 mmol·l\(^{-1}\), which are basal glucose levels in rats; 13 mmol·l\(^{-1}\) and 22 mmol·l\(^{-1}\), as in our hyperglycemic clamps *in vivo*. As expected, increasing glucose concentration resulted in enhanced insulin secretion from isolated islets (*Figure 4.9.A-B*). The insulin secretory response of islets isolated from rats treated with a prolonged oleate (OLE) or olive oil (OLO) infusion was markedly decreased compared with control (SAL), both at 13 (\(p<0.01\), OLE or OLO vs SAL) and 22 mmol·l\(^{-1}\) (\(p<0.001\), OLE or OLO vs SAL) glucose. We also performed *in vivo* infusion of the IKK\(\beta\) inhibitor salicylate (SLY) alone, and in combination with oleate or olive oil, followed by evaluation of insulin secretion in isolated islets. Similar to our results *in vivo*, salicylate prevented the oleate/olive oil induced decrease in \(\beta\)-cell function at at both 13 (\(p=\text{NS}\), OLE+SLY or OLO+SLY vs. SAL) and 22 mmol·l\(^{-1}\) (\(p=\text{NS}\), OLE+SLY or OLO+SLY vs. SAL, *Figure 4.9.A-B*). Exposure of islets to oleate or olive oil for 48h increased the level of ROS (\(p<0.01\), OLE or OLO vs SAL), as detected by the DCF method, however addition of SLY (*Figure 4.10.*) did not prevent the increase in ROS (\(p<0.01\), SAL vs. OLE+SLY or OLO+SLY). These results indicate that salicylate has no antioxidant effects in islets in our model, and that the protective effect of salicylate on insulin secretion might be unrelated or act beyond the impairing effect of oxidative stress.
No significant change in ROS was observed with salicylate alone, as compared to control.

4.4.3. IKKβ and the activation of the NFκB pathway

Infusion of oleate or olive oil caused a significant increase in phosphorylated IκBα, which reflects IKKβ activation (301). This was prevented by co-infusion of salicylate (Figure 4.11.A-B). Furthermore, both oleate or olive oil infusions increased the amount of active nuclear NFκB (Figure 4.12.A-B) which was also prevented by salicylate. Interestingly, salicylate alone has no effect on either IκBα or activated nuclear NFκB.

A consequence of IKKβ activation, which is thought to play a major role in the pathogenesis of insulin resistance, is serine phosphorylation of IRS-1 (18;302-305). Both oleate and olive oil increased IRS-1 serine 307 phosphorylation in islets, an effect which was prevented by co-infusion of salicylate (Figure 4.13.A-B).

4.4.4. In vivo clamp studies in mice and the mouse lipotoxicity model

Following the same lipotoxicity model developed in rats, we used a similar 48h oleate infusion in mice, and evaluated the in vivo effect of BMS, which is a more specific IKKβ inhibitor than salicylate (300) at a dose that was previously found to inhibit IKKβ in vivo (298). After the 48h infusions and prior to the hyperglycemic clamp the groups
treated with oleate had higher plasma FFA than mice infused with saline or BMS alone (Figure 4.14.A). During the clamp, the FFA levels declined as expected (Figure 4.14.A).

Following the 48h infusions, the glucose infusion rate (GINF) necessary to maintain the hyperglycemic clamps (Figure 4.14.B) was lower in oleate infused mice as compared with control but was restored to control levels when BMS was used in combination with oleate (Figure 4.14.C). Basal insulin and C-peptide levels were higher in the groups treated with BMS as compared to control or oleate (p<0.05 for both OLE+BMS and BMS vs. SAL or OLE, Figure 4.15.A-B). Different from our oleate model in rats, but similar to our olive oil model in rats, plasma insulin and C-peptide did not decrease as compared to control, in the oleate-treated mice, during the hyperglycemic clamp. For the groups treated with BMS, both insulin and C-peptide levels were higher than saline or oleate treated mice (p<0.05 for both OLE+BMS or BMS vs SAL).

The sensitivity index M/I=GINF/Insulin (287) was significantly lower in oleate-treated mice (Figure 4.16.A). BMS had no significant effect on M/I when added to oleate (p=NS, OLE+BMS vs. OLE), but had an effect by itself in decreasing M/I (p<0.05, BMS vs. SAL). The disposition index (Di) was decreased with oleate infusion, but completely restored with BMS (Figure 4.16.B), whereas BMS alone had no effect on Di (Di=2.510±0.393 μmol·kg⁻¹·min⁻¹/pmol·l⁻¹ for SAL; Di=1.893±0.429 μmol·kg⁻¹·min⁻¹/pmol·l⁻¹ for BMS, p=NS BMS vs SAL). The C-Peptide/Insulin ratio taken as an index of insulin clearance was not significantly different among the groups (Table 4.3).
4.4.5. *In Vitro* studies in islets

To completely rule out that the effect of salicylate or BMS-345545 on GSIS was indirect and mediated by its effect on whole body insulin sensitivity, we performed *in vitro* studies in islets. 0.4 mmol·l\(^{-1}\) oleate in 0.5% BSA decreased GSIS (p<0.05 OLE vs CON, at both 13 and 22 mmol·l\(^{-1}\) glucose, Figure 4.17), whereas coincubation with 0.25 mmol·l\(^{-1}\) salicylate (Figure 4.17.A) or BMS (Figure 4.17.B) restored GSIS (p=NS, CON vs. OLE+SLY at both 13 and 22 mmol·l\(^{-1}\) glucose). Salicylate or BMS alone had no effect on insulin secretion (p=NS, SLY vs CON).
4.5. Discussion

In this study we have examined the effects of prolonged FFA exposure with or without IKK inhibitors, on β-cell function in vivo, ex vivo, and in vitro. We used our model of lipotoxicity in rat, and we also developed a new model of lipotoxicity in mice. During the hyperglycemic clamp in vivo, both insulin and C-peptide levels were lower in rats treated with oleate, indicating reduced insulin secretion. Insulin secretion in vivo should be interpreted in the context of insulin sensitivity. FFA induce insulin resistance, and this effect was observed in the present study when olive oil was infused in normal Wistar rats. In our oleate infusion model in rats there was no change in insulin sensitivity, and because β-cells did not have to compensate for insulin resistance, our results are similar in vivo and ex vivo. However, when we used olive oil, although the insulin and C-peptide levels were similar during the hyperglycemic clamp, the GINF was still impaired by the 48h olive oil infusion, suggesting insulin resistance. Thus, although the same degree of impairment of GSIS with both oleate and olive oil infusions was noticed in isolated islets, the in vivo studies demonstrated that 48h infusion of olive oil did not impair the absolute insulin secretion, as measured by the C-peptide levels, during the hyperglycemic clamp. This was because the olive oil infusion decreased insulin sensitivity, as measured by the SI index. This effect of olive oil may be due to the amount of saturated fat contained in olive oil (16%), as opposed to oleate, which is a monounsaturated fatty acid. Also, the plasma triglyceride elevation induced by olive oil, which is a mixture of triglycerides may impair insulin sensitivity independent of plasma FFA (306). It should also be noted that the elevated plasma FFA induced by olive oil
actually are due to spillover of FFA not taken up immediately by tissue after release by LPL, which implicates higher intracellular levels of fat with olive oil than oleate in tissues where LPL is high (muscle and fat).

\textit{In vivo}, the \(\beta\)-cell compensates for insulin resistance by increasing secretion thus DI, rather than absolute insulin secretion should be taken as a measure of \(\beta\)-cell function. Our results showed that DI was impaired by both oleate and olive oil, showing a decrease in \(\beta\)-cell function with both types of fat, consistent with the results \textit{ex vivo} in isolated islets. \(\beta\)-cell function was completely restored by Salicylate, \textit{in vivo} and \textit{ex vivo} in isolated islets, suggesting a potential role for activation of inflammatory pathways involving IKK\(\beta\) in lipid-induced \(\beta\)-cell dysfunction.

Inflammatory pathways are known to be activated in \(\beta\)-cell glucotoxicity (307) and may enhance lipotoxicity, as suggested by previous data from our laboratory showing that prediabetic BB rats with insulitis display severe \(\beta\)-cell lipotoxicity (308). Our results in \textbf{Chapter 3} show that oxidative stress plays a causal role in \(\beta\)-cell lipotoxicity (309). The effect of ROS to activate inflammatory pathways involves IKK\(\beta\), which in addition to phosphorylating IKB\(\alpha\), thus activating the transcription factor NF\(\kappa\)B, phosphorylates IRS, thus inhibiting insulin signalling. Studies from our laboratory showed that FFA induced oxidative stress activated the IKK/NF\(\kappa\)B inflammatory pathway and induced serine 307 phosphorylation of IRS-1 in the liver (310), suggesting that the same mechanism might be involved in lipotoxicity in islets. In our \textit{ex vivo} study, oleate and olive oil increased the levels of phosphorylated IKB\(\alpha\) which was prevented by salicylate, suggesting that salicylate did indeed prevent fat-induced IKK\(\beta\) activation, although, interestingly, salicylate alone had no effect at this dose, as
previously seen in the liver (311). An increase in ROS was induced by oleate, but salicylate did not prevent this effect, suggesting that salicylate did not act as an antioxidant. The fat-induced activation of IKKβ may be secondary to oxidative stress, but could also be unrelated to oxidative stress, and due to an effect of fatty acid binding to TLR4 in islets. As was described in the Introduction of this thesis, TLR4 is present in islets (135) and FFA can bind to this receptor. TLR-null mice were found to be protected from FFA-induced insulin resistance (133). The same study has shown that although these mice have increased obesity, they are partially protected against high fat diet-induced insulin resistance, possibly due to reduced inflammatory gene expression in liver and fat. Salicylate has not been previously studied in a β-cell lipotoxicity model, however, it did restore GSIS in an in vitro glucotoxicity model (245). It has also been reported that high-dose salicylate (inhibitor of IKKβ) increases insulin secretion in obese individuals (312), and partially restores β-cell glucose sensitivity in type 2 diabetes (246). This effect has been initially attributed to inhibition of COX2 (247;248), a gene under the control of NFκB, and the consequently decreased synthesis of PGE2, a prostaglandin which inhibits insulin secretion. Salicylate is also a direct inhibitor of both COX 1 and COX 2, although a weak one, compared to other NSAID (299). Because of this issue, we also used BMS, which has not been reported to directly inhibit COX, and we obtained the same results as with salicylate, on β-cell function, indicating that the effect of salicylate is likely due to IKKβ inhibition. The mechanism whereby IKKβ inhibition may restore β-cell function in the context of β-cell lipotoxicity has to be determined. In addition to inhibition of COX2 gene transcription level, the inhibition of iNOS, another gene under NFκB control, as well
as enhancement of insulin signalling in β-cells by salicylate may also have played a role. An important role of iNOS induction in β-cell lipotoxicity has been postulated by Unger (66;200;204) but has been disputed by others (78). In our studies, salicylate also prevented the fat-induced serine phosphorylation of IRS1, which is known to decrease tyrosine phosphorylation, therefore playing a potential role in β-cell insulin signaling, or protecting against the possible β-cell “insulin resistance” induced by fat. As alluded to before, the results with salicylate were confirmed by our in vitro studies with BMS. When BMS was used in mice, our results were similar to the rat studies, and BMS behaved in the same way as salicylate, by preventing the decrease in β-cell function induced by 48h oleate infusion. However, two important differences were noticed with respect to the rat studies. First, 48h exposure to oleate did induce insulin resistance in mice. The reason behind this finding may be greater FFA levels attained with oleate infusion, but also the strain of mice, as C57/Bl6 mice have a higher degree of native insulin resistance as compared to other strains. Oleate infusion inducing insulin resistance in mice did not result in lower absolute insulin secretion during the clamps, but decreased β-cell function (DI), similar to our results with olive oil in rats. Second, BMS did restore DI but did not affect oleate-induced insulin resistance, as it probably had an effect of itself, in decreasing insulin sensitivity. Decreased insulin sensitivity with an IKKβ inhibitor may be dose-related and due to the inhibition of COX2-derived prostaglandins that increase insulin action, as was described by a number of studies (313-322). With salicylate, decreased insulin sensitivity has been described in one recent study from our group (323), an effect which was also attributed to aspirin (324-327).
In summary, our study demonstrates that prolonged exposure to oleate which induces oxidative stress in islets, decreases glucose stimulated insulin secretion both in vitro and in vivo and activates the IKK/NFκB inflammatory pathway. IKKβ inhibitors were effective in restoring β-cell function. These findings are the first direct demonstration that the activation of the IKK inflammatory pathway plays a causal role in the FFA-induced decrease in β-cell secretory function, and that anti-inflammatory drugs may be useful in its prevention.
### Table 4.1.A.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>FFA (µmol·l⁻¹)</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>0</td>
<td>634±67</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>489±73</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>407±70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>693±26</td>
<td>-</td>
</tr>
<tr>
<td>OLE</td>
<td>0</td>
<td>646±67</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>773±92</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>864±103</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1050±118</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>OLE+SLY</td>
<td>0</td>
<td>745±118</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>916±168</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>893±168</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1123±168</td>
<td>p&lt;0.05</td>
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<tr>
<td>SLY</td>
<td>0</td>
<td>667±149</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>643±146</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>597±93</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>580±95</td>
<td>NS</td>
</tr>
</tbody>
</table>
**Table 4.1.A** Plasma FFA levels during the 48h infusion period. Rats were treated with: 1) Saline alone (SAL, n=12), 2) Oleate alone (OLE, 1.3 µmol·min⁻¹, n=10), 3) Oleate+Salicylate (OLE+SLY, oleate-1.3µmol·min⁻¹, SLY-0.7µmol·kg⁻¹·min⁻¹, n=8), 4) Salicylate alone (SLY, n=9). Data are means±SE. For groups infused with oleate alone (OLE) or oleate in combination with the IKKβ inhibitor salicylate (OLE+SLY) the plasma FFA concentrations were raised to levels higher than control rats (SAL) or rats treated with salicylate alone (SLY).
### Table 4.1.B.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>FFA (µmol·L⁻¹)</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>0</td>
<td>634±67</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>489±73</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>407±70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>693±26</td>
<td>-</td>
</tr>
<tr>
<td>OLO</td>
<td>0</td>
<td>686±124</td>
<td>NS</td>
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<td></td>
<td>18</td>
<td>902±77</td>
<td>p&lt;0.01</td>
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<td>24</td>
<td>934±61</td>
<td>p&lt;0.01</td>
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<tr>
<td></td>
<td>48</td>
<td>925±104</td>
<td>p&lt;0.01</td>
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<tr>
<td>OLO+SLY</td>
<td>0</td>
<td>660±87</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>937±70</td>
<td>p&lt;0.01</td>
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<td></td>
<td>24</td>
<td>985±99</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1027±95</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>SLY</td>
<td>0</td>
<td>667±149</td>
<td>NS</td>
</tr>
<tr>
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<td>18</td>
<td>643±146</td>
<td>NS</td>
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<tr>
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<td>24</td>
<td>597±93</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>580±95</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 4.1.B Plasma FFA levels during the 48h infusion period. Rats were treated with:

1) Saline alone (SAL, n=12), 2) Olive oil alone (OLO, 5.5 μl·min⁻¹, n=7), 3) Olive oil+Salicylate (OLO+SLY, olive oil-5.5 μl·min⁻¹, SLY-0.7μmol·kg⁻¹min⁻¹, n=11), 4) Salicylate alone (SLY, n=9). Data are means±SE. For groups infused with olive oil alone (OLO) or olive oil in combination with the IKKβ inhibitor salicylate (OLO+SLY) the plasma FFA concentrations were raised to levels higher than control rats (SAL) or rats treated with salicylate alone (SLY).
<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose (mM)</th>
<th>C-peptide/Insulin</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>13</td>
<td>0.0039±0.0003</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.0035±0.0002</td>
<td>-</td>
</tr>
<tr>
<td>OLE</td>
<td>13</td>
<td>0.0038±0.0002</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.0039±0.0003</td>
<td>NS</td>
</tr>
<tr>
<td>OLO</td>
<td>13</td>
<td>0.0043±0.0004</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.0031±0.0004</td>
<td>NS</td>
</tr>
<tr>
<td>OLE+SLY</td>
<td>13</td>
<td>0.0047±0.0003</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.0039±0.0002</td>
<td>NS</td>
</tr>
<tr>
<td>OLO+SLY</td>
<td>13</td>
<td>0.0049±0.0005</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.0043±0.0004</td>
<td>NS</td>
</tr>
<tr>
<td>SLY</td>
<td>13</td>
<td>0.0062±0.0009</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.0038±0.0009</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 4.2. The C-Peptide/Insulin ratio was taken as an index of insulin clearance. Data are means±SE. There was no significant difference in this ratio among the groups.
Table 4.3.

<table>
<thead>
<tr>
<th>Group</th>
<th>C-peptide/Insulin</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>0.0056±0.0006</td>
<td>-</td>
</tr>
<tr>
<td>OLE</td>
<td>0.0047±0.0007</td>
<td>NS</td>
</tr>
<tr>
<td>OLE+BMS</td>
<td>0.0048±0.0005</td>
<td>NS</td>
</tr>
<tr>
<td>BMS</td>
<td>0.0046±0.0005</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 4.3. The C-Peptide/Insulin ratio was taken as an index of insulin clearance. Treatment groups are described in the legend of Figure 4.14. Data are means±SE. There was no significant difference in this ratio among the groups.
48 h Infusion Protocol

| SALINE | OLEATE 0.4µmol/min |

48 h i.v. infusion period (groups as above) followed by evaluation of insulin secretion in vivo during hyperglycemic clamps

Hyperglycemic Clamp Protocol

hyperglycemic clamp (22 mM glucose)

Figure 4.1. The β-cell lipotoxicity model in mice. 48h i.v. saline control/oleate infusion, followed by the hyperglycemic clamp protocol
Figure 4.2. Effects of salicylate on plasma FFA during two-step hyperglycemic clamps with/without 48h oleate (4.2.A.) or olive oil (4.2.B.) infusion. Rats were treated with: 4.2.A.1) Saline alone (SAL, n=12), 2) Oleate alone (OLE, 1.3 µmol·min⁻¹, n=10), 3) Oleate+Salicylate (OLE+SLY, oleate-1.3µmol·min⁻¹, SLY-0.7µmol·kg⁻¹·min⁻¹, n=8), 4) Salicylate alone (SLY, n=9). 4.2.B. 1) Saline alone (SAL, n=12), 2) Olive oil alone (OLO, 5.5 µl·min⁻¹, n=7), 3) Olive oil+Salicylate (OLO+SLY, olive oil-5.5 µl·min⁻¹, SLY-0.7µmol·kg⁻¹·min⁻¹, n=11), 4) Salicylate alone (SLY, n=9). Data are means±SE. Both oleate and olive oil increased plasma FFA, as expected.

# oleate and olive oil infused groups vs. non-oleate/olive oil infused groups, p<0.01 throughout the hyperglycemic clamp
Figure 4.3. Effects of salicylate on plasma glucose during two-step hyperglycemic clamps with/without 48h oleate (4.3.A.) or olive oil (4.3.B.) infusion. Rats were treated with: 4.3.A.1) Saline alone (SAL, n=12), 2) Oleate alone (OLE, 1.3 μmol·min⁻¹, n=10), 3) Oleate+Salicylate (OLE+SLY, oleate 1.5μmol·min⁻¹, SLY 0.7μmol·kg⁻¹·min⁻¹, n=8), 4) Salicylate alone (SLY, n=9). 4.3.B. 1) Saline alone (SAL, n=12), 2) Olive oil alone (OLO, 5.5 μl·min⁻¹, n=7), 3) Olive oil+Salicylate (OLO+SLY, olive oil 5.5 μl·min⁻¹, SLY 0.7μmol·kg⁻¹·min⁻¹, n=11), 4) Salicylate alone (SLY, n=9). Data are means±SE. Plasma glucose levels were superimposable among groups.
Figure 4.4: Effects of salicylate on plasma GINF during two-step hyperglycemic clamps with/without 48h oleate (4.4.A.) or olive oil (4.4.B.) infusion. Rats were treated with: 4.4.A.1) Saline alone (SAL, n=12), 2) Oleate alone (OLE, 1.3 μmol·min⁻¹, n=10), 3) Oleate+Salicylate (OLE+SLY, oleate-1.3μmol·min⁻¹, SLY-0.7μmol·kg⁻¹·min⁻¹, n=8), 4) Salicylate alone (SLY, n=9). 4.4.B. 1) Saline alone (SAL, n=12), 2) Olive oil alone (OLO, 5.5 μl·min⁻¹, n=7), 3) Olive oil+Salicylate (OLO+SLY, olive oil-5.5 μl·min⁻¹, SLY-0.7μmol·kg⁻¹·min⁻¹, n=11), 4) Salicylate alone (SLY, n=9). Data are mean±SE. Both oleate and olive oil impaired GINF during the two-step hyperglycemic clamp, whereas salicylate completely prevented the impairment. † OLE vs. all, p<0.001 throughout the hyperglycemic clamp.
Figure 4.5. Effects of salicylate on insulin during two-step hyperglycemic clamps with/without 48h oleate (4.5.A.) or olive oil (4.5.B.) infusion. Groups are described in the legend of Figure 4.2. Oleate, but not olive oil impaired the insulin levels during the two-step hyperglycemic clamp, whereas salicylate completely prevented the impairment.

# OLE vs all, p<0.01 during the first and p<0.001 during the second step of the hyperglycemic clamp
Figure 4.6

Figure 4.6. Effects of salicylate on C-peptide during two-step hyperglycemic clamps with/without 48h oleate (4.6.A.) or olive oil (4.6.B.) infusion. Groups are described in the legend of Figure 4.2. Oleate, but not olive oil impaired the C-peptide levels during the two-step hyperglycemic clamp, whereas salicylate completely prevented the impairment.

# OLE vs all, p<0.01 during the first and p<0.001 during the second step of the hyperglycemic clamp
Figure 4.7. Effects of salicylate on Sensitivity Index (M/I = GINF/Insulin) during two-step hyperglycemic clamps with/without 48h oleate (4.7.A) or olive oil (4.7.B) infusion. Groups are described in the legend of Figure 4.2. Data are means ± SE. Olive oil, but not oleate induced insulin resistance during the two-step hyperglycemic clamp, whereas salicylate completely prevented the impairment.

# OLO vs all, p<0.01 during the first and the second step of the hyperglycemic clamp
Figure 4.8. Effects of salicylate on Disposition Index (DI = M/I x C-peptide, index of β-cell function) during two-step hyperglycemic clamps with/without 48h oleate (4.8.A.) or olive oil (4.8.B.) infusion. Groups are described in the legend of Figure 4.2. Data are means ± SE. Both oleate and olive oil impaired the β-cell function during the two-step hyperglycemic clamp, whereas salicylate completely prevented the impairment.

# OLE and OLO vs all, p<0.01 during the first and the second step of the hyperglycemic clamp
**Figure 4.9**

A

- SAL
- OLE
- OLE+SLY
- SLY

B

- SAL
- OLO
- OLO+SLY
- SLY

**Figure 4.9.** Insulin secretory response to glucose of freshly isolated islets of 12 week old normal female Wistar rats treated for 48h with: 4.9.A) 1) Saline alone (SAL n=16), 2) Oleate alone (OLE 1.3 μEq·min⁻¹, n=14), 3) Oleate + Salicylate (OLE+SLY, oleate-1.3μ Eq·min⁻¹, SLY-0.7 μmol·kg⁻¹·min⁻¹, n=8), 4) Salicylate alone, (SLY, n=10); 4.9.B) 1) Saline alone (SAL n=16), 2) Olive oil alone (OLO 5μl·min⁻¹, n=12), 3) Olive oil + Salicylate (OLO+SLY, olive oil-5μl·min⁻¹, SLY-0.7μmol·kg⁻¹·min⁻¹, n=6), 4) Salicylate alone, (SLY, n=10); Data are means ± SE. Both oleate and olive oil impaired the insulin secretion at both 13 and 22 mmol·l⁻¹ glucose; salicylate completely prevented the effect of oleate at both 13 mmol·l⁻¹ glucose and 22 mmol·l⁻¹ glucose.  *p <0.001, SAL vs. OLE or OLO

#p<0.01, SAL vs. OLE or OLO
**Figure 4.10.**

A

![Graph showing ROS Levels (% of Control)](image)

B

![Images of cells: SAL, OLE, OLE+SLY, SLY](image)

**Figure 4.10.A.** ROS levels in cells exposed to treatments in Figure 4.9.A. (SAL, n=5; OLE, n=6; OLE+SLY, n=4; SLY, n=4). Data are means±SE, calculated as fold change from control. Infusion of olate increased ROS levels as compared to saline control, whereas coinfusion of salicylate did not prevent the increase in ROS induced by olate. Salicylate alone had no significant effect. **Figure 4.10.B.** Representative fluorescent images for ROS (200X). Light images are available upon request.

* p <0.001, vs. SAL
Figure 4.11. Phosphorylated IkB alpha levels in islets of 12 week old normal female Wistar rats treated for 48h with: 4.11.A. 1) Saline alone (SAL, n=7), 2) Olate alone (OLE 1.3 µEq min⁻¹, n=4), 3) Oleate + Salicylate (OLE+SLY, oleate-1.3µ Eq·min⁻¹, SLY-0.7 µmol·kg⁻¹·min⁻¹, n=3), 4) Salicylate alone, (SLY, n=6); 4.11.B. 1) Salicylate alone (SAL, n=7), 2) Olive oil alone (OLO 5µ1·min⁻¹, n=5), 3) Olive oil + Salicylate (OLO+SLY, olive oil-5µ1·min⁻¹, SLY-0.7 µmol·kg⁻¹·min⁻¹, n=4), 4) Salicylate alone, (SLY, n=6) Data are means±SE. Infusion of oleate or olive oil caused a significant increase in phosphorylated IkBα, which reflects IKKβ activation. This was prevented by co-infusion of salicylate.

* p <0.05, SAL vs. OLE or OLO
**Figure 4.12.** Active nuclear NFκB levels in islets of 12 week old normal female Wistar rats treated for 48h with: 4.12.A. 1) Saline alone (SAL n=7, 2) Oleate alone (OLE 1.3 μEq min⁻¹ n=4), 3) Oleate + Salicylate (OLE+SLY, oleate-1.3μ Eq min⁻¹, SLY-0.7 μmol·kg⁻¹·min⁻¹, n=5), 4) Salicylate alone, (SLY, n=6); 4.12.B. 1) Saline alone (SAL n=7, 2) Olive oil alone (OLO 5μl·min⁻¹ n=5), 3) Olive oil + Salicylate (OLO+SLY, olive oil-5μl·min⁻¹, SLY-0.7 μmol·kg⁻¹·min⁻¹, n=4), 4) Salicylate alone, (SLY, n=6) Data are means±SE. Infusion of oleate or olive oil increased the amount of active nuclear NFκB which was prevented by salicylate.

* p <0.05, SAL vs. OLO
Figure 4.13. IRS-1 serine 307 phosphorylation levels in islets of 12 week old normal female Wistar rats treated for 48h with: 1) Saline alone (SAL n=7), 2) Olate alone (OLE 1.3 μEq·min⁻¹ n=4), 3) Olate + Salicylate (OLE+SLY, olate-1.3μ Eq·min⁻¹, SLY-0.7 μmol·kg⁻¹·min⁻¹, n=5), 4) Salicylate alone, (SLY, n=3); Data are means±SE. Infusion of olate increased IRS-1 serine 307 phosphorylation in islets, an effect which was prevented by co-infusion of salicylate.

* p<0.01, vs. All.
Figure 4.14. Plasma FFA (A), Glucose (B) and GINF (C) during the hyperglycemic clamps with/without 48h oleate infusion. Mice were treated with: 1) Saline alone (SAL, n=7), 2) Oleate alone (OLE, 3.9 µmol·min⁻¹, n=6), 3) Oleate+BMS (OLE+BMS, oleate-3.9 µmol·min⁻¹, BMS 30 mg·kg⁻¹·day⁻¹, n=5), 4) BMS alone (BMS, n=3). Data are means±SE. For groups infused with oleate alone (OLE) or oleate in combination with the IKKβ inhibitor BMS (OLE+BMS) the plasma FFA concentrations were raised to levels higher than control rats (SAL) or rats treated with BMS alone (BMS). Oleate impaired GINF during the hyperglycemic clamp, whereas BMS completely prevented the impairment.

* OLE vs. SAL, p<0.01
# OLE vs. BMS, p<0.05
Figure 4.15. Effects of BMS-345541 on plasma insulin and C-peptide during the hyperglycemic clamps with/without 48h oleate infusion. Treatment groups are described in the legend of Table 4.3. Different from our oleate model in rats, but similar to our olive oil model in rats, plasma insulin and C-peptide did not decrease as compared to control, in the oleate-treated mice, during the hyperglycemic clamp. For the groups treated with BMS, both insulin and C-peptide levels were higher than saline or oleate treated mice.

* OLE+BMS and BMS vs SAL and OLE, p<0.05 during the hyperglycemic clamp
Figure 4.16. Effects of BMS on Sensitivity Index (M/I = GINF/Insulin, 4.16.A) and Disposition Index (DI = M/I x C-peptide, 4.16.B) during hyperglycemic clamps with/without 48h oleate infusion. Groups are described in the legend of Table 4.3. Data are means ± SE. Oleate induced insulin resistance during the two-step hyperglycemic clamp, an effect which was not prevented by BMS; oleate also impaired the β-cell function during the hyperglycemic clamp, and BMS completely prevented the impairment.

* OLE vs All, p<0.01 during the hyperglycemic clamp
**Figure 4.17**

**A**
- CON
- OLE
- OLE+SLY
- SLY

**B**
- CON
- OLE
- OLE+BMS
- BMS

**Figure 4.17.** Effects of oleate+IKKβ inhibitors on insulin secretion in cultured islets. Islets were exposed for 48h to: 4.17.A. (CON, n=7, black); Olate (OLE, 0.4 mmol/l in BSA, n=14, white); Olate + Salicylate, (OLE+SLY, oleate-0.4 mmol/l in BSA, Salicylate 0.04 mg.ml⁻¹, n=10, checker); Salicylate (SLY 0.04 mg.ml⁻¹, n=7, large grid); 4.17.B. (CON, n=7, black); Olate (OLE, 0.4 mmol.l⁻¹ in BSA, n=14, white); Olate + BMS, (OLE+BMS, oleate-0.4 mmol.l⁻¹ in BSA, BMS 3 μmol.l⁻¹, n=10, light grey); BMS (BMS 3 μmol.l⁻¹, n=7, dark grey); Olate impaired the insulin secretion at both 13 and 22 mmol.l⁻¹ glucose, an effect completely prevented by both salicylate and BMS.

* OLE vs All, p<0.01
Study 3

Bisperoxovanadate Prevents Fat-Induced β-Cell Dysfunction \textit{In Vitro} and \textit{In Vivo}
5.1. Abstract

**Objective** Fatty acids induce insulin resistance and chronically decrease β-cell function. The insulin signaling pathway plays a role in insulin secretion, however it is still unclear whether the FFA-induced decrease in β-cell function involves ‘β-cell insulin resistance’.

**Research Design and Methods** To address the role of generalized and β-cell insulin resistance in FFA-induced β-cell dysfunction we coinfused with fat a peroxovanadium compound, bisperoxovanadate (BPV), in normal Wistar rats. BPV, a tyrosine phosphatase inhibitor, acts as insulin mimetic and is devoid of any antioxidant effect that could prevent β-cell dysfunction, unlike most insulin sensitizers. Two fat infusions were given iv to elevate plasma FFA by approximately 2-fold: a direct infusion of oleate in bovine serum albumin, or an emulsion of 20% olive oil (5 µl·min⁻¹)+heparin (50 U·ml⁻¹) for 48h in Wistar rats, with or without BPV at 0.0025 μmol·kg⁻¹min⁻¹. At the end of the 48h infusions we performed two-step hyperglycemic clamps to evaluate glucose stimulated insulin secretion (GSIS) *in vivo*, or we isolated islets to evaluate GSIS *ex vivo*. We also exposed the islets in culture conditions to oleate ± BPV for 48h to evaluate GSIS *in vitro*.

**Results** 48h infusion of oleate or olive oil impaired β-cell function *in vivo* (p<0.01) as measured with the disposition index during a two-step hyperglycemic clamp (13 and 22 mmol·l⁻¹ glucose), whereas coinfusion of BPV prevented the effect of oleate or olive oil. GSIS *ex vivo* in isolated islets of oleate or olive oil-treated rats was impaired (oleate: 1.033±0.097 pmol·islet⁻¹h⁻¹ at 22 mmol·l⁻¹, n=9, olive oil: 0.908±0.128 pmol·islet⁻¹h⁻¹, at 22mM, n=9, both p<0.001 vs. control: 1.505±0.131 pmol·islet⁻¹h⁻¹, at 22 mmol·l⁻¹, n=8),
but was restored by coinfusion of BPV (oleate+BPV: 1.700±0.350 pmol·islet⁻¹h⁻¹ at 22 mmol·l⁻¹, n=7, olive oil+BPV: 1.740±0.264 pmol·islet⁻¹h⁻¹ at 22 mmol·l⁻¹, n=7, both p=NS vs control), while BPV alone had no effect. Similar results were obtained in cultured islets, where 48h exposure to oleate impaired the GSIS at 13 mmol·l⁻¹ and 22 mmol·l⁻¹, an effect prevented by BPV.

**Conclusion** Our results suggest a role of FFA in decreasing β-cell function by impairing tyrosine phosphorylation at the β-cell level, consistent with the induction of ‘β-cell insulin resistance’.
5.2. Introduction

Numerous studies have established a close relationship between obesity, insulin resistance, and type 2 diabetes. This link is attributed to a greater release of various adipocyte-derived products, such as cytokines, resistin, and free fatty acids (FFA), from the expanded adipose tissue in obesity. In particular, elevated circulating levels of FFA cause insulin resistance (11;236;328-333).

Impaired insulin signaling in the main insulin-target tissues can lead to β-cell dysfunction, by promoting compensatory hypersecretion of insulin leading to β-cell exhaustion. In addition, the mechanisms of lipotoxicity may involve impairment of the insulin signaling cascade directly in islets, and this leads to impaired β-cell function in vitro and in vivo. The insulin receptor and the insulin signalling molecules are present in the β-cell and can be activated not only by insulin and IGF-1, but also by glucose and other hormones (255;256). The insulin signalling cascade has been found important not only for β-cell growth, but also secretion, including insulin gene transcription (257). Therefore, it can be postulated that lipotoxicity might impair insulin signaling not only in peripheral tissues, but also at the β-cell level. Results from the study in Chapter 4 clearly showed that salicylate improved β-cell function possibly by preventing serine phosphorylation of the IRS1, and therefore perhaps by increasing ‘β-cell insulin sensitivity’.

Reports from in vitro studies suggest a possible role for ‘β-cell insulin resistance’ in lipotoxicity (206). Because increased insulin signaling in the pancreatic
islets may prevent lipotoxicity, we studied the effects of the phospho-tyrosine phosphatase inhibitor peroxovanadate (BPV) on insulin secretion in Wistar rats. The same in vivo, ex vivo and in vitro models were used as in our previous studies, i.e. β-cell function was evaluated in vivo during the hyperglycemic clamps, ex vivo in islets of fat-treated rats with/without BPV, and in vitro in isolated islets exposed to oleate with and without BPV.

Vanadium compounds, although tyrosine phosphatase inhibitors which are nonspecific for the insulin signalling cascade, have the advantage over other insulin sensitizers such as metformin and thiazolidinediones of inducing rather than decreasing oxidative stress (334). Therefore, a positive effect in preventing lipotoxicity could not be ascribed to reduction of oxidative stress.
5.3. Materials and Methods

5.3.1. Animals and surgery

Female Wistar rats (250-300g, Charles River, Canada) were cannulated as previously described in the General Methods section and in (92).

5.3.2. Intravenous infusions

The rats were fasted overnight and randomized to the following infusion groups (n=6-12/group): 1) Saline control (SAL, 5 μl·min⁻¹); 2) Oleate at the same dose used in the study presented in the Chapter 3 of this thesis (OLE) or olive oil plus heparin (OLO, 50 U·ml⁻¹ heparin; 5.5 μl·min⁻¹); 3) Oleate plus bisperoxovanadum (0.0025 μmol·kg⁻¹·min⁻¹, the dose that increased insulin sensitivity in (335)) or Olive oil plus bisperoxovanadium (0.0025 μmol·kg⁻¹·min⁻¹) and 4) Bisperoxovanadium alone. A 2-step hyperglycemic clamp was performed after the 48h infusion period, using the same protocol described in the General Methods.
5.3.3. Islet isolation and secretion studies

Islets of *in vivo* infused rats were isolated as in (277), and preincubated for 1h at 37°C in Krebs Ringer buffer containing 10 mmol·l⁻¹ HEPES (KRBH), and 2.8 mmol·l⁻¹ glucose. Thereafter, 5 islets of approximately the same size were incubated in triplicate at 2.8, 6.5, 13 and 22 mmol·l⁻¹ glucose for 2h, 37°C. Insulin was measured in the supernatant with Linco’s kit. For *in vitro* studies, before preincubation, islets of untreated rats were cultured for 48h in RPMI 1640 without antioxidants, containing 0.4 mmol·l⁻¹ oleate in 0.5% BSA with or without 4 µmol·l⁻¹ BPV, a dose based on (336) as well as on our pilot studies.

5.3.4. Plasma Assays

Plasma glucose, insulin, and C-peptide was measured as described in the General Methods section of this thesis.

5.3.5. Calculations

Please refer to the General Methods section for calculations used in this study.
5.3.6. Evaluation of GSIS and β-Cell Function *In Vivo*

Similar to the study in Chapter 3, C-peptide levels were taken as indices of absolute insulin secretion. As explained in the General Methods section, β-cell function *in vivo* (insulin secretion corrected for insulin sensitivity) was assessed by calculating the Disposition Index (DI = C-peptide multiplied by an index of insulin sensitivity). Insulin sensitivity during hyperglycemic clamps was calculated as the M/I index, that is the glucose infusion rate required to maintain the glycemic target divided by the insulin concentration.

5.3.7. Statistical Analysis

Data are means±SE. One way non-parametric ANOVA for repeated measurements, followed by Tukey’s t-test was used to compare treatments. Calculations were performed using SAS (Cary, NC).
5.4. Results

5.4.1. In vivo clamp studies

Throughout the 48h infusion period both oleate and olive oil treated rats had higher plasma FFA than rats that were treated with saline or BPV alone (Table 5.1.A-B, tables and figures are at the end of the chapter). The infusion of oleate, olive oil or BPV did not affect plasma glucose or insulin (data not shown).

Following the 48h infusions, we evaluated insulin secretion in vivo using hyperglycemic clamps. As shown in Figures 5.1.A and 5.1.B, basal plasma FFA prior to the clamps were higher in oleate or olive oil-treated than control rats, or rats treated with BPV alone. FFA levels decreased during the clamp because of hyperglycemia and hyperinsulinemia, but remained higher in oleate or olive oil treated rats.

Plasma glucose was similar in all groups in the basal state and during the clamp, as per experimental design (Figure 5.2.A-B). The glucose infusion rate (GINF) necessary to maintain the target glucose level was lower in both oleate and olive oil than saline-treated group (Figures 5.3.A-B). BPV alone had no effect on GINF. When BPV was added to the oleate infusion, GINF was partially restored during the first step of the hyperglycemic clamp (Figure 5.3.A), but was not affected during the second step of the clamp. When the insulin sensitizer BPV was used in combination with olive oil, GINF was completely restored to control levels (Figure 5.3.B).

Basal insulin and C-peptide levels were similar in all groups. During the clamp, plasma insulin and C-peptide were lower than saline and similar to oleate in rats treated
with BPV alone or BPV in combination with oleate, but not in the group treated with BPV and olive oil (Figures 5.4.A-B and 5.5.A-B).

As evidenced by Figures 5.6.A-B, the M/I showed a tendency to increase with BPV alone, or in combination with oleate, however the increase was only significant during the second step of the hyperglycemic clamp. In the group treated with olive oil M/I was reduced, an effect which was prevented by the addition of BPV.

β-cell function calculated as DI was impaired with both oleate and olive oil infusions, but completely restored with the addition of BPV (Figure 5.7.A-B). BPV alone also had an effect to improve β-cell function throughout the clamp. Similar to the study presented in Chapter 4, we calculated the C-Peptide/Insulin ratio as an index of insulin clearance. In the groups exposed to vanadate, we noticed a significant increase in this ratio (Figure 5.8.A-B).

5.4.2. Ex vivo studies in islets

We also performed in vivo infusion of BPV/saline, with and without oleate or olive oil, followed by evaluation of insulin secretion in isolated islets. BPV infusion prevented the GSIS decrease induced by oleate or olive oil infusion at both 13 mmol·l⁻¹ glucose (p=NS, both OLE+BPV and OLO+BPV vs. SAL) and 22 mmol·l⁻¹ (p=NS, both OLE+BPV and OLO+BPV vs. SAL, Figure 5.9.A-B). No significant effect of BPV alone was observed at any glucose concentration, although a tendency to increase the insulin response was observed at 13 mmol·l⁻¹ glucose as compared to saline.
5.4.3. *In Vitro* studies in islets

We performed *in vitro* studies in cultured islets, to investigate whether addition of BPV to the media can prevent oleate-induced β-cell dysfunction directly in islets. Similar to our previous studies, 0.4 mmol·l\(^{-1}\) oleate in 0.5% BSA decreased GSIS (*Figure 5.10*). Coincubation with 4 µmol·l\(^{-1}\) BPV restored GSIS. BPV alone had no significant effect on insulin secretion.
5.5. Discussion

Inflammatory and stress-associated kinases could affect β-cell function via inducing insulin resistance directly at the β-cell level. Insulin receptor and insulin signalling molecules are expressed in the β-cell, and it has recently become evident that insulin may affect β-cell function and growth. Short-term effect on GSIS can be either inhibitory or stimulatory (249-252), whereas effects on proinsulin biosynthesis and insulin gene transcription appear to be stimulatory (253-255). Although there is still debate about the importance of insulin for β-cell function, there is consensus about the concept that insulin signalling molecules, however activated (i.e., by insulin, directly by glucose or other hormones (256), play an important and mainly stimulatory role in β-cell function and growth (257;258). This raises the possibility that known inducers of insulin resistance at peripheral sites, such as oxidative stress, may also induce “insulin resistance” at the β-cell level.

In this study we have examined the effects of prolonged FFA exposure with or without the tyrosine phosphatase inhibitor bisperoxovanadium on β-cell function in vivo, ex vivo, and in vitro.

In our in vivo clamp studies both insulin and C-peptide responses to glucose were lower in rats treated with oleate, but not olive oil, as in the study of Chapter 4. This is because with olive oil the β-cell attempted to compensate for insulin resistance. As explained in Chapter 4, this effect of olive oil to induce insulin resistance may be due to the amount (16%) of saturated fat contained by olive oil, as opposed with oleate, which is a monounsaturated fatty acid and also to the nature of olive oil, which is a mixture of
triglycerides. At the same time, the FFA elevation in plasma with olive oil is actually due to tissue spillover, which implicates an even higher intracellular level of fat.

The glucose infusion rate (GINF) is a measurement of the glucose amount required for i.v. infusion during the hyperglycemic clamp, in order to keep a desired glucose concentration in plasma. Changes in GINF reflect changes in insulin secretion, clearance and/or sensitivity. In our model, the GINF was reduced with both oleate and olive oil infusion. This effect was only prevented by addition of BPV to olive oil, but not to oleate, during the second step of the hyperglycemic clamp (22 mmol·l⁻¹). BPV alone did not affect the GINF. When DI was used to assess β-cell function, we noticed a decrease in both groups exposed to oleate or olive oil. This effect was completely prevented by addition of BPV to both fat infusions. An interesting finding was the effect of BPV, which seemed to increase the β-cell function, as measured by the DI index. β-cell function in vivo has to be considered not only in relationship with insulin resistance, but also with insulin clearance. Since BPV alone increased C-peptide/insulin ratio (index of insulin clearance), the β-cell presumably had to secrete more to compensate for insulin degradation, despite the fact that insulin sensitivity was, if anything, enhanced. Since insulin clearance is mainly a liver function and is dependent on the insulin receptor tyrosine phosphorylation, these results suggest that liver is a primary target tissue for BPV. However, the M/I index of insulin sensitivity during the hyperglycemic clamp mainly reflects the insulin sensitivity in muscle, which was apparently less affected than liver by BPV infusion alone. In spite of normalizing the DI, coinfusion of BPV did not normalize GINF in the oleate treated rats. This is likely because at 22 mmol·l⁻¹ insulin clearance increased in the oleate plus BPV treated group more than the β-cell could
compensate for, but did not increase in the olive oil plus BPV group. Since similar signaling mechanisms (i.e. insulin receptor tyrosine phosphorylation) mediate both insulin clearance and insulin sensitivity, this is consistent with hepatic insulin resistance with olive oil.

To assess β-cell function independent of systemic factors influencing the β-cell response to glucose in vivo (e.g. notably the prevailing insulin sensitivity and clearance increased by BPV), we examined GSIS ex vivo in isolated islets. Furthermore, to eliminate any indirect effect of in vivo treatments in the 48h preceding islet isolation on GSIS, we exposed islets directly to fat and BPV in vitro. 48h infusion of oleate, or olive oil, which contains mostly oleate, impaired GSIS ex vivo in isolated islets, consistent with our in vivo findings. 48h infusion of BPV protected the islets from the FFA-mediated impairment in GSIS. Direct exposure of islets for 48h to oleate decreased the insulin response at high glucose, and in vitro exposure to BPV restored GSIS, similar to our ex vivo model. Previously vanadate has been used in vitro in islets of GK rats where it was found to increase both basal as well as GSIS. However it only increased insulin secretion at basal or low stimulatory glucose levels in islets of Wistar control rats (336;337). Another study, using the same dose as our study, showed that 72h treatment with vanadate had no effect on cellular insulin content but improved GSIS (338). The mechanism of the effect of vanadate needs further study, however, vanadate did not affect islet glucose metabolism in one study (337), increased IRS-1 and IRS-2 tyrosine phosphorylation and serine phosphorylation of Akt and MAPK in another study (336), and its effect on basal insulin secretion was prevented by the PI3K inhibitor Wortmannin (337). Thus, it is likely that vanadate upregulated the activity of the IGF-1 insulin
signaling cascade which was decreased by fat. In INS-1 cells (206) fatty acids decreased IGF-1 stimulated Akt activation, and Akt prevented fatty acids-induced apoptosis (339).

The β-cell insulin signaling cascade was also impaired by prolonged fat exposure in another study (304), an effect which was linked to JNK activation, serine-phosphorylation of IRS 1 and 2 and impaired insulin gene transcription in mouse pancreatic islets. These data are in accordance with our results from Chapter 4, where we also noticed an increase in serine phosphorylation of IRS1, induced by oleate, and prevented by the IKKβ inhibitor salicylate.

In conclusion, our results show that both fat-induced whole body insulin resistance and the decrease in β-cell function are prevented by an insulin mimetic. Further studies are required to evaluate the activity of the insulin signaling cascade in islets in our model. Furthermore, as BPV is a non-specific tyrosine phosphatase inhibitor, other mechanisms may be involved in the overall effect noticed in our studies. Studies in other models of upregulation of the insulin/IGF-I signaling cascade will be required to consolidate the concept that the mechanisms whereby fat affects β-cell function involve the induction of ‘β-cell insulin resistance’.
### Table 5.1.A.

<table>
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<tr>
<th>Group</th>
<th>Time (h)</th>
<th>FFA (µmol·l⁻¹)</th>
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Table 5.1.A Plasma FFA levels during the 48h infusion period. Rats were treated with: 1) Saline alone (SAL, n=12), 2) Oleate alone (OLE, 1.3 µmol·min⁻¹, n=10), 3) Oleate+Bisperoxovanadate (OLE+BPV, oleate-1.3µmol·min⁻¹, BPV-0.0025 µmol·kg⁻¹·min⁻¹, n=8), 4) Bisperoxovanadate alone (BPV, n=7). Data are means±SE. For groups infused with oleate alone (OLE) or oleate in combination with the insulin sensitizer bisperoxovanadate (OLE+BPV) the plasma FFA concentrations were raised to levels higher than control rats (SAL) or rats treated with salicylate alone (BPV).
**Table 5.1.B.**

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Table 5.1.B Plasma FFA levels during the 48h infusion period. Rats were treated with:

1) Saline alone (SAL, n=12), 2) Olive oil alone (OLO, 5.5 \( \mu l\cdot min^{-1} \), n=9), 3) Olive oil+Bisperoxovanadate (OLO+BPV, OLO-5.5 \( \mu l\cdot min^{-1} \), BPV-0.0025 \( \mu mol\cdot kg^{-1}\cdot min^{-1} \), n=8), 4) Bisperoxovanadate alone (BPV, n=7). Data are means±SE. For groups infused with olive oil alone (OLO) or olive oil in combination with the insulin sensitizer bisperoxovanadate (OLO+BPV) the plasma FFA concentrations were raised to levels higher than control rats (SAL) or rats treated with salicylate alone (BPV).
Figure 5.1. Effects of bisperoxovanadate on plasma FFA during two-step hyperglycemic clamps with/without 48h oleate (5.1.A.) or olive oil (5.1.B.) infusion. Groups are described in the legend of Table 5.1 Data are means±SE. Both oleate and olive oil increased plasma FFA, as expected.

# oleate and olive oil infused groups vs. non-oleate/olive oil infused groups, p<0.01 throughout the hyperglycemic clamp
Figure 5.2. Effects of bisperoxovanadate on plasma glucose during two-step hyperglycemic clamps with/without 48h oleate (5.2.A.) or olive oil (5.2.B.) infusion. Groups are described in the legend of Table 5.1. Data are means±SE. Plasma glucose levels were superimposable among groups.
Figure 5.3. Effects of bisperoxovanadate on plasma GINF during two-step hyperglycemic clamps with/without 48h oleate (5.3.A.) or olive oil (5.3.B.) infusion. Groups are described in the legend of Table 5.1. Data are means±SE. Both oleate and olive oil impaired GINF during the two-step hyperglycemic clamp, whereas bisperoxovanadate completely prevented the impairment induced by olive oil.

† OLE, OLE+BPV, OLO vs. SAL, p<0.01 throughout the hyperglycemic clamp
Figure 5.4. Effects of bisperoxovanadate on insulin during two-step hyperglycemic clamps with/without 48h oleate (5.4.A.) or olive oil (5.4.B.) infusion. Groups are described in the legend of Table 5.1. Oleate, but not olive oil impaired the insulin levels during the two-step hyperglycemic clamp.

# OLE vs SAL, p<0.01 during the first and p<0.001 during the second step of the hyperglycemic clamp.
Figure 5.5. Effects of bisperoxovanadate on C-peptide during two-step hyperglycemic clamps with/without 48h oleate (5.5.A.) or olive oil (5.5.B.) infusion. Groups are described in the legend of Table 5.1. Oleate, but not olive oil impaired the C-peptide levels during the two-step hyperglycemic clamp.

# OLE or BPV vs SAL, p<0.01 during the first and p<0.001 during the second step of the hyperglycemic clamp
Figure 5.6. Effects of bisperoxovanadate on Sensitivity Index (M/I - GILF/Insulin) during two-step hyperglycemic clamps with/without oleate (5.6.A.) or olive oil (5.6.B.) infusion. Groups are described in the legend of Table 5.1. Data are means ± SE. Olive oil, but not oleate induced insulin resistance during the two-step hyperglycemic clamp, whereas bisperoxovanadate completely prevented the impairment.

# p<0.01 vs. SAL during the first and the second step of the hyperglycemic clamp
Figure 5.7. Effects of bisperoxovanadate on Disposition Index (DI = M/I x C-peptide, index of β-cell function) during two-step hyperglycemic clamps with/without 48h olate (5.7.A.) or olive oil (5.7.B.) infusion. Groups are described in the legend of Table 5.1. Data are means ± SE. Both olate and olive oil impaired the β-cell function during the two-step hyperglycemic clamp, whereas bisperoxovanadate completely prevented the impairment.

# OLE and OLO vs all, p<0.01 during the first and the second step of the hyperglycemic clamp
Figure 5.8. The C-Peptide/Insula ratio was taken as an index of insulin clearance. Groups are described in the legend of Table 5.1. Data are means±SE. This ratio was increased in the groups containing BPV.

* BPV and OLE+BPV vs SAL or OLE, p<0.05
**Figure 5.9.** Insulin secretory response to glucose of freshly isolated islets of 12 week old normal female Wistar rats treated for 48h with: 5.9.A 1) Saline alone (SAL n=16), 2) Oleate alone (OLE 1.3μEq·min⁻¹ n=14), 3) Oleate + Bisperoxovanadate (OLE+BPV, oleate-1.3μ Eq·min⁻¹, BPV 0.0025 μmol·kg⁻¹·min⁻¹, n=8), 4) Bisperoxovanadate alone, (BPV, n=10); 5.9.B. 1) Saline alone (SAL n=16), 2) Olive oil alone (OLO 5μl·min⁻¹ n=12), 3) Olive oil + Bisperoxovanadate (OLO+BPV, olive oil-5μl·min⁻¹, BPV 0.0025 μmol·kg⁻¹·min⁻¹, n=6), 4) Bisperoxovanadate alone, (BPV, n=10); Data are means ± SE. Both oleate and olive oil impaired the insulin secretion at both 13 and 22 mmol·l⁻¹ glucose; bisperoxovanadate completely prevented the effect of oleate at both 13 mmol·l⁻¹ glucose and 22 mmol·l⁻¹ glucose.

* p <0.001, SAL vs. OLE or OLO;
# p <0.01, SAL vs. OLE or OLO.
Figure 5.10: Effects of oleate on insulin secretion in cultured islets. Islets were exposed for 48h to: BSA (CON, n=7, black); Oleate (OLE, 0.4 mmol·l⁻¹ in BSA, n=14, white); Oleate + Bisperoxovanadate, (OLE+BPV, oleate-0.4 mmol·l⁻¹ in BSA, Bisperoxovanadate-4 μmol·l⁻¹, n=10, dark grey); Bisperoxovanadate (BPV, 4 μmol·l⁻¹, n=7, Light Grey); Oleate impaired the insulin secretion at both 13 and 22 mmol·l⁻¹ glucose, an effect completely prevented by bisperoxovanadate.

* OLE vs All, p<0.01
General Discussion

6.1. Lipotoxicity and Oxidative Stress

An important mechanism which may be involved in the pathogenesis of type 2 diabetes in obese individuals is elevation of plasma free fatty acids (FFA) which induce insulin resistance and may impair both β-cell function and mass (β-cell lipotoxicity). Oxidative stress has been shown to play an important role in β-cell glucotoxicity, i.e. the impairment in β-cell function and mass induced by glucose, which is in many aspects similar to that induced by FFA. The first objective of my thesis was to investigate the role of oxidative stress (ROS) in β-cell lipotoxicity, using in vivo, ex vivo, and in vitro models.

In my studies I found that the antioxidants taurine, N-Acetylcysteine and TEMPOL prevented the impairment in β-cell function induced by prolonged exposure to
FFA in vivo during hyperglycemic clamps and ex vivo in isolated islets of fat-treated rats. My in vitro studies showed that both oleate and palmitate induced the same degree of β-cell dysfunction, an effect that was prevented by addition of taurine. These findings confirmed my ex vivo results in islets of fat-treated rats, with or without antioxidants. I also performed in vitro studies using H₂O₂ as a ROS generator. My data showed that the impairment in GSIS of islets exposed for 48h to H₂O₂ was similar to that induced by oleate or palmitate. Therefore, my results did support the hypothesis that oxidative stress plays a role in the FFA-induced impairment of β-cell function. These results lead us to build a hypothetical scheme (Figure 6.1, figures are at the end of the chapter) which suggests possible mechanisms of action involved in the impairing effect of FFA on β-cell function and was partly validated by the results from our studies with salicylate and vanadate. This scheme will be further discussed in this chapter.

A number of studies have shown the deleterious effect of oxidative stress on β-cell function, and not all these effects are necessarily linked to lipotoxicity. ROS can decrease glucose oxidation via inhibition of mitochondrial (166) and glycolytic enzymes (211), and can induce uncoupling by activating (146;171) and/or upregulating (169;170) uncoupling protein 2 (UCP2). Both of these processes influence the amount of ATP derived from glucose, and therefore GSIS. A 72h exposure of mouse islets to glucose increases mitochondrial superoxide, which activates UCP2 without altering UCP2 mRNA (171). However, whether oxidative stress increases UCP2 activity in vivo in selective models of lipotoxicity remains to be determined. In our simplified scheme of Figure 6.1 these ROS mediated effects of FFA on glucose oxidation/UCP2 (Figure 1.1 of introduction) can be considered as direct effect of ROS on β-cell function. Both Figure
6.1 and Figure 1.1 are incomplete in that FFA can decrease glucose oxidation and UCP2 activity/expression independent of ROS. In fact, it would be interesting to investigate whether, and how much antioxidants prevent the FFA-induced effect on glucose oxidation and UCP2 expression/activity in our *ex-vivo* model. In MIN6 cells, upregulation of UCP2 by oleate was not affected by the antioxidant taurine, suggesting that this upregulation is independent of ROS. Although antioxidants completely prevented FFA-induced β-cell dysfunction in our model, it is still possible that not all pathways of β-cell lipotoxicity diverge or converge into oxidative stress. In the rat model, it is not unusual that all treatments are completely effective, presumably because inhibitors can be given at doses high enough to oppose the effect of induction of parallel pathways; however, our results do suggest that oxidative stress plays a pivotal role in fat-induced β-cell dysfunction. Another level of impairment induced by oxidative stress or independent of oxidative stress might be the induction of endoplasmic reticulum (ER) stress. However, in our preliminary studies (not published), ER stress was not affected by oleate or lard-oil infusion as quantified by measurement of ER stress markers. In contrast, ER stress markers were induced in an *in vivo* model of glucotoxicity used in our lab (340). Glucose-induced oxidative stress can impair β-cell function also at the level of insulin gene transcription (77;78), because of decreased binding of the transcription factors PDX-1 (172;173) and MafA (174;175) to the insulin promoter. Impairment of insulin gene transcription also occurs after prolonged islet exposure to palmitate (160;193;341-343), but not oleate (136;161), or a physiologic mixture of oleate + palmitate (163). The palmitate effect has been attributed to ceramides (162), which can induce oxidative stress (192), however the role of oxidative stress in this effect has not
been investigated. Oxidative stress can have direct effects on β-cell transcription factors (172;174;175), such as FoxO-1. This direct effect would also be included in the arrow directly linking ROS to β-cell function of Figure 6.1. Recently, a number of oxidative stress mediated effects which were considered to be direct have been linked to NAD depletion via activation of PARP, which are DNA repair enzymes activated by ROS-induced DNA damage. NAD depletion decreases Sirt1, which can act as transcription factors (for example, Sirt1 binds to the UCP2 promoter, thus indirectly increasing insulin secretion, (344)). Sirt1 also deacetylases other transcription factors such as FoxO1, the activity of which can be either increased or decreased, depending on the tissue. In the β-cell oxidative stress increases FoxO-1 nuclear retention (176;177) which results in nuclear exclusion of PDX-1 (178) and consequent inhibition of insulin gene transcription. However, in contrast with the prevalent notion that FoxO-1 activation decreases insulin gene transcription, it should also be mentioned that one study found that FoxO-1 upregulates transcription factors that are important for β-cell function (185). At least part of the effect of oxidative stress to activate FoxO-1 is currently thought to be mediated by JNK (179;180). In addition to impairing insulin gene transcription via FoxO-1, activation of JNK by glucose can induce serine phosphorylation of insulin receptor substrates (IRS) (345). This results in further activation of FoxO-1 and inhibition of the mTOR pathway required for (pro)insulin biosynthesis (345) as a result of impairment of β-cell IGF-I/insulin signaling. Recently, JNK has been reported to mediate the inhibition of insulin gene transcription and the serine phosphorylation of IRS by palmitate (193). This is the reason for which we cannot exclude JNK as an alternate pathway of β-cell lipotoxicity to IKKβ/NFκB, and we have included it in our summarizing scheme. Thus, there is
evidence that JNK can impair β-cell function at the level of insulin gene transcription and that JNK is activated by lipotoxicity (at least by palmitate, this remains to be shown with oleate), however whether JNK is important in vivo is not known.

Using an in vivo model of glucolipotoxicity consisting of cyclic infusion of glucose and Intralipid (346) it has recently been demonstrated that insulin gene transcription is decreased in islets of glucose and fat-infused rats. In our MIN6 cell model, insulin gene transcription was not affected by oleate (81), however insulin content was decreased and partially restored by NAC. Future studies will be necessary to determine the effect of fat on insulin gene transcription and insulin biosynthesis on our vivo model, and the effect of antioxidants and JNK inhibitors in this model.

Oxidative stress is a known activator of IKKβ, which, by phosphorylating the inhibitor IκBα, activates NFκB. The role of NFκB on β-cell function is more controversial than that of JNK, because there are no reports that NFκB affects FoxO-1 and insulin gene transcription. IKKβ can induce serine phosphorylation of IRS (18;297), which we demonstrated in β-cells in the study performed in Chapter 4. It is also currently controversial whether fat (205;240) activates the IKKβ/NFκB pathway in vitro in β-cells, although from our results, this seems to be the case in vivo. To further address this pathway and better understand if this effect is dependent on oxidative stress, future studies should be designed exposing islets to fat and antioxidants, to assess the serine phosphorylation of IκBα in these conditions.
6.1.1. Additional Data Regarding the Source of ROS in Lipotoxicity

My results showed that β-cell lipotoxicity is likely caused by ROS that are detected by the dye DCFH-DA, such as lipid peroxides. This is because antioxidants that did not scavenge superoxide, such as NAC and taurine, were effective. TEMPOL was also effective and its effect may be due to direct reduction of superoxide and/or to decrease in other ROS formed from superoxide. One important site of production of superoxide is the mitochondria. In order to determine the type and cellular location of ROS involved in β-cell lipotoxicity in vivo, I performed a few pilot experiments in which two different types of dyes were used, hydroethidine, to measure the total superoxide involved in lipotoxicity, and MitoSOX, to measure the mitochondrial superoxide. My preliminary data show that total, but not mitochondrial superoxide is increased in islets of oleate-infused rats (Figure 6.2). Based also on the results obtained in the first study presented in this thesis in Chapter 3, where the measurement of the mitochondrial SOD through RT-PCR showed that the expression level of the mRNA for this enzyme was not increased in MIN6 cells, it is possible that cytosolic superoxide plays a major role in β-cell lipotoxicity. However, since the antioxidants NAC and taurine, which do not decrease superoxide, were also effective in restoring β-cell function, other ROS must be implicated, in addition to superoxide. These other ROS appear to be detected by DCFH-DA, since the level of DCFH-DA-measured ROS correlated with the impairment in β-cell function. Unsaturated fatty acids in membranes, including plasma and mitochondrial membranes, are substrates for lipid peroxidation. Lipid peroxides are detected by DCFH-DA and lipid peroxidation products are known to cause impaired insulin secretion (166).
Thus, lipid peroxides are candidate mediators of fat-induced β-cell dysfunction, although we cannot exclude that other ROS are implicated. To address the type and site of production of ROS involved, total lipid peroxides will be assessed with cis-parinaric acid (347), or other dye specific for lipid peroxides such as BODIPY (348) or DPPP (349), and mitochondrial localization of lipid peroxides will be assessed by co-localization with rhodamine123 by other students in the lab. In addition, the role of NADPH oxidase in cytosolic superoxide production will be addressed by using the NADPH-oxidase specific inhibitor, apocynin. This enzyme is probably implicated in the ROS production in lipotoxicity as it is activated by PKC, which is activated by lipid metabolites. PKC may be involved in β-cell lipotoxicity, as shown by a recent paper which implicated PKCε in the β-cell dysfunction induced by lipid treatment (350;351) and by a recent study which has also implicated PKCδ (352). PKC might be also involved in our animal model of lipotoxicity, and this will have to be analyzed in future studies. Interestingly we have implicated PKCδ, NADPH-oxidase and oxidative stress in fat-induced hepatic insulin resistance (353) and other investigators have implicated PKC-ε (354). It is possible that similar mechanisms mediate lipotoxicity at multiple sites, including liver, muscle, and β-cell.
6.2. Inflammation and Oxidative Stress

In the study described in Chapter 4, we determined whether the effect of FFA to induce β-cell dysfunction involves activation of the IKK/NFκB inflammatory pathway in islets. For this purpose, we infused i.v. oleate or an emulsion of 20% olive oil+heparin (50 U·ml⁻¹) for 48h in normal Wistar rats ± the IKKβ inhibitor salicylate and we found that salicylate restored β-cell function in vivo and ex vivo in both models. Furthermore, both salicylate and the specific IKKβ inhibitor BMS prevented β-cell dysfunction induced by oleate in cultured islets, and BMS restored β-cell function in oleate-infused mice. Another study implicating the IKK/NFκB pathway has shown that both pioglitazone and sodium salicylate had a protective effect in human islets against the detrimental effects of IL-1β and high glucose, by preventing NFκB activation (245). This study also suggested that preventing the activation of this inflammatory pathway might be a useful approach in retarding the manifestation and progression of diabetes. Interestingly, results from a recent clinical trial have shown that a synthetic IL-1 receptor antagonist was also useful in improving β-cell function and insulin secretion in type 2 diabetes, and possibly in preventing some of its secondary effects (355). This study brings further proof of a possible inflammatory state in type 2 diabetes at the β-cell level.

In previous studies, we found that lipid infusion phosphorylates IRS on serine residues in the liver, inducing insulin resistance and both serine phosphorylation of IRS and insulin resistance were prevented by coinfusion of the IKKβ inhibitor salicylate (236). A similar pathway was confirmed by our studies in islets, where 48h exposure to fat, which decreased β-cell function, increased the serine phosphorylation of IRS1, while
salicylate restored normal levels of IRS-1 phosphorylation and \( \beta \)-cell function. These results implicate the effect of IKK\( \beta \) on \( \beta \)-cell IGF-I/insulin signaling in fat-induced \( \beta \)-cell dysfunction as shown in **Figure 6.1**. However, NF\( \kappa \)B itself may be directly implicated because of induction of iNOS and COX2 and these effects are described in the arrow connecting IKK\( \beta \)/NF\( \kappa \)B directly to \( \beta \)-cell function of **Figure 6.1**. We are currently addressing these effects using iNOS and COX2 inhibitors in studies in islets *in vitro*. NF\( \kappa \)B mediated transcription also leads to induction of cytokines which may result in a vicious cycle by further enhancing IKK\( \beta \)/NF\( \kappa \)B and maybe JNK pathways. Glucotoxicity increased IL-1 beta expression (245), however the effect of lipotoxicity on cytokine expression is currently unknown. We are now assaying mRNA samples from our studies to address the possibility that transcriptional effects of NF\( \kappa \)B on iNOS, COX2 and cytokines may be involved in fat-induced \( \beta \)-cell dysfunction. We will also test c-JUN phosphorylation in rats exposed to oleate *in vivo* or use JNK inhibitors *in vitro* in cultured islets to address the effect of JNK. In the scheme of Figure 6.1, the JNK mediated effect on FoxO-1 could be a direct effect in decreasing \( \beta \)-cell function, whereas JNK induced serine phosphorylation of IRS-1 would be an indirect effect, linked to \( \beta \)-cell insulin signaling.

Another possible mechanism to consider as downstream of oxidative stress is the p38 MAPK pathway. However, although oxidative stress activates p38 MAPK in rat islets, p38 MAPK inhibition does not protect insulin gene expression and secretion from oxidative stress (213). Thus, further studies of the p38 MAPK pathways seem to be currently unjustified.
6.3. Role of β-cell Insulin Resistance in Fat-Induced β-cell Dysfunction

Other authors’ data showed that high-dose salicylate prevented FFA-induced peripheral insulin resistance through its inhibitory effect on IKKβ, which enhanced insulin signalling (236;356), while our data show that salicylate prevented FFA-induced hepatic insulin resistance by inducing serine phosphorylation of IRS1 and IRS2 (353). Generally, it is thought that any improvement of insulin sensitivity in the main insulin-target tissues can indirectly improve β-cell function in the long-term, by diminishing FFA release from adipose tissue, and by relieving β-cell exhaustion, or allowing the β-cell to “rest”. However, the hypothesis that we addressed in Chapter 5 is that the mechanism of β-cell lipotoxicity involves impairment of the insulin signaling cascade directly in islets, which leads to impaired β-cell function in vitro and in vivo. As mentioned above in Chapter 5, lipotoxicity might impair insulin signaling not only in peripheral tissues, but also at the β-cell level, due to the presence of the insulin receptor and the insulin signaling molecules in the β-cell (256). The insulin signaling cascade has been found important not only for β-cell growth, but also secretion, including insulin gene transcription (257). Accordingly, β-cell insulin receptor knockout (βIRKO) mice have a reduction in glucose-stimulated insulin secretion (357-359) although it cannot be excluded that this is in part due to deletion of IR in the brain.

To address the role of generalized and β-cell insulin resistance in β-cell lipotoxicity, we coinfused with fat a peroxovanadium compound, BPV a tyrosine phosphatase inhibitor with no antioxidant activity (334). Two fat infusions were given iv
to elevate plasma FFA by approximately 2-fold: a direct infusion of oleate in bovine serum albumin, or an emulsion of 20% olive oil for 48h in Wistar rats, with or without BPV. We also exposed the islets in culture conditions to oleate±BPV for 48h to evaluate GSIS \textit{in vitro}. 48h infusion of oleate or olive oil impaired β-cell function \textit{in vivo} as measured with the Disposition Index during a two-step hyperglycemic clamp, whereas coinfusion of BPV prevented the effect of oleate or olive oil. GSIS \textit{ex vivo} in isolated islets of oleate or olive oil-treated rats was impaired, but was restored by coinfusion of BPV, while BPV alone had no effect. Similar results were obtained in cultured islets, where 48h exposure to oleate impaired the GSIS at 13 mmol·l\(^{-1}\) and 22 mmol·l\(^{-1}\), an effect prevented by BPV. Combined with our findings from \textbf{Chapter 4}, our results suggest a role of FFA in decreasing β-cell function by impairing the insulin/IGF-I signaling pathway and induction of β-cell insulin resistance. However, since vanadate is a non-specific tyrosine phosphatase inhibitor we cannot totally exclude that BPV might have some direct effect in islets which are unrelated to IGF-1/insulin signaling. It is well known that vanadate is not an antioxidant, if anything it increases oxidative stress (360;361), therefore we expect that oxidative stress is even more increased in our vanadate treated islets, despite restoration of β-cell function and increased tyrosine phosphorylation of IR and IRS. In future studies we will measure these parameters with vanadate exposure \textit{in vitro}, and if the above expected results are found, this would suggest that the impairment of insulin signaling is independent of or downstream of oxidative stress in the pathway leading to fat-induced β-cell dysfunction.

Other studies have found that BPV increases insulin secretion in islets of GK rats and that this effect is in part mediated by PI3K (337). These findings prompt further
studies of the role of ‘β-cell insulin resistance’ in β-cell lipotoxicity, for example by examining Akt and MAPK activation in islets. Other more specific models of upregulation of IGF-I/insulin signaling should be used in future studies i.e., the β-cell specific PTEN-null mouse, which has an upregulated insulin signaling cascade in islets and we expect will be protected from β-cell lipotoxicity. We could also consider investigating the effect of GLP-1, which is an important activator of PI3K in β-cells and prevents apoptosis induced by glucoinipotoxicity in β-cell lines (362). However, these experiments may be difficult to interpret because of the effect of GLP-1 alone on insulin secretion and because GLP-1 may also directly reduce oxidative stress (363).
6.4. Validity of the proposed scheme in humans

The question of the validity of the proposed scheme in humans is very important, due to obvious reasons. These data may be confirmed mostly by in vivo clamps studies which are feasible in humans to evaluate insulin secretion, as human islets are difficult to find for in vitro studies. A recent paper from our group showed promising results with the antioxidant taurine. Obese and overweight, non-diabetic men (221) were studied, as they were found to be the most susceptible to β-cell lipotoxicity (364). The methods used in this paper are very similar to our rat studies, one major difference being the 48h Intralipid infusion (standard method of increasing plasma FFA in humans), as opposed to our oleate infusion, followed by hyperglycemic clamps (221). Taurine or NAC were given orally, which is the main difference from our i.v. infusions in rats. In the taurine study, participants ingested taurine at a dosage of 3 g/day divided into three equal doses for 2 weeks prior to and during the Intralipid infusion. NAC was administered with a loading dose of 140 mg/kg followed by 70 mg/kg every 4h during the 48h Intralipid infusion. Oral treatment with taurine improved FFA-induced impairment in insulin sensitivity as well as β-cell function, NAC had no effect, except for an improvement in insulin clearance, presumably because of its effect in the liver and low systemic bioavailability after oral administration. Our group also studied the effect of salicylate in the same category of patients. The results showed that high dose salicylate (1 week oral treatment with 4.5 g/day) did not improve insulin sensitivity but it did improve first phase insulin response in the presence and absence of chronically elevated plasma FFA (323) an effect which was due to the salicylate induced decrease in insulin clearance (data not
published). The results with salicylate are in contrast with the promising results on both insulin sensitivity and insulin secretion in obese and diabetic individuals found by other authors (323;364). The reasons for these discrepant results are unclear but it is possible that they are related to the different doses, formulations, or modalities of treatment. Overall, these in vivo studies have brought further evidence as to the presence of oxidative stress in lipotoxicity in humans, and represent a direct translation of our findings in animal models to humans.

Metformin and thiazolidinediones (TZD) insulin sensitizers have also been used in human islets and they were found to prevent β-cell lipotoxicity, although a link to β-cell insulin signaling upregulation has not been investigated. These drugs are also antioxidants and AMP kinase activators, and they will therefore deplete islets from fat (365).
6.5. Relevance of studies

These studies provide new insights into the physiology and pathophysiology of insulin secretion and into the mechanisms of the β-cell dysfunction caused by lipotoxicity; they also establish the rationale for FFA lowering therapies in preventing deterioration of β-cell function and identify novel targets susceptible to nutritional and/or pharmacological intervention to prevent and treat β-cell dysfunction in obesity associated type 2 diabetes.

Regarding pharmacologic intervention, although *in vitro* and animal studies have shown that antioxidants have beneficial effects in models of type 2 diabetes, data from clinical studies are limited, and mostly involve attempts to treat diabetes complications with antioxidants, rather than the initial physiopathologic event that triggers the β-cell damage or insulin resistance.

The Insulin Resistance and Atherosclerosis Study (IRAS) investigated the association between plasma vitamin E and incidence of type 2 diabetes in individuals who did and those who did not report regular use of vitamin supplementation. It was hypothesized that vitamin E may reduce the risk of diabetes. However, results of this prospective study showed only an effect limited to subjects who did not take vitamin E supplements, and thus a positive effect may exist only within the range of intake available from food (366). Furthermore, results of the HOPE study showed that diabetes was not prevented by vitamin E; also, vitamin E had no apparent effect on cardiovascular outcomes in people with high cardiovascular risk (367), and with/without mild-to-moderate renal insufficiency (368). Thus, despite the evidence that oxidative stress is
present in islets of type 2 diabetic subjects (369), the potential of antioxidant treatment remains to be demonstrated. Similarly, despite the overwhelming experimental evidence that oxidative stress is a critical mediator of diabetes complications (295), current studies have not provided strong evidence that treatment with antioxidants can prevent such complications (367).

To clarify the role of oxidative stress in type 2 diabetes, and improve future studies with possible benefits for human disease, previous experimental data should be closely examined. High doses of single-antioxidant supplements may perturb the antioxidant-prooxidant balance of cell systems. Therefore, mixtures of antioxidants, or antioxidants combined with trace elements and vitamins, as well as dietary increases of cysteine, as the rate limiting factor for the synthesis of glutathione, may constitute a better therapeutic option. SOD-mimetics are a novel class of potent antioxidants which showed promising results in animal models of diabetes (369), and may be considered for future human studies. Our results with salicylate in rats suggest that anti-inflammatory agents may be developed into treatments for β-cell dysfunction. Although oral salycilate was not effective to prevent Intralipid induced β-cell dysfunction in humans in our study, in another recent study performed in young obese patients proved that salsalate (salycilate dimer), was useful in prevention of hyperglycemia and brought further proof for a role of subacute-chronic inflammation in the pathogenesis of obesity-related dysglycemia (355).

It is well known that insulin sensitizers may be beneficial at the β-cell level mainly because of β-cell rest and reduction of glucolipotoxicity. A number of studies have proved that metformin and TZDs are also useful to prevent β-cell lipotoxicity (283;364;370;371), for their direct effect in islets, however as it has been refered to
before, insulin sensitizers are also AMP kinase activators and antioxidants. The combination of these properties may be advantageous clinically. In fact, it is most probable that the effective therapy will be a combination therapy. Such combination would include not only inhibition of oxidative stress and inflammatory pathways downstream of oxidative stress, but also modification of the other pathways involved (ER stress, PKC activation, etc.).
Figure 6.1. Possible mechanisms involved in the impairing effect of FFA on β-cell function. This scheme was partly validated by the results from our studies with antioxidants, salicylate and vanadate, and is discussed in Chapter 6 of this thesis. Thick arrows show the working hypothesis based on our results; thin arrows show additional links between the mechanisms addressed by our studies; dashed arrows show possible mechanisms not addressed by our studies. Decreased β-cell insulin signaling will occur due to increased serine phosphorylation of IRS.
Figure 6.2. Effects of oleate and taurine on total (hydroethidine, A) and mitochondrial (MitoSOX, B) superoxide levels in islets. Rats were treated for 48h with: A. SAL, n=2, black; OLE, n=3, white; OLE+TAU, n=3, chrome; B. SAL, n=2, black; OLE, n=3, white. Data are means±SE, calculated as % of saline. Infusion of oleate increased total, but not mitochondrial superoxide levels as compared to saline control, whereas coinfusion of taurine did not prevent the increase in total superoxide levels induced by oleate. C-D. Representative fluorescent images for total superoxide (C) and mitochondrial superoxide (D) (200X). Light images are available upon request.

* p<0.05, SAL vs. OLE or OLE+TAU
Summary and Conclusions

7.1. Summary of Each Study in the Thesis

Our first study demonstrates that prolonged exposure to oleate which induces oxidative stress in islets, decreases glucose stimulated insulin secretion both in vitro and in vivo. These findings are the first direct demonstration that oxidative stress is involved in the FFA-induced decrease in β-cell secretory function, and that antioxidants may be useful in its prevention.

In our second study, the mechanisms whereby fat affects β-cell function suggest a possible role for inflammation and the IKK/NFκB pathway. An increase in ROS was induced by oleate, but salicylate did not prevent this effect, suggesting that IKKβ activation was unrelated or downstream of ROS. Oleate increased the levels of
phosphorylated IkBα (indicator of IKKβ activity) which was prevented by the IKK inhibitor salicylate. Another IKK inhibitor, BMS, had the same effect as salicylate. In summary, our study demonstrates that prolonged exposure to oleate, which induces oxidative stress in islets, decreases glucose stimulated insulin secretion both in vitro and in vivo and activates the IKK/NFκB inflammatory pathway. These findings are the first to demonstrate that the IKK inflammatory pathway in islets is involved in the FFA-induced decrease in β-cell secretory function, and that anti-inflammatory drugs may be useful in its prevention. Furthermore, 48h oleate infusion increased the levels of serine phosphorylation of IRS1 in β-cells, showing that fat may affect the insulin signaling in the β-cells.

Our third study demonstrates that the effect of oleate or olive oil to decrease β-cell function was prevented by the insulin mimetic BPV in vitro and in vivo. Our results thus suggest that maneuvers that upregulate the insulin signaling cascade directly in islets do prevent β-cell dysfunction.
7.2. Conclusions

The studies in this thesis have demonstrated that a 48h exposure to free fatty acids decreasing β-cell function caused an increase in oxidative stress, activated the IKK/NFκB inflammatory pathway and induced IRS-1 serine phosphorylation in islets. β-cell function was restored by the addition of antioxidants, anti-inflammatory, and insulin sensitizers, which support the hypothesis that all these agents may be useful in the prevention and treatment of obesity related type 2 diabetes.
Limitations of the Studies and Future Directions

Ideally, these studies should be performed in humans, however, infusion of pure oleate-as per the Bezman Tarker method is impossible in human studies, due to the risk of hemolysis, clotting and inflammation at the infusion site induced by oleate, which requires infusion into a central line, and is still too risky in humans. Olive oil can be prepared as an emulsion (268) from olive oil of chemical grade purity (Sigma Chemicals), as virgin olive oil contains phenolic antioxidants (372), however, olive oil infusion lacks the sterility required. A reason against the use of olive oil infusions in human studies is the fact that the amount of saturated fat in olive oil may be too high and induce arrhythmia. A better and safer fat infusion option for human studies is Intralipid, which is a commercially available fat mixture containing mostly...
polyunsaturated fat (PUFA) available for infusion. However, a number of studies have shown that the decrease in β-cell function induced by Intralipid is a mild one both in animal models (92), as well as in human studies (373), possibly due to the type and proportion of fat content. Monounsaturated fat (MUFA) appears to be the most effective to decrease β-cell function, at least ex vivo in rats. Our in vitro studies show that palmitate is very effective as well, however in vivo (268) and ex vivo treatment with lard oil, which has a greater content of saturated fat (SFA) than olive oil, is not effective (374). The reason for these discrepancies are unclear, however it is possible that initial changes in β-cell volume and number in response to insulin resistance affect the islet response not only in vivo but also ex vivo in lard-oil treated rats. Regarding the effect of oleate vs. olive oil in our rat studies, it is only olive oil that induces insulin resistance, contrary to oleate infusion. Therefore, it seems that olive oil is a different model of lipotoxicity than oleate. Both models have advantages and disadvantages. From our studies, it seems that a direct effect of fat on insulin secretion in vivo may be better studied with oleate; however, olive oil allows a more complete analysis of the relationship between the insulin secretion and insulin resistance.

Studies in humans have been performed where different fat were given enterally as it is impossible to give i.v. certain types of fat as indicated above (221;373). In these studies, PUFA ingestion resulted in an absolute reduction in insulin secretion, whereas SFA ingestion induced insulin resistance. In the same study, the failure of insulin secretion to compensate for insulin resistance implicated impaired β-cell function also in the study using SFA ingestion. The disposition index, a measurement of
β-cell function, was similarly reduced in all groups including MUFA. Enteral fat administration is unavoidable in human studies because of the reasons explained above, however it has a number of disadvantages (373), as compared to i.v. infusions. Using enteral fat, it is relatively impossible to reach higher plasma levels of fat, and therefore the expected effects will be minimal. Another possibility which might explain the differences between the human and rat model might be a faster increase in β-cell mass in the rat.

Regarding the fat type, in real life the circulating fatty acids are mostly a mixture of oleate and palmitate in a ratio slightly below 2:1. However, palmitate cannot be solubilized for i.v. infusion in rat studies and given the above-described limitations of fat infusion in humans, it would appear that models of pharmacological reductions of elevated fatty acids in obese animals or humans could be preferable from a pathophysiological standpoint. The problem with this approach is that antilipolytic treatment is not very effective because of short half-life of drugs and frequent rebounds. Moreover, nicotinic acid (the major antilipolytic agent) is known to decrease insulin sensitivity (375).

In real life, patients with type 2 diabetes have both plasma FFA and glucose levels elevated; therefore, the combined effects of lipotoxicity and glucotoxicity are of great clinical importance. Moreover, in obese glucose intolerant subjects, postprandial hyperglycemia is added over the already high levels of FFA. Glucotoxicity due to chronic hyperglycemia might add to the lipotoxicity, or even have a permissive or synergistic effect on lipotoxicity as it favors fat esterification, generating lipotoxic signals (376). The outcome of this combination, known as glucolipotoxicity, or the
synergistic interaction between glucotoxicity and lipotoxicity is associated with islet triglyceride accumulation (53;376) and decrease in insulin gene expression (342;376). Although it would be tempting to investigate a combined model of glucolipotoxicity, in obesity the glucose levels are not necessarily increased from the very beginning, and therefore, it seems more appropriate to separate the two effects, and study their mechanisms separately. Future studies will address their combination.

One limitation of our studies is that our experimental design lacks the chronic effects of fat exposure observed in obesity due to the short duration (48 h) of fat exposure. A better model of chronic lipotoxicity might be a prolonged high-fat diet animal model, but in that case the effect will be different. Although high fat diet is a convenient and widely used model for the investigation of lipotoxicity in animal models, this approach is not selective, involving for example all the incretin effects of food and weight gain (high fat diet), making therefore difficult the task of distinguishing a particular effect due to high plasma FFA only.

Regarding our study with salicylate, it should be recognized that inhibitors have non-specific effects. To further consolidate our findings of the involvement of IKKβ in fat induced β-cell dysfunction, studies will be performed using oleate/control infusions in β-cell specific IKKβ-null mice as in (377). These mice will be generated by crossing IKKβ (flox/flox) mice provided by Dr. M. Karin with mice expressing Rip-Cre or inducible PDX-1 Cre provided by our collaborator Dr. M. Woo. GSIS will also be evaluated in cultured islets of IKKβ-null mice exposed to fatty acids and oxidants in vitro (superoxide generators (171) and lipid peroxidation products (166))
As an alternative to the mechanistic pathway that we propose in Chapter 6, it is also possible to use a JNK inhibitor, instead of salicylate, in order to address the JNK pathway. As mentioned elsewhere in this thesis, previous *in vitro* studies have shown that fat activates JNK in islets (378), although this effect was mostly due to palmitate (378). In a recent study, palmitate was found to decrease insulin gene transcription via JNK activation in islets (193). Again, the role of oxidative stress in palmitate-induced JNK activation has not been explored. In our future studies, we will first find out whether c-Jun is phosphorylated (as marker of JNK activation) in our *in vivo* models and expose cultured islets to oleate with/out the JNK inhibitor SP600125 *in vitro* (379). To directly assess the role of JNK in β-cell lipotoxicity *in vivo*, 48h oleate or olive oil/control infusions will be performed in rats with/out the JNK inhibitor SP600125 (15 mg·kg⁻¹·day⁻¹) (379;380), followed by evaluation of GSIS *in vivo* via hyperglycemic clamps, and *ex vivo* in isolated islets. We have recently found that this inhibitor prevents β-cell glucotoxicity in an *in vivo* rat model (374). If the JNK inhibitor is effective in β-cell lipotoxicity in our *in vivo* studies, we will study JNK-null mice. As JNK1 seems to be the isoform involved in diabetes (304), we will use JNK1-null mice which are available from Jackson lab (Stock # 004319) and have no unstimulated phenotype, except for increased susceptibility to infections. Cultured islets of JNK1-null mice may also be exposed to fatty acids and oxidants *in vitro*, followed by GSIS evaluation.

As BPV is a non-specific tyrosine phosphatase inhibitor, further studies will have to consider other insulin sensitizers, although these compounds usually are antioxidants. Studies with rapamycin, which increases insulin signalling pathways through inhibition of p70 S6 kinase (381;382) may also be performed using the same “three models” setting.
as before (\textit{in vivo, ex vivo, in vitro}). We may also consider activators of the PI3K-Akt or PKB signalling pathway, such as GLP-1, which prevented apoptosis induced by glucolipotoxicity in β-cell lines, (362) at a dose in which the prevailing effect is not the stimulation of insulin secretion. A more promising approach is through studies in islets of mice with genetic deficiency or over-expression of insulin signalling molecules. Acute overexpression or knockout of insulin signaling molecules in islets by using adenoviral techniques could also be considered. Studies could be performed with oleate/control infusions followed by hyperglycemic clamps in β-cell specific PTEN-null mice or their heterozygous available from our collaborator Dr. M. Woo. PTEN (phosphatase with tensin homology) is a potent negative regulator of phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. These mice have an upregulated IGF-I-insulin signaling cascade downstream of PI3K (383).

A recent paper from our collaborator Dr. M. Rozakis provided new data regarding PHIP1 (pleckstrin homology domain-interacting protein 1) attributing this protein with a role as a novel positive regulator of β-cell function, which may be involved in promoting β-cell mitogenesis and survival. Moreover, PHIP1 overexpression blocks free fatty acid-induced apoptosis in INS-1 cells in parallel with marked activation of phosphoprotein kinase B (PKB)/Akt (384). Mice overexpressing this protein are now available, and could be used in future studies regarding lipotoxicity.


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