**The impairing effect of a prolonged Free Fatty Acid elevation on Glucose Stimulated Insulin Secretion**

by

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for the degree of Master of Science

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## ABSTRACT

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Reduced glucose-stimulated insulin secretion (GSIS) characterizes both type 1 and type 2 diabetes mellitus. An elevation in plasma free fatty acids (FFA) is characteristic of both obesity and absolute or relative insulin deficiency. Previous work by our laboratory has demonstrated that prolonged elevation of plasma FFA impairs GSIS. We hypothesized that preexisting islet inflammation/oxidative stress modulates the FFA effect on GSIS (Study#1) and that this is mediated by FFA-induced oxidative stress (Study#2). Our results indicate that the FFA-induced defect in GSIS is accentuated in pre-diabetic BB rats (model of type 1 diabetes) with insulitis (Study#1), suggesting that islet inflammation modulates the effect of FFA. We also found that some antioxidants prevent the impairing effect of a prolonged FFA elevation on GSIS, suggesting that oxidative stress plays a role in mediating this effect (Study#2). Thus, antioxidants/anti-inflammatory agents may have therapeutic potential in type 2 diabetes and in slow-onset type 1 diabetes.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ACC | Acetyl coenzyme A Carboxylase |
| ADP | Adenosine 5'-diphosphate |
| AMP | Adenosine 5'-monophosphate |
| APC | Antigen Presenting cell |
| ATP | Adenosine Triphosphate |
| CL | Citrate Lyase |
| CoA | Coenzyme A |
| COX | Cyclooxygenase |
| CPT | Carnitine palmitoyltransferase |
| dpBB | Diabetes Prone BioBreeding |
| DI | Disposition Index |
| DNA | Deoxyribonucleic acid |
| drBB | Diabetes Resistant BioBreeding |
| ERK | Extracellular signal-regulated kinase |
| FFA | Free Fatty Acids |
| FLICE | Fas-Associated Death Domain-Like Interleukin-1beta-Converting Enzyme |
| GAD | Glutamic Acid Decarboxylase |
| GINF | Glucose Infusion (rate) |
| GK | Glucokinase |
| GLUT | Glucose Transporter |
| GSIS | Glucose Stimulated Insulin Secretion |
| GTP | Guanosine 5'-triphosphate |
| HLA | Human Leukocyte Antigen |
| IAA | Insulin Autoantibody |
| IA2 | Protein Tyrosine Phosphatase |
| IAN | Immune Associated Nucleotide |
| ICA | Islet Cell Autoantibody |
| IDDM | Insulin Dependent Diabetes Mellitus |
| IFN | Interferon |
| IGF | Insulin-like Growth Factor |
| IGT | Impaired Glucose Tolerance |
| IH | Intralipid + heparin |
| IKK | Inhibitor kappa B Kinase |
| IL | Interleukin |
| IL-1RacP | Interleukin-1 Receptor Accessory Protein |
| IRF | Interferon Regulatory Factor |
| IRS | Insulin Receptor Substrate |
| INOS | Inducible Nitric Oxide Synthase |
| IRAK | Interleukin-1 Receptor Associated Kinase |
| JAK | Janus Kinase |
| JNK | c-Jun NH2-terminal kinase |
| LADA | Latent Autoimmune Diabetes of the Adult |
| LCCoA | Long Chain Acyl coenzyme A |
| MAP | Mitogen Activated Protein |
| MAPK | Mitogen Activated Protein Kinase |
| MHC | Major Histocompatibility Complex |
| NAC | N-Acetylcysteine |
| NFkB | Nuclear Factor-kappaB |
| NIK | Nuclear Factor-kappaB-Inducing Kinase |

| | |
|---|---|
| NK | Natural Killer |
| NOD | Non-Obese Diabetic |
| OLE | Oleate |
| PDH | Pyruvate Dehydrogenase |
| PKC | Protein Kinase C |
| PKB | Protein Kinase B |
| PPAR | Peroxisome Proliferator-Activated Receptor |
| ROS | Reactive Oxygen Species |
| RNA | Ribonucleic Acid |
| SAL | Saline |
| SI | Sensitivity Index |
| SOCS-3 | Suppressor of Cytokine Signalling-3 |
| STAT | Signal Transducers and Activators of Transcription |
| TAU | Taurine |
| TCA | Tricarboxylic Acid |
| TIR | Toll/Interleukin-1 Receptor |
| TNF | Tumor Necrosis Factor |
| TNFR | Tumor Necrosis Factor Receptor |
| TRADD | Tumor Necrosis Factor Receptor Associated Death Domain |
| TRAF | Tumor Necrosis Factor Receptor-Associated Factors |
| UCP | Uncoupling Protein |
| VNTR | Variable Number of Tandem Repeats |

# 1) INTRODUCTION

## 1.1) DIABETES MELLITUS

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). The increase in the prevalence of diabetes has been referred to as an epidemic by many. A survey of the U.S. population found that diabetes affects 7.8% of adults 20 years of age and older, with the greatest prevalence in individuals older than the age of 60 (2). The prevalence of diabetes appears to be similar in other Western industrialized nations, including Canada (3;4). 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) (5). Alterations in any of these factors 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 (6). 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 shown to delay the onset of these complications (7;8).

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 a 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.

### 1.1.1) Type 1 Diabetes Mellitus

The classical form of type 1 diabetes mellitus, which accounts for approximately 10% of all cases of diabetes worldwide, is characterized by the autoimmune destruction of the pancreatic β-cells (9), which leads to severe insulin deficiency. The lack of insulin results in elevated plasma glucose levels, both because of hepatic overproduction of glucose, and of decreased glucose uptake by muscles and adipocytes. Furthermore, in the absence of circulating plasma insulin, there is an increase in fat breakdown and fatty acid oxidation, resulting in the excessive production of ketones. If left untreated, these metabolic disturbances lead progressively to central nervous system depression, coma, and death. Therefore, this form of the disease requires life long treatment with exogenous insulin for survival. The rate of ß cell destruction varies, but tends to be more aggressive in infants and young children (10). Type 1 diabetes usually presents during childhood or adolescence, although it may develop much later in life.

#### 1.1.1.1) Genetic and Environmental Factors

The susceptibility to type 1 diabetes is determined by both genetic and environmental factors. It is widely accepted that this form of diabetes is strongly genetically linked with the major histocompatibility complex (MHC) (11). These genetic loci, known as human leukocyte antigen (HLA) complex in humans (H-2 complex in mice and RT1 in rats), codes for proteins that control the immune response. There exist 3 known classes of MHC. The class II genes (HLA-DQ, HLA-DR, HLA-DP in humans, H-2 IA, H-2 IE in mice, and RT1.B, RT1.D in rats) are of particular interest because they are strongly associated both with protection from and predisposition to type 1 diabetes. These genes code for dimeric polypeptides that are expressed on the surface of antigen presenting cells (APCs) such as B lymphocytes, macrophages, and dendritic cells. The APCs present a foreign antigen in association with the MHC class II molecule to CD4+ T cells (T-helper cells). The majority of patients (90%) with type 1 diabetes express either the DR3 or DR4 HLA allele, suggesting that these alleles confer susceptibility to this form of diabetes. Certain DQ alleles have also been found to influence the development of this disease. One explanation for the association between the HLA alleles and diabetes susceptibility is that the different allelic variants show a great deal of variability in their three dimensional structure, which may alter the way which the HLA molecule interacts with both the diabetogenic antigen and the autoreactive T cell receptor (11). Furthermore, type 1 diabetes has been associated to polymorphisms within the insulin gene region. The primary association has been thought to be within the variable number of tandem repeats (VNTR) region, which is upstream of the insulin gene translation start site (12). The

mechanism by which the insulin VNTR polymorphisms influence the risk of type 1 diabetes remain unclear. However, one possibility is that allelic variants result in varying degrees of transcription of the insulin gene in the human thymus (13). This results in differences in the deletion of autoreactive T cells specific for insulin, which therefore affects immune tolerance to this key autoantigen in the pathogenesis of type 1 diabetes.

Despite the existence of a genetic link to type 1 diabetes mellitus, it has been suggested that a genetic predisposition to the disease is insufficient to trigger the development of the disease. This theory is supported by studies that demonstrate a concordance rate of 55 % for type 1 diabetes in monozygotic twins (14). This lack of concordance in genetically identical individuals indicates that this disease is not entirely due to genetic causes. Multiple environmental factors may play a role in promoting the development of type 1 diabetes. For example, infection with the rubella virus (15) and the coxsackie B4 (16) virus have been associated with an increased risk of developing the disease. Exposure to certain toxins, such as nitrosamines (17), have also been suggested to contribute to the risk of developing type 1 diabetes mellitus. Finally, dietary factors, such as the ingestion of cow's milk protein has been suggested as a possible trigger for the development of this disease (18).

### 1.1.1.2) Pathophysiology of Type 1 Diabetes Mellitus

The clinical manifestations of the disease (hyperglycemia and ketoacidosis) occur late in the course the disease process, when at least 80 % of β cells have been destroyed (19). Interestingly, only the insulin-secreting β cells appear to be targeted during the progression of this disease (20). By contrast, glucagon (α cells), somatostatin (δ cells), and pancreatic polypeptide (PP cells) secreting cells of the pancreatic islet are preserved. The loss of β cell mass is preceded by an infiltration of lymphocytes into the Islets of Langerhans, commonly referred to as insulitis. This inflammatory infiltrate consists of CD8+ and CD4+ T-lymphocytes, B-lymphocytes, natural killer (NK) cells, and macrophages. Insulitis is a hallmark of the development of autoimmune (type 1) diabetes not only in humans (21;22) but also in animal models of the disease (23-30). However, it should be noted that there do exist some forms of benign ("non-destructive") insulitis that do not lead to the complete destruction of the β cells and to overt insulin-dependent diabetes (31).

Type 1 diabetes is often considered a disease that has an acute clinical onset characterized by the sudden appearance of metabolic abnormalities in otherwise healthy individuals. However, there exists evidence to indicate that a prolonged pre-diabetic period underlies the development of the disease. During this period, the immune system is actively targeting the pancreatic β cells. This is supported by findings that islet-cell autoantibodies (ICA) (32), autoantibodies to insulin (IAA) (33), autoantibodies to glutamic acid decarboxylase (GAD) (34), and autoantibodes to the tyrosine phosphatase IA2 (35) are present in 85-90% of individuals at the onset of overt diabetes (36). Some

studies report that of these autoantibodies, both ICA and IAA may precede the development of overt diabetes by more than a decade, suggesting that the autoimmune process responsible for the destruction of the β cells is occurring years before the appearance of any clinical or metabolic findings (37;38). The rate of β cell loss varies between individuals. However, there exists a small but significant proportion of diabetic patients in which this process occurs at a remarkably slow pace. These patients often present to their physician during adulthood with symptoms resembling those of type 2 diabetes mellitus. However, β cell function is noted to be lost at a relatively rapid pace in these patients (i.e. < 5 years), when compared to patients suffering from the classical form of type 2 diabetes. This form of "adult-onset" autoimmune diabetes is referred to as latent autoimmune diabetes in adults (LADA) or type 1.5 diabetes mellitus.

Throughout the development of type 1 diabetes, there is a progressive loss in insulin secretion, eventually resulting in the complete dependence on exogenous insulin administration for survival. Studies have found detectable impairments in β cell function prior to the onset of overt type 1 diabetes. Long-term follow-up studies of initially discordant monozygotic twins and triplets for type 1 diabetes mellitus, and of antibody-positive first-degree relative of patients with the disease, have documented a slowly but progressive loss of the early (first-phase) insulin response to intravenous glucose several years before the onset of overt type 1 diabetes (39;40). As the disease progresses, circulating plasma insulin levels fall, fasting plasma glucose levels begin to rise, and glucose intolerance ensues. The progressive loss of circulating insulin levels is accompanied by a corresponding increase in plasma glucose levels, resulting in a

decrease in insulin sensitivity (insulin resistance due to glucotoxicity). Insulin resistance may be an important component for the development of type 1 diabetes (41).

It is clear that the β cell defect is a pathogenic feature of type 1 diabetes mellitus. However, there exists evidence to indicate that insulin resistance, which is commonly associated to type 2 diabetes mellitus, may also play a role in the development and/or progression of type 1 diabetes. Several studies have demonstrated an association between insulin resistance and impaired glucose tolerance in the presence of preexisting β cell damage. In one study conducted in a group of normoglycemic ICA positive first-degree relatives, 2 weeks of nicotinamide therapy induced insulin resistance and impaired glucose tolerance (42). In otherwise normal individuals, the induction of insulin resistance would stimulate a compensatory increase in insulin secretion to maintain normal glucose tolerance.

### 1.1.1.3) Mediators of the autoimmune destruction of the β cell

Several different mechanisms may be involved in the immune-mediated destruction of the pancreatic β cells. One potential mechanism is the release of granzyme B and perforins from cytotoxic T lymphocytes, which are compounds known to induce apoptosis in tumor and pathogen containing cells (43). Perforin permeabilizes the plasma membrane and/or the endosomal membrane of the target cell. Alone, this alters the target cell's hydrostatic balance, resulting in osmotic cell death. The pores also allow granzyme B to enter the cell. Once in the cell, granzyme B induces cell death through activation of the caspase cascade, which triggers β cell apoptosis. The importance of the granzyme/perforin pathway in mediating the autoimmune destruction of pancreatic β cells has been the subject of numerous investigations. In one study, the expression of perforin was detected in the pancreatic islets of NOD mice (animal model of type 1 diabetes mellitus) at 15, but not at 5 weeks of age (44). In another study in the same animal model, the incidence of spontaneous diabetes over a one year period was reduced from 77% in perforin +/+ mice to 16% in perforin deficient animals (45). Taken together, these findings suggest that that the granzyme B/perforin pathway may play an important role in the destruction of β cells in this particular animal model.

Another mechanism that may also be involved in the autoimmune-mediated destruction of the pancreatic β cell is the Fas/Fas ligand (FasL) pathway. FasL is normally expressed on the surface of activated CD4+ and CD8+ lymphocytes. Apoptosis is triggered when a Fas-expressing cell meets a T cell that has FasL on its surface. Normally, pancreatic β cells do not express the Fas receptor on their cell surface, and

therefore, are not usually prone to FasL mediated apoptosis. However, insulitis has been shown to promote the expression of the Fas receptor on the β cell in both humans with type 1 diabetes and in animal models of the disease. In one study, it was suggested that CD8+ cytotoxic T lymphocytes kill β cells in a Fas-dependent manner in NOD mice (46). In the same animal model, the incidence of diabetes was significantly reduced in Fas -/- animals (47). However, Thomas et al. found that only a limited number of β cells were Fas-positive prior to the development of diabetes in the NOD mouse, raising doubts about the significance of Fas-mediated cell death in this particular animal model (48). In humans, a study that examined pancreatic biopsy specimens from patients recently diagnosed with type 1 diabetes revealed that Fas was in fact expressed on islet cells in specimens that showed signs of insulitis (49). This finding suggest that the autoimmune destruction of β cells may occur through a Fas-dependent mechanism in humans.

The role of cytokines in mediating the autoimmune destruction of β cells in type 1 diabetes has been the topic of numerous investigations. The secretion of cytokines by lymphocytes present in the insulitis lesion may play an important role in mediating the destruction of pancreatic β cells. Studies suggest that the presence of both pro-inflammatory cytokines (IL-1α/β, TNF-α, and IFN-α), as well as type 1 cytokines (IL-2, IFN-γ, TNF-β, and IL-12) correlate with β cell destruction in both the NOD mouse, and the diabetes prone BB rat, however no such correlation has been elucidated in humans (50-54). There exists evidence to suggest that cytokines play a role in causing β cell destruction in vitro both in animal and in human islets. Early studies that investigated the effect of cytokines on cells of the pancreatic islet examined the effect of crude cytokine preparations, rather than any one isolated cytokine. Exposure to these preparations

resulted in both destruction and dysfunction of the β cell (55). Further studies have demonstrated that IL-1 (56) alone, or IL-1, TNF-α, and IFN-γ in combination (57), destroy β cells in both rodent and human pancreatic islets (58). The following paragraphs will examine the mechanisms involved in mediating these effects.

Exposure of pancreatic islets to the cytokine IL-1 results in β cell destruction and dysfunction. Cell surface IL-1 receptors have been identified on the surface of pancreatic islet cells, including β cells (6;59-62). IL-1 has been found to activate several signaling pathways (63;64). Binding of IL-1 to its receptor promotes the recruitment of the IL-1 receptor accessory protein (IL-1RAcP) (65;66). This triggers the recruitment of the adapter protein MyD88, which associates with the IL-1 receptor through a TIR domain (Toll/IL-1 Receptor) (67;68). Once recruited, the MyD88 interacts with the serine/threonine kinase known as the IL-1 receptor associated kinase (IRAK) (69). IRAK then stimulates the activation of a kinase cascade that ultimately leads to the activation of NFκB (70) and mitogen activated protein kinase (MAPK) subfamilies (5;71). NFκB is constitutively bound to its inhibitor I kappa B in the cytosol (67;72). This interaction prevents the transcription factor from translocating to the nucleus. The activation of the NFκB transcription factor occurs as a consequence of the phosphorylation, and consequent degradation of its inhibitor I kappa B (73). In addition to activating NFκB, IL-1 also activates several MAPK pathways such as ERK, JNK, and p38. However, the most important MAPK pathway involved in IL-1 mediated cell destruction appears to be JNK (74). The addition of cell permeable inhibitors of JNK signaling were shown to prevent IL-1 induced AP-1 induction (downstream of JNK) and protect cell from apoptosis in the insulin secreting BTC3 cell line (75). GTP-binding proteins have been

found to play a role in IL-1 signaling in β cells. Exposure of freshly isolated rat islets to agents that are known to interfere with GTP binding protein signaling mechanisms, such as pertussis toxin and cholera toxin, protect the β cells from the cytotoxic effects induced by IL-1 (76). Low molecular weight GTP binding proteins such as Cdc42, Rac-1, and Ras, have been found to be involved in IL-1 signaling and are believed to be important for IL-1 activation of the JNK, p38, and ERK MAPK (77-81). The mechanism by which IL-1 activates GTP-binding proteins is not known. Another mechanism by which IL-1 binding to its receptor may activate the MAPK pathway involves the sphingomyelin/ceramide pathway. Binding of ligand to the IL-1 receptor has been shown to activate the sphingomyelinase enzyme, releasing ceramide from membrane sphingomyelin in the RINm5f insulin producing cell line (82). The mechanism by which the IL-1 receptor stimulates the activation of the enzyme has yet to be determined. Studies suggest that ceramides mimic the cytostatic and cytotoxic effects of IL-1 on β cells (83). Exposure to cell-permeable ceramides induced the phosphorylation and increased activity of the protein kinase JNK1 and the upregulation of the transcription factor ATF2 and c-jun in the RINm5f cell line (84). However, the importance of this pathway has been brought into question by Major et al., who demonstrated that IL-1 failed to stimulate sphingomyelin metabolism in the BTC3 cell line (85).

TNF-α is a pro-inflammatory cytokine that acts through the TNF receptor 1 (TNFR1) on the surface of pancreatic β cells (86). TNF-α induces β cell death through a variety of different mechanisms. The TNF receptor, like the Fas receptor, contains an intracellular "death domain" (TRADD), which upon activation interacts with various adapter proteins (87). One of these adapter proteins (FLICE) is associated with a pathway

that activates the caspase signaling cascade, while another (TRAF2) is involved in a pathway that activates NFκB (88). Malinin et al. suggest that a MAP3K related kinase known as NIK is recruited to the TRAF2 adapter protein where it is activated (89). NIK is a kinase that activates a kinase cascade, which eventually leads to the phosphorylation of the I kappa B protein, thereby activating the NFκB transcription factor.

IFN-γ is also a pro-inflammatory cytokine that has been found to mediate the destruction of pancreatic β cells. This cytokine upregulates MHC class I, intracellular adhesion molecule-1, and iNOS. Binding of IFN-γ to the IFN-γ cell surface receptors activates the JAK/STAT pathway (90-92). Activated JAK kinase phosphorylates tyrosine residues on the intracellular domains of the receptor, which acts as a docking site for the STAT1 transcription factor. Recruited STAT1 are activated by phosphorylation and then dimerize and translocate to the nucleus where they bind to transcription elements within the promoters of IFN-γ responsive genes. In addition, IFN-γ binding to its receptor induces the expression and de novo synthesis of interferon regulatory factor-1 (IRF-1), which is also thought to be important in gene regulation (93). Both the STAT1 transcription factor and the IRF-1 binding protein may be involved in the upregulation of the iNOS gene (21;93;94).

The transcription factor NFκB appears to play a central role in regulating immune and inflammatory responses. A variety of different genes are regulated by NFκB including those coding for cytokines, chemokines, adhesion molecules, colony stimulating factors, immunoreceptors, and inflammatory enzymes (95). Several of the genes regulated by NFκB are involved in mediating apoptosis. As previously described, NF kappa B is usually stored in the cytosol in its inactive form bound to the inhibitory

unit I kappa B. Phosphorylation of the inhibitor promotes its subsequent degradation. NFκB translocates to the nucleus once it has been released by I kappa B. Several studies have demonstrated that NFκB has primarily pro-apoptotic functions in β cells. There exists evidence to suggest that this transcription factor may be involved in mediating the development of type 1 diabetes mellitus. This is suggested by the fact that studies have shown that the transgenic overexpression of the I kappa B inhibitor protects the β cells from cytokine mediated destruction in isolated pancreatic islets from both animals (96) and humans (97).

Cytokines also induce oxidative stress in the β cell. The insulin secreting β cells are especially vulnerable to injury by free radicals due to their low antioxidant capacity (98). Cytokines have been found to induce the formation of nitric oxide in islets. Exposure to the proinflammatory cytokines, IL-1, IFN-γ, and TNF-α, alone or in combination has been found to stimulate the production of nitric oxide in rodent (99;100) and human (92;101;102) islets, through a mechanism that involves an increase in the expression of inducible nitric oxide synthase (iNOS). It has been suggested that cytokines upregulate the expression of this enzyme through the activation of the transcription factor NFκB (101;103). The iNOS enzyme primarily synthesizes nitric oxide, however it also has been found to synthesize detectable amounts of the superoxide radical (104). The nitric oxide molecule may react with superoxide to form the peroxynitrite radical, which has been suggested to mediate the cytokine induced destruction of the pancreatic β cell (105).

It is interesting to note that not all of the cytokines secreted in the inflamed islets contribute to β cell dysfunction and destruction. Some cytokines have been shown to

protect the β cell by regulating the autoimmune and/or inflammatory processes that would otherwise result in the loss of these insulin-producing cells. This is supported by the fact that the expression of certain type 2 cytokines (such as IL-4 and IL-10) dominates over the expression of type 1 cytokines in the islets of animal models of autoimmune diabetes with benign ("non-destructive") insulitis (51).

### 1.1.1.4) Animal Models of Type 1 Diabetes

Two animal models are commonly used to study type 1 diabetes: the non obese diabetic (NOD) mouse, and the diabetes prone BioBreeding (dpBB) rat. Both animal models develop autoimmune type 1 diabetes spontaneously during their lifetime. The diabetes prone BioBreeding rat will be discussed further as it is relevant to Study #1 described in this thesis.

#### 1.1.1.4.a) The BioBreeding Rat

The diabetes prone BioBreeding (dpBB) is a recognized animal model of autoimmune diabetes. This model was first recognized in 1974 by Drs. Reginald and Clifford Chappel at the BioBreeding Laboratories in Ottawa, Canada. The syndrome first appeared in a commercial outbred colony of Wistar-derived albino rats. However, since then, the colony remained closed, and recent genotyping indicates the animals are ~80% identical at the DNA level (106). The diabetes resistant BioBreeding (drBB) rats are a subline derived from dpBB rats that were found to be resistant to the development of autoimmune diabetes.

The onset of diabetes in the dpBB rats is spontaneous, occurring in both sexes between 60 and 120 days of age, and is preceded by a period of insulitis that is followed by the selective destruction of the β cells (107). The onset of the disease is characterized by the abrupt development of hyperglycemia, ketosis, hypoinsulinemia, and weight loss. In addition, severe T-cell lymphopenia, which is essential for the development of the

diabetic phenotype, is present since birth in the dpBB rats (108;109). The lymphopenia is characterized by a reduction in CD4+ peripheral T-cells, a severe reduction in CD8+ peripheral T-cells (110), and an almost total absence of RT6+ T-cells (111). Interestingly, RT6+ T-cell depletion of the non-lymphopenic and non-diabetic diabetes-resistant (drBB) rat induces diabetes in 50% of the animals (112), whereas transfusion of CD4+ T-cells from drBB rats to young dpBB rats reduces the incidence of diabetes in a manner dependent on the number of RT6+ T-cells transferred (113).

Two susceptibility loci have been identified in the dpBB rat. The first locus, referred to as *iddm1/lyp*, which was mapped to rat chromosome 4, is linked to the lymphopenic phenotype and is essential for the development of diabetes in this particular animal model. It has been shown that this locus codes for Ian5, a member of the immune-associated nucleotide (IAN) gene family, a new and largely uncharacterized family of GTP-binding proteins (114). Members of this gene family were found to be primarily expressed in B and T lymphocytes (115). Findings from a recent in vivo study demonstrate that the Ian5 protein has an antiapoptotic effect, protecting cells against a broad range of apoptogens (Fas, TNF-α, staurosporine, and etoposide), suggesting that this protein plays an important role in determining cell viability (116). In the dpBB rats, a frameshift mutation in the gene coding for Ian5 results in the synthesis of a truncated form of the protein. It may therefore be hypothesized that the accompanying reduction / loss of protein function may reduce T-cell viability, resulting in lymphopenia.

The second susceptibility locus, known as *iddm2* has been mapped to the *u* haplotype of the RT.1 gene in the rat (analogous to MHC class II in the human). As in human type 1 diabetes, the locus that codes for proteins responsible for antigen

presentation is also involved in conferring susceptibility to autoimmune disease in this animal model.

Despite the observed similarities between autoimmune diabetes in humans and the dpBB rat, there exist certain important differences. The most notable difference is that the disease is accompanied by severe lymphopenia in the dpBB rat, whereas this is not the case in humans. This obvious difference in the disease phenotype clearly implies that there exists a limit to the degree of analogy that can be ascribed to the two disease states.

Furthermore, when interpreting data from the dpBB rat it is important to keep in mind that there is genetic variability among the various colonies around the world. There exists evidence to suggest that colonies of dpBB rats in Ottawa, Worcester, Edinburgh etc. are not all genetically identical (117). Therefore, it is important to exercise caution when generalizing observations that may have been made in animals from only a single location.

### 1.1.2) 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 (118). Type 2 diabetes is characterized by defects both in insulin sensitivity and in insulin secretion. Unfortunately, the exact cause and pathogenesis of type 2 diabetes is not well understood.

### 1.1.2.1) Genetic and Environmental factors

The development of type 2 diabetes 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 (14). 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.

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 (119). In particular, upper body (central) obesity is closely linked to insulin resistance and type 2 diabetes mellitus (120).

### 1.1.2.2) 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 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. Several hypotheses have been introduced in an attempt to explain this association. One mechanism that was proposed to explain this phenomenon is that fatty acids, which are often elevated in obesity, 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 (121;122), which phosphorylate serine residues on the insulin receptor and IRS-1, 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 intracellular ceramides and also of free radicals (123;124). However, 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 (125).

In addition to being an energy storehouse, adipose tissue has been shown to be secretory organ. Among other things, adipocytes secrete the cytokine TNFα. The levels of this particular cytokine correlate with the adipose tissue mass. Interestingly, TNFα has been found to reduce insulin sensitivity (126), and therefore this cytokine may serve as a mediator of obesity induced insulin resistance. It has been suggested that 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 (127). One possibility is that TNF-α induces serine phosphorylation of IRS-1 through the activation of the inhibitor kappa B kinase (IKK) complex (128). Another cytokine secreted by adipocytes, interleukin-6 (IL-6), has also been associated with obesity induced insulin resistance (129). A recent in vivo study conducted in mice demonstrated 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) (130). 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 (131).

The exact mechanisms by which resistin induces insulin resistance have yet to be determined.

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 (132) and adiponectin (133) are known to enhance insulin sensitivity. Leptin has been shown to enhance insulin sensitivity by depleting the intracellular triglyceride stores, which are known to impair insulin action (134), by activating AMP-activated kinase (135). Adiponectin is thought to act by increasing both the phosphorylation of AMP-activated kinase (136) and the activity of peroxisome proliferator-activated receptor α (PPARα) (137). Activation of these pathways results in increased glucose uptake and fatty acid oxidation in muscle, and decreased hepatic glucose production.

### 1.1.2.3) 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. However, IGT already features β cell dysfunction. 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.

### 1.1.2.4) Latent Autoimmune Diabetes in Adults

Interestingly, between 5-10% of patients who are initially diagnosed with type 2 diabetes display some immunological and clinical features typically associated with type 1 diabetes mellitus (138). Individuals with this form of diabetes often present during adulthood with symptoms of type 2 diabetes accompanied by circulating autoantibodies to either ICA (139) or GAD (140). These immunological findings suggest the presence of an autoimmune process targeted to the pancreatic β cell, similar to that which occurs in type 1 diabetes. This slowly progressive form of type 1 diabetes is commonly referred to as Latent Autoimmune Diabetes in Adults (LADA) or type 1.5 diabetes. Individuals diagnosed with this disease have been found to require insulin much sooner than individuals with typical type 2 diabetes (141). Patients with LADA share insulin resistance with type 2 diabetic patients, which presumably develops secondarily to impaired glucose tolerance, but display a more severe defect in insulin secretory function than patients with type 2 diabetes (142).

## 1.2) THE EFFECT OF FREE FATTY ACIDS ON INSULIN SECRETION

### 1.2.1) 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 (GK). In the β cell, glucose-6-phosphate is further metabolized through glycolysis and the tricarboxylic acid (TCA) 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 ($K+_{ATP}$ channels), resulting in a depolarization of the cell. Voltage gated $Ca^{2+}$ channels ($Ca^{2+}{}_{v}$ 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 nataglinide, increase insulin release by closing the $K^{+}{}_{ATP}$ 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 $K^{+}{}_{ATP}$ channels. This $K^{+}{}_{ATP}$ 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) (143). Glucose is metabolized through the glycolytic pathway to pyruvate, which is then be 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 (CL) and acetyl-CoA carboxylase (ACC). 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 (144). 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 (145). 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-CoA have been shown to mainly stimulate the activity of PKC isoforms in β cells (146). Activation of PKC stimulates glucose-induced insulin secretion in these insulin producing cells (147). 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-CoA 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 (148), thereby preventing the membrane depolarization required to trigger insulin exocytosis.

In addition to glucose, FFAs are also insulin secretagogues. FFAs 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 and animals, 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 response is due to release of insulin stores, whereas the sustained release of insulin is due to the release of newly synthesized insulin. Another interesting characteristic of insulin secretion is that it occurs in an oscillatory manner. It is thought that oscillations in glucose metabolism, which lead to oscillations intracellular calcium levels, are at least in part responsible for this phenomenon. However, the physiological benefit of such a secretion pattern has yet to be determined.

## 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 (119). Plasma free fatty acids (FFA), which are often elevated in obesity, play an important role in the development of diabetes. Plasma free fatty acids (FFA) are derived from lipolysis of adipose tissue triglyceride (TG) 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 (149). 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) (150). Upper body obesity, which is associated with higher circulating FFA levels, is more closely associated to the development of diabetes (120), strengthening the hypothesis that FFA play an important role in the development of this disease. Experimental studies have also found 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.

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 (125), whereas these subjects appeared to be particularly susceptible to an FFA-induced impairment in β cell function (151).

### 1.2.3) Free Fatty Acids and the β cell

FFAs have been shown to have a time-dependent effect on β cells. Early studies conducted by Crespin et al. showed that acute exposure to elevated plasma FFA levels enhanced insulin secretion, in fasted animals (152). 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 (153). On the other hand, 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 (154;155), but not human (156) or mouse (157) 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 (154;157), perfused rat pancreas (155), and in β cell lines (158;159) 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 (160). 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 (161). 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 (162). 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 (163). 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 corrected for the fatty acid induced defect in insulin action, a prolonged FFA infusion was shown to actually impair β cell function (164).

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 (165), and Kahn et al

(166) have introduced the concept that the relationship between insulin sensitivity and β cell function is hyperbolic so that the disposition index (DI), which is the product of insulin sensitivity and β cell function, is constant. That is, when insulin sensitivity is low (insulin resistance), β cell function 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 β cell function, even an unchanged 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 several studies, prolonged exposure to elevated FFA levels was found to induce β cell apoptosis in vitro (167-170). Furthermore, there exists evidence that long term FFA exposure also affects β cell proliferation (see following paragraphs) (170;171). The mechanisms responsible for the observed changes in both β cell function and mass will be discussed in the following paragraphs.

### 1.2.4) Chronic effects of FFA on GSIS

The mechanisms that mediate the impairing effect of a prolonged FFA elevation on insulin secretion have been the focus of intense research. It has been suggested that the observed lipid induced defects in insulin secretion may be the consequence of an impairment in islet glucose metabolism. Studies have demonstrated that the FFA-induced impairment in insulin secretion is associated with an exaggerated rate of fatty acid oxidation (158;172). This observed increase in fatty acid metabolism has been accounted for by 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 (158). 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 (159). It has been suggested that 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 (173). According to this concept, increased fatty acid oxidation enhances the generation of both NADH and acetyl-CoA, which inhibit pyruvate dehydrogenase (PDH), and thus glucose oxidation. Furthermore, the Randle cycle also predicts an elevation in the in the intracellular concentration of citrate. This metabolite is known to negatively regulate the key rate limiting enzymes involved in glucose metabolism known as

phosphofruktokinase (PFK), resulting in a lower glucose utilization rate. There is some in vitro evidence suggesting that this 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 (154). 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 (174). In other studies fatty acids did not affect the β cell glucose utilization rate (175), or the glucose-6-phosphate and citrate levels (172), 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.

As alluded to in the previous paragraph, exposure long-term exposure to free fatty acids has been found to alter gene expression. Exposure of isolated rat pancreatic islets to fatty acids induced a significant decrease in mRNA and protein expression of IDX-1 (176), a transcription factor expressed in the pancreatic β cells. This resulted in an expected decrease in the expression of genes transactivated by IDX-1 such as those for GLUT2 and glucokinase (176). 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.

It has been suggested that the FFA-induced defect in GSIS may be mediated by a decrease in insulin production. Several studies have reported that long-term fatty acid exposure reduced islet insulin content (154;156;177). This may be the result of a fatty acid induced decrease in the transcription of the preproinsulin gene (62;176;178), through

a mechanism that may involve the transcription factor IDX-1 (176), perhaps through ceramide synthesis (62). Furthermore, prolonged exposure to fatty acids may impair insulin production by inhibiting proinsulin biosynthesis (154;177). 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 (179).

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 (180), the only member of the UCP family located in the pancreatic islet. The findings obtained from overexpression (181) and knockout (182) studies are consistent with the hypothesis that alterations in the protein levels of UCP2 modulate GSIS.

Oxidative stress may also play a role in the FFA-induced impairment in insulin secretion. The term oxidative stress is used to describe an environment where there is an excess concentration of free radicals, such as reactive oxygen species (ROS). ROS and other free radicals damage cellular proteins, lipids, and DNA. It has been shown that exposure to FFAs enhance ROS production in islets (183). The pancreatic β cells, which have a low antioxidant capacity, are especially vulnerable to oxidative stress (98). This

sensitivity has been found to affect both β cell function and viability. Exposure to a source of ROS (i.e. hydrogen peroxide) (184) or to lipid peroxidixation products (i.e. aldehydes such as 4-hydroxy-2-noneal) (185) results in a marked impairment in GSIS. Taken together, these findings suggest that FFA may impair GSIS by enhancing oxidative stress in the islets.

The precise mechanism by which oxidative stress impairs β cell function 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 (186), and mitochondrial DNA (187) 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 (185). Oxidative stress may also reduce the mitochondrial ATP production by inducing the expression of a mitochondrial uncoupling protein (UCP-2) in the pancreatic β cell (188). Finally, oxidative stress may also affect insulin gene expression. 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 (189).

### 1.2.5) Chronic effect of FFA on β cell mass

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 both in vitro (171) and in vivo (190). 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 (191), 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) (192).

Long-term FFA exposure has also been found to promote β cell apoptosis (167;169). 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

(193). Studies have noted that ceramide levels are elevated in fat rich islets (167;194), 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 (167) and human (169) 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 (195), and they have also been found to upregulate the expression of the iNOS gene (196). However, fatty acids have also been shown to enhance the production of ROS, independent of ceramides (197). Free radicals have been implicated as important regulators of the apoptotic pathway (198). 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 (183;199). Treatment with either antioxidants or inhibitors of inducible nitric oxide synthase (iNOS) has been found to prevent FFA-induced β cell apoptosis (167;168;199). This suggests that fatty acids depend on oxidative stress to induce β cell apoptosis. Finally, activation of PKC-δ 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 (200).

# 2) GENERAL HYPOTHESIS

The general hypothesis of this thesis is that prolonged exposure to FFA impairs glucose stimulated insulin secretion via mechanisms that involve inflammation and oxidative stress. This thesis consists of two studies. The first study investigated the effects of a prolonged elevation of plasma FFA on glucose stimulated insulin secretion in vivo in diabetes prone BioBreeding rats, an established model of autoimmune diabetes. The second study investigated the effects of prolonged exposure to FFA, alone and in combination with antioxidants, on glucose stimulated insulin secretion, in the whole animal and also in isolated islets.

# 3) SPECIFIC OBJECTIVES

**Study # 1**

To examine whether islet inflammation during autoimmune insulitis sensitizes glucose stimulated insulin secretion to the impairing effect of a prolonged (48-h) free fatty acid elevation. Previous work by our laboratory has shown that a prolonged infusion of Intralipid impaired glucose stimulated insulin secretion to a lesser extent than an infusion of oleate (160). Since the objective of this study was to determine whether the FFA-induced impairment in glucose stimulated insulin secretion is accentuated in pre-diabetic model of autoimmune diabetes, we decided to use an infusion of Intralipid to elevate plasma FFA levels.

**Study # 2**

To examine whether antioxidants can prevent the effects of prolonged exposure to FFA on glucose stimulated insulin secretion. Previous work by our laboratory has shown that a prolonged infusion of oleate impaired GSIS to a greater extent than an infusion of Intralipid and heparin (162). Since the objective of this study was to examine whether antioxidants can prevent the FFA-induced impairment in glucose stimulated insulin secretion, we decided to use an oleate infusion to elevate plasma FFA levels.

# 4) MATERIALS AND METHODS

## 4.1) PROCEDURES

**Cannulation.** Rats were anesthetized with ketamine:xylazine:acepromazine (100:0.1:0.5 mg/ml, 1ul/g body wt), and under sterile conditions, indwelling catheters were inserted into the right internal jugular vein and the left carotid artery. 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 for vascular catherization. The venous catheter was extended to the level of the aortic arch. Both catheters were tunneled subcutaneously, fed through a subcutaneous interscapular implant, and exteriorized. Catheters were filled with a mixture of 60% polyvinylpyrrolidone and heparin (1,000 U/ml) to maintain patency and were closed at the end with a metal pin. Cathethers were flushed with saline every 2-3 days. The rats were allowed a minimum 3- 4 days period of post-surgery recovery before experiments.

**Chronic Infusions.** At least 2-3 days after surgery, PE-50 infusion tubing was connected to each of the catheters. 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 run through a swivel, which was suspended on top of the cage. This procedure protected the infusion tubing and allowed the rat complete freedom of movement. Rats were allowed a 30-min adaptation period during which slow saline infusions (5ul/min) kept the lines patent. Thereafter, a basal nonfasting (random) sample was taken for FFAs, glucose, and insulin. A slow infusion of heparinized saline (4U/ml) was started at 5ul/min to maintain the arterial line patent between samples in all rats. The saline/fat (Intralipid or

oleate) infusions were given through the jugular catheter. Throughout the infusion period, rats had free access to water and to their standard pelleted food. 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 1900 the day before the two-step hyperglycemic clamp.

**Two-step hyperglycemic clamp.** Glucose stimulated insulin secretion was determined by measuring insulin and C-peptide responses to a two-step (~13 and 22 mmol/L) hyperglycemic clamp. The two-step hyperglycemic clamp was performed in conscious rats fasted overnight. 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. Two basal samples were taken at –20 and 0 min, after which an infusion of 37.5% glucose was started (time = 0 min) in order to approximately double the plasma glucose levels (first step of hyperglycemic clamp). 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. The target plasma glucose level of ~13 mmol/L was achieved and maintained by adjusting the rate of the glucose infusion according to frequent (every 5-10 min) plasma glucose determinations. At 120 min, the glucose infusion was again raised to achieve and maintain plasma glucose levels of ~22 mmol/L (the second step of the hyperglycemic clamp) until the end of the experiment (time = 240 min). Samples for insulin, C-peptide, and FFAs 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 heprinized saline (4U/ml) and reinfused into the rats.

**Pancreatic Islet Isolation.** Pancreatic islets were isolated in overnight fasted rats, after the 48-h 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 carefully removed and placed in a sterile 50 ml Falcon tube. The pancreas was then incubated in a water bath (37oC). 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 300um filter. Again the mixture was centrifuged. The pellet was resuspended and the islets were isolated by a Histopaque 1077 density gradient.

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.

## 4.2) LABORATORY METHODS

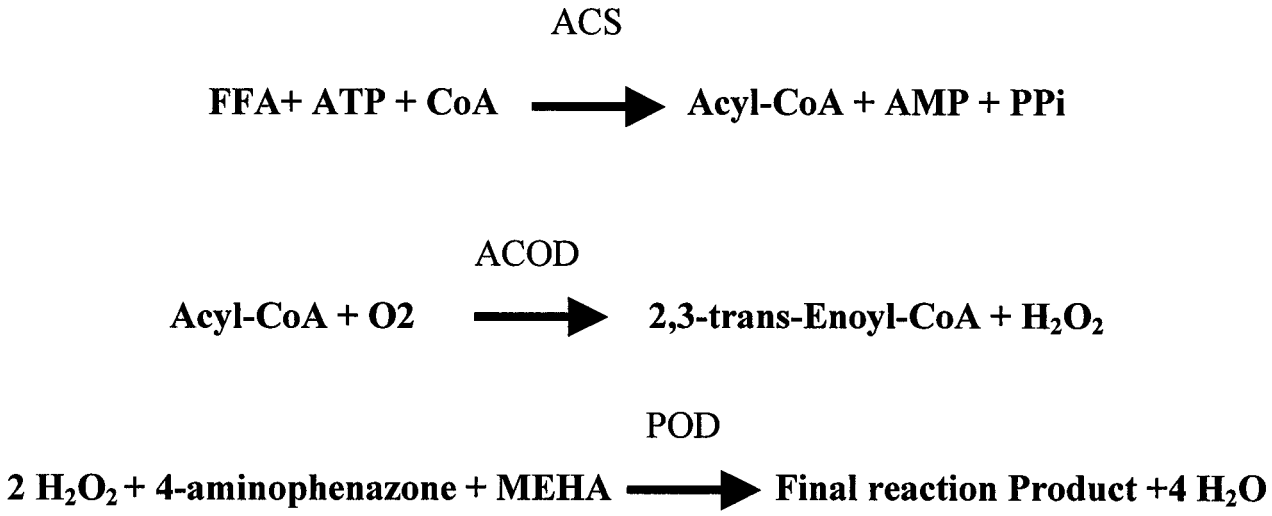
**Plasma Glucose.** Plasma glucose concentrations were measured by the glucose oxidase method using a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). A 10 ul sample of plasma containing D-glucose is pipetted into a solution containing oxygen and glucose oxidase. The glucose reacts with oxygen in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

$$\textbf{D-Glucose} + O_2 + H_2O \longrightarrow \textbf{Gluconic Acid} + H_2O_2$$

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. 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.

**Plasma FFA Assay.** Plasma FFA were analyzed using a colorimetric kit under enzymatic reaction from Wako Industries (Osaka, Japan). 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 then oxidized by adding acyl-CoA oxidase (ACOD), which generates $H_2O_2$. $H_2O_2$ in the presence of peroxidase (POD) permits the oxidative

condensation of 3-methyl-N-ethyl-N-(B-hydroxyethl)-aniline (MEHA) with 4-aminophenazone to form the final reaction product, which is a purple coloured adduct. This can be measured colorimetrically at 550 nm. The results are corrected to within 1.1%. The reactions of this assay is shown below:




**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 uU/ml 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 (50ul to all tubes) and rat insulin antibody (50ul to all tubes) were mixed with plasma sample (100ul to all tubes). The tubes were then vortexed and

incubated overnight at 4 degrees. 1000ul of precipitating reagent was added to all tubes followed by vortexing and incubating for 20 minutes at 4 degrees. The tubes then spinned at 1500g 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):

**% activity bound = B (Standard or sample) / Bo x 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 7 %.

**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 degrees Celsius. On the second day, $^{125}$I-rat C-peptide was added followed by vortexing and overnight incubation at 4 degrees. On the last day, 1000 ul of precipitating reagent was added to all tubes followed by vortexing and incubation for 20 min at 4 degrees. 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 interassay variation determined on reference plasma less than 10.5% .

## 4.3) CALCULATIONS

**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.

**Insulin sensitivity index.** The insulin sensitivity index (SI) 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:

$$SI_{(period)} = \frac{GINF_{(period)}}{(Insulin_{(Period)} - Insulin_{(Basal)}) \times Glucose_{(period)}}$$

Where GINF (Period) is the rate of glucose infusion, Insulin (Period) is the insulin concentration, and Glucose (Period) is the plasma glucose level at individual time points during the last 40 minutes of each step of the hyperglycemic clamp. Insulin (Basal) is the mean insulin level during the baseline period. 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. SI is reported in units of deciliter per kilogram per minute per microunit per milliliter. No correction was made for the urine glucose loss, which was assumed to be equal between studies for the same individual because plasma glucose levels were similar. Unfortunately, there are

limitiations to using this method to assess insulin sensitivity at elevated insulin and glucose levels. It has been reported that the association between circulating insulin levels and insulin action is not linear at insulin concentrations higher than 180uU/ml (68) and at high glucose levels.

**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 experimental period as an index of correction of the insulin secretory rate (ISR) for the ambient degree of insulin sensitivity as the product of SI and ISR. The C-peptide response was taken as a measure of absolute insulin secretion, as the ISR cannot be calculated in rats because C-peptide kinetic parameters have yet to be determined (this is due to the fact that rat C-peptide (species specific) is not available for injection.)

$$\mathbf{SI_{(period)} \times C\ Peptide_{(Period)} = DI_{(period)}}$$

## 4.4) STATISTICAL ANALYSIS

All data are presented as means ± SE. First, one-way Analysis Of Variance (ANOVA) for repeated measurements was used to examine the difference between treatments within each experimental group. Then, ANOVA for repeated measurements was used to compare differences between experimental groups and treatments, and to examine interactions between the two. The statistical calculations were performed using SAS software (SAS Institute, Cary, NC). Significance was accepted at $P<0.05$. Please refer to each study for further details.

# 5) STUDY # 1: The effect of a prolonged infusion of Free Fatty Acids on Glucose Stimulated Insulin Secretion is accentuated in the diabetes prone BioBreeding rat.

## 5.1) ABSTRACT (Study #1)

The impairing effect of prolonged elevation of plasma free fatty acids (FFA) on both β cell function and turnover (β cell lipotoxicity) has been suggested to play an important role in the development of type 2 diabetes. However, no study to date has examined the effect of prolonged FFA elevation in the context of autoimmune diabetes. Reports of enhanced cytokine toxicity in fat-laden islets are consistent with the hypothesis that lipid and cytokine toxicity may be synergistic. Thus, β cell lipotoxicity could be enhanced in models of autoimmune diabetes. To determine this, we examined the effects of prolonged FFA elevation on glucose stimulated insulin secretion (GSIS) in the prediabetic diabetes-prone BioBreeding rat (dpBB), an established model of autoimmune diabetes, and its diabetes-resistant control (drBB). DpBB and drBB rats were treated with a 48 h intravenous infusion of either saline (SAL: n=9 dpBB; n=8 drBB), or Intralipid + Heparin (IH: n=10 dp-BB; n=7 dr-BB). At the end of the 48h infusion, after an overnight fast, GSIS was evaluated with a two-step hyperglycemic clamp (13 and 22 mM glucose). IH elevated FFA levels to the same extent in both the dpBB and the drBB rats (~2 fold). At 13mM, IH increased absolute insulin secretion, as determined by plasma C-peptide levels in the drBB rats ($p<0.001$), but not in the dpBB rats. At 22 mM, IH increased plasma C-peptide levels from $4.24 \pm 0.74$ to $8.31 \pm 0.91$ nM in the drBB rats ($p<0.001$), but decreased C-peptide levels from $3.40 \pm 0.67$ to $2.34 \pm 0.54$ nM in the dpBB rats ($p<0.001$). In this study, the disposition index (DI) was used as a measure of relative insulin secretion. Treatment with IH induced a decrease in DI in the dpBB rats ($p < 0.01$) but not in the drBB rats throughout the hyperglycemic clamp.

Therefore, prolonged FFA elevation impaired GSIS in prediabetic dpBB rats, but actually increased GSIS in the drBB rats, suggesting that lipotoxicity in the BB rat may require the presence of a preexisting β cell impairment and/or the synergistic effect of cytokine toxicity in islets.

## 5.2) INTRODUCTION (Study # 1)

Type 1 diabetes mellitus is characterized by the specific autoimmune-mediated destruction of the pancreatic β-cells, which results in hypoinsulinemia. The autoimmune nature of the disease is corroborated by histological studies that confirm that at the time of diagnosis, islets from patients with type 1 diabetes are infiltrated with a variety of immune cells in a process known as insulitis (201).

The diabetes-prone BioBreeding rat is a recognized animal model of autoimmune type 1 diabetes. The onset of diabetes in these rats is spontaneous, occurring in both sexes between 60 and 120 days of age (107). Similar to human type 1 diabetes, the development of diabetes in the diabetes-prone BioBreeding rat is preceded by the appearance of auto-antibodies (202) and a brief period of insulitis (25;203) followed by the selective destruction of the pancreatic β cells. This results in hypoinsulinemia, which leads to hyperglycemia, ketosis, and weight loss. In contrast to the human form of the disease, diabetes in this animal model is associated with severe T-cell lymphopenia (204). The diabetes resistant BioBreeding rats are a subline derived from diabetes prone rats that were found to be resistant to the development of autoimmune diabetes.

The presence of proinflammatory (IL-1, TNFα, and IFNα), as well as type 1 (IL-12, TNFβ, IFNγ, and IL-2) cytokines correlate with β cell destruction in the diabetes-prone BioBreeding rat. These molecules have been hypothesized to play an important role also in the pathogenesis of type 1 diabetes. The results of in vitro studies conducted in isolated islets suggest that cytokines such as IL-1β alone, or IL-1β, TNFα, and IFNα, in combination, impair insulin secretion and trigger β cell death through a mechanism

that may depend on the induction of oxidative stress (92;205). In particular, the enzyme that synthesizes the free radical nitric oxide (NO), known as inducible nitric oxide synthase (iNOS), is expressed in the insulitis lesion of adult diabetes prone BioBreeding rats (206). Both inhibitors of iNOS (207) and antioxidants (208) were found to significantly reduce the incidence of autoimmune diabetes in diabetes prone BioBreeding rat.

Similar to cytokines, Free Fatty Acids (FFAs) induce alterations in both β cell function and mass. Acutely, FFA stimulate glucose stimulated insulin secretion (GSIS). In contrast, numerous studies have shown that prolonged (i.e. > 24-h) exposure to FFA leads to a reversible impairment in GSIS in vitro (154-156;209) and also in vivo (162), when insulin secretion is corrected for insulin sensitivity (164). Furthermore, long term fatty acids exposure also causes an irreversible reduction in β cell mass via apoptosis. The term "lipotoxicity" was first coined by Unger to characterize both the reversible and irreversible alterations in β cell function and mass induced by prolonged exposure to fatty acids (210). Similarly to cytokines, prolonged exposure to FFA induces oxidative stress both by increasing the generation of NO and other reactive oxygen species (ROS), such as hydrogen peroxide and the hydroxyl radical (183;199).

Lipotoxicity is most often associated with type 2 diabetes mellitus. However, there is some evidence that fat may also contribute to the pathogenesis of autoimmune type 1 diabetes. For example, a dietary intervention study conducted in the non obese diabetic (NOD) mouse found that fat restriction reduced the incidence of diabetes in this animal model of autoimmune type 1 diabetes (211). In addition, troglitazone, which has both lipopenic and antioxidant properties, reduced the incidence of diabetes in this animal

model (212). Thus, lipotoxicity may be accentuated in autoimmune type 1 diabetes. This may be particularly relevant in patients with slow onset type 1 diabetes (LADA), because fat may play a role in accelerating the development of the disease. The accentuation of lipotoxicity in autoimmune diabetes may be due to a synergy between the effects of cytokines and lipids on the β cell. Shimabukuro and his colleagues conducted an in vitro study that demonstrated that the toxicity of cytokines, namely interleukin-1 (IL-1β), which plays a role in the autoimmune destruction of the β cells in autoimmune diabetes, is enhanced in triglyceride rich islets, and reduced in triglyceride-depleted islets (213). This finding suggests that fat may sensitize the β cell to the cytotoxic effect of cytokines involved in the autoimmune destruction of the pancreatic β cells, perhaps by providing more substrate for cytokine induced ROS production (i.e. lipid peroxidation).

The aim of this study is to determine whether the converse is true. That is, whether the presence of autoimmune insulitis, such as that found in the islets of prediabetic diabetes-prone BioBreeding rats, sensitizes the β cell to the lipotoxic effect of fat. In order to achieve this, we evaluated the insulin secretory response to glucose by means of a two-step hyperglycemic clamp after prolonged (i.e. 48-h) intravenous infusion of a synthetic triglyceride emulsion (Intralipid) together with heparin (which stimulates the breakdown of Intralipid to fatty acids by the enzyme lipoprotein lipase) in both prediabetic diabetes-prone and diabetes-resistant BioBreeding rats.

## 5.3) MATERIALS AND METHODS (Study #1)

**Animal Models.** Nine week old Diabetes-Prone (dpBB) and Diabetes-Resistant (drBB) female BioBreeding rats were obtained from the Animal Resources Division of Health Canada (Ottawa). The mean incidence of diabetes in dpBB rats from this colony fed a standard cereal-based diet has remained constant over the past 5 years at 65.3 ± 14.9% (mean ± S.D.) (106). This colony is directly descended from the original diabetic rats discovered at BioBreeding laboratories near Ottawa in 1974 and transferred to Health Canada in 1977. The colony is not completely inbred but has remained a closed colony for the past 25 years, and recent genotyping for selected markers indicates the animals are ~ 80% identical at the DNA level (106;106). These animals carry the same mutation at the Iddm1/lyp locus as BB/W rats that is attributable to a frameshift deletion in a novel member of the immune-associated nucleotide-related gene family, Ian5 (114). The drBB rats are derived from an early subline of animals from the original BB rat colony that does not spontaneously develop diabetes. The rats were housed in the University of Toronto's Department of Comparitive Medicine. They were exposed to a 12 h light/dark cycle and were fed rat chow (Purina 5001, 4.5% fat; Ralston Purina, St. Louis, MO) and water ad libitum. To evaluate the onset of diabetes, random plasma glucose (RPG) levels were tested at least twice a weak using handheld Glucometer (Bayer, Toronto, Ontario). Only rats eleven week old rats with random plasma glucose (RPG) values below 11 mmol/L were used in this study. This excluded the rats that had already developed diabetes (~ 50%).

**Surgical procedures.** See General Materials and Methods section.

**Preclamp (48 hour infusion) period.** Both dpBB and drBB rats were randomized to one of the following protocols: Saline (SAL; dpBB=9 , drBB=8) or Intralipid plus heparin (IH; dpBB n= 10,drBB n= 7). At least 2-3 days after surgery, PE-50 infusion tubing was connected to each of the catheters. 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 run through a swivel, which was suspended on top of the cage. This procedure protected the infusion tubing and allowed the rat complete freedom of movement. Rats were allowed a 30-min adaptation period during which slow saline infusions (5ul/min) kept the lines patent. Thereafter, a basal nonfasting (random) sample was taken for FFAs, glucose, and insulin. A slow infusion of heparinized saline (4U/ml) was started at 5ul/min to maintain the arterial line patent between samples in all rats. The saline/intralipid infusions were given through the jugular catheter; 20% Intralipid was infused at 2.5ul/min in the dpBB rat and 3ul/min in the drBB rats to maintain the plasma FFA levels. Heparin had been added to the Intralipid to reach a final concentration of 20 U heparin/ml of infusate. Intralipid consists of a fat emulsion made of sybean oil and an emulsifier from egg phospholipids. According to the manufacturer it typically contains 13% palmitic acid, 4.5% stearic acid, 22% oleic acid, 52% linoleic acid, 8% linolenic acid, and 1% other fatty acids. The composition may vary somewhat with different batches. Throughout the infusion period, rats had free access to water and to their standard pelleted food.

Samples for FFAs, glucose, and insulin were taken at 18, 24, and 46 hours after the onset of the Saline or Intralipid infusion, i.e. –30, -24, and –2 h before the onset of the hyperglycemic clamp (time = 0). Food was removed at 1900 the day before the two-step hyperglycemic clamp.

**Two-step hyperglycemic clamp.** Glucose stimulated insulin secretion was determined by measuring insulin and C-peptide responses to a two-step (~13 and 22 mmol/L) hyperglycemic clamp. The two-step hyperglycemic clamp was performed in conscious rats fasted overnight. 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/Intralipid was continued throughout the experiment. Intralipid was given at the same rate, however, without added heparin, since the same total amount of heparin (0.1 U/min) that was infused during the preclamp period was used to flush the arterial catheter when sampling during the experiment. Two basal samples were taken at –20 and 0 min, after which an infusion of 37.5% glucose was started (time = 0 min) in order to approximately double the plasma glucose levels (first step of hyperglycemic clamp). The glucose infusion was given through the jugular catheter. Both the glucose and the Saline/Intralipid infusion lines were connected to the jugular line through a Y shaped connector. The target plasma glucose level of 13 mmol/L was achieved and maintained by adjusting the rate of the glucose infusion according to frequent (every 5-10 min) plasma glucose determinations. At 120 min, the glucose infusion was again raised to achieve and maintain plasma glucose levels of ~22 mmol/L

(the second step of the hyperglycemic clamp) until the end of the experiment (time = 240 min). Samples for insulin, C-peptide, and FFAs 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. At the end of the two step hyperglyemic clamp, the rats were anesthetized and the whole pancreas was rapidly removed. The collected pancreas samples were then fixed overnight in Bock's solution (formalin + acetic acid) and then stored in 70% ethanol. The samples were processed and embedded in paraffin wax within 5 days of tissue collection.

**Laboratory methods**. See General Materials and Methods section.

**Calculations.**

*Insulin clearance, Insulin sensitivity index and Disposition Index.* See General Materials and Methods section.

**Statistical analysis.** All data are presented as means ± SE. First, a one-way Analysis Of Variance (ANOVA) for repeated measurements was used to examine the difference between treatments (SAL vs. ILH) within each experimental group (i.e. dpBB and drBB rats). Then, a two-way ANOVA for repeated measurements was used to examine the effect of experimental groups and treatments, and to examine interactions between the two. The statistical calculations were performed using SAS software (SAS Institute, Cary, NC). Significance was accepted at $P<0.05$.

## 5.4) RESULTS (Study #1)

**Preclamp (48 hour infusion) period.** Table 1 presents the fed preinfusion plasma FFA, glucose, and insulin levels. Fed plasma FFA and glucose levels were significantly elevated ($p < 0.05$ for both FFA and glucose) in the dpBB rats (vs. drBB rats) at baseline before starting the infusions, a finding consistent with abnormalities in glucose tolerance in this group. Insulin levels were not significantly different between dpBB and drBB rats.

Figures 1, 2 and 3 present the plasma FFA, glucose and insulin levels during the pre clamp period. Intralipid treatment rapidly elevated plasma FFAs, to the same extent in both dpBB and drBB rats, to levels that were approximately twofold higher than those seen in saline treated rats of the same group (IH vs. SAL dpBB, $p< 0.001$ ; drBB, $p< 0.001$ ) (Figure 1). During the pre clamp infusion period, plasma glucose (Figure 2) and plasma insulin (Figure 3) levels were similar in both dpBB and drBB rats. Intralipid treatment did not significantly affect either glucose or insulin levels in either group of rat.

**Two-step hyperglycemic clamp.** During the basal period (-20 min to 0 min), plasma FFA levels were elevated to the same extent (~ 2 fold) with a prolonged Intralipid infusion at different rates for the dpBB (2.5 ul/min) and drBB (3 ul/min) rats. FFA levels declined throughout the two step hyperglycemic clamp as a result of the progressive hyperglycemia and hyperinsulinemia. However, FFAs remained higher in all Intralipid-treated rats throughout the experiment.

During the basal period, plasma glucose levels (fasting) where elevated in dpBB rats treated with Intralipid ($p<0.01$). During the first step of the two-step hyperglycemic

clamp, plasma glucose levels gradually rose to the target value of 13 mmol/L, which was maintained until 120 min. During the second step of the hyperglycemic clamp, plasma glucose levels gradually rose to the target level of 22 mmol/L, which was maintained until 240 min. During both steps of the hyperglycemic clamp, the glucose levels were superimposable in all groups (Figure 5).

During both steps of the hyperglycemic clamp, the glucose infusion (GINF) rate was lower in the dpBB rats than in the drBB rats ($p<0.05$) (Figure 6). Furthermore, Intralipid treatment induced a significantly lower GINF rate in dpBB ($p<0.01$) rats throughout the clamp (Figure 6A). In contrast, Intralipid treatment increased the GINF rate in the drBB rats ($p<0.01$) during both steps of the clamp (Figure 6B).

Plasma insulin levels during the basal period were similar in both dpBB and drBB rats. When compared to saline-treated dpBB rats, Intralipid-treated dpBB rats had higher basal plasma insulin levels ($p<0.05$) (Figure 7A)(not evident due to the scale of the graphs). This effect was not observed in the drBB rats. As expected, plasma insulin concentrations rose in response to increasing glucose levels throughout the two steps of the hyperglycemic clamp. Circulating insulin levels were lower in the dpBB rat than in the drBB rat ($p<0.01$) throughout both the first and second step of the hyperglycemic clamp. In the dpBB rat, plasma insulin levels were similar in both Intralipid-treated and saline-treated rats, during both the first and second step of the hyperglycemic clamp (Figure 7A). In the drBB rat, plasma insulin levels were higher in Intralipid-treated rats than in saline-treated rats, during both the first ($p<0.05$) and second step ($p<0.01$) of the hyperglycemic clamp (Figure 7B).

Fasting C-peptide levels measured during the basal period were similar in both dpBB and drBB rats. Intralipid-treated dpBB rats had higher basal plasma C-peptide levels ($p<0.05$) than saline-treated controls (Figure 8A) (not evident due to the scale of the graphs). This effect was not observed in the drBB rats. Throughout both the first and second step of the hyperglycemic clamp, plasma C-peptide levels were lower in the dpBB rat than in the drBB rat ($p<0.01$) (Figure 8). In the dpBB rat, plasma C-peptide levels were lower in Intralipid-treated than in saline-treated rats during both the first and second step of the hyperglycemic clamp (Figure 8A), however this was only significant during the second step ($p<0.01$). In contrast, in the drBB rat, plasma C-peptide levels were significantly higher in the Intralipid-treated rats than in saline-treated animals during both steps of the hyperglycemic clamp ($p<0.01$ for both seps of the clamp) (Figure 8B).

The C-Peptide/Insulin ratio was taken as an index of insulin clearance. The basal C-Peptide/Insulin ratio was higher in the dpBB rats than in the drBB rats ($p<0.05$), but appeared unaffected by Intralipid treatment. Similarly, this index was higher in the dpBB rats during both steps of the clamp ($p<0.01$ for both steps). However, during the hyperglycemic clamp, treatment with Intralipid tended to decrease the C-Peptide/Insulin ratio in both dpBB (Figure 9A) and drBB (Figure 9B) rats but this was significant only during the second step of the hyperglycemic clamp ($p<0.05$ for both groups of rats).

The sensitivity index (SI), which is a measure of insulin sensitivity, was calculated as previously described in the Methods section. The SI was significantly higher in the dpBB (Figure 10A) rats than in the drBB (Figure 10B) rats ($p<0.05$). Intralipid appeared to induce a decrease in the SI in both dpBB and drBB rats during both

steps of the clamp, however this effect was only significant in the drBB rats during the first step of the clamp ($p<0.05$).

The disposition index (DI), which is an established measure of relative insulin secretion, was calculated as previously described in the Methods section. Treatment with Intralipid induced a decrease in DI in dpBB rats (Figure 11A) throughout the two-step hyperglycemic clamp ($p<0.01$ for both steps of the clamp). In contrast, Intralipid treatment did not induce any change in the DI in the drBB rats during either step of the clamp (Figure 11B).

The examination of pancreas specimens collected at the end of each experiment revealed a lymphocytic infiltration of the islets of Langerhans (i.e. insulitis) in sections of pancreas retrieved from all the dpBB rats (Figure 12A). As expected, insulitis was notably absent in sections of pancreas collected from drBB rats (Figure 12B).

**Table 1:** FFA, glucose and insulin levels at setup (time = 0). The rats were in the fed state when these results were obtained.

| | dpBB | drBB |
|---|---|---|
| **FFA (nmol/L)** | | |
| SAL | 0.53 ± 0.07 **a** | 0.42 ± 0.06 |
| IH | 0.58 ± 0.06 **a** | 0.41 ± 0.06 |
| **Glucose (mmol/L)** | | |
| SAL | 8.3 ± 1 **a** | 6.6 ± 0.3 |
| IH | 7.4 ± 0.3 **a** | 6.6 ± 0.3 |
| **Insulin (nmol/L)** | | |
| SAL | 0.34 ± 0.05 | 0.48 ± 0.06 |
| IH | 0.39 ± 0.04 | 0.37 ± 0.05 |

Data are means ± SE.

**a.** dpBB vs. drBB, $p<0.05$ for both FFA and Glucose levels

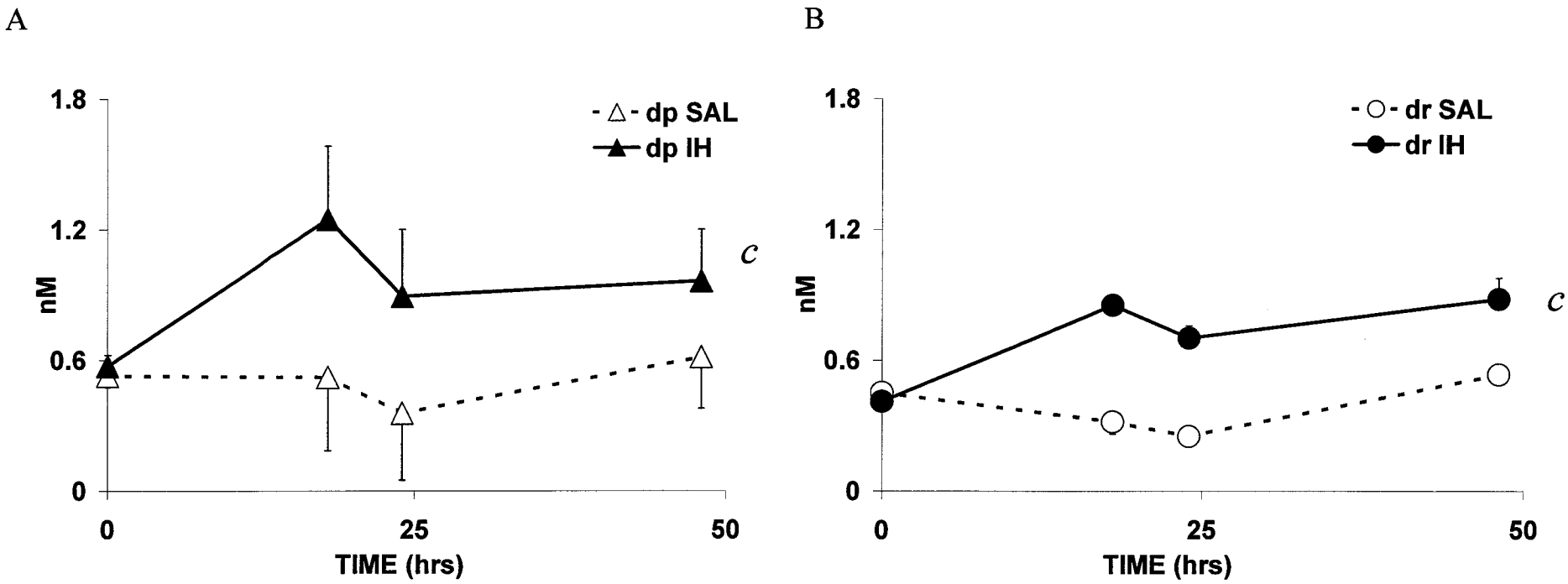



**Figure 1.** Plasma FFA levels during pre clamp period. A) Plasma FFA levels during pre clamp period in both Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Plasma FFA levels during the pre clamp period both in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. All rats were in the fed state at 0, 18 and 24h and in the fasted state at 48h. Data are in means ± SE.

***c.*** SAL vs. IH, $p<0.001$, in both drBB and dpBB rats

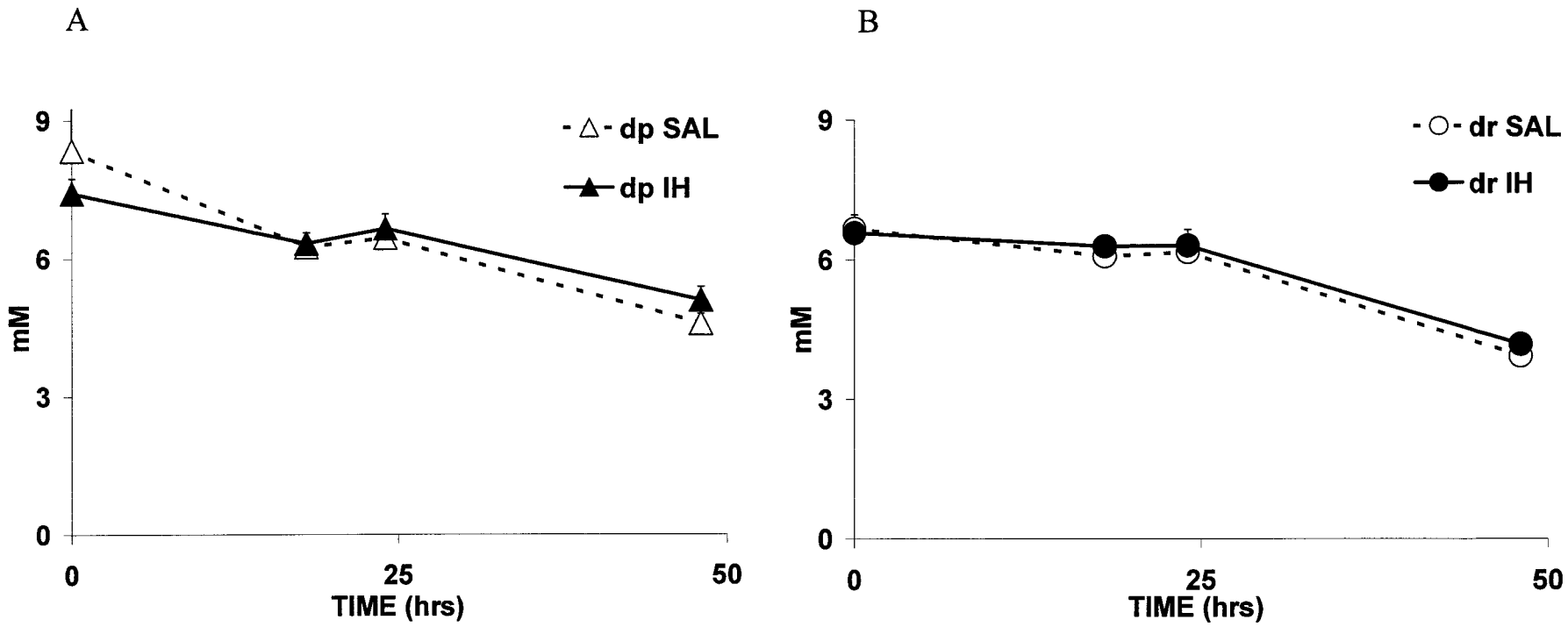



**Figure 2.** Plasma glucose levels during the pre clamp period. A) Plasma glucose levels during the pre clamp period in both Saline (*n* = *9*) and Intralipid (*n* = *10*) treated dpBB rats. B) Plasma glucose levels during the pre clamp period in both Saline (*n* = *8*) and Intralipid (*n* = *7*) treated drBB rats. All rats were in the fed state at 0, 18 and 24h and in the fasted state at 48h.Data are in means ± SE.

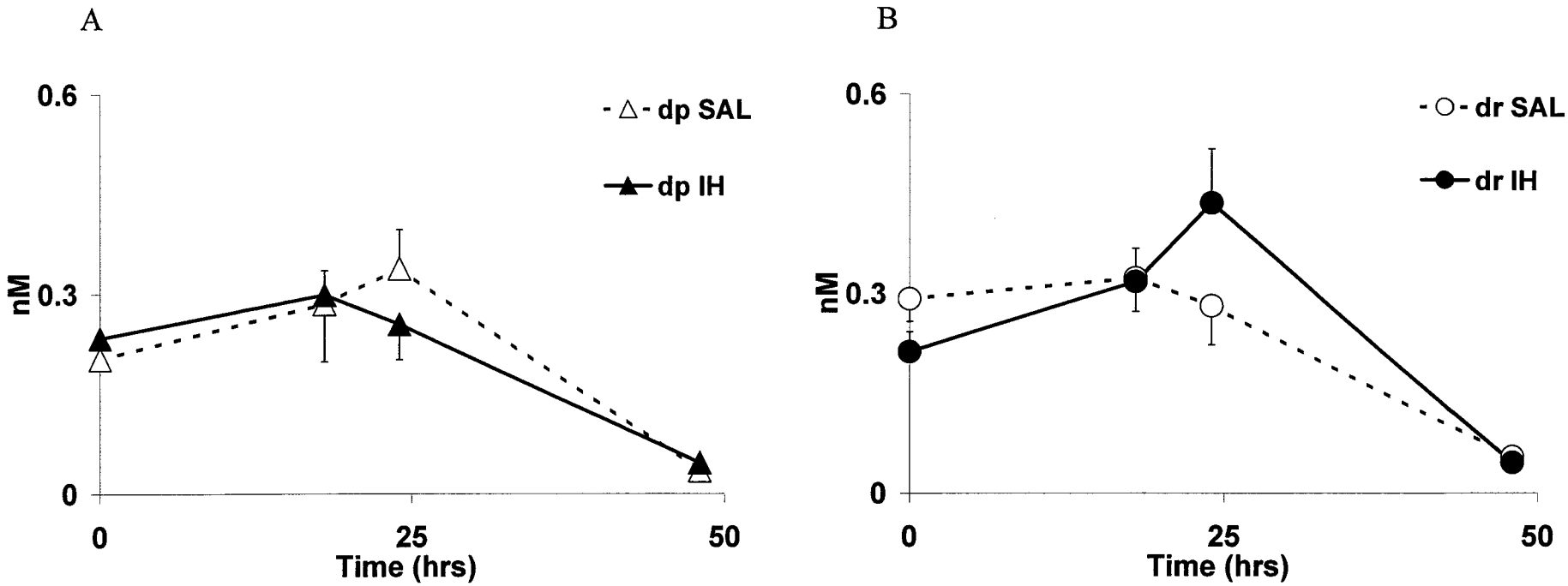



**Figure 3.** Plasma insulin levels during the pre clamp period. A) Plasma insulin levels during the pre clamp period in both Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Plasma insulin levels during the pre clamp period in both Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. All rats were in the fed state at 0, 18 and 24h and in the fasted state at 48h. Data are in means ± SE.

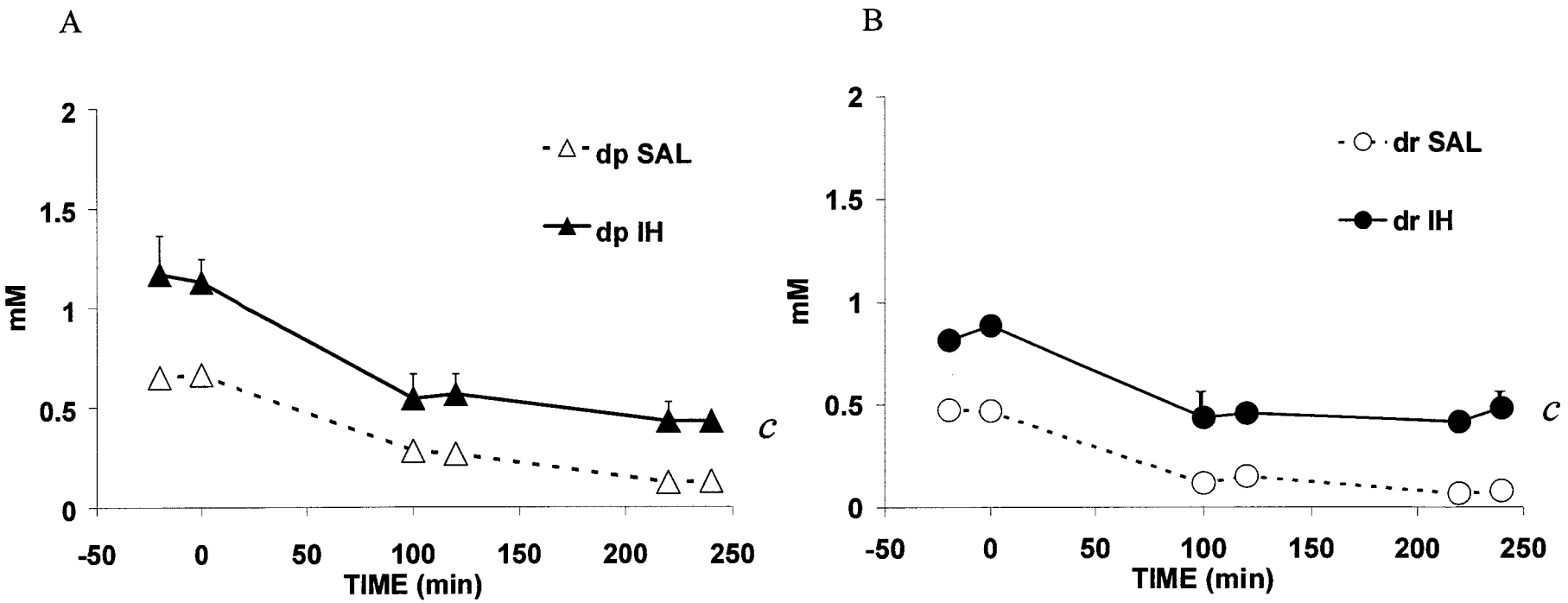



**Figure 4.** Plasma FFA levels during the two-step hyperglycemic clamp. A) Plasma FFA levels during the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Plasma FFA levels during the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

*c.* SAL vs. IH, $p<0.05$ during first and second step of the hyperglycemic clamp in both dpBB and drBB rats

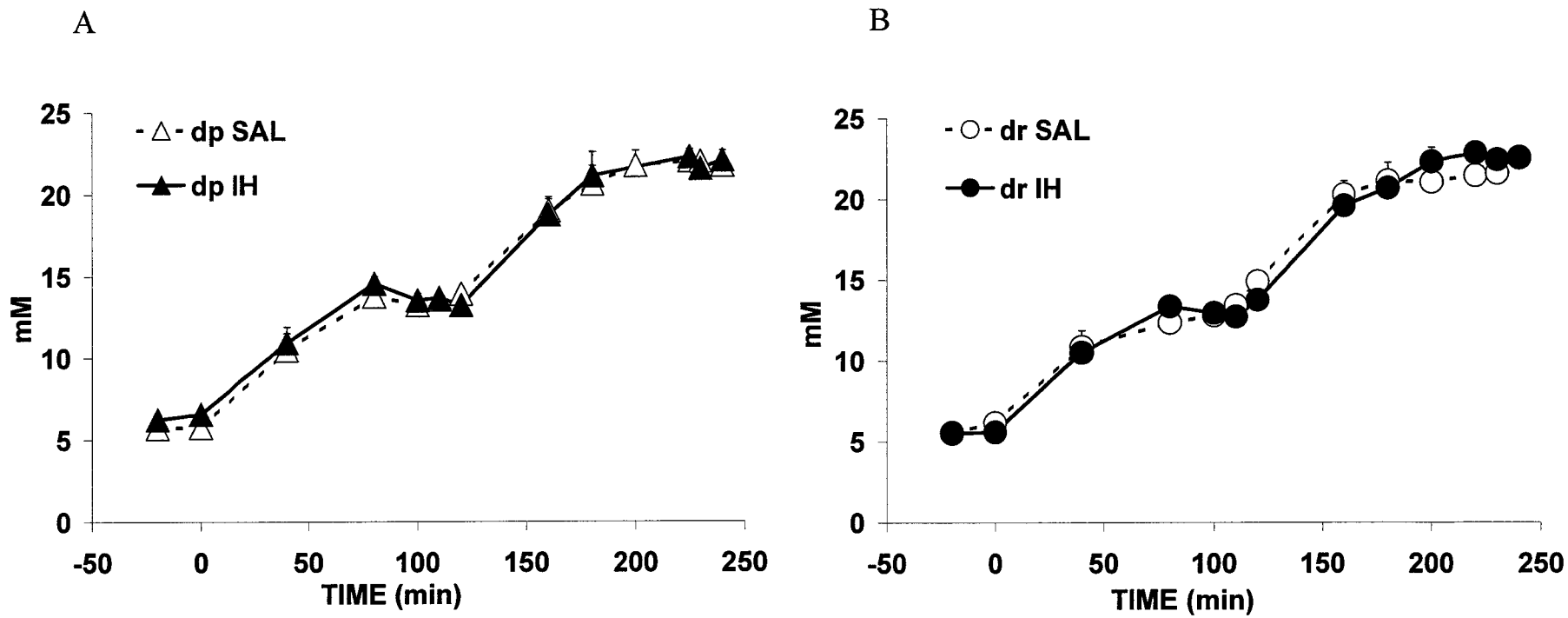



**Figure 5.** Plasma glucose levels during the two-step hyperglycemic clamp. A) Plasma glucose levels during the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Plasma glucose levels during the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

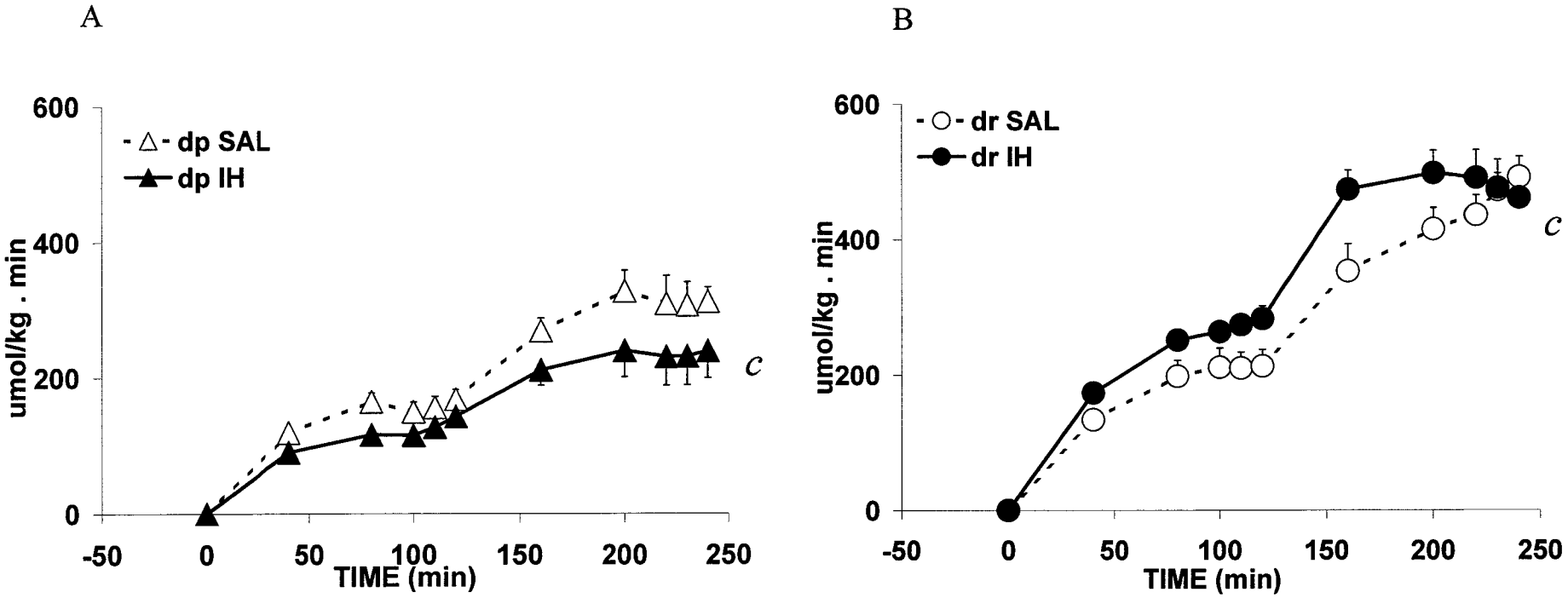



**Figure 6.** Glucose Infusion (GINF) rate during the two-step hyperglycemic clamp. A) Glucose Infusion (GINF) rate during the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) GINF rate during the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

*c*. SAL vs. IH, $p<0.01$ during both the first and second step of the clamp for both the dpBB and drBB rat

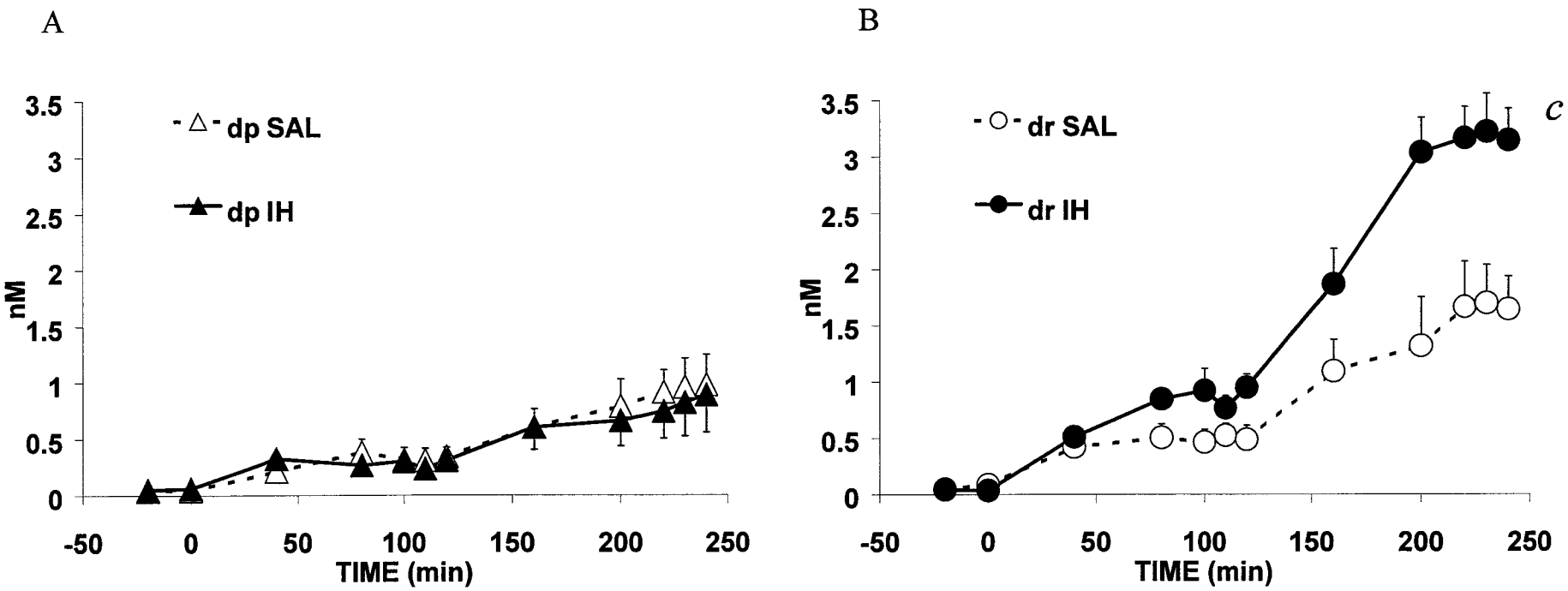



**Figure 7.** Plasma Insulin levels during the two-step hyperglycemic clamp. A) Plasma Insulin levels during the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Plasma Insulin levels during the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

***c.*** SAL vs IH, $p<0.05$ for the first step and $p<0.01$ for the second step of the hyperglycemic clamp in the drBB rat

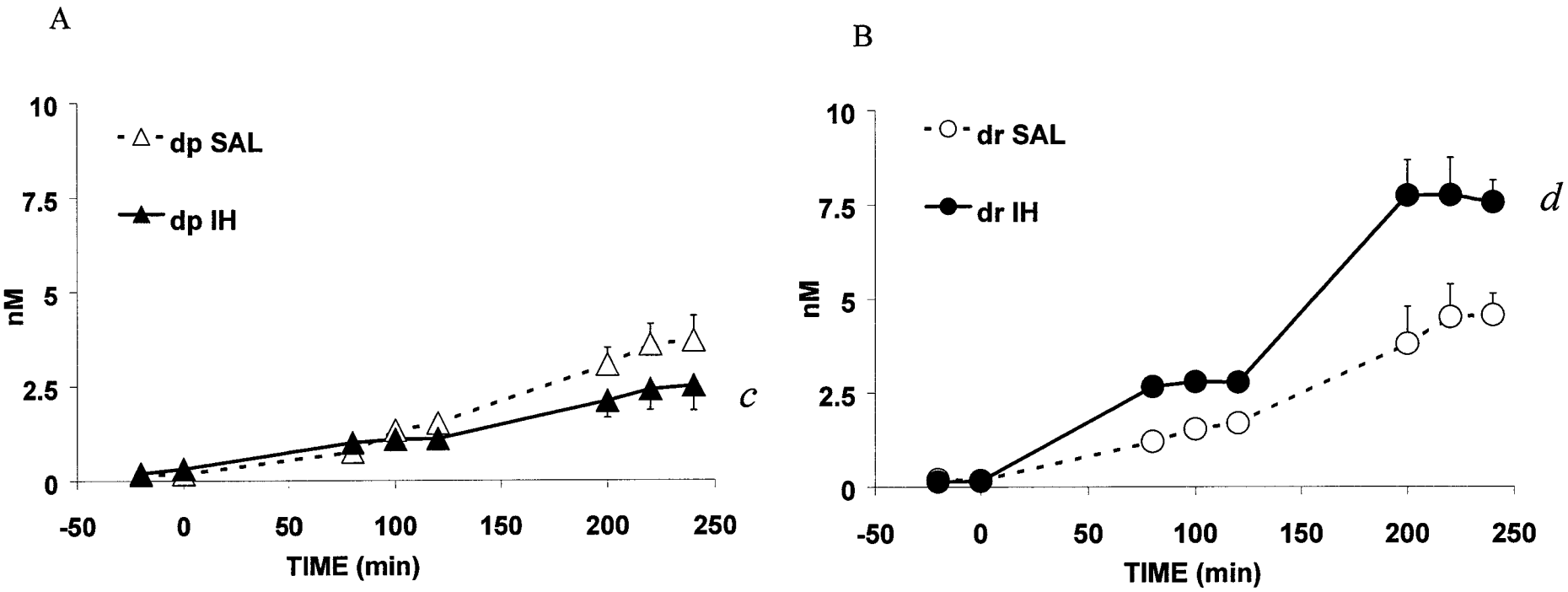



**Figure 8.** Plasma C-Peptide levels during the two-step hyperglycemic clamp. A) Plasma C-Peptide levels during the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Plasma C-Peptide levels during the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

***c.*** SAL vs. IH, $p < 0.01$ during the second step of the clamp for dpBB rats

***d.*** SAL vs. IH, $p < 0.01$ during both the first and second step of the clamp for drBB rats

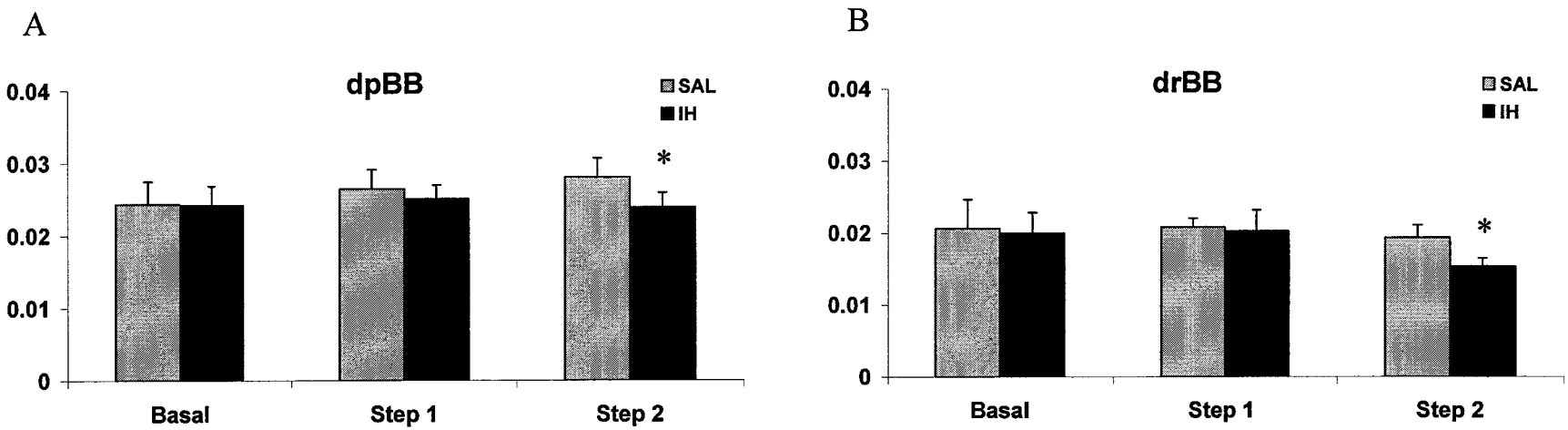



**Figure 9.** C-Peptide/Insulin ratio. A) C-Peptide/Insulin ratio during the basal period and during both the first and second step of the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) C-Peptide/Insulin ratio during both the first and second step of the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

* $p<0.05$, SAL vs. IH

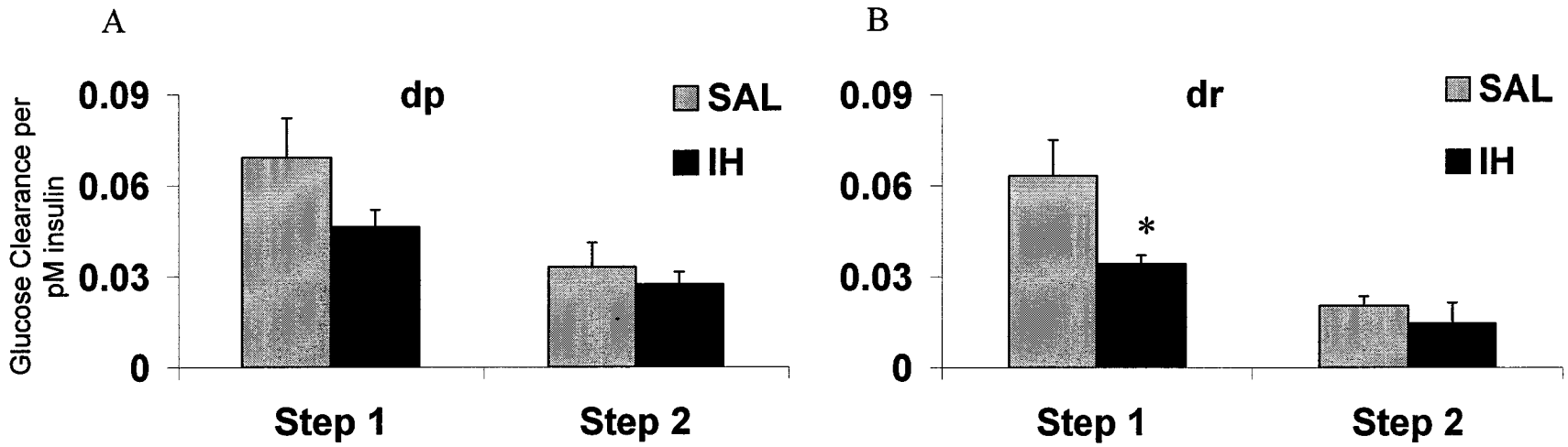



**Figure 10.** Sensitivity Index . A) Sensitivity Index (SI) during both the first and second step of the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Sensitivity Index (SI) during both the first and second step of the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

* $p<0.05$, SAL vs. IH

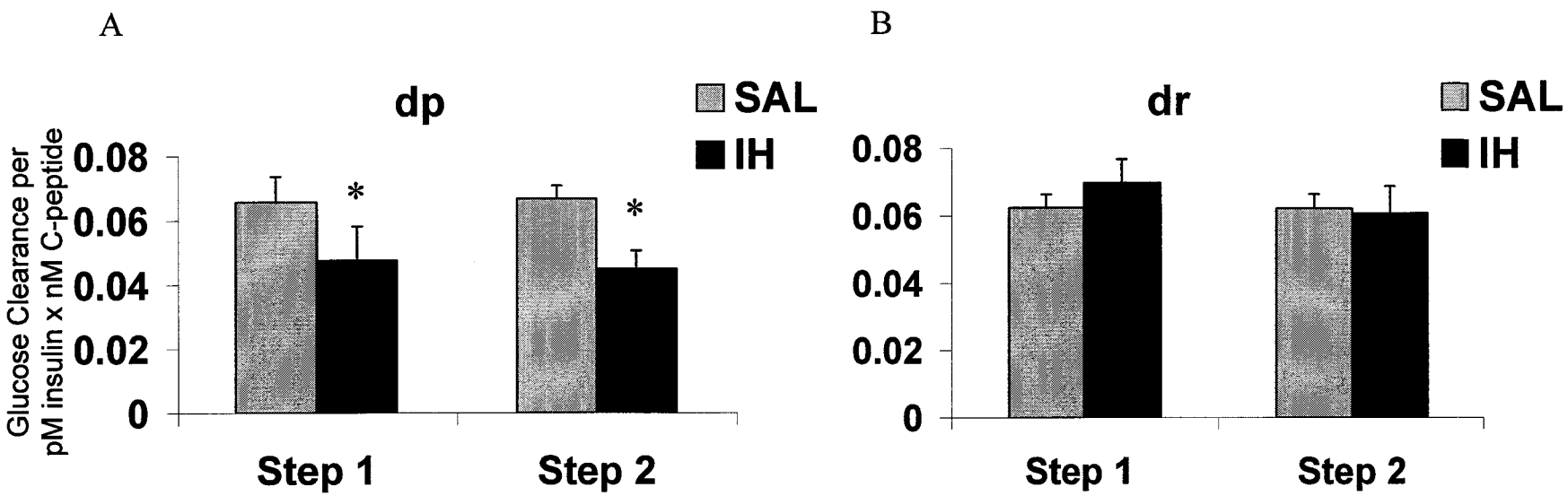
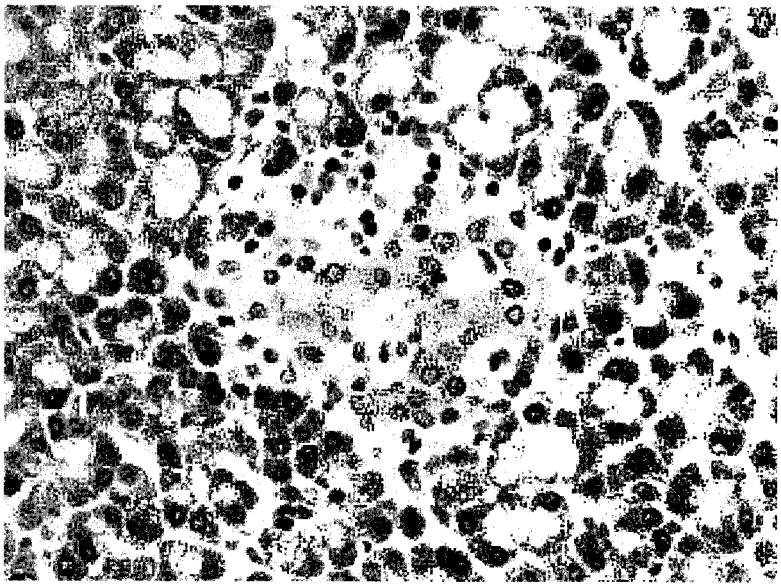



**Figure 11.** Disposition Index.A) Disposition Index (DI) during both the first and second step of the two-step hyperglycemic clamp in Saline (*n = 9*) and Intralipid (*n = 10*) treated dpBB rats. B) Disposition Index (DI) during both the first and second step of the two-step hyperglycemic clamp in Saline (*n = 8*) and Intralipid (*n = 7*) treated drBB rats. Data are in means ± SE.

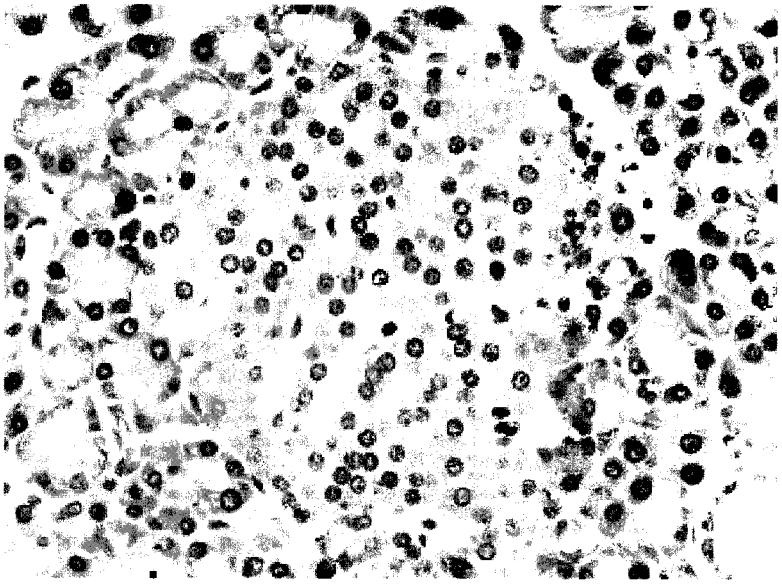
* $p<0.01$, SAL vs. IH

**Figure 12.** Photograph of islets of both dpBB (A) and drBB (B) rats (magnification 200x). Islets of dpBB and drBB rats stained for insulin (red) and counterstained with hematoxylin. The islet of the dpBB (A) rat appears to be infiltrated with non-islet cells and appears to have lost a significant proportion of its insulin producing β cells. In contrast the islet of the drBB rat (B) does not appear to be any cellular infiltration and there does not appear to be any loss of insulin secretory tissue (β cell).

A



B



## 5.5) DISCUSSION (Study #1)

The aim of the present study was to determine whether the presence of autoimmune insulitis, such as that found in the islets of prediabetic diabetes-prone BioBreeding rats, sensitizes the β cell to the lipotoxic effect of fat. The examination of pancreas specimens collected at the end of each experiment revealed a lymphocytic infiltration of the islets of Langerhans (i.e. insulitis) in sections of pancreas retrieved from the dpBB rats (Figure 12A). As expected, insulitis was notably absent in sections of pancreas collected from drBB rats (Figure 12B). As reported above, the C-peptide response (at 13 and 22 mmol/L glucose) and the insulin response (at 22 mmol/L glucose) were both lower in the dpBB rat after a 48-h Intralipid infusion. In contrast, both the C-peptide and the insulin response were markedly enhanced in Intralipid-treated drBB rats. These findings suggest that a prolonged FFA elevation impairs the insulin secretory response to glucose in the dpBB rat, and enhances insulin secretion in the drBB rat. However, in order to properly interpret the results from this in vivo study, it is important to realize that the circulating insulin levels are not only determined by insulin secretion, but also by insulin sensitivity and insulin clearance.

In the results from the dpBB rats, the insulin responses were not as affected as the C-peptide responses by a prolonged Intralipid infusion. Because insulin levels are determined not only by insulin secretion but also by insulin clearance, this finding suggests that Intralipid impairs insulin clearance in accordance with our previous findings (214). This impairment in insulin clearance has been postulated to be a compensatory response to the FFA-induced defect in insulin sensitivity. In this study, the C-

peptide/Insulin ratio was used as an estimate of insulin clearance. Based on this index, Intralipid was found to impair insulin clearance in both the dpBB and drBB rats.

The prolonged elevation of plasma FFA levels produced the expected decrease in insulin sensitivity index (SI) (119), in all rats. It is clear however, that during the second step of the two step hyperglycemic clamp, the SI was markedly reduced, and the effect of the prolonged Intralipid infusion on SI was also less. This estimate of insulin sensitivity depends on a linear relationship between GINF and both insulin and glucose levels. However, this relationship is not linear at elevated insulin levels (> 1000 pM), such as those observed during the second step of the hyperglycemic clamp (68).

The relationship between insulin sensitivity (SI) and β cell function is hyperbolic, so that the disposition index (DI), which is a product of insulin sensitivity and β cell function, remains constant in subjects with normal glucose tolerance (165;166). That is, in individuals with normal β cell function, a decline in insulin sensitivity should be followed by a compensatory increase in insulin secretion, thus maintaining the ability of the body to dispose of glucose. Despite the decrease in insulin sensitivity, the insulin and the C-peptide response during a two step hyperglycemic clamp was significantly impaired by a prolonged elevation of plasma FFA in the dpBB rat. In contrast, in the drBB rat, insulin secretion increased to compensate for the loss in insulin sensitivity. Accordingly, the DI (an index of β cell function) was significantly lower in the Intralipid-treated dpBB rats, but was unchanged in the drBB rats. This suggests that the β cells in the dpBB rats, but not in the drBB rats, are susceptible to the lipotoxic effects of a prolonged elevation of plasma FFA levels on β cell function. The results from the drBB rats are surprising, as they imply an absence of β cell lipotoxicity in this model, despite

previously published results by our laboratory that show that a prolonged FFA elevation impairs GSIS in vivo in normal Wistar rats (162). It should be noted that the different effect of prolonged FFA infusion on GSIS observed in drBB rats and in normal Wistar rats may be due to their difference in weight (drBB rats weigh less than normal Wistar rats) or in rat strain (dpBB rats are derived from Wistar-Furth rats)

It can also be postulated that the mechanisms that protect the β cell from autoimmune diabetes (for example antioxidants and protective cytokines) may also protect the drBB rats from the FFA-mediated impairment in β cell function. In addition, the enhanced ability of the drBB rat (compared to dpBB and Wistar-Furth rats) to mount a protective response to cellular stress (i.e. heat shock) may also help protect the drBB rat from both autoimmune diabetes and lipotoxicity (215).

As previously alluded to, insulitis was present in all the dpBB rats used in this study. The results of this study suggest that pre-existing inflammation/oxidative stress present in the insulitis lesion may be permissive for β cell lipotoxicity. It is possible that cytokines sensitize the β cell to the toxic effect of fat. Work by Shimabukuro and Aarnes demonstrate that the converse holds true (i.e. the toxicity of cytokines is enhanced in fat rich islets (213)). Similarly, it has been demonstrated that exposure to FFA enhance the toxicity of cytokines in β cells in vitro (81). As previously described, both cytokines (99) and fat (199) enhance oxidative stress. It has been well established that the β cells are especially vulnerable to oxidative stress due to their low antioxidant capacity (98). Oxidative stress has been clearly shown to impair β cell function, namely glucose-induced insulin secretion, and to trigger apoptotic β cell death. Therefore, at first glance, the interaction between fat and cytokines on the β cell may be explained by the fact that

both share a common signaling pathway, namely oxidative stress. However, it is important to note that shared/common signaling pathways would be predicted to cause a less than additive effect, rather than the synergistic effect observed in this study. Therefore, other mechanisms may be suggested to account for the synergistic nature of the relationship between fat and cytokines on β cells. One possibility may be that fat is a substrate for cytokine-induced free radical production. It is also possible that cytokines upregulate inflammatory pathways in a manner to magnify the cytotoxic effect of fat. Finally, fat or fat derived free radicals may upregulate the expression of cytokines or cytokine receptor expression. That is, prolonged exposure of β cells to fat may activate the inflammatory process (release of cytokines and upregulation of receptors) in a manner similar to that observed in β cell glucotoxicy, where prolonged exposure to elevated glucose levels has been linked to higher levels of IL-1 (216) and Fas (217) expression in pancreatic β cells. However, we can not exclude that the effects observed may be due to mild hyperglycemia or to pre-existing β cell impairment.

In summary, the results of the present study suggest that a prolonged elevation of plasma FFAs impairs glucose-induced insulin secretion in the diabetes prone, but not in the diabetes resistant BioBreeding rat. This suggests that the presence of insulitis is permissive for β cell lipotoxicity in the BioBreeding rat model. Further studies are required to determine the mechanisms that facilitate the FFA-mediated impairment in GSIS in the diabetes prone BioBreeding rat.

# 6) **STUDY #2:** The antioxidant Taurine prevents the impairing effect of a prolonged FFA infusion on Glucose Stimulated Insulin Secretion.

## 6.1) ABSTRACT (Study #2)

Prolonged exposure of pancreatic islets to free fatty acids (FFA) impairs β cell function both *in vitro* and *in vivo*. FFA exposure induces oxidative stress in islets, and oxidative stress has been implicated in the impairment in β cell function induced by glucotoxicity. To investigate whether oxidative stress plays a role in the impairment in β cell function induced by FFA, we first examined glucose stimulated insulin secretion (GSIS) in vivo with a two-step hyperglycemic clamp (13 and 22 mM) in rats treated with a 48h intravenous infusion of saline (SAL) or oleate (OLE), with or without co-infusion of the antioxidants N-acetylcysteine (NAC; 0.35 mg/kg/min) (a dose that reversed FFA induced insulin resistance in our previous studies). Treatment with OLE, alone and in combination with NAC, elevated plasma FFA levels approximately two-fold. The C peptide secretory responses to both 13 and 22 mM glucose was diminished in all rats treated with NAC. Treatment with OLE, in rats that did not receive NAC, impaired the C peptide response to 13 mM glucose from 2.28 ± 0.17 to 1.4 ± 0.20 nM ($p<0.01$) and also at 22 mM glucose from 6.5 ± 0.55 to 3.83 ± 0.54 ($p<0.01$). Treatment with OLE, in rats that also received NAC, did not affect the C peptide secretory response to either glucose concentration. Calculation of the disposition index (a measure of relative insulin secretion) revealed that a prolonged oleate infusion impaired insulin secretion to a lesser extent in the presence of the antioxidant NAC. To further investigate the effects of antioxidants on β cell function, we examined insulin secretion in freshly isolated islets from rats that received a 48h infusion of SAL and OLE, alone or in combination with the antioxidants NAC (0.35 mg/kg/min) or Taurine (TAU; 0.35 mg/kg/ml). Plasma FFA

levels were raised to a similar extent (~ 2 fold) in rats treated with OLE alone and in combination with antioxidants. GSIS was similar in islets from rats treated with SAL, NAC, and TAU, suggesting that antioxidants alone do not affect insulin secretion in this in vivo model. GSIS in islets from OLE treated rats was lower than that in islets from SAL treated rats at both 13 (OLE vs SAL: 0.194 ± 0.031 vs. 0.294 ± 0.038 pmol/islet/h, $p<0.01$) and 22 (0.254 ± 0.026 vs. 0.341 ± 0.061 pmol/islet/h, $p<0.01$) mM glucose. The impairing effect of OLE on GSIS was prevented by NAC at 13 but not 22 (OLE+NAC vs. NAC: 0.309 ± 0.041 vs. 0.402 ± 0.040 pmol/islet/h, $p<0.01$) mM glucose. The impairing effect of OLE on GSIS was prevented by TAU at both 13 and 22 mM glucose. The results from this study i) confirm that a prolonged FFA elevation impairs GSIS, and ii) suggest a role for antioxidants in preventing the impairing effects of a prolonged FFA elevation on GSIS.

## 6.2) INTRODUCTION (Study #2)

More than 85 % of type 2 diabetic individuals are obese. Free fatty acids (FFAs), which are often elevated in obese individuals, have been found to induce defects in both insulin secretion and action, similar to those observed in individuals with type 2 diabetes. Elevation of plasma FFA levels can induce peripheral and hepatic insulin resistance (119). Prolonged exposure (>24-h) to FFAs has also been shown to impair glucose stimulated insulin secretion (GSIS) *in vitro* (154;156;174;175;210;218) and *in vivo* (162) when insulin secretion is corrected for FFA induced impairment in insulin sensitivity (164). The mechanisms involved in the impairing effect of a prolonged FFA elevation on GSIS are still unclear.

One possibility is that the impairing effect of FFAs on GSIS is mediated by oxidative stress. The term oxidative stress is used to describe an environment where there is an excess concentration of free radicals and other reactive oxygen species (ROS). ROS damage cellular proteins, lipids, and DNA (219;220). Prolonged exposure to FFA generates ROS in islets (183). The pancreatic β cells, which have a low antioxidant capacity, are especially vulnerable to oxidative stress (98;221). This sensitivity has been found to affect both β cell function and viability. Exposure to a source of ROS (i.e. hydrogen peroxide) (184) or to lipid peroxidixation products (i.e. 4-hydroxy-2-noneal) (185) resulted in a marked impairment in GSIS. Furthermore, Kaneto and his colleagues demonstrated, through scanning electron microscopic examination of the pancreatic islets, that oxidative stress promotes morphological changes consistent with apoptosis (222).

Oxidative stress has also been associated with the insulin secretory impairment induced by chronic hyperglycemia (223) and cytokines exposure (224;225). Studies conducted in both these models suggest that either the use of antioxidants (226;227) or the overexpression of antioxidant enzymes (228;229) prevent, at least in part, the defect in β cell function. However, the role of oxidative stress in preventing the lipotoxic effect of FFA on β cell function has not been investigated. Therefore, the aim of this study was to examine the effects of a prolonged (48-h) FFA elevation, alone and in combination with antioxidants, on GSIS.

Two antioxidants, N-acetylcysteine (NAC) and Taurine (TAU), were used in this study. N-Acetylcysteine is not only a scavenger of free radicals and aldehydes, but is also a precursor of intracellular glutathione, an integral part of the intracellular antioxidant defense system. Taurine, also known as 2-amino ethanesulfonic acid, is the most abundant intracellular free amino acid present in mammalian cells, including in the cells of the endocrine pancreas (230). Similarly to other amino acids, taurine is transported into mammalian cells by specific taurine transport proteins (231-234). In addition, taurine can be synthesized using a variety of precursors including from both glutathione and cysteine (and therefore NAC). However, controversy still surrounds the question as to whether taurine is actually present in β cells (235). Taurine has been shown to be an effective aldehyde scavanger (236) and therefore reduce oxidative stress. (237). Since reactive aldehydes are products of lipid peroxidation, taurine is an interesting antioxidant in the context of lipotoxicity.

## 6.3) METHODS (Study #2)

**Animal Models.** Female Wistar rats (Charles River, Quebec, Canada), weighing 250-300g, were used for these experiments. The rats were housed in the University of Toronto's Department of Comparative Medicine. They were exposed to a 12-h light/dark cycle and were fed rat chow (Purina 5001, 4.5% fat; Ralston Purina, St. Louis, MO) and water ad libitum. All procedures were approved by the animal care committee of the University of Toronto.

**Surgical procedures.** See General Materials and Methods section.

**48 h Infusion Period.** Rats were infused for 48 h with either: 1) saline (SAL); 2) N-acetylcysteine (NAC); 3) Taurine (TAU); 4) Oleate (OLE); 5) oleate as above + NAC; 6) oleate as above + TAU. At least 2-3 days after surgery, PE-50 infusion tubing was connected to each of the catheters. 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 run through a swivel, which was suspended on top of the cage. This procedure protected the infusion tubing and allowed the rat complete freedom of movement. Rats were allowed a 30-min adaptation period during which slow saline infusions (5ul/min) kept the lines patent. Thereafter, a basal nonfasting (random) sample was taken for FFAs, glucose, and insulin. A slow infusion of heparinized saline (4U/ml) was started at 5ul/min to maintain the arterial line patent between samples in all rats. The oleate, antioxidant, or control infusions were given through the jugular catheter. Oleate was prepared in fatty acid free BSA (Sigma, St. Louis, MO) according to the Bezman-Tarcher method as

modified by Miles et al., and given at a rate of 1.3uEq/min. Oleate is, with palmitate, the most prevalent circulating fatty acid. Unfortunately, palmitate has low solubility for intravenous infusion. The antioxidants NAC or TAU were given at a dose of 0.35mg/kg/min (pH 7.4). Throughout the infusion period, rats had free access to water and to their standard pelleted food.

Samples for FFAs, glucose, and insulin were taken at 18, 24, and 46 hours after the onset of the saline/oleate infusion, i.e. –30, -24, and –2 h before the onset of the hyperglycemic clamp or the islet isolation procedure (time = 0). Food was removed at 1900 the day before.

**Two-step hyperglycemic clamp.** Glucose stimulated insulin secretion was determined by measuring insulin and C-peptide responses to a two-step (~13 and 22 mmol/l) hyperglycemic clamp. The two-step hyperglycemic clamp was performed in conscious rats fasted overnight. 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. Two basal samples were taken at –20 and 0 min, after which an infusion of 37.5% glucose was started (time = 0 min) to approximately double the plasma glucose levels (first step of hyperglycemic clamp). The glucose infusion was given through the jugular catheter. Both the glucose and the saline/oleate infusion lines were connected to the jugular line through a Y shaped connector. We did not use a glucose prime to rapidly elevate plasma glucose levels because we wished to avoid the adverse effects (arrythmia) caused by a bolus of oleate from the dead space of the venous line. The target plasma

glucose level of 13 mmol/L was achieved and maintained by adjusting the rate of the glucose infusion according to frequent (every 5-10 min) plasma glucose determinations. At 120 min, the glucose infusion was again raised to achieve and maintain plasma glucose levels of ~22 mmol/L (the second step of the hyperglycemic clamp) until the end of the experiment (time = 240 min). Samples for insulin, C-peptide, and FFAs 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.

**Pancreatic Islet Isolation.** Pancreatic islets were isolated in overnight fasted rats, after the 48-h 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 descending aorta. The common bile duct was quickly isolated and a collagenase solution was infused into the pancreas (~15 ml). The pancreas was then carefully removed and placed in a sterile 50 ml Falcon tube. The pancreas was then incubated in a water bath (37oC). Following this incubation, the islets were isolated through a series of centrifugation steps followed by Histopaque 1077 density gradient.

**Insulin secretion.** The freshly isolated islets were equilibrated for 1 h at 37oC in Krebs Ringer containing 10 mmol/l HEPES, and 2.8 mmol/l glucose. To measure insulin release, 5 islets of approximately the same size were transferred to vials containing fresh

medium plus glucose (2.8, 6.5, 13, 22 mmol/l) for 2 h at 37oC. All experiments were conducted in triplicate. Supernatant was retained and stored at –20oC.

**Laboratory methods**. See General Materials and Methods section.

**Calculations.** *Insulin sensitivity index and Disposition Index.* See General Materials and Methods section.

**Statistical analysis.** All data are presented as means ± SE. *In vivo experiment:* First, one-way Analysis Of Variance (ANOVA) for repeated measurements was used to examine the difference between treatments (SAL vs. OLE) within each experimental group (SAL and NAC treated rats). Then, a two-way ANOVA for repeated measurements was used to look at the effect of antioxidants and fatty acid treatments, and to examine any interaction between the two. *Ex-vivo experiments:* One-way analysis of variance (ANOVA) for repeated measurements followed by Tukey's *t* test for multiple comparisons was used to compare differences between treatment groups at different glucose concentrations. The statistical calculations were performed using SAS software (SAS Institute, Cary, NC).

## 6.4) RESULTS (Study #2)

**Two Step Hyperglycemic Clamp.** In this paper, oleate was used to elevate plasma FFA levels because we have previously shown that oleate had a more pronounced impairing effect on GSIS than Intralipid and heparin (162). The 48-h oleate infusion, alone and in combination with the antioxidant NAC, rapidly elevated plasma FFAs when compared to rats treated with either saline or NAC alone. As expected, plasma FFA levels in the basal period (-20 min to 0 min) were higher in rats treated with oleate than in rats treated with saline (Figure 13). Similarly, plasma FFA levels were also higher in rats treated with oleate in combination with NAC than in rats treated with NAC alone (Figure 13). FFA levels declined throughout the clamp as a result of the progressive hyperglycemia and hyperinsulinemia. However, FFA remained higher in all oleate treated rats throughout the experiment.

During the first step of the hyperglycemic clamp, plasma glucose levels gradually rose to the target value of 13 mmol/L, which was maintained until 120 min. During the second step of the hyperglycemic clamp, plasma glucose levels gradually rose to the target level of 22 mmol/L, which was maintained until 240 min. During both steps of the hyperglycemic clamp, the glucose levels were superimposable in all groups (Figure 14).

The rate of glucose infusion (GINF) throughout the hyperglycemic clamp, which is a measure of whole body glucose tolerance, was unaffected by treatment with NAC. The GINF rate in oleate-treated rats was lower than in saline-treated animals throughout the hyperglycemic clamp, however, this was only significant in the absence of NAC ($p < 0.05$) (Figure 15).

As expected, both plasma insulin and C-peptide levels rose in response to increasing glucose levels throughout the two steps of the hyperglycemic clamp (Figure 16 and 17). Plasma insulin and C-peptide levels were both lower in the NAC-treated rats (both with and without oleate) throughout both the first ($p < 0.01$ for both insulin and C-peptide) and second step ($p < 0.01$ for insulin and $p < 0.05$ for C-peptide) of the two-step hyperglycemic clamp (Figure 16 and 17). In the absence of NAC, plasma insulin and C-peptide levels were significantly lower in oleate-treated rats than in saline-treated animals during both the first ($p < 0.05$ for both insulin and c-peptide) and second ($p < 0.001$ for insulin and $p < 0.05$ for C-peptide) step of the two step hyperglycemic clamp (Figure 16 and 17). In contrast, in the presence of NAC, plasma insulin and C-peptide levels were not significantly different between rats treated with oleate and those treated with NAC alone, in either step of the two step hyperglycemic clamp (Figure 16 and 17).

The C-peptide/Insulin ratio was taken as an index of insulin clearance. This index was higher in the NAC treated rats during both steps of the hyperglycemic clamp ($p<0.05$ for both steps of the clamp) (Figure 18). Treatment with oleate only decreased this ratio in NAC treated rats only during the first step of the hyperglycemic clamp ($p<0.05$).

The insulin sensitivity index (SI), was calculated as previously described in the above Methods section. The SI was significantly higher in all NAC treated rats, when compared to saline treated animals, during both the first ($p < 0.01$) and second ($p < 0.01$) step of the two-step hyperglycemic clamp (Figure 19). The SI was significantly lower in rats treated with oleate in combination with NAC, compared to rats treated with NAC alone, during both the first ($p < 0.01$) and second ($p < 0.01$) steps of the two-step

hyperglycemic clamp. In contrast, the SI was not significantly affected in rats treated with oleate alone during either step of the clamp (Figure 19).

The disposition index (DI), which is an established measure of insulin secretion corrected for insulin sensitivity, was calculated as previously described in the above Methods section. The DI was significantly higher in all NAC treated rats during both the first ($p < 0.01$) and second ($p < 0.01$) step of the two-step hyperglycemic clamp (Figure 20). The DI was significantly lower in rats treated with oleate alone when compared to saline treated rats, both during the first ($p < 0.01$) and second ($p < 0.01$) steps of the clamp (Figure 20). However, the DI was lower in rats treated with oleate in the presence of NAC when compared to the DI in rats treated with NAC alone only during the first step of the hyperglycemic clamp ($p < 0.01$).

**Glucose Stimulated Insulin Secretion in Isolated Islets.** As expected, increasing glucose concentration resulted in enhanced insulin secretion from isolated islets in all treatment groups (Figure 21). The insulin secretory response of islets isolated from rats treated with a prolonged oleate infusion was markedly impaired both at 13 and 22 mmol/l glucose ($p< 0.01$ for both). The insulin secretory response of islets isolated from rats treated with NAC in combination with oleate was lower at 22 mmol/l ($p < 0.01$), but not at 13 mmol/l glucose, than that of islets from rats treated with NAC alone. However, when compared with islets from rats treated with saline alone, insulin secretion was significantly lower in islets isolated from rats treated with oleate + NAC, at both 13 and 22 mmol/L ($p < 0.05$ and $p < 0.01$ respectively). In contrast, the antioxidant TAU prevented the oleate-induced decrease in insulin secretion at both 13 and 22 mmol/l.

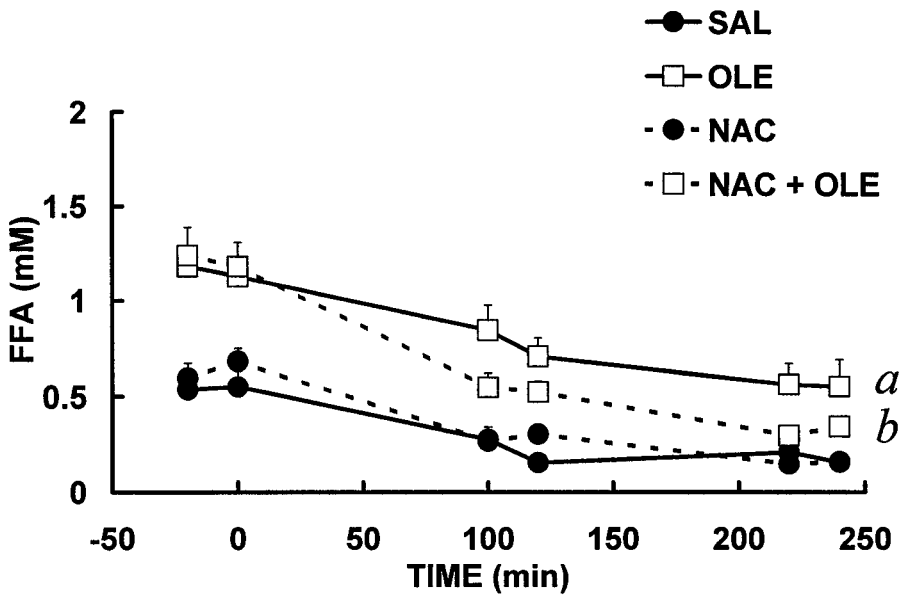



**Figure 13.** Plasma FFA levels during the two-step hyperglycemic clamp in rats treated with i) saline alone (*n* = *9*); ii) oleate alone (1.3 uEq/min, *n* = *8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n* = *8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n* = *8*). Data are means ± SE.

***a.*** effect of OLE, $p<0.01$ during both the first and second step of the clamp

***b.*** effect of NAC + OLE, $p<0.01$ during both the first and second step of the clamp

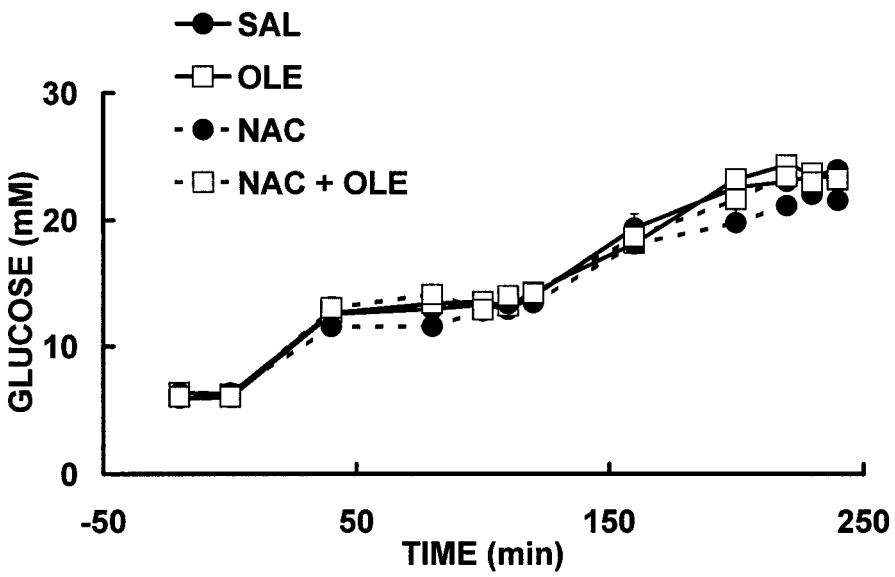



**Figure 14.** Plasma glucose levels during the two-step hyperglycemic clamp rats in treated with i) saline alone (*n* = *9*); ii) oleate alone (1.3 uEq/min, *n* = *8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n* = *8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n* = *8*). Data are means ± SE.

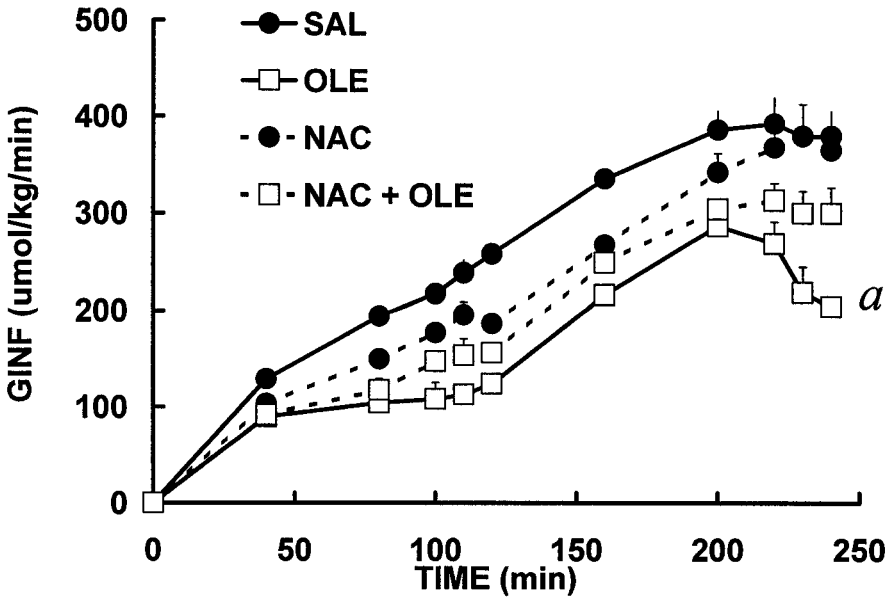



**Figure 15.** Glucose Infusion (GINF) rate during the two-step hyperglycemic clamp in rats treated with i) saline alone (*n* = *9*); ii) oleate alone (1.3 uEq/min, *n* = *8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n* = *8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n* = *8*). Data are means ± SE.

***a.*** effect of OLE, $p<0.05$ during both the first and second step of the hyperglycemic clamp

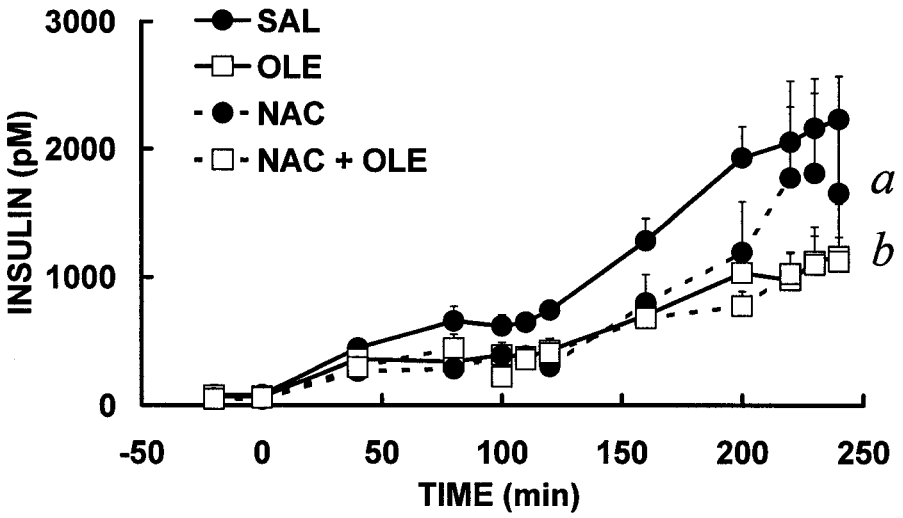



**Figure 16.** Plasma Insulin levels during the two-step hyperglycemic clamp in rats treated with i) saline alone (*n* = *9*); ii) oleate alone (1.3 uEq/min, *n* = *8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n* = *8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n* = *8*). Data are means ± SE.

***a.*** effect of NAC, $p < 0.01$ during both the first and second step of the clamp

***b.*** effect of OLE, $p<0.05$ during the first sep and $p<0.001$ during the second step

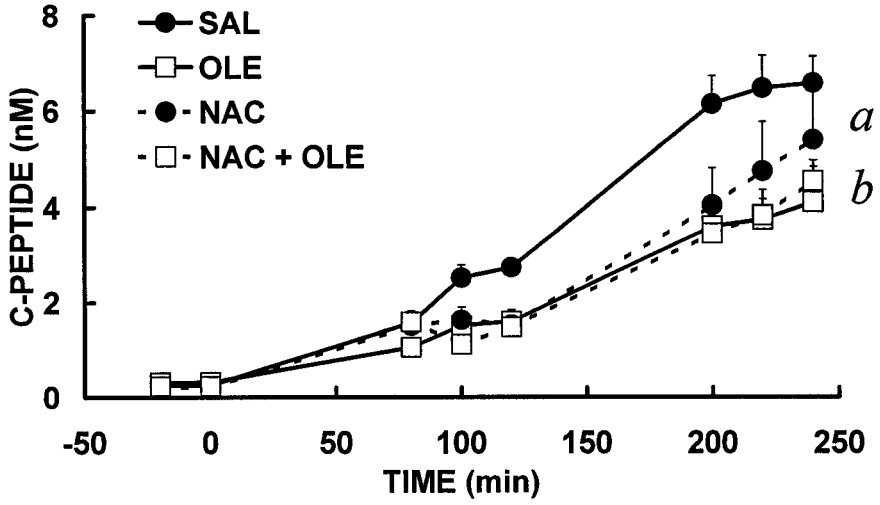



**Figure 17.** Plasma C-Peptide levels during the two-step hyperglycemic clamp in rats treated with i) saline alone (*n* = *9*); ii) oleate alone (1.3 uEq/min, *n* = *8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n* = *8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n* = *8*). Data are means ± SE.

***a.*** effect of NAC, $p < 0.01$ during the first and $p < 0.05$ during the second step of the clamp
***b.*** effect of OLE, $p < 0.05$ during both the first and second step of the clamp

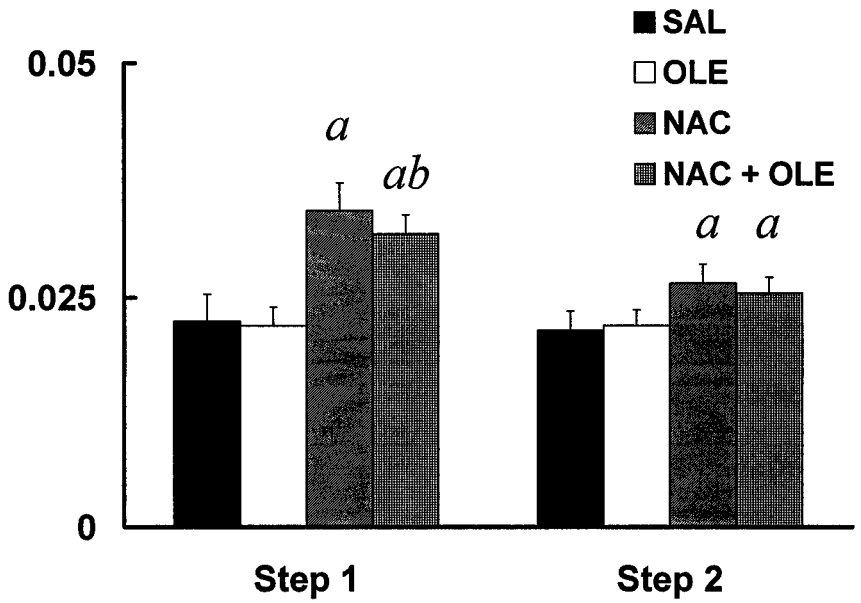



**Figure 18.** C-Peptide/Insulin ratio during both the first and second step of the two step hyperglycemic clamp in rats treated with i) saline alone (*n* = *9*); ii) oleate alone (1.3 uEq/min, *n* = *8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n* = *8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n* = *8*). Data are means ± SE.

***a.*** effect of NAC, $p<0.05$ during both the first and second step of the clamp

***b.*** effect of NAC+OLE, $p<0.05$ during the first step of the clamp

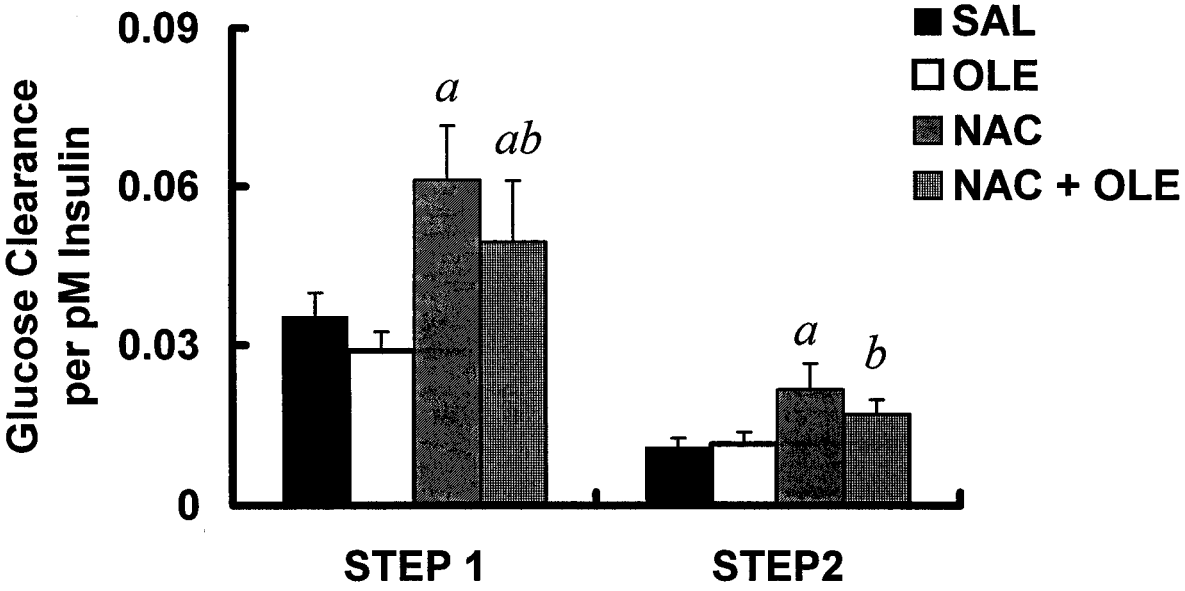



**Figure 19.** Sensitivity Index (SI) during the two-step hyperglycemic clamp in rats treated with i) saline alone (*n = 9*); ii) oleate alone (1.3 uEq/min, *n = 8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n = 8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n = 8*). Data are means ± SE.

***a.*** effect of NAC, $p<0.01$ during both the first and second step of the clamp

***b.*** effect of NAC+OLE, $p<0.01$ during both the first and second step of the clamp

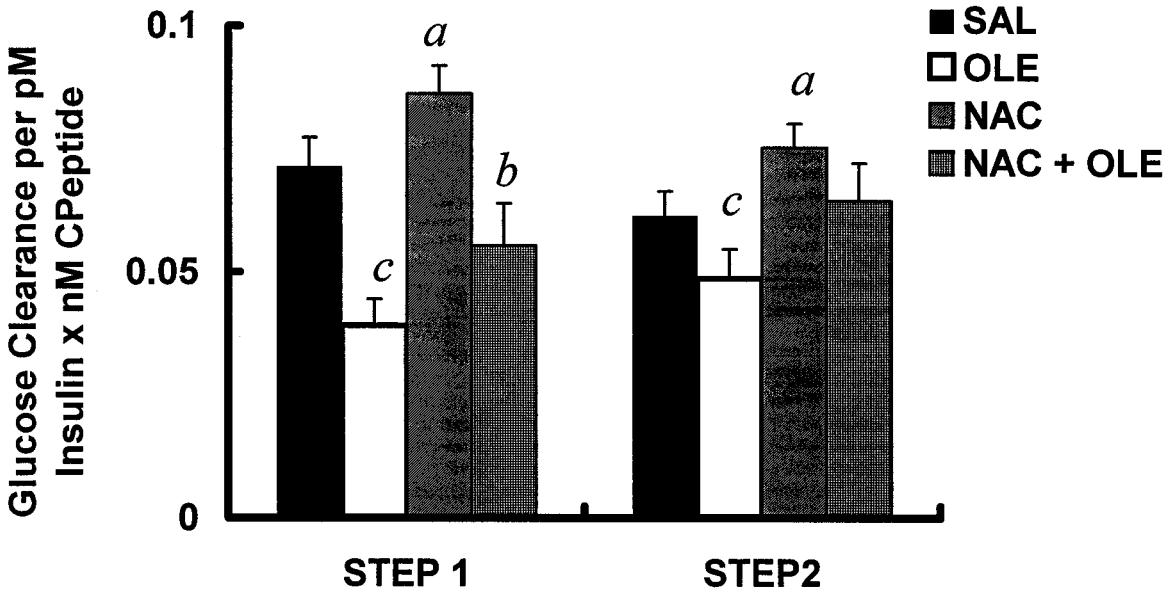



**Figure 20.** Disposition Index (DI) during the two-step hyperglycemic clamp in rats treated with i) saline alone (*n* = *9*); ii) oleate alone (1.3 uEq/min, *n* = *8*); iii) N-Acetylcysteine alone (0.35 mg/kg/min, *n* = *8*); and iv) N-Acetylcysteine (0.35 mg/kg/min) + oleate (1.3 uEq/min, *n* = *8*). Data are means ± SE.

***a.*** effect of NAC, $p<0.01$ during both the first and second step of the clamp

***b.*** effect of NAC+OLE, $p<0.01$ during both the first and second step of the clamp

***c.*** effect of OLE, $p<0.01$ during both the first and second step of the clamp.

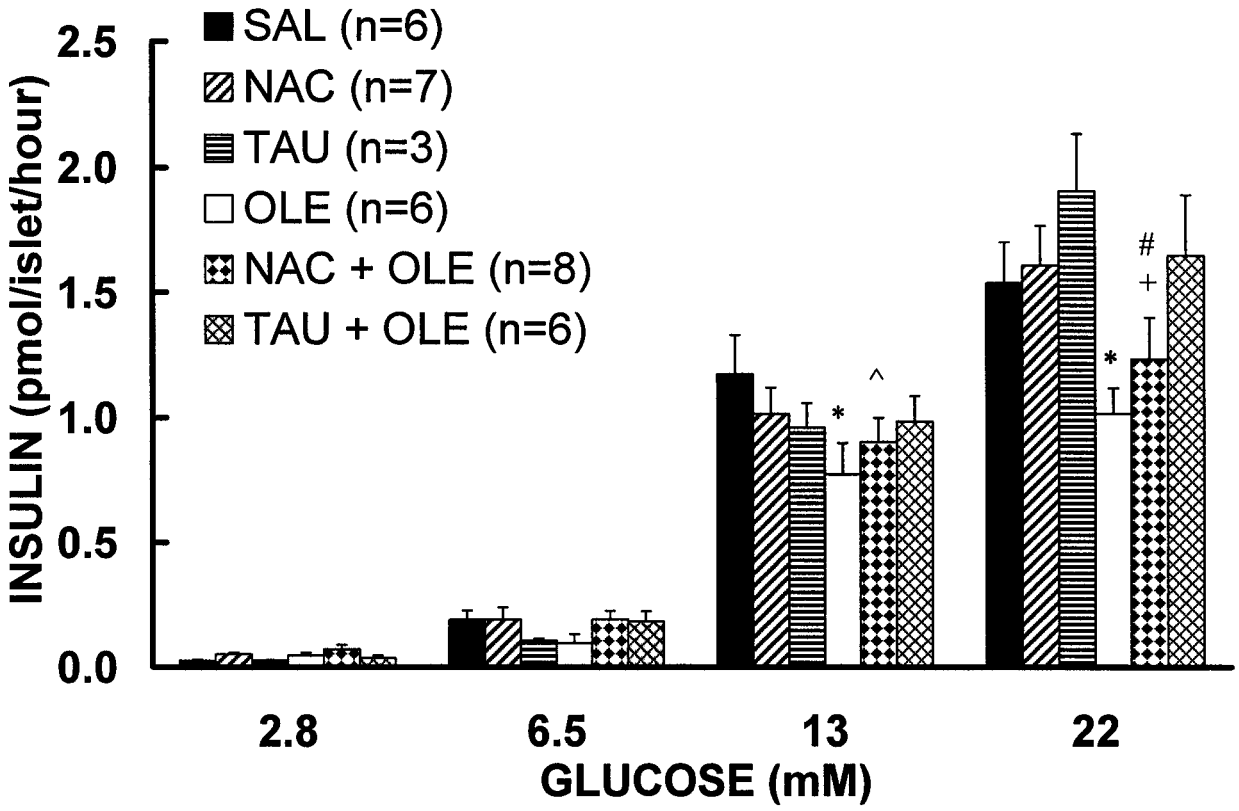



**Figure 21.** Insulin secretory response to glucose of freshly isolated islets of 12 week old normal female Wistar rats treated with saline (*n=6*), oleate alone (1.3 uEq/min , *n=6*), N-Acetylcysteine alone (0.35mg/kg/min, *n=7*), N-Acetylcysteine (0.35 mg/kg/min) in combination with oleate (1.3 uEq/min , *n=8*), Taurine alone (0.35mg/kg/min, *n=3*), and Taurine (0.35 mg/kg/min) in combination with oleate (1.3 uEq/min , *n=6*). Data are means ± SE.

* $p < 0.01$, SAL vs. OLE
\# $p < 0.01$, NAC vs. NAC+OLE
^ $p < 0.05$, SAL vs. NAC+OLE
\+ $p < 0.01$, SAL vs. NAC+OLE

## 6.5) DISCUSSION (Study #2)

In the present study, we examined the effects of a prolonged FFA infusion, alone and in combination with antioxidants, on β cell function. Chronic exposure to FFA has been previously shown to impair GSIS both in vivo and in vitro. Consistent with these findings, we found that both the C-peptide and the insulin responses to 13 and to 22-mmol/L glucose were lower in rats treated with a 48-h oleate infusion than in saline-treated rats. As previously discussed, elevated plasma FFAs induce insulin resistance in vivo. Therefore, it is not surprising that in our study, a prolonged infusion of oleate was associated with a reduction in SI during the first step of the clamp. Our insulin secretion 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. Bergman (165) and Khan (238) have introduced the concept that the relationship between insulin sensitivity and β cell function is hyperbolic so that the disposition index (DI), which is the product of insulin sensitivity and β cell function, remains constant in subjects with normal glucose tolerance (165;238). That is, when insulin sensitivity is low (i.e. as in the case of insulin resistance), β cell function is high and vice versa in normal subjects. Despite the decrease in insulin sensitivity (i.e. SI) that was observed in our study, insulin secretion, as assessed by the insulin and C-peptide secretory response during the two step hyperglycemic clamp, was significantly impaired by a prolonged oleate infusion. Accordingly, the DI, which is an estimate of β cell function corrected for insulin sensitivity, was significantly lower in oleate-treated rats than in the saline treated animals. In other words, our results suggest that in oleate-treated animals, the pancreatic β cell was

unable to adequately compensate for the FFA-induced reduction in SI by hypersecreting insulin – a finding that can be interpreted as a FFA-mediated impairment of β cell function.

The mechanism by which fatty acids impair β cell function is not completely understood. Studies have shown that i) fatty acids promote the generation of reactive oxygen species in islets (183), and ii) both lipid peroxides (185) and free radicals (such as ROS) (184) impair GSIS. Taken together, these findings suggest that the FFA-mediated impairment in glucose-induced insulin secretion may depend on oxidative stress. Cytokine toxicity (224;225) and chronic hyperglycemia (223) are two other instances where β cell dysfunction was found to be associated to oxidative stress. Interestingly, antioxidants were found to prevent the impairing effects of these factors on GSIS (226-229). Therefore, in an attempt to determine whether the FFA-mediated impairment in β cell function is dependent on oxidative stress, GSIS was examined in rats that received a prolonged infusion of oleate, both with and without a co-infusion of the antioxidant NAC. As indicated in our results, treatment with NAC alone reduced absolute insulin secretion in vivo, possibly due to the effect of the antioxidant to improve insulin sensitivity. In NAC treated rats, oleate treatment did not impair absolute insulin secretion, contrary to findings in animals that were not treated with the antioxidant. This suggests that i) the antioxidant NAC prevented the oleate-induced impairment in GSIS, and/or ii) the impairing effect of oleate on absolute insulin secretion was masked by the effect of the antioxidant on insulin sensitivity. The impairing effect of oleate in the presence of NAC on the disposition index suggests that the latter possibility is in fact true. However, the results of the disposition index also suggest that there may be a partial effect of NAC

in preventing the impairing effect of oleate on β cell function. Notably, increased insulin sensitivity with NAC was not only compensated by a decrease in insulin sensitivity but also by an increase in clearance.

In an attempt to more accurately assess the direct effect fat and antioxidants on β cell function, we examined glucose-induced insulin secretion isolated pancreatic islets (i.e. in a model where insulin sensitivity cannot acutely affect β cell function). As described in our results, a 48-h oleate infusion impaired GSIS at both 13 and 22 mmol/L glucose in isolated islets, consistent with our in vivo results. Infusion of the antioxidants NAC or TAU alone did not affect GSIS, suggesting that antioxidants do not directly influence β cell function at the concentrations used in our model. Treatment with the antioxidant TAU was found to protect the islets from the FFA-mediated impairment in GSIS at all glucose concentrations. On the other hand, NAC (the antioxidant that was also studied in vivo) was only found to prevent the fatty acid mediated impairment in GSIS at 13 mmol/L. Taken together, our findings suggest that antioxidants may play a role in preventing the FFA-mediated impairment in glucose induced insulin secretion. Consistent with our findings, a recent in vitro study demonstrated that NAC failed to prevent the impairment in GSIS induced by chronic exposure to oleate. Interestingly, they also found that NAC restored the impairment in insulin content induced by chronic oleate exposure, reinforcing the suggestion that antioxidants may play a role in protecting the β cells from lipotoxicity (239).

The mechanisms by which oxidative stress influences β cell function and viability have yet to be elucidated. Oxidative stress has been found to reduce insulin secretion by impairing mitochondrial ATP synthesis. Oxidative stress can inhibit glucose metabolism

in the β cell. Miwa and colleagues have shown that both glucose utilization and glucose oxidation are significantly reduced in isolated islets exposed to lipid peroxidation products (i.e. aldehydes), which are the end product of a reaction between free radicals and membrane lipids (185). Furthermore, donors of ROS, such as hydrogen peroxide, have been shown to reduce the generation of ATP by affecting mitochondrial membrane permeability and by altering mitochondrial membrane potential in the INS-1 β cell line (184). Free radicals have also been found to reduce the generation of ATP by inducing the expression of the uncoupling protein (UCP-2) in β cells (188). It has also been suggested that oxidative stress may impair insulin gene expression and insulin gene promoter activity (227).

The antioxidants used in this study share several common properties that are related to their function as antioxidants and aldehyde scavengers. A TAU derivative, known as taurine chloroamine, is particularly effective in inhibiting the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX2) (240), through inhibition of the NFkB pathway (241). NAC also inhibits the NFkB pathway (242;242) and the expression of the iNOS gene (243) although its effect may be less than that of TAU (236;244).

The reasons for the different ability of TAU and NAC to prevent the FFA-induced impairment in β cell function have yet to be determined. One possible explanation for the discrepancy between the two antioxidants may be that TAU is a better antioxidant at the level of the β cell; that is, TAU may enter the β cell and reach its site of action more readily than NAC, although β cell uptake of TAU may not be significant (235). Another possibility for the different protective effect of TAU and NAC on the β cell is that

although both antioxidants are free radical and aldehyde scavengers, and also inhibit iNOS gene expression and NFkB activation, these agents may differ in their potency at these different sites of action. Further biochemical studies in islets could address these hypotheses. It is also possible that the beneficial effects of TAU on insulin secretion are unrelated to the antioxidant and antiinflamatory properties of this molecule. TAU has a number of other effects that could influence insulin secretion. In high concentrations (greater than ten times those used in the present study) TAU can alter calcium flux (245;246) and interact with GABA receptors (247), however whether this occurs in the β cell is not known. Recent studies have also suggested that TAU at high concentrations can close $K+_{ATP}$ channels in β cells (248). Since NAC can be converted to TAU, its effects may also be unrelated to its antioxidant properties.

In summary, our study demonstrates that a prolonged oleate infusion impairs GSIS both in vivo and in isolated islets. Furthermore, antioxidants such as TAU, and to a lesser extent NAC, prevent the FFA-induced impairment in GSIS. The reasons for the enhanced ability of TAU to protect GSIS from the impairing effects of a prolonged FFA elevation have yet to be determined.

# 7) GENERAL DISCUSSION

Diabetes mellitus is a debilitating and often lethal disease affecting millions of individuals worldwide (249). The prevalence of diabetes mellitus is expected to increase over the coming decades, resulting in an extra strain on health care resources in countries around the world. Unfortunately, the greatest increase in the prevalence of this disease is anticipated to occur in the developing world, where health care resources are already lacking (3). It is thought that the major reason for this escalation is the increasing number of people who are overweight and obese. As previously described, free fatty acid (FFAs), which are often elevated in obese individuals, have been found to impair both insulin sensitivity and insulin secretion. In this thesis, we have explored some of the pathophysiology underlying the fatty acid-mediated impairment in glucose stimulated insulin secretion (GSIS).

Prolonged exposure to FFAs impairs GSIS, both in vitro and in vivo. This finding is particularly relevant in the pathogenesis of type 2 diabetes, a disease typically associated with obesity and elevated plasma FFA levels (119). A characteristic feature of obesity associated type 2 diabetes mellitus is insulin resistance. Interestingly, FFAs also impair insulin sensitivity in vitro and in vivo. Because insulin sensitivity and β cell function are inversely related, a defect in insulin action is compensated for by an increase in insulin secretion in normal subjects. However, chronically elevated plasma FFA levels present in obese and insulin resistant individuals prevent this compensation by triggering a defect in insulin secretory capacity, resulting in impaired glucose tolerance and eventually overt hyperglycemia and diabetes. It is interesting to note that not all obese individuals develop diabetes. This suggests that certain individuals are predisposed to develop the disease in the face of obesity and insulin resistance. Interestingly, individuals

with a family history of type 2 diabetes mellitus may be particularly sensitive to the impairing effect of FFA on GSIS (250).

In addition to playing an important role in the pathogenesis of type 2 diabetes mellitus, β cell lipotoxicity may also play some role in autoimmune type 1 diabetes. Several studies conducted in the NOD mouse model of autoimmune diabetes have demonstrated that lipids may also play a role in the pathogenesis of this form of the disease. In one study, differences in fat feeding were found to influence the incidence of diabetes in the NOD mouse. In this particular study, 65% of animals fed a low fat diet developed diabetes by 30 weeks of age, whereas 80% of rats fed a high fat diet developed diabetes at the same time point (211). In another study in the NOD mouse, depletion of the islet triglyceride content with pharmacotherapy (troglitazone) reduced the diabetes incidence in this animal model of autoimmune diabetes (212). Unfortunately, in addition to having a lipopenic effect, troglitazone also possesses anti-inflammatory and antioxidant properties, which may confound the results. Furthermore, in vitro studies showing that lipids can modulate the toxicity of cytokines also suggest that fat may play a role in the pathogenesis of autoimmune diabetes. In a study conducted by Shimaburkuro, the toxicity of cytokines was found to be enhanced in fat rich islets, and was found to be diminished in fat depleted islets (199). Similarly, in a separate study, prolonged exposure to fatty acids was shown to enhance the toxicity of cytokines in β cells (81). There also exists evidence suggesting that fat is associated with the development of type 1 diabetes in humans. In one study high weight gain during infancy was associated with an increased incidence of type 1 diabetes (251). Taken together, these findings all suggest that fat may also play some role in the pathogenesis of autoimmune diabetes.

It has been established that insulin is an important hormone in the regulation of lipid metabolism. Insulin stimulates fatty acid re-esterification, and inhibits lipolysis. A reduction in insulin secretion or action will therefore be expected to increase circulating plasma FFA levels. The findings of the first study presented in this thesis suggest that cytokines and FFA interact synergistically, through one or more putative mechanisms, to impair β cell function. The findings derived from the studies described in this thesis may have significant implications in the pathogenesis of LADA, the slowly progressing form of autoimmune diabetes that presents in adults and that is often misdiagnosed for type 2 diabetes. In this form of diabetes elevated levels of circulating FFA (either due to obesity or decreasing insulin levels (natural history of LADA)) may interact with cytokines present in the insulitis lesion to enhance the pace of β cell destruction, accelerating the dependence for insulin in these individuals. Therefore, it may be possible that aggressive management of dyslipidemia in individuals with impaired glucose tolerance who are also ICA+ can slow down or halt the progression to full blown diabetes.

Oxidative stress was suggested as a possible mechanism to explain the synergistic interaction between fat and cytokines on GSIS. It has been previously established that oxidative stress may play a role in mediating cytokine induced tissue dysfunction. The second study presented in this thesis attempted to further elaborate on the importance of oxidative stress in mediating the FFA-induced impairment in β cell function. As described above, this was accomplished by observing the effect of prolonged FFA exposure alone, and in combination with antioxidants, on glucose-induced insulin secretion. As in previous studies, long-term exposure to fatty acids was shown to have an impairing effect on GSIS. Our results also demonstrated that simultaneous treatment with

the antioxidant taurine prevented this effect, suggesting a role for oxidative stress in mediating the impairing effects of FFAs on glucose-induced insulin secretion.

The importance of oxidative stress as a mediator of β cell dysfunction and destruction has only been recently recognized. The pancreatic β cells are especially vulnerable to oxidative stress due to their low antioxidant capacity. A study conducted by Lenzen et al, which examined antioxidant gene expression in various tissues in mice, suggested that levels of cytoplasmic Cu/Zn superoxide dismutase, mitochondrial superoxide dismutase, and glutathione peroxidase gene expression were all lower in the pancreatic islets than in any other tissues (98). Oxidative stress is thought to induce β cell dysfunction and destruction through a variety of different mechanisms such as i) modifying cellular macromolecules (i.e. protein, lipids, and DNA), ii) activating the pro-inflammatory transcription factor NF kappa B, iii) inducing mitochondrial dysfunction, and iv) promoting cellular apoptosis.

The finding that oxidative stress may be involved with β cell abnormalities associated with the onset of diabetes mellitus offers the possibility of a new therapeutic target for this disease. By interfering in the oxidative stress pathway, it may be possible to help in the prevention and the treatment of diabetes mellitus. New strategies could include a number of "low tech" approaches that are feasible in countries around the world. These strategies include dietary modification, in order to incorporate more antioxidants into the diet, and also the administration of antioxidant supplements. In order to investigate this, there exist a small number of studies that have been completed or that are currently underway. The majority of studies in this area have focused on protecting additional β cell loss in newly diagnosed patients with type 1 diabetes. In one large

multicenter study (IMDIAB IV) both vitamin E (tocopherol) and nicotinamide were shown to protect residual β cell function in patients with recent onset type 1 diabetes mellitus (252). However, the results from another study suggested that the administration of a mixture of antioxidants (namely nicotinamide, vitamin C, vitamin E, β carotene, and selenium) to newly diagnosed children with type 1 diabetes mellitus did not preserve β cell function (253). Unfortunately, these disparate results leave the question of whether antioxidants can help preserve β cell function in patients diagnosed with autoimmune diabetes unanswered.

In the area of type 2 diabetes research, there exists evidence from the Insulin Resistance Atherosclerosis Study (IRAS) suggesting that Vitamin E (tocopherol) may prevent the development of type 2 diabetes mellitus (254). However, there exists contradictory evidence from the Heart Outcome Prevention Evaluation (HOPE) trial suggesting that the administration of Vitamin E to patients older than 55 years of age without known diabetes did not prevent the development of diabetes mellitus in this cohort (255). With these disparate results, it is clear that additional studies must be conducted in order to determine the therapeutic potential of antioxidants in the prevention or adjuvant treatment of diabetes.

# 8) CONCLUSION

In summary, this thesis focused on examining the mechanisms that mediate the impairing effect of a prolonged FFA elevation on glucose stimulated insulin secretion. The first study presented in this thesis demonstrated that the presence of insulitis has a permissive effect on the FFA mediated impairment in GSIS. Our results indicate that the impairing effect of a prolonged fatty acid elevation is accentuated in the diabetes-prone BB rat with insulitis. It was therefore hypothesized that inflammation and oxidative stress may play a role in the mechanism of fat induced impairment of β cell function. In the second study presented, we examined GSIS in animals treated with a prolonged fat infusion in combination with antioxidants. This study demonstrated that antioxidants prevented the impairing effect of a prolonged FFA elevation on GSIS, suggesting a possible role for oxidative stress in mediating the observed β cell lipotoxicity. Taken together, the results of the two studies presented in this thesis strengthen the hypothesis that oxidative stress may play a role in mediating β cell dysfunction.

# 9) STUDY LIMITATIONS

One limitation of both of the studies presented is that they were conducted in a specific animal model. The results therefore, cannot be immediately generalized to other species, including humans. However, there exist studies in the literature confirming the impairing effect of a prolonged FFA infusion on GSIS in humans (164). Another caveat of our studies is that, plasma FFA levels were elevated for a shorter period, and to a slightly higher level than that observed in the pathologic condition (i.e. obesity) that we were trying to mimic. This is because the feasibility of an intravenous lipid infusion lasting longer than 48 hours is low in rats. A chronic high fat diet model could have been used to experimentally elevate post-prandial plasma FFA levels, however the dietary model also has certain limitations (difficult to control, does not mimic endogenous FFA release from adipose tissue etc.). A final caveat is that the method used to calculate insulin sensitivity depends on assumptions such as the validity of the hyperbolic relationship between insulin secretion and insulin sensitivity (our laboratory has found this to be true in rats). Also, the measurment of insulin sensitivity using the sensitivity index (SI) from the two step hyperglycemic clamp is not the gold standard method of evaluating insulin sensitivity. A hyperinsulinemic euglycemic clamp could have been performed in every rat to accurately measure the insulin sensitivity in each animal. However, performing both clamps in the same animals also has its limitation related to the stress of prolonging the experiment and to interference of each of the clamps with each other. If the hyperinsulinemic euglycemic clamp is performed prior to the two step hyperglycemic clamp, the transient elevation in plasma insulin levels required to assess insulin sensitivity may affect the measurement of GSIS. Also, if the hyperinsulinemic euglycemic clamp is performed after the two step hyperglycemic clamp, the measure of

insulin sensitivity may be altered by the transient episode of hyperglycemia required to assess GSIS.

# 10) FUTURE DIRECTIONS

Several important findings were presented in this thesis, however there are still questions that remain to be answered. Based on the results of the first study presented in this thesis, the impairing effect of a prolonged FFA elevation on GSIS appears to be accentuated in the context of autoimmune diabetes. However, the mechanism responsible for this effect remains to be uncovered. It is our belief that oxidative stress may play an important role in mediating this effect. Therefore, a strategic starting point would be to examine markers of oxidative stress in insulin secretory tissue in order to determine whether a prolonged elevation of fatty acids increases free radical production, and whether this increase is accentuated in the context of autoimmune diabetes. Additionally, it would be valuable to determine whether antioxidants and/or anti-inflammatory agents (i.e. IL-1 antagonists) prevent the observed effect of a prolonged FFA elevation on GSIS in the diabetes-prone animals.

The results of the second study presented in this thesis suggest that antioxidants may prevent the impairing effect of a prolonged FFA elevation on GSIS, suggesting a role for oxidative stress. Further experiments remain to be conducted in order to confirm that i) FFAs enhance oxidative stress in insulin secretory tissue, and that ii) this is prevented by the systemic delivery of antioxidant compounds. Also, the mechanisms accounting for the observed difference in the protective effect of the antioxidants NAC and TAU on pancreatic islets remains to be elucidated. Such studies will not only further our understanding of the mechanisms that may be involved in mediating the impairing effect of FFA on GSIS, but also provide the possibility for a novel therapeutic target in the treatment of diabetes mellitus.

# REFERENCES

Reference List


1. Zimmet,P, Alberti,KG, Shaw,J: Global and societal implications of the diabetes epidemic. *Nature* 414:782-787, 2001

2. Harris,MI, Flegal,KM, Cowie,CC, Eberhardt,MS, Goldstein,DE, Little,RR, Wiedmeyer,HM, Byrd-Holt,DD: Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults. The Third National Health and Nutrition Examination Survey, 1988-1994. *Diabetes Care* 21:518-524, 1998

3. King,H, Aubert,RE, Herman,WH: Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 21:1414-1431, 1998

4. Health Canada, Laboratory Centre for Disease Control. Diabetes in Canada: National Statistics and Opportunities for Improved Surveillance, Prevention, and Control. 1999. Ottawa, Canada, Minister of Public Works and Government Services.
Ref Type: Report

5. Ganong W: Review of Medical Physiology. Stamford, Connecticut, Appleton & Lange, 1999, p. 324-328

6. Panzram,G: Mortality and survival in type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 30:123-131, 1987

7. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N.Engl.J.Med.* 329:977-986, 1993

8. Nasr,CE, Hoogwerf,BJ, Faiman,C, Reddy,SS: United Kingdom Prospective Diabetes Study (UKPDS). Effects of glucose and blood pressure control on complications of type 2 diabetes mellitus. *Cleve.Clin.J.Med.* 66:247-253, 1999

9. Eisenbarth,GS: Type I diabetes mellitus. A chronic autoimmune disease. *N.Engl.J.Med.* 314:1360-1368, 1986

10. Couper,JJ, Hudson,I, Werther,GA, Warne,GL, Court,JM, Harrison,LC: Factors predicting residual beta-cell function in the first year after diagnosis of childhood type 1 diabetes. *Diabetes Res.Clin.Pract.* 11:9-16, 1991

11. Kelly,MA, Rayner,ML, Mijovic,CH, Barnett,AH: Molecular aspects of type 1 diabetes. *Mol.Pathol.* 56:1-10, 2003

12. Bennett,ST, Lucassen,AM, Gough,SC, Powell,EE, Undlien,DE, Pritchard,LE, Merriman,ME, Kawaguchi,Y, Dronsfield,MJ, Pociot,F, .: Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus. *Nat.Genet.* 9:284-292, 1995

13. Vafiadis,P, Bennett,ST, Todd,JA, Nadeau,J, Grabs,R, Goodyer,CG, Wickramasinghe,S, Colle,E, Polychronakos,C: Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. *Nat.Genet.* 15:289-292, 1997

14. Barnett,AH, Eff,C, Leslie,RD, Pyke,DA: Diabetes in identical twins. A study of 200 pairs. *Diabetologia* 20:87-93, 1981

15. Forrest,JM, Menser,MA, Burgess,JA: High frequency of diabetes mellitus in young adults with congenital rubella. *Lancet* 2:332-334, 1971

16. Varela-Calvino,R, Ellis,R, Sgarbi,G, Dayan,CM, Peakman,M: Characterization of the T-cell response to coxsackievirus B4: evidence that effector memory cells predominate in patients with type 1 diabetes. *Diabetes* 51:1745-1753, 2002

17. Dahlquist,GG, Blom,LG, Persson,LA, Sandstrom,AI, Wall,SG: Dietary factors and the risk of developing insulin dependent diabetes in childhood. *BMJ* 300:1302-1306, 1990

18. Karjalainen,J, Martin,JM, Knip,M, Ilonen,J, Robinson,BH, Savilahti,E, Akerblom,HK, Dosch,HM: A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N.Engl.J.Med.* 327:302-307, 1992

19. Foulis,AK, Liddle,CN, Farquharson,MA, Richmond,JA, Weir,RS: The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. *Diabetologia* 29:267-274, 1986

20. Gepts,W: Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 14:619-633, 1965

21. Itoh,N, Hanafusa,T, Miyazaki,A, Miyagawa,J, Yamagata,K, Yamamoto,K, Waguri,M, Imagawa,A, Tamura,S, Inada,M, . : Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *J.Clin.Invest* 92:2313-2322, 1993

22. Imagawa,A, Hanafusa,T, Tamura,S, Moriwaki,M, Itoh,N, Yamamoto,K, Iwahashi,H, Yamagata,K, Waguri,M, Nanmo,T, Uno,S, Nakajima,H, Namba,M, Kawata,S, Miyagawa,JI, Matsuzawa,Y: Pancreatic biopsy as a procedure for detecting in situ autoimmune phenomena in type 1 diabetes: close correlation between serological markers and histological evidence of cellular autoimmunity. *Diabetes* 50:1269-1273, 2001

23. Voorbij,HA, Jeucken,PH, Kabel,PJ, De Haan,M, Drexhage,HA: Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. *Diabetes* 38:1623-1629, 1989

24. Lee,KU, Kim,MK, Amano,K, Pak,CY, Jaworski,MA, Mehta,JG, Yoon,JW: Preferential infiltration of macrophages during early stages of insulitis in diabetes-prone BB rats. *Diabetes* 37:1053-1058, 1988

25. Hanenberg,H, Kolb-Bachofen,V, Kantwerk-Funke,G, Kolb,H: Macrophage infiltration precedes and is a prerequisite for lymphocytic insulitis in pancreatic islets of pre-diabetic BB rats. *Diabetologia* 32:126-134, 1989

26. Jansen,A, Homo-Delarche,F, Hooijkaas,H, Leenen,PJ, Dardenne,M, Drexhage,HA: Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta- cell destruction in NOD mice. *Diabetes* 43:667-675, 1994

27. Nagata,M, Santamaria,P, Kawamura,T, Utsugi,T, Yoon,JW: Evidence for the role of CD8+ cytotoxic T cells in the destruction of pancreatic beta-cells in nonobese diabetic mice. *J.Immunol.* 152:2042-2050, 1994

28. Signore,A, Pozzilli,P, Gale,EA, Andreani,D, Beverley,PC: The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. *Diabetologia* 32:282-289, 1989

29. Lee,KU, Amano,K, Yoon,JW: Evidence for initial involvement of macrophage in development of insulitis in NOD mice. *Diabetes* 37:989-991, 1988

30. Timsit,J, Debray-Sachs,M, Boitard,C, Bach,JF: Cell-mediated immunity to pancreatic islet cells in the non-obese diabetic (NOD) mouse: in vitro characterization and time course study. *Clin.Exp.Immunol.* 73:260-264, 1988

31. Kolb,H: Benign versus destructive insulitis. *Diabetes Metab Rev.* 13:139-146, 1997

32. Baekkeskov,S, Nielsen,JH, Marner,B, Bilde,T, Ludvigsson,J, Lernmark,A: Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. *Nature* 298:167-169, 1982

33. Atkinson,MA, Maclaren,NK, Riley,WJ, Winter,WE, Fisk,DD, Spillar,RP: Are insulin autoantibodies markers for insulin-dependent diabetes mellitus? *Diabetes* 35:894-898, 1986

34. Kaufman,DL, Erlander,MG, Clare-Salzler,M, Atkinson,MA, Maclaren,NK, Tobin,AJ: Autoimmunity to two forms of glutamate decarboxylase in insulin- dependent diabetes mellitus. *J.Clin.Invest* 89:283-292, 1992

35. Lan,MS, Wasserfall,C, Maclaren,NK, Notkins,AL: IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proc.Natl.Acad.Sci.U.S.A* 93:6367-6370, 1996

36. Summary of Revisions for the 2003 Clinical Practice Recommendations. *Diabetes Care* 26:S3, 2003

37. Gorsuch,AN, Spencer,KM, Lister,J, McNally,JM, Dean,BM, Bottazzo,GF, Cudworth,AG: Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus. *Lancet* 2:1363-1365, 1981

38. Vardi P TMGMeal: Consistency of anti-islet autoimmunity in "pre-type 1 diabetics" and genetically susceptible subjects: evidence from an ultrasensitive competitive insulin autoantibody (CIAA) radioimmunoassay (Abstract). *Diabetes* 35(Suppl 1), 1986

39. Vardi,P, Crisa,L, Jackson,RA: Predictive value of intravenous glucose tolerance test insulin secretion less than or greater than the first percentile in islet cell antibody positive relatives of type 1 (insulin-dependent) diabetic patients. *Diabetologia* 34:93-102, 1991

40. Srikanta,S, Ganda,OP, Eisenbarth,GS, Soeldner,JS: Islet-cell antibodies and beta-cell function in monozygotic triplets and twins initially discordant for Type I diabetes mellitus. *N.Engl.J.Med.* 308:322-325, 1983

41. Greenbaum,CJ: Insulin resistance in type 1 diabetes. *Diabetes Metab Res.Rev.* 18:192-200, 2002

42. Greenbaum,CJ, Kahn,SE, Palmer,JP: Nicotinamide's effects on glucose metabolism in subjects at risk for IDDM. *Diabetes* 45:1631-1634, 1996

43. Talanian,RV, Yang,X, Turbov,J, Seth,P, Ghayur,T, Casiano,CA, Orth,K, Froelich,CJ: Granule-mediated killing: pathways for granzyme B-initiated apoptosis. *J.Exp.Med.* 186:1323-1331, 1997

44. Ingelsson,E, Saldeen,J, Welsh,N: Islet expression of perforin, Fas/Apo-1 and interleukin-1 converting enzyme (ICE) in non-obese diabetic (NOD) mice. *Immunol.Lett.* 63:125-129, 1998

45. Kagi,D, Odermatt,B, Seiler,P, Zinkernagel,RM, Mak,TW, Hengartner,H: Reduced incidence and delayed onset of diabetes in perforin-deficient nonobese diabetic mice. *J.Exp.Med.* 186:989-997, 1997

46. Amrani,A, Verdaguer,J, Anderson,B, Utsugi,T, Bou,S, Santamaria,P: Perforin-independent beta-cell destruction by diabetogenic CD8(+) T lymphocytes in transgenic nonobese diabetic mice. *J.Clin.Invest* 103:1201-1209, 1999

47. Chervonsky,AV, Wang,Y, Wong,FS, Visintin,I, Flavell,RA, Janeway,CA, Jr., Matis,LA: The role of Fas in autoimmune diabetes. *Cell* 89:17-24, 1997

48. Thomas,HE, Darwiche,R, Corbett,JA, Kay,TW: Evidence that beta cell death in the nonobese diabetic mouse is Fas independent. *J.Immunol.* 163:1562-1569, 1999

49. Moriwaki,M, Itoh,N, Miyagawa,J, Yamamoto,K, Imagawa,A, Yamagata,K, Iwahashi,H, Nakajima,H, Namba,M, Nagata,S, Hanafusa,T, Matsuzawa,Y: Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus. *Diabetologia* 42:1332-1340, 1999

50. Pilstrom,B, Bjork,L, Bohme,J: Demonstration of a TH1 cytokine profile in the late phase of NOD insulitis. *Cytokine* 7:806-814, 1995

51. Rabinovitch,A: Cytokines in the pathogenesis of type 1 diabetes. *Canadian Journal of Diabetes Care* 23:40-50, 1999

52. Rabinovitch,A, Suarez-Pinzon,W, El Sheikh,A, Sorensen,O, Power,RF: Cytokine gene expression in pancreatic islet-infiltrating leukocytes of BB rats: expression of Th1 cytokines correlates with beta-cell destructive insulitis and IDDM . *Diabetes* 45:749-754, 1996

53. Seidell,JC: Time trends in obesity: an epidemiological perspective. *Horm.Metab Res.* 29:155-158, 1997

54. Flegal,KM, Carroll,MD, Kuczmarski,RJ, Johnson,CL: Overweight and obesity in the United States: prevalence and trends, 1960-1994. *Int.J.Obes.Relat Metab Disord.* 22:39-47, 1998

55. Mandrup-Poulsen,T, Bendtzen,K, Nerup,J, Egeberg,J, Nielsen,JH: Mechanisms of pancreatic islet cell destruction. Dose-dependent cytotoxic effect of soluble blood mononuclear cell mediators on isolated islets of Langerhans. *Allergy* 41:250-259, 1986

56. Mandrup-Poulsen,T, Bendtzen,K, Nerup,J, Dinarello,CA, Svenson,M, Nielsen,JH: Affinity-purified human interleukin I is cytotoxic to isolated islets of Langerhans. *Diabetologia* 29:63-67, 1986

57. Rabinovitch,A, Suarez-Pinzon,WL: Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochem.Pharmacol.* 55:1139-1149, 1998

58. Rabinovitch,A: Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immunostimulation? *Diabetes* 43:613-621, 1994

59. Jafarian-Tehrani,M, Amrani,A, Homo-Delarche,F, Marquette,C, Dardenne,M, Haour,F: Localization and characterization of interleukin-1 receptors in the islets of Langerhans from control and nonobese diabetic mice. *Endocrinology* 136:609-613, 1995

60. Majumdar,S, Rossi,MW, Fujiki,T, Phillips,WA, Disa,S, Queen,CF, Johnston,RB, Jr., Rosen,OM, Corkey,BE, Korchak,HM: Protein kinase C isotypes and signaling in neutrophils. Differential substrate specificities of a translocatable cal. *J.Biol.Chem.* 266:9285-9294, 1991

61. Hammonds,P, Beggs,M, Beresford,G, Espinal,J, Clarke,J, Mertz,RJ: Insulin-secreting beta-cells possess specific receptors for interleukin- 1 beta. *FEBS Lett.* 261:97-100, 1990

62. Kelpe,CL, Moore,PC, Parazzoli,SD, Wicksteed,B, Rhodes,CJ, Poitout,V: Palmitate inhibition of insulin gene expression is mediated at the transcriptional level via ceramide synthesis. *J.Biol.Chem.* 2003

63. Dinarello,CA: The interleukin-1 family: 10 years of discovery. *FASEB J.* 8:1314-1325, 1994

64. Rissanen,AM, Heliovaara,M, Knekt,P, Reunanen,A, Aromaa,A: Determinants of weight gain and overweight in adult Finns. *Eur.J.Clin.Nutr.* 45:419-430, 1991

65. Wesche,H, Korherr,C, Kracht,M, Falk,W, Resch,K, Martin,MU: The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). *J.Biol.Chem.* 272:7727-7731, 1997

66. Bottazzo,GF, Dean,BM, McNally,JM, MacKay,EH, Swift,PG, Gamble,DR: In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. *N.Engl.J.Med.* 313:353-360, 1985

67. Burns,K, Martinon,F, Esslinger,C, Pahl,H , Schneider,P, Bodmer,JL, Di Marco,F, French,L, Tschopp,J: MyD88, an adapter protein involved in interleukin-1 signaling. *J.Biol.Chem.* 273:12203-12209, 1998

68. Bergman,RN, Finegood,DT, Ader,M: Assessment of insulin sensitivity in vivo. *Endocr.Rev.* 6:45-86, 1985

69. Wesche,H, Henzel,WJ, Shillinglaw,W, Li,S , Cao,Z: MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity.* 7:837-847, 1997

70. Croston,GE, Cao,Z, Goeddel,DV: NF-kappa B activation by interleukin-1 (IL-1) requires an IL-1 receptor- associated protein kinase activity. *J.Biol.Chem.* 270:16514-16517, 1995

71. Clerk,A, Harrison,JG, Long,CS, Sugden,PH : Pro-inflammatory cytokines stimulate mitogen-activated protein kinase subfamilies, increase phosphorylation of c-Jun and ATF2 and upregulate c-Jun protein in neonatal rat ventricular myocytes. *J.Mol.Cell Cardiol.* 31:2087-2099, 1999

72. Bowie,A, O'Neill,LA: The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *J.Leukoc.Biol.* 67:508-514, 2000

73. Beg,AA, Finco,TS, Nantermet,PV, Baldwin,AS, Jr.: Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol.Cell Biol.* 13:3301-3310, 1993

74. Ammendrup,A, Maillard,A, Nielsen,K, Aabenhus,AN, Serup,P, Dragsbaek,MO , Mandrup-Poulsen,T, Bonny,C: The c-Jun amino-terminal kinase pathway is preferentially activated by interleukin-1 and controls apoptosis in differentiating pancreatic beta- cells. *Diabetes* 49:1468-1476, 2000

75. Bonny,C, Oberson,A, Negri,S, Sauser,C, Schorderet,DF: Cell-permeable peptide inhibitors of JNK: novel blockers of beta-cell death. *Diabetes* 50:77-82, 2001

76. Rabuazzo,AM, Buscema,M, Caltabiano,V, Anello,M, Degano,C, Patane,G, Vigneri,R, Purrello,F: Interleukin-1 beta inhibition of insulin release in rat pancreatic islets: possible involvement of G-proteins in the signal transduction pathway. *Diabetologia* 38:779-784, 1995

77. Bagrodia,S, Derijard,B, Davis,RJ, Cerione,RA: Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen- activated protein kinase activation. *J.Biol.Chem.* 270:27995-27998, 1995

78. Palsson,ME, O'Neill,LA: Ras participates in the activation of p38 MAPK by interleukin-1 by associating with IRAK, IRAK2, TRAF6 and TAK-1. *J.Biol.Chem.* 2001

79. Zhang,S, Han,J, Sells,MA, Chernoff,J, Knaus,UG, Ulevitch,RJ, Bokoch,GM: Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. *J.Biol.Chem.* 270:23934-23936, 1995

80. Corbett,JA, Mikhael,A, Shimizu,J, Frederick,K, Misko,TP, McDaniel,ML, Kanagawa,O, Unanue,ER: Nitric oxide production in islets from nonobese diabetic mice: aminoguanidine-sensitive and -resistant stages in the immunological diabetic process. *Proc.Natl.Acad.Sci.U.S.A* 90:8992-8995, 1993

81. Aarnes,M, Schonberg,S, Grill,V: Fatty acids potentiate interleukin-1beta toxicity in the beta-cell line INS-1E. *Biochem.Biophys.Res.Commun.* 296:189-193, 2002

82. Welsh N: Interleukin-1beta induces ceramide and diacylglycerol generation does not lead to NF-kappaBeta activation in RINm5F cells. (Abstract). *Diabetologia* 38 [Suppl1], 1995

83. Sjoholm,A: Ceramide inhibits pancreatic beta-cell insulin production and mitogenesis and mimics the actions of interleukin-1 beta. *FEBS Lett.* 367:283-286, 1995

84. Welsh,N: Interleukin-1 beta-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F. *J.Biol.Chem.* 271:8307-8312, 1996

85. Major,CD, Gao,ZY, Wolf,BA: Activation of the sphingomyelinase/ceramide signal transduction pathway in insulin-secreting beta-cells: role in cytokine-induced beta-cell death. *Diabetes* 48:1372-1380, 1999

86. Ishizuka,N, Yagui,K, Tokuyama,Y, Yamada,K, Suzuki,Y, Miyazaki,J, Hashimoto,N, Makino,H, Saito,Y, Kanatsuka,A: Tumor necrosis factor alpha signaling pathway and apoptosis in pancreatic beta cells. *Metabolism* 48:1485-1492, 1999

87. Hsu,H, Shu,HB, Pan,MG, Goeddel,DV: TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell* 84:299-308, 1996

88. Hsu,H, Xiong,J, Goeddel,DV: The TNF receptor 1-associated protein TRADD signals cell death and NF- kappa B activation . *Cell* 81:495-504, 1995

89. Malinin,NL, Boldin,MP, Kovalenko,AV, Wallach,D: MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature* 385:540-544, 1997

90. Darnell,JE, Jr., Kerr,IM, Stark,GR: Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415-1421, 1994

91. Greenlund,AC, Farrar,MA, Viviano,BL, Schreiber,RD: Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). *EMBO J.* 13:1591-1600, 1994

92. Corbett,JA, Sweetland,MA, Wang,JL, Lancaster,JR, Jr., McDaniel,ML: Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc.Natl.Acad.Sci.U.S.A* 90:1731-1735, 1993

93. Flodstrom,M, Eizirik,DL: Interferon-gamma-induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. *Endocrinology* 138:2747-2753, 1997

94. Dell'Albani,P, Santangelo,R, Torrisi,L, Nicoletti,VG, de Vellis,J, Giuffrida Stella,AM: JAK/STAT signaling pathway mediates cytokine-induced iNOS expression in primary astroglial cell cultures. *J.Neurosci.Res.* 65:417-424, 2001

95. Ho,E, Bray,TM: Antioxidants, NFkappaB activation and Diabetogenesis. *Experimental Biology and Medicine* 222:205-213, 1999

96. Heimberg,H, Heremans,Y, Jobin,C, Leemans,R, Cardozo,AK, Darville,M, Eizirik,DL: Inhibition of cytokine-induced NF-kappaB activation by adenovirus- mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. *Diabetes* 50:2219-2224, 2001

97. Giannoukakis,N, Rudert,WA, Trucco,M, Robbins,PD: Protection of human islets from the effects of interleukin-1beta by adenoviral gene transfer of an Ikappa B repressor. *J.Biol.Chem.* 275:36509-36513, 2000

98. Lenzen,S, Drinkgern,J, Tiedge,M: Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic.Biol.Med.* 20:463-466, 1996

99. Corbett,JA, Kwon,G, Turk,J, McDaniel,ML: IL-1 beta induces the coexpression of both nitric oxide synthase and cyclooxygenase by islets of Langerhans: activation of cyclooxygenase by nitric oxide. *Biochemistry* 32:13767-13770, 1993

100. Cetkovic-Cvrlje,M, Eizirik,DL: TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly via generation of nitric oxide. *Cytokine* 6:399-406, 1994

101. Flodstrom,M, Welsh,N, Eizirik,DL: Cytokines activate the nuclear factor kappa B (NF-kappa B) and induce nitric oxide production in human pancreatic islets. *FEBS Lett.* 385:4-6, 1996

102. Eizirik,DL, Sandler,S, Welsh,N, Cetkovic-Cvrlje,M, Nieman,A, Geller,DA, Pipeleers,DG, Bendtzen,K, Hellerstrom,C: Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *J.Clin.Invest* 93:1968-1974, 1994

103. Eizirik,DL, Flodstrom,M, Karlsen,AE, Welsh,N: The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic beta cells. *Diabetologia* 39:875-890, 1996

104. Alderton,WK, Cooper,CE, Knowles,RG: Nitric oxide synthases: structure, function and inhibition. *Biochem.J.* 357:593-615, 2001

105. Lakey,JR, Suarez-Pinzon,WL, Strynadka,K, Korbutt,GS, Rajotte,RV, Mabley,JG, Szabo,C, Rabinovitch,A: Peroxynitrite is a mediator of cytokine-induced destruction of human pancreatic islet beta cells. *Lab Invest* 81:1683-1692, 2001

106. MacFarlane,AJ, Burghardt,KM, Kelly,J, Simell,T, Simell,O, Altosaar,I, Scott,FW: A type 1 diabetes-related protein from wheat (Triticum aestivum). cDNA clone of a wheat storage globulin, Glb1, linked to islet damage. *J.Biol.Chem.* 278:54-63, 2003

107. Like,AA, Butler,L, Williams,RM, Appel,MC, Weringer,EJ, Rossini,AA: Spontaneous autoimmune diabetes mellitus in the BB rat. *Diabetes* 31:7-13, 1982

108. Yale,JF, Grose,M, Marliss,EB: Time course of the lymphopenia in BB rats. Relation to the onset of diabetes. *Diabetes* 34:955-959, 1985

109. Awata,T, Guberski,DL, Like,AA: Genetics of the BB rat: association of autoimmune disorders (diabetes, insulitis, and thyroiditis) with lymphopenia and major histocompatibility complex class II. *Endocrinology* 136:5731-5735, 1995

110. Woda,BA, Padden,C: BioBreeding/Worcester (BB/Wor) rats are deficient in the generation of functional cytotoxic T cells. *J.Immunol.* 139:1514-1517, 1987

111. Greiner,DL, Handler,ES, Nakano,K, Mordes,JP, Rossini,AA: Absence of the RT-6 T cell subset in diabetes-prone BB/W rats. *J.Immunol.* 136:148-151, 1986

112. Greiner,DL, Mordes,JP, Handler,ES, Angelillo,M, Nakamura,N, Rossini,AA: Depletion of RT6.1+ T lymphocytes induces diabetes in resistant biobreeding/Worcester (BB/W) rats. *J.Exp.Med.* 166:461-475, 1987

113. Burstein,D, Mordes,JP, Greiner,DL, Stein,D, Nakamura,N, Handler,ES, Rossini,AA: Prevention of diabetes in BB/Wor rat by single transfusion of spleen cells. Parameters that affect degree of protection. *Diabetes* 38:24-30, 1989

114. MacMurray,AJ, Moralejo,DH, Kwitek,AE, Rutledge,EA, Van Yserloo,B, Gohlke,P, Speros,SJ, Snyder,B, Schaefer,J, Bieg,S, Jiang,J, Ettinger,RA, Fuller,J, Daniels,TL, Pettersson,A, Orlebeke,K, Birren,B, Jacob,HJ, Lander,ES, Lernmark,A: Lymphopenia in the BB rat model of

type 1 diabetes is due to a mutation in a novel immune-associated nucleotide (Ian)-related gene. *Genome Res.* 12:1029-1039, 2002

115. Cambot,M, Aresta,S, Kahn-Perles,B, de Gunzburg,J, Romeo,PH: Human immune associated nucleotide 1: a member of a new guanosine triphosphatase family expressed in resting T and B cells. *Blood* 99:3293-3301, 2002

116. Sandal,T, Aumo,L, Hedin,L, Gjertsen,BT, Doskeland,SO: Irod/Ian5: an inhibitor of gamma-radia. *Mol.Biol.Cell* 14:3292-3304, 2003

117. Rossini,AA, Handler,ES, Mordes,JP, Greiner,DL: Human autoimmune diabetes mellitus: lessons from BB rats and NOD mice--Caveat emptor. *Clin.Immunol.Immunopathol.* 74:2-9, 1995

118. Harris,MI: Impaired glucose tolerance in the U.S. population. *Diabetes Care* 12:464-474, 1989

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

120. Bjorntorp,P: Regional obesity and NIDDM. *Adv.Exp.Med.Biol.* 334:279-285, 1993

121. Lam,TK, Yoshii,H, Haber,CA, Bogdanovic,E, Lam,L, Fantus,IG, Giacca,A: Free fatty acid-induced hepatic insulin resistance: a potential role for protein kinase C-delta. *Am.J.Physiol Endocrinol.Metab* 283:E682-E691, 2002

122. Griffin,ME, Marcucci,MJ, Cline,GW, Bell,K, Barucci,N, Lee,D, Goodyear,LJ, Kraegen,EW, White,MF, Shulman,GI: Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48:1270-1274, 1999

123. Paolisso,G, Gambardella,A, Tagliamonte,MR, Saccomanno,F, Salvatore,T, Gualdiero,P, D'Onofrio,MV, Howard,BV: Does free fatty acid infusion impair insulin action also through an increase in oxidative stress? *J.Clin.Endocrinol.Metab* 81:4244-4248, 1996

124. Chavez,JA, Knotts,TA, Wang,LP, Li,G, Dobrowsky,RT, Florant,GL, Summers,SA: A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. *J.Biol.Chem.* 278:10297-10303, 2003

125. Kashyap,SR, Belfort,R, Berria,R, Suraamornkul,S, Pratipranawatr,T, Finlayson,J, Barrentine,A, Bajaj,M , Mandarino,L, DeFronzo,R, Cusi,K: Discordant Effects of a Chronic Physiologic Increase in Plasma Free Fatty Acids on Insulin Signaling in Healthy Subjects With or Without a Family History of Type 2 Diabetes. *Am.J.Physiol Endocrinol.Metab* 2004

126. Hotamisligil,GS, Shargill,NS, Spiegelman,BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87-91, 1993

127. Hotamisligil,GS, Peraldi,P, Budavari,A, Ellis,R, White,MF, Spiegelman,BM: IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-a. *Science* 271:665-668, 1996

128. Gao,Z, Hwang,D, Bataille,F, Lefevre,M , York,D, Quon,MJ, Ye,J: Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. *J.Biol.Chem.* 277:48115-48121, 2002

129. Kern,PA, Ranganathan,S, Li,C, Wood,L, Ranganathan,G: Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am.J.Physiol Endocrinol.Metab* 280:E745-E751, 2001

130. Klover,PJ, Zimmers,TA, Koniaris,LG, Mooney,RA: Chronic exposure to interleukin-6 causes hepatic insulin resistance in mice. *Diabetes* 52:2784-2789, 2003

131. Steppan,CM, Bailey,ST, Bhat,S, Brown,EJ , Banerjee,RR, Wright,CM, Patel,HR, Ahima,RS, Lazar,MA: The hormone resistin links obesity to diabetes. *Nature* 409:307-312, 2001

132. Sivitz,WI, Walsh,SA, Morgan,DA, Thomas,MJ, Haynes,WG: Effects of leptin on insulin sensitivity in normal rats. *Endocrinology* 138:3395-3401, 1997

133. Goldfine,AB, Kahn,CR: Adiponectin: linking the fat cell to insulin sensitivity. *Lancet* 362:1431-1432, 2003

134. Shimabukuro,M, Koyama,K, Chen,G, Wang,MY, Trieu,F, Lee,Y, Newgard,CB, Unger,RH: Direct antidiabetic effect of leptin through triglyceride depletion of tissues. *Proc.Natl.Acad.Sci.U.S.A* 94:4637-4641, 1997

135. Minokoshi,Y, Kim,YB, Peroni,OD, Fryer,LG, Muller,C, Carling,D, Kahn,BB: Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415:339-343, 2002

136. Tomas,E, Tsao,TS, Saha,AK, Murrey,HE, Zhang,CC, Itani,SI, Lodish,HF, Ruderman,NB: Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *Proc.Natl.Acad.Sci.U.S.A* 99:16309-16313, 2002

137. Yamauchi,T, Kamon,J, Ito,Y, Tsuchida,A, Yokomizo,T, Kita,S, Sugiyama,T, Miyagishi,M, Hara,K, Tsunoda,M, Murakami,K, Ohteki,T, Uchida,S: Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* 423:762-769, 2003

138. Schernthaner,G, Hink,S, Kopp,HP, Muzyka,B, Streit,G, Kroiss,A: Progress in the characterization of slowly progressive autoimmune diabetes in adult patients (LADA or type 1.5 diabetes). *Exp.Clin.Endocrinol.Diabetes* 109 Suppl 2:S94-108, 2001

139. Groop,LC, Bottazzo,GF, Doniach,D: Islet cell antibodies identify latent type I diabetes in patients aged 35-75 years at diagnosis. *Diabetes* 35:237-241, 1986

140. Zimmet,PZ, Tuomi,T, Mackay,IR, Rowley,MJ, Knowles,W, Cohen,M, Lang,DA: Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabet.Med.* 11:299-303, 1994

141. Niskanen,L, Karjalainen,J, Sarlund,H, Siitonen,O, Uusitupa,M: Five-year follow-up of islet cell antibodies in type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 34:402-408, 1991

142. Carlsson,A, Sundkvist,G, Groop,L, Tuomi,T: Insulin and glucagon secretion in patients with slowly progressing autoimmune diabetes (LADA). *J.Clin.Endocrinol.Metab* 85:76-80, 2000

143. Prentki,M, Vischer,S, Glennon,MC, Regazzi,R, Deeney,JT, Corkey,BE: Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion. *J.Biol.Chem.* 267:5802-5810, 1992

144. Deeney,JT, Gromada,J, Hoy,M, Olsen,HL, Rhodes,CJ, Prentki,M, Berggren,PO, Corkey,BE: Acute stimulation with long chain acyl-CoA enhances exocytosis in insulin-secreting cells (HIT T-15 and NMRI beta-cells). *J.Biol.Chem.* 275:9363-9368, 2000

145. Haber,EP, Ximenes,HM, Procopio,J, Carvalho,CR, Curi,R, Carpinelli,AR : Pleiotropic effects of fatty acids on pancreatic beta-cells. *J.Cell Physiol* 194:1-12, 2003

146. Yaney,GC, Korchak,HM, Corkey,BE: Long-chain acyl CoA regulation of protein kinase C and fatty acid potentiation of glucose-stimulated insulin secretion in clonal beta- cells. *Endocrinology* 141:1989-1998, 2000

147. Deeney,JT, Cunningham,BA, Chheda,S, Bokvist,K, Juntti-Berggren,L, Lam,K, Korchak,HM, Corkey,BE, Berggren,PO: Reversible Ca2+-dependent translocation of protein kinase C and glucose-induced insulin release. *J.Biol.Chem.* 271:18154-18160, 1996

148. Larsson,O, Deeney,JT, Branstrom,R, Berggren,PO, Corkey,BE: Activation of the ATP-sensitive K+ channel by long chain acyl-CoA. A role in modulation of pancreatic beta-cell glucose sensitivity. *J.Biol.Chem.* 271:10623-10626, 1996

149. Bjorntorp,P, Bergman,H, Varnauskas,E: Lipid mobilization in relation to body composition in man. *Metabolism* 18:840, 1969

150. Jensen,MD, Haymond,MW, Rizza,RA, Cryer,PE, Miles,JM: Influence of body fat distribution on free fatty acid metabolism in obesity. *J.Clin.Invest* 83:1168-1173, 1989

151. Kashyap,S, Belfort,R, Gastaldelli,A, Pratipanawatr,T, Berria,R, Pratipanawatr,W, Bajaj,M, Mandarino,L, DeFronzo,R, Cusi,K: A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes. *Diabetes* 52:2461-2474, 2003

152. Crespin,SR, Greenough,WB, III, Steinberg,D: Stimulation of insulin secretion by long-chain free fatty acids. A direct pancreatic effect. *J.Clin.Invest* 52:1979-1984, 1973

153. Dobbins,RL, Chester,MW, Stevenson,BE, Daniels,MB, Stein,DT, McGarry,JD: A fatty acid-dependent step is critically important for both glucose and non-glucose stimulated insulin secretion. *J.Clin.Invest* 101:2370-2376, 1998

154. Zhou,YP, Grill,VE: Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle. *J.Clin.Invest* 93:870-876, 1994

155. Sako,Y, Grill,VE: A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* 127:1580-1589, 1990

156. Zhou,YP, Grill,V: Long term exposure to fatty acids and ketones inhibits B-cell functions in human pancreatic islets of Langerhans. *J.Clin.Endocrinol.Metab* 80:1584-1590, 1995

157. Zhou,YP, Berggren,PO, Grill,V: A fatty acid-induced decrease in pyruvate dehydrogenase activity is an important determinant of beta-cell dysfunction in the obese diabetic db/db mouse. *Diabetes* 45:580-586, 1996

158. Brun,T, Assimacopoulos-Jeannet,F, Corkey,BE, Prentki,M: Long-chain fatty acids inhibit acetyl-CoA carboxylase gene expression in the pancreatic beta-cell line INS-1. *Diabetes* 46:393-400, 1997

159. Assimacopoulos-Jeannet,F, Thumelin,S, Roche,E, Esser,V, McGarry,JD, Prentki,M: Fatty acids rapidly induce the carnitine palmitoyltransferase I gene in the pancreatic beta-cell line INS-1. *J.Biol.Chem.* 272:1659-1664, 1997

160. Magnan,C, Collins,S, Berthault,MF, Kassis,N, Vincent,M, Gilbert,M, Penicaud,L, Ktorza,A, Assimacopoulos-Jeannet,F: Lipid infusion lowers sympathetic nervous activity and leads to increased beta-cell responsiveness to glucose. *J.Clin.Invest* 103:413-419, 1999

161. Boden,G, Chen,X, Rosner,J, Barton,M: Effects of a 48-h fat infusion on insulin secretion and glucose utilization. *Diabetes* 44:1239-1242, 1995

162. Mason,TM, Goh,T, Tchipashvili,V, Sandhu,H, Gupta,N, Lewis,GF, Giacca,A: Prolonged elevation of plasma free fatty acids desensitizes the insulin secretory response to glucose in vivo in rats. *Diabetes* 48:524-530, 1999

163. Paolisso,G, Gambardella,A, Amato,L, Tortoriello,R, D'Amore,A, Varricchio,M, D'Onofrio,F: Opposite effects of short- and long-term fatty acid infusion on insulin secretion in health subjects. *Diabetologia* 38:1295-1299, 1995

164. Carpentier,A, Mittelman,SD, Lamarche,B, Bergman,RN, Giacca,A, Lewis,GF: Acute enhancement of insulin secretion by FFA in humans is lost with prolonged FFA elevation. *Am.J.Physiol* 276:E1055-E1066, 1999

165. Bergman,RN, Phillips,LS, Cobelli,C: Physiologic evaluation of factors controlling glucose tolerance in man: measurement of insulin sensitivity and beta-cell glucose sensitivity from the response to intravenous glucose. *J.Clin.Invest* 68:1456-1467, 1981

166. Kahn,SE, Prigeon,RL, McCulloch,DK, Boyko,EJ, Bergman,RN, Schwartz,MW, Neifing,JL, Ward,WK, Beard,JC, Palmer,JP, .: Quantification of the relationship between insulin sensitivity and beta- cell function in human subjects. Evidence for a hyperbolic function. *Diabetes* 42:1663-1672, 1993

167. Shimabukuro,M, Zhou,YT, Levi,M, Unger,RH: Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes. *Proc.Natl.Acad.Sci.U.S.A* 95:2498-2502, 1998

168. Piro,S, Anello,M, Di Pietro,C, Lizzio,MN, Patane,G, Rabuazzo,AM, Vigneri,R, Purrello,M, Purrello,F: Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: possible role of oxidative stress. *Metabolism* 51:1340-1347, 2002

169. Lupi,R, Dotta,F, Marselli,L, Del Guerra,S, Masini,M, Santangelo,C, Patane,G, Boggi,U, Piro,S, Anello,M, Bergamini,E, Mosca,F, Di Mario,U, Del Prato,S, Marchetti,P: Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated . *Diabetes* 51:1437-1442, 2002

170. Maedler,K, Spinas,GA, Dyntar,D, Moritz,W, Kaiser,N, Donath,MY: Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* 50:69-76, 2001

171. Milburn,JL, Jr., Hirose,H, Lee,YH, Nagasawa,Y, Ogawa,A, Ohneda,M, BeltrandelRio,H, Newgard,CB, Johnson,JH, Unger,RH: Pancreatic beta-cells in obesity. Evidence for induction of functional, morphologic, and metabolic abnormalities by increased long chain fatty acids. *J.Biol.Chem.* 270:1295-1299, 1995

172. Segall,L, Lameloise,N, Assimacopoulos-Jeannet,F, Roche,E, Corkey,P, Thumelin,S, Corkey,BE, Prentki,M: Lipid rather than glucose metabolism is implicated in altered insulin secretion caused by oleate in INS-1 cells. *Am.J.Physiol* 277:E521-E528, 1999

173. Randle,PJ, Kerbey,AL, Espinal,J: Mechanisms decreasing glucose oxidation in diabetes and starvation: role of lipid fuels and hormones. *Diabetes Metab Rev.* 4:623-638, 1988

174. Zhou,YP, Grill,VE: Palmitate-induced beta-cell insensitivity to glucose is coupled to decreased pyruvate dehydrogenase activity and enhanced kinase activity in rat pancreatic islets. *Diabetes* 44:394-399, 1995

175. Liang,Y, Buettger,C, Berner,DK, Matschinsky,FM: Chronic effect of fatty acids on insulin release is not through the alteration of glucose metabolism in a pancreatic beta-cell line (beta HC9). *Diabetologia* 40:1018-1027, 1997

176. Gremlich,S, Bonny,C, Waeber,G, Thorens,B: Fatty acids decrease IDX-1 expression in rat pancreatic islets and reduce GLUT2, glucokinase, insulin, and somatostatin levels. *J.Biol.Chem.* 272:30261-30269, 1997

177. Bollheimer,LC, Skelly,RH, Chester,MW, McGarry,JD, Rhodes,CJ: Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *J.Clin.Invest* 101:1094-1101, 1998

178. Ritz-Laser,B, Meda,P, Constant,I, Klages,N, Charollais,A, Morales,A, Magnan,C, Ktorza,A, Philippe,J: Glucose-induced preproinsulin gene expression is inhibited by the free fatty acid palmitate. *Endocrinology* 140:4005-4014, 1999

179. Furukawa,H, Carroll,RJ, Swift,HH, Steiner,DF: Long-term elevation of free fatty acids leads to delayed processing of proinsulin and prohormone convertases 2 and 3 in the pancreatic beta- cell line MIN6. *Diabetes* 48:1395-1401, 1999

180. Lameloise,N, Muzzin,P, Prentki,M, Assimacopoulos-Jeannet,F: Uncoupling protein 2: a possible link between fatty acid excess and impaired glucose-induced insulin secretion? *Diabetes* 50:803-809, 2001

181. Chan,CB, MacDonald,PE, Saleh,MC, Johns,DC, Marban,E, Wheeler,MB: Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. *Diabetes* 48:1482-1486, 1999

182. Zhang,CY, Baffy,G, Perret,P, Krauss,S, Peroni,O, Grujic,D, Hagen,T, Vidal-Puig,AJ, Boss,O, Kim,YB, Zheng,XX, Wheeler,MB, Shulman,GI, Chan,CB, Lowell,BB: Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell* 105:745-755, 2001

183. Carlsson,C, Borg,LA, Welsh,N: Sodium palmitate induces partial mitochondrial uncoupling and reactive oxygen species in rat pancreatic islets in vitro. *Endocrinology* 140:3422-3428, 1999

184. Maechler,P, Jornot,L, Wollheim,CB: Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells. *J.Biol.Chem.* 274 :27905-27913, 1999

185. Miwa,I, Ichimura,N, Sugiura,M, Hamada,Y , Taniguchi,S: Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. *Endocrinology* 141 :2767-2772, 2000

186. Yan,LJ, Levine,RL, Sohal,RS: Oxidative damage during aging targets mitochondrial aconitase. *Proc.Natl.Acad.Sci.U.S.A* 94:11168-11172, 1997

187. Yakes,FM, Van Houten,B: Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc.Natl.Acad.Sci.U.S.A* 94:514-519, 1997

188. Li,LX, Skorpen,F, Egeberg,K, Jorgensen,IH, Grill,V: Uncoupling protein-2 participates in cellular defense against oxidative stress in clonal beta-cells. *Biochem.Biophys.Res.Commun.* 282:273-277, 2001

189. Tanaka,Y, Gleason,CE, Tran,PO, Harmon,JS, Robertson,RP: Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants. *Proc.Natl.Acad.Sci.U.S.A* 96:10857-10862, 1999

190. Steil,GM, Trivedi,N, Jonas,JC, Hasenkamp,WM, Sharma,A, Bonner-Weir,S, Weir,GC: Adaptation of beta-cell mass to substrate oversupply: enhanced function with normal gene expression. *Am.J.Physiol Endocrinol.Metab* 280:E788-E796, 2001

191. Roche,E, Buteau,J, Aniento,I, Reig,JA, Soria,B, Prentki,M: Palmitate and oleate induce the immediate-early response genes c-fos and nur-77 in the pancreatic beta-cell line INS-1. *Diabetes* 48:2007-2014, 1999

192. Cousin,SP, Hugl,SR, Wrede,CE, Kajio,H, Myers,MG, Jr., Rhodes,CJ: Free fatty acid-induced inhibition of glucose and insulin-like growth factor I-induced deoxyribonucleic acid synthesis in the pancreatic beta-cell line INS-1. *Endocrinology* 142:229-240, 2001

193. Kolesnick,RN, Kronke,M: Regulation of ceramide production and apoptosis. *Annu.Rev.Physiol* 60 :643-665, 1998

194. Shimabukuro,M, Higa,M, Zhou,YT, Wang,MY , Newgard,CB, Unger,RH: Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression. *J.Biol.Chem.* 273:32487-32490, 1998

195. Garcia-Ruiz,C, Colell,A, Mari,M, Morales,A, Fernandez-Checa,JC: Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. *J.Biol.Chem.* 272:11369-11377, 1997

196. Pahan,K, Sheikh,FG, Khan,M, Namboodiri,AM, Singh,I: Sphingomyelinase and ceramide stimulate the expression of inducible nitric-oxide synthase in rat primary astrocytes. *J.Biol.Chem.* 273:2591-2600, 1998

197. Listenberger,LL, Ory,DS, Schaffer,JE: Palmitate-induced apoptosis can occur through a ceramide-independent pathway. *J.Biol.Chem.* 276:14890-14895, 2001

198. Gottlieb,E, Vander Heiden,MG, Thompson,CB: Bcl-x(L) prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis. *Mol.Cell Biol.* 20:5680-5689, 2000

199. Shimabukuro,M, Ohneda,M, Lee,Y, Unger,RH: Role of nitric oxide in obesity-induced beta cell disease. *J.Clin.Invest* 100:290-295, 1997

200. Wrede,C, Dickson,L, Lingohr,M, Briaud,I , Rhodes,C: Fatty Acid and Phorbol Ester-mediated interference of mitogenic signaling via novel protein kinase C isoforms in the pancreatic beta-cells (INS-1). *J.Mol.Endocrinol.* 30:271-286, 2003

201. Atkinson,MA, Maclaren,NK: The pathogenesis of insulin-dependent diabetes mellitus. *N.Engl.J.Med.* 331:1428-1436, 1994

202. Dyrberg,T, Poussier,P, Nakhooda,F, Marliss,EB, Lernmark,A: Islet cell surface and lymphocyte antibodies often precede the spontaneous diabetes in the BB rat. *Diabetologia* 26:159-165, 1984

203. Logothetopoulos,J, Valiquette,N, Madura,E, Cvet,D: The onset and progression of pancreatic insulitis in the overt, spontaneously diabetic, young adult BB rat studied by pancreatic biopsy. *Diabetes* 33:33-36, 1984

204. Ramanathan,S, Poussier,P: BB rat lyp mutation and Type 1 diabetes. *Immunol.Rev.* 184:161-171, 2001

205. Corbett,JA, Wang,JL, Misko,TP, Zhao,W, Hickey,WF, McDaniel,ML: Nitric oxide mediates IL-1 beta-induced islet dysfunction and destruction: prevention by dexamethasone. *Autoimmunity* 15:145-153, 1993

206. Kleemann,R, Rothe,H, Kolb-Bachofen,V, Xie,QW, Nathan,C, Martin,S, Kolb,H: Transcription and translation of inducible nitric oxide synthase in the pancreas of prediabetic BB rats. *FEBS Lett.* 328:9-12, 1993

207. Lindsay,RM, Smith,W, Rossiter,SP, McIntyre,MA, Williams,BC, Baird,JD : N omega-nitro-L-arginine methyl ester reduces the incidence of IDDM in BB/E rats. *Diabetes* 44:365-368, 1995

208. Prasad,K: Oxidative stress as a mechanism of diabetes in diabetic BB prone rats: effect of secoisolariciresinol diglucoside (SDG). *Mol.Cell Biochem.* 209:89-96, 2000

209. Elks,ML: Chronic perifusion of rat islets with palmitate suppresses glucose-stimulated insulin release. *Endocrinology* 133:208-214, 1993

210. Lee,Y, Hirose,H, Ohneda,M, Johnson,JH, McGarry,JD, Unger,RH: Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships. *Proc.Natl.Acad.Sci.U.S.A* 91:10878-10882, 1994

211. Linn,T, Strate,C, Schneider,K: Diet promotes beta-cell loss by apoptosis in prediabetic nonobese diabetic mice. *Endocrinology* 140:3767-3773, 1999

212. Beales,PE, Liddi,R, Giorgini,AE, Signore,A, Procaccini,E, Batchelor,K , Pozzilli,P: Troglitazone prevents insulin dependent diabetes in the non-obese diabetic mouse. *Eur.J.Pharmacol.* 357:221-225, 1998

213. Shimabukuro,M, Koyama,K, Lee,Y, Unger,RH: Leptin- or troglitazone-induced lipopenia protects islets from interleukin 1beta cytotoxicity. *J.Clin.Invest* 100:1750-1754, 1997

214. Giacca,A, Fisher,SJ, Shi,ZQ, Gupta,R, Lickley,HL, Skottner,A, Anderson,GH, Efendic,S, Vranic,M: Insulin-like growth factor-I and insulin have no differential effects on glucose production and utilization under conditions of hyperglycemia. *Endocrinology* 134:2251-2258, 1994

215. Bellmann,K, Hui,L, Radons,J, Burkart,V, Kolb,H: Low stress response enhances vulnerability of islet cells in diabetes-prone BB rats. *Diabetes* 46:232-236, 1997

216. Maedler,K, Sergeev,P, Ris,F, Oberholzer,J, Joller-Jemelka,HI, Spinas,GA, Kaiser,N, Halban,PA, Donath,MY: Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets . *J.Clin.Invest* 110:851-860, 2002

217. Maedler,K, Spinas,GA, Lehmann,R, Sergeev,P, Weber,M, Fontana,A, Kaiser,N, Donath,MY: Glucose induces beta-cell apoptosis via upregulation of the Fas receptor in human islets. *Diabetes* 50:1683-1690, 2001

218. Man,ZW, Zhu,M, Noma,Y, Toide,K, Sato,T, Asahi,Y, Hirashima,T, Mori,S, Kawano,K, Mizuno,A, Sano,T, Shima,K: Impaired beta-cell function and deposition of fat droplets in the pancreas as a consequence of hypertriglyceridemia in OLETF rat, a model of spontaneous NIDDM. *Diabetes* 46:1718-1724, 1997

219. Melov,S, Coskun,P, Patel,M, Tuinstra,R, Cottrell,B, Jun,AS, Zastawny,TH, Dizdaroglu,M, Goodman,SI, Huang,TT, Miziorko,H, Epstein,CJ, Wallace,DC: Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc.Natl.Acad.Sci.U.S.A* 96:846-851, 1999

220. Melov,S: Mitochondrial oxidative stress. Physiologic consequences and potential for a role in aging. *Ann.N.Y.Acad.Sci.* 908:219-225, 2000

221. Tiedge,M, Lortz,S, Drinkgern,J, Lenzen,S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733-1742, 1997

222. Kaneto,H, Fujii,J, Seo,HG, Suzuki,K, Matsuoka,T, Nakamura,M, Tatsumi,H, Yamasaki,Y, Kamada,T, Taniguchi,N: Apoptotic cell death triggered by nitric oxide in pancreatic beta-cells. *Diabetes* 44:733-738, 1995

223. Ihara,Y, Toyokuni,S, Uchida,K, Odaka,H, Tanaka,T, Ikeda,H, Hiai,H, Seino,Y, Yamada,Y: Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes. *Diabetes* 48:927-932, 1999

224. Suarez-Pinzon,WL, Strynadka,K, Rabinovitch,A: Destruction of rat pancreatic islet beta-cells by cytokines involves the production of cytotoxic aldehydes. *Endocrinology* 137:5290-5296, 1996

225. Rabinovitch,A, Suarez-Pinzon,WL, Strynadka,K, Lakey,JR, Rajotte,RV: Human pancreatic islet beta-cell destruction by cytokines involves oxygen free radicals and aldehyde production. *J.Clin.Endocrinol.Metab* 81:3197-3202, 1996

226. Kaneto,H, Kajimoto,Y, Miyagawa,J, Matsuoka,T, Fujitani,Y, Umayahara,Y, Hanafusa,T, Matsuzawa,Y, Yamasaki,Y, Hori,M: Beneficial effects of antioxidants in diabetes: possible protection of pancreatic beta-cells against glucose toxicity. *Diabetes* 48:2398-2406, 1999

227. Tanaka,Y, Gleason,CE, Tran,PO, Harmon,JS, Robertson,RP: Prevention of glucose toxicity in HIT-T15 cells and Zucker diabetic fatty rats by antioxidants. *Proc.Natl.Acad.Sci.U.S.A* 96:10857-10862, 1999

228. Tanaka,Y, Tran,PO, Harmon,J, Robertson,RP: A role for glutathione peroxidase in protecting pancreatic beta cells against oxidative stress in a model of glucose toxicity. *Proc.Natl.Acad.Sci.U.S.A* 99:12363-12368, 2002

229. Lortz,S, Tiedge,M, Nachtwey,T, Karlsen,AE, Nerup,J, Lenzen,S: Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. *Diabetes* 49:1123-1130, 2000

230. Bustamante,J, Alonso,FJ, Lobo,MV, Gine,E, Tamarit-Rodriguez,J, Solis,JM, Martin,dR: Taurine levels and localization in pancreatic islets. *Adv.Exp.Med.Biol.* 442:65-69, 1998

231. Tchoumkeu-Nzouessa,GC, Rebel,G: Characterization of taurine transport in human glioma GL15 cell line: regulation by protein kinase C. *Neuropharmacology* 35:37-44, 1996

232. Ramamoorthy,S, Leibach,FH, Mahesh,VB, Han,H, Yang-Feng,T, Blakely,RD: Functional characterization and chromosomal localization of a cloned taurine transporter from human placenta. 300 ( Pt 3):893-900, 1994

233. Jhiang,SM, Fithian,L, Smanik,P, McGill,J, Tong,Q, Mazzaferri,EL: Cloning of the human taurine transporter and characterization of taurine uptake in thyroid cells. *FEBS Lett.* 318:139-144, 1993

234. Shimizu,M, Satsu,H: Physiological significance of taurine and the taurine transporter in intestinal epithelial cells. *Amino.Acids* 19:605-614, 2000

235. Bustamante,J, Lobo,MV, Alonso,FJ, Mukala,NT, Gine,E, Solis,JM, Tamarit-Rodriguez,J, Martin,dR: An osmotic-sensitive taurine pool is localized in rat pancreatic islet cells containing glucagon and somatostatin. *Am.J.Physiol Endocrinol.Metab* 281:E1275-E1285, 2001

236. Hansen,SH: The role of taurine in diabetes and the development of diabetic complications. *Diabetes Metab Res.Rev.* 17:330-346, 2001

237. Devamanoharan,PS, Ali,AH, Varma,SD: Oxidative stress to rat lens in vitro: protection by taurine. *Free Radic.Res.* 29:189-195, 1998

238. Meyerhoff,WL, Paparella,MM, Gudbrandsson,FK: Clinical evaluation of Meniere's disease. *Laryngoscope* 91:1663-1668, 1981

239. Wang,X, Li,H, De Leo,D, Guo,W, Koshkin,V, Fantus,IG, Giacca,A, Chan,CB, Der,S, Wheeler,MB: Gene and protein kinase expression profiling of reactive oxygen species-associated lipotoxicity in the pancreatic beta-cell line MIN6. *Diabetes* 53:129-140, 2004

240. Liu,Y, Tonna-DeMasi,M, Park,E, Schuller-Levis,G, Quinn,MR: Taurine chloramine inhibits production of nitric oxide and prostaglandin E2 in activated C6 glioma cells by suppressing inducible nitric oxide synthase and cyclooxygenase-2 expression. *Brain Res.Mol.Brain Res.* 59:189-195, 1998

241. Kanayama,A, Inoue,J, Sugita-Konishi,Y, Shimizu,M, Miyamoto,Y: Oxidation of Ikappa Balpha at methionine 45 is one cause of taurine chloramine-induced inhibition of NF-kappa B activation. *J.Biol.Chem.* 277:24049-24056, 2002

242. Blackwell,TS, Blackwell,TR, Holden,EP, Christman,BW, Christman,JW: In vivo antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. *J.Immunol.* 157:1630-1637, 1996

243. Bergamini,S, Rota,C, Canali,R, Staffieri,M, Daneri,F, Bini,A, Giovannini,F, Tomasi,A, Iannone,A: N-acetylcysteine inhibits in vivo nitric oxide production by inducible nitric oxide synthase. *Nitric.Oxide.* 5:349-360, 2001

244. Jiang,B, Brecher,P: N-Acetyl-L-cysteine potentiates interleukin-1beta induction of nitric oxide synthase : role of p44/42 mitogen-activated protein kinases. *Hypertension* 35:914-918, 2000

245. Porter,DW, Martin,WG: Taurine regulation of Ca2+ uptake and (Ca(2+)+Mg2+)-ATPase in developing chick B-cells. *Comp Biochem.Physiol Comp Physiol* 106:309-312, 1993

246. Punna,S, Ballard,C, Hamaguchi,T, Azuma,J, Schaffer,S: Effect of taurine and methionine on sarcoplasmic reticular Ca2+ transport and phospholipid methyltransferase activity. *J.Cardiovasc.Pharmacol.* 24:286-292, 1994

247. Kirchner,A, Breustedt,J, Rosche,B, Heinemann,UF, Schmieden,V: Effects of taurine and glycine on epileptiform activity induced by removal of Mg2+ in combined rat entorhinal cortex-hippocampal slices. *Epilepsia* 44:1145-1152, 2003

248. Park,EJ, Bae,JH, Kim,SY, Lim,JG, Baek,WK, Kwon,TK, Suh,SI, Park,JW, Lee,IK, Ashcroft,FM, Song,DK: Inhibition of ATP-sensitive K+ channels by taurine through a benzamido-binding site on sulfonylurea receptor 1. *Biochem.Pharmacol.* 67:1089-1096, 2004

249. Amos,AF, McCarty,DJ, Zimmet,P: The rising global burden of diabetes and its complications: estimates and projections to the year 2010. *Diabet.Med.* 14 Suppl 5:S1-85, 1997

250. Kashyap,S, Belfort,R, Gastaldelli,A, Pratipanawatr,T, Berria,R, Pratipanawatr,W, Bajaj,M, Mandarino,L, DeFronzo,R, Cusi,K: A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes. *Diabetes* 52:2461-2474, 2003

251. Johansson,C, Samuelsson,U, Ludvigsson,J: A high weight gain early in life is associated with an increased risk of type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 37:91-94, 1994

252. Pozzilli,P, Visalli,N, Cavallo,MG, Signore,A, Baroni,MG, Buzzetti,R, Fioriti,E, Mesturino,C, Fiori,R, Romiti,A, Giovannini,C, Lucentini,L, Matteoli,MC, Crino,A, Teodonio,C, Paci,F, Amoretti,R, Pisano,L, Suraci,C, Multari,G, Suppa,M, Sulli,N , De Mattia,G, Faldetta,MR, Suraci,MT: Vitamin E and nicotinamide have similar effects in maintaining residual beta cell function in recent onset insulin-dependent diabetes (the IMDIAB IV study). *Eur.J.Endocrinol.* 137:234-239, 1997

253. Ludvigsson,J, Samuelsson,U, Johansson,C, Stenhammar,L: Treatment with antioxidants at onset of type 1 diabetes in children: a randomized, double-blind placebo-controlled study . *Diabetes Metab Res.Rev.* 17:131-136, 2001

254. Mayer-Davis,EJ, Costacou,T, King,I, Zaccaro,DJ, Bell,RA: Plasma and dietary vitamin E in relation to incidence of type 2 diabetes: The Insulin Resistance and Atherosclerosis Study (IRAS). *Diabetes Care* 25:2172-2177, 2002

255. Yusuf,S, Gerstein,H, Hoogwerf,B, Pogue,J, Bosch,J, Wolffenbuttel,BH , Zinman,B: Ramipril and the development of diabetes. *JAMA* 286 :1882-1885, 2001