EXERCISE TRAINING ATTENUATES PANCREATIC $\beta$-CELL DECOMPENSATION AND HEPATIC INFLAMMATION IN THE MALE ZUCKER DIABETIC FATTY RAT

By

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A thesis submitted in conformity with the requirements for the Doctor of Philosophy Degree Program.

Department of Physiology

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ABSTRACT

TITLE: EXERCISE TRAINING ATTENUATES PANCREATIC $\beta$-CELL DECOMPENSATION AND HEPATIC INFLAMMATION IN THE MALE ZUCKER DIABETIC FATTY RAT


We hypothesized that with exercise training and the subsequent attenuation of hyperglycemia, $\beta$-cell adaptation to worsening insulin resistance would be maintained. Also, because classical stress-activated systems and oxidative stress are involved in hepatic insulin resistance we examined if exercise would be associated with improvements in hepatic markers of oxidative stress and inflammation.

Exercise maintained fasted hyperinsulinemia and preserved normoglycemia in male Zucker diabetic fatty (ZDF) rats. $\beta$-cell function calculations indicate prolonged $\beta$-cell adaptation in exercised animals. Such improved $\beta$-cell function was associated with increased $\beta$-cell mass. Hypertrophy and replication contributed to expansion of $\beta$-cell mass; exercised animals had increased $\beta$-cell size and bromodeoxyuridine (BrdU) incorporation rates versus controls. Furthermore, we observed augmented $\beta$-cell-specific immunohistochemical staining of GLUT2 and Akt/PKB in exercised versus sedentary controls.

We also observed large cytoplasmic ubiquitinated structures which form in response to oxidative stress in pancreatic tissue samples from hyperglycemic ZDF rats. In the exercised groups such aggregate numbers were reduced to numbers compared to those seen in younger non-diabetic basal ZDF animals and age-matched lean Zucker rats.

With respect to the liver we investigated whether exercise alters kinases such as c-Jun NH$_2$-terminal kinase (JNK) and IκK$\beta$ (as evidenced by IκB$\alpha$ levels) and related insulin
receptor substrate-1 (IRS-1) serine phosphorylation which are associated with hepatic insulin resistance in obesity. On average, exercised animals ran 5250m/day which improved insulin sensitivity based on the homeostasis model assessment for insulin resistance (HOMA-IR) calculations, and maintained fed and fasted glucoregulation and glucose tolerance. Ten weeks of running decreased whole-body markers of inflammation and oxidative stress in the blood and in the liver. Exercise lowered circulating interleukin-6 (IL-6), haptoglobin, malondialdehyde (MDA) levels, and protein oxidation in the liver. Exercise reduced phosphorylated JNK (pJNK) indicating decreased JNK activity; in accordance serine phosphorylated IRS-1 was reduced in exercised rats.

In conclusion, improvements in glucoregulation were associated with increased β-cell compensation at least in part due to a reduction in oxidative stress. Furthermore, we show exercise attenuates development of hyperglycemia in ZDF rats in association with decreases in plasma and hepatic markers of inflammation, oxidative stress, JNK activation, and serine phosphorylation of IRS-1.
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Special thanks go to my parents for their support and understanding. My father has shown me over the years how to solve a problem and to enjoy the process. My mother has shown me how to identify one. Dad/mom, I simply would not be where I am now; if it weren’t for your contagious keenness, active minds, and support in every sense of the word. Lastly I would like to dedicate this opus to my sister Sarah and the late Jess Debeck—They’re the reason I started this.
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1.1 The Epidemiology and Etiology of Type 2 Diabetes Mellitus

Obesity is a heritable disease which is defined as one having a body mass index (BMI) of $\geq 30$ kg/m$^2$ (1). This rapidly growing metabolic disorder has reached epidemic proportions in industrialized and developing countries (403; 491; 633), and in particular in the United States (99). For example, in the late 1990’s, 50% adults were overweight and 25% were obese in United States (514). Approximately 70% of the individual variance in BMI in obese people is related to genetically pre-determined factors each contributing its own predisposing risk and each modified by environmental factors (425; 829). For these reasons it is more accurate to think of obesity as the result of interactions between obesity susceptibility genes and an obesogenic environment such as physical inactivity and readily available high-fat foods (725).

T2DM is its own chronic metabolic disorder caused by deficient insulin production and/or secretion, or by the ineffectiveness of the insulin produced. T2DM accounts for 90-95% of diabetic cases (438; 633; 836). The cause of T2DM is unknown, but is characterized by obesity, a progressive decline in insulin sensitivity, and eventual insulin insufficiency leading to hyperglycemia.

It is now estimated that the number of people with T2DM affects 16-17 million people in the United States alone, while an equal number are thought to be pre-diabetic (99; 491). The numbers pertaining to the global population are equally disconcerting. In industrialized countries alone, the World Health Organization projects that the number will increase by 170% between 1995 and 2025 from 84 to 228 million people (829). To make matters
worse, the disease has begun to affect children (543; 544) and people in developing countries (425; 829). To put this in perspective; 189 million adults were estimated to have T2DM in the year 2003 (403; 862), but by 2025, the projected total number of persons will rise to 300 million (403; 862).

Obesity is the major etiological factor in T2DM because it contributes to insulin resistance (95; 235; 358; 701; 836). Studies of various populations indicate a strong association between the prevalence of T2DM and mean percentage standard weight or BMI (808), such that ~80% of type 2 diabetic patients are obese (56). Furthermore, cross-sectional studies have established that duration and degree of obesity is a risk factor for T2DM (810). The risk of T2DM is twice as high for those who have been obese for 10 years compared to those who have been clinically defined as obese for less than 5 years (191). Visceral fat is most strongly associated with progression to T2DM as people with this disease often have a more central distribution of body fat (767) and manifest greater body fat at the waist relative to the hips (273).

Normally, insulin signaling involves insulin binding and activation of tyrosine kinase activity of its cell-surface receptor (652). When the receptor kinase is activated, tyrosine residues on cytosolic protein substrates are phosphorylated (652) which signal other proteins that serve as “adapter” molecules forming intracellular signaling protein complexes (652). Insulin signaling facilitates the movement of glucose across the cell membrane by translocation of glucose transporter-4 (GLUT4) to the plasma membrane (452) which allows entry of glucose into the cell and facilitates the storage of glucose as glycogen, glycolysis to lactate, and mitochondrial oxidation to form energy (652). In this way insulin action directly and indirectly affects glucose and lipid metabolism. Insulin resistance
describes a defect in one or more of these normal actions of insulin, and can be broken
down into peripheral and central perturbations. Peripherally, insulin resistance is
manifested as impaired glucose and lipid uptake at the adipocyte and impaired glucose
uptake and storage in skeletal muscle (586) and is ultimately the cause of defective insulin
signaling and GLUT4 translocation to the sarcolemma (586). Central insulin resistance is
associated with dysfunctional counter regulatory hormonal action, which exert their effects
primarily on the liver and pancreas, resulting in increased hepatic gluconeogenesis and
glycogenolysis (324). Insulin resistance may occur at the level of the insulin receptor or at
the level of any of the downstream pathways in insulin target tissues (235; 290; 829). The
result is a decreased glucose disposal into muscle and adipose tissue at a given insulin
concentration as well as increased hepatic glucose output and overt hyperglycemia (290;
470).

Figure 1. Pathogenesis of T2DM (Adapted from (425)).
There are two general mechanisms that associate excessive adiposity with insulin resistance. Excessive lipids in the body provide one mechanism linking obesity to insulin resistance by a process called lipotoxicity (235). In obese individuals, there are elevated plasma free fatty acids (FFA) that are released from adipose tissue (95). With chronic elevations in FFA, non-adipocyte depots, such as liver and muscle, become laden with triglycerides (TG) which work in concert with elevated plasma FFA to inhibit insulin action (95; 235). Therefore, fat accumulation in liver and muscle mediates obesity-induced insulin resistance (95; 235). The second mechanism that associates obesity to insulin resistance involves the secretion of peptides, complement factors, and cytokines into circulation by adipocytes and/or adipocyte/macrophage consortiums (235). Adipose tissue releases inflammatory mediators such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) which increase insulin resistance (235). The secretion of these factors depends on overall adiposity and more importantly the distribution of fat depots; visceral or intra-abdominal adipose tissue contributes more to the adverse effects of excessive adiposity than subcutaneous adipose tissue (235).

Obese insulin resistant individuals do not progress to T2DM because their β-cells function normally to secrete adequate amounts of insulin (95). However, a small proportion or approximately 33%, of insulin resistant individuals cannot secrete sufficient insulin as their insulin resistance worsens (95); these people eventually develop T2DM. Therefore the main attribute that differentiates obese individuals from those with T2DM is β-cell function (357).

Following is a review of how obesity and T2DM lead to pancreatic β-cell decompensation and hepatic insulin resistance. More specifically, the purpose of the review is to outline the
pathogenesis of T2DM specifically as it pertains to the pancreatic β-cell, while focusing on topics of obesity, hepatic insulin resistance, inflammation, β-cell adaptation, and exercise.

1.2 Insulin Signaling

Insulin signaling takes place once insulin is carried to tissues expressing the membrane receptor for insulin. The insulin receptor belongs to a family of growth factor receptors with intrinsic tyrosine kinase activity (452). Signaling by this molecule requires the participation of many cellular intermediates which belong to the insulin signaling cascade. In normal, healthy individuals, insulin signaling brings about intracellular tyrosine kinase activity and autophosphorylation of the receptor (452). Once the receptor phosphorylates itself by intrinsic processes, the insulin receptor substrate (IRS) is phosphorylated on tyrosine residues (452). It is these phosphorylated tyrosine residues on IRS complexes that then serve as active sites whereby other signaling proteins involved in the insulin signaling cascade are bound (452). In this way, tyrosine phosphorylated IRS complexes then associate with the next player in the insulin signaling cascade which is phosphatidylinositol-3 kinase (PI3K). PI3K is an SH₂ domain-characterized lipid kinase that is able to phosphorylate phosphotidylinositol phosphate-2 to phosphotidylinositol phosphate-3 which then, along with phosphoinositide-dependent kinase, activates Akt/protein kinase B (PKB) and protein kinase C (PKC) (452). These activated molecules then go on to stimulate the translocation of GLUT4 from intracellular pools to the cell surface (651; 652). This signaling pathway not only plays a role in the entry of glucose into the cell but also increases glycogen synthesis and decreases glycogenolysis via inactivation of glycogen synthase kinase 3 (GSK3) (143) and activation of protein phosphatase-1 in an Akt/PKB dependent pathway (70). This complex process describes the sequence of events whereby insulin regulates glucose and lipid metabolism, facilitates the movement of glucose across cellular
membranes by increasing GLUT4 content at the membrane (227), and governs intracellular glucose metabolism (452).

1.3 Insulin Resistance

Glucose clearance is reduced by more than 55% in patients with T2DM (413). *In vivo* studies indicate this reduction in glucose clearance is due to decreases in cellular insulin receptor numbers and post-binding defects in the insulin signaling cascade (413). Interestingly differences in glucose tolerance that exist between obese individuals with and without T2DM are not due to reductions in total GLUT4 protein content as mRNA and total protein content for this transporter is normal in T2DM (226; 574). Insulin-stimulated translocation of GLUT4 to the plasma membrane, however, is decreased in individuals with T2DM (385). Such differences indicate there are defects with respect to the insulin signaling cascade in terms of normal tyrosine phosphorylation and/or protein expression of insulin signaling intermediates. *In vitro* studies of monocytes (413), erythrocytes (165), and adipocytes (551) do in fact indicate a reduced number of insulin receptors, but the key regulator of insulin resistance is perturbation to normal tyrosine phosphorylation of the insulin receptor and/or IRS at serine/threonine sites (4; 5; 221; 222; 570). Multiple hormones and metabolites have been implicated in this process including TNFα and FFA and c-jun-NH2-terminal kinase (JNK). In adipose and muscle tissue, elevated FFAs result in increased fat oxidation relative to glucose oxidation, leading to reduced insulin-stimulated muscle glucose disposal (56). Recent studies of the role of FFA in insulin resistance in skeletal muscle tissue have proposed that FFA act by inhibiting insulin signaling (58) which results in reduced muscle glycogen synthesis (58). It has also been suggested that elevated plasma FFA increase membrane-bound protein kinase C-θ (PKCθ) which then stimulates the serine/threonine kinase signaling cascade, resulting in IRS-1/IRS-2
serine/threonine phosphorylation and corresponding decreases in tyrosine phosphorylation (58). Decreased IRS-1 tyrosine phosphorylation suppresses PI3K activity which decreases GLUT4 translocation, reducing glucose uptake (58). In people with insulin resistance, and to a much greater extent people with T2DM, there is a delayed reduction in hepatic glucose production (198; 379) following glucose ingestion. Increased hepatic glucose production present in patients with T2DM is attributed to impairment of insulin and glucose to suppress hepatic glucose release (198; 379).

1.4 Glucose and Lipid Metabolism during Insulin Resistance

Glucocorticoids and catecholamines are the two primary catabolic stress hormones (716). In obesity and T2DM, glucocorticoid and catecholamine levels are high, leading to elevated FFA, TG and glucose in the blood (364; 468). As well as mobilizing substrate for gluconeogenesis, glucocorticoids decrease peripheral glucose uptake (540) in order to spare blood glucose for utilization by the brain (555). The hormones play a role in energy balance and homeostasis by influencing a number of metabolic pathways such as hepatic glucose production. They increase hepatic glucose production (gluconeogenesis) by stimulating release of gluconeogenic amino acids from peripheral tissues (555), by activating glucose-6-phosphatase (G6Pase) (555), and inducing gene transcription of phosphoenolpyruvate carboxykinase (PEPCK) (480; 555). Stress hormones inhibit glycogen synthesis by inactivating glycogen synthase (61; 431) and activating glycogen phosphorylase (61). Glucocorticoids also promote adipose tissue lipolysis by enhancing the sensitivity of target tissues to catecholamines (555) thus regulating plasma and tissue lipid concentrations. Despite the catabolic actions of glucocorticoids and catecholamines, such stress hormones have been implicated in the development of visceral obesity (468) as increased circulating levels of FFA and TG from subcutaneous adipocytes are redistributed.
throughout the body and in the presence of glucocorticoids, saturate visceral fat cells (455; 468; 836).

Intracellular lipid metabolism is under the control of enzymes such as acetyl CoA carboxylase (ACC), malonyl-CoA (MCoA) and carnitine palmitoyl-transferase-1 (CPT-1). CPT-1 is responsible for the shuttling of FFA into mitochondria where they are subsequently β-oxidized (499). Inhibition of CPT-1 and reduced FFA β-oxidation occurs when ACC is activated and increases the expression and activity of MCoA which is an allosteric inhibitor of CPT-1 (499). When proper rates of β-oxidation are inhibited by MCoA and reductions in CPT-1, cytosolic FFA derivatives accumulate (170). Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is involved in this process as it is activated in response to a number of signals including hypoxia and decreased adenosine triphosphate (ATP)/(AMP) ratios, both of which are common during exercise (264; 384). When AMPK is activated, ACC is inhibited and CPT-1 activity is restored through decreased MCoA levels resulting in increased fat β-oxidation (817; 818; 826). Increased AMPK activity is associated with increased hepatic insulin action stemming primarily from decreased hepatic lipid accumulation (134; 321; 834). Recently, acute inhibition of liver ACC1 and ACC2 was found to reverse high-fat diet-induced hepatic steatosis and hepatic insulin resistance in rats (658). Further supporting the role of ACC in lipid metabolism is the fact that adiponectin and 5-aminoimidazole-4-carboxamide riboside (AICAR) are effective to activate AMPK, decreasing fat content and increasing insulin sensitivity which results in improved hepatic insulin sensitivity (134; 321; 834).
1.5 Obesity, Free Fatty Acids, and Inflammation in Insulin Resistance

Obesity and T2DM are now recognized as diseases characterized by a state of low-grade chronic inflammation and are both accompanied by the acute-phase response and the production of C-reactive protein (CRP) and haptoglobin (579). The production and secretion of these acute-phase proteins is stimulated by pro-inflammatory cytokines such as TNFα, interleukin-1β (IL-1β), and IL-6 (11; 12; 168). In conditions of chronic low-grade systemic inflammation a 2-3 fold increase in concentrations of TNFα, IL-1β, IL-6 (138; 168; 93; 566), interleukin-1 receptor antagonist (IL-1ra) (171), soluble TNF receptor (sTNF-R) (90; 91; 116) and CRP (29) are reported.

TNFα and IL-6 are released from expanded adipose tissue and co-localized macrophages (138; 311) and cause insulin resistance (579; 778). TNFα increases in proportion to obesity (389) and patients with T2DM demonstrate high levels of TNFα in skeletal muscle (647) and plasma (197; 507; 819-822). TNFα affects insulin signaling (260; 305-311; 576) and glucose uptake (579; 843) which is attenuated by anti-TNFα treatment (67; 68) in rats. TNFα has been shown to impair insulin-stimulated rates of glucose storage in cultured human muscle cells (260) and to impair insulin-mediated glucose uptake in rats (843). Also, obese mice with a gene knockout (KO) for TNFα (766) show improved insulin sensitivity. The mechanism whereby TNFα causes insulin resistance ultimately involves the serine phosphorylation of IRS-1 at serine residue 307 (309). Additionally it has been shown that TNFα disturbs insulin signaling possibly by decreasing IRS-1 and GLUT4 expression and by increasing intracellular calcium concentrations (714).

In vivo, TNFα causes insulin resistance independently by increasing release of FFA from adipose tissue (69; 228; 250; 699; 700), as TNFα increases lipolysis in human (641; 851),...
rat (69; 228; 250), and 3T3-L1 adipocytes (542; 607; 699; 700). Recently it has also been shown that TNFα increased FFA incorporation into diacylglycerol (DAG), which is involved in the development of skeletal muscle insulin resistance (84). Currently there is discussion that TNFα may mediate these affects through its potent action to stimulate JNK (466; 776; 811).

Chronic elevations in circulating markers of inflammation such as CRP, haptoglobin, and TNFα occur both in obesity and T2DM (151). Like TNFα, IL-6 secretion is increased by the adipose tissues of obese subjects (115; 206; 512; 751). TNFα and IL-1β stimulate the production of IL-6 and a linear relationship between plasma levels of TNFα and IL-6 is reported (579). The function of IL-6 remains elusive as it has both pro-inflammatory and anti-inflammatory functions. For example, IL-6 exerts inhibitory effects on TNFα and IL-1β production (744; 745). Levels of TNFα are markedly elevated in anti-IL-6-treated mice and in IL-6 KO mice (496; 509), indicating that circulating IL-6 is involved in the regulation of TNFα levels. In addition, recombinant human (rh) IL-6 infusion inhibits the endotoxin-induced increase in circulating levels of TNFα in healthy humans (707). The anti-inflammatory effects of IL-6 are also demonstrated by the fact that IL-6 stimulates the production of IL-1ra and interleukin-10 (IL-10) which are anti-inflammatory cytokines (710). Furthermore, IL-6 but not IL-1β and TNFα, (744) stimulates the release of sTNF-R, and appears to be the primary inducer of the hepatocyte-derived acute-phase proteins, many of which have anti-inflammatory properties (11).

IL-6 seems to have primarily an anti-inflammatory effect. However; the role of IL-6 in insulin resistance and glucoregulation is unclear, to say the least. IL-6 increases hepatic glucose production and secretion of insulin, glucagon and cortisone, and inhibits insulin signal
transduction in hepatocytes (847). For example individuals with elevated levels of IL-6 and IL-1β are at increased risk of developing T2DM (702; 703). In vitro studies demonstrate that IL-6 can induce insulin resistance in isolated 3T3-L1 adipocytes (634) and in mice (394). Other human studies show patients with T2DM given rhIL-6 respond with decreased insulin levels comparable with that in age and body mass index-matched healthy controls, indicating that IL-6 enhances insulin sensitivity (580). IL-6 also enhances lipolysis (84; 534; 569; 580; 718) and lipid β-oxidation. In one study IL-6 increased fat oxidation (palmitate) in L6 myotubes comparably to AICAR, a compound known to increase lipid oxidation (580). In accordance, Wallenius et al. (788) demonstrated that IL-6 KO mice developed mature-onset obesity, insulin resistance and impaired glucose tolerance. In addition, when mice were treated with IL-6, there was a significant decrease in body fat and insulin resistance in the IL-6 KO but not in the wild-type mice. In short, circulating IL-6 levels may (35; 36; 588) or may not (109; 581) be associated with insulin resistance.

Elevated levels of circulating FFA in obesity can activate inflammatory and/or stress-activated pathways in the liver (57) and skeletal muscle (328). Boden et al. showed that hepatic insulin resistance caused by short-term lipid infusion is also associated with activation of the IKKβ/NF-κB inflammatory pathway (57). In humans, FFA-induced muscle insulin resistance was found to be associated with increased muscle PKCδ and PKCβII plasma membrane translocation, and decreased muscle IκBα, an indication of increased IKKβ activation (328). Further evidence for the role of FFA levels in the induction of inflammatory processes comes from studies investigating the effects of administration of salicylate which is an IKKβ inhibitor. Salicylate at high doses prevents insulin resistance caused by lipid infusion in rat skeletal muscle (396). Moreover, Yuan et al. demonstrated that high-dose aspirin and salicylate dramatically improved insulin sensitivity in Zucker fatty
(ZF) rats (846), while heterozygous deletion of IKKβ protected mice from developing insulin resistance due to lipid infusion (396), high-fat feeding, or genetic (ob/ob) obesity (846).

1.6 Oxidative Stress in Insulin Resistance

Reactive oxygen species (ROS) are highly reactive atoms and molecules that form in response to oxidative stress; defined as a persistent imbalance between the production of ROS and antioxidant defenses (190). It has been shown that oxidative stress is involved in the progression of insulin resistance as well as pancreatic β-cell dysfunction (189; 190). Oxidative stress has been shown to disrupt IRS-1, PI3K and GLUT4 translocation in 3T3-L1 adipocytes (746). ROS are elevated in diabetic patients (38; 539) and antioxidants can prevent toxic effects of high glucose and lipid levels on insulin sensitivity (258; 332; 367; 476; 527). For example, Paolisso et al. showed that intravenous infusion of glutathione was effective to attenuate insulin resistance in humans (565), while treatments with N-acetyl cysteine (NAC) or taurine prevented glucose-mediated insulin resistance in rodents (258). Oxidative stress leads to tissue damage by altering the structure of proteins, lipids, and DNA (190) and has been suggested to play a causal role in the pathogenesis (189) as well as complications (38; 426) of T2DM. Antioxidant treatments (NAC, taurine, and α-lipoic acid) have also been shown to have been shown to prevent hyperglycemia induced insulin resistance in vivo (258; 415). One possibility as a downstream target of oxidative stress is IKKβ (367). Kamata et al. (366) demonstrated that H₂O₂ has stimulatory effects on NF-κB through the activation of IKKβ, which impairs insulin signaling by phosphorylating IRS-1 on serine residue 307 (221). Superoxide radicals (O₂⁻) are also involved in activation of NF-κB (146).
1.7 Players in Hepatic Insulin Resistance

Accumulation of lipid in the liver (hepatic steatosis) is closely associated with hepatic insulin resistance (88; 256; 482; 643; 657; 668) however the exact mechanism remains unclear (485). Release of FFA by lipolysis of abdominal adipocytes are directed to the portal vein where they cause hepatic insulin resistance, increased gluconeogenesis, and increased lipoprotein and TG export to other tissues (296). Some investigators have postulated that with insulin resistance, the combination of elevated plasma concentrations of glucose and FFA promote hepatic lipid synthesis and impair β-oxidation leading to hepatic steatosis (485; 656). These relationships have since been confirmed in humans by a study which employed acipimox, a nicotinic acid analogue, which lowered FFA in obese individuals (655). Administration of acipimox resulted in 60-70% reduction of plasma FFA and two-fold increase in insulin-stimulated glucose uptake (655). In contrast, others have proposed that hepatic fat accumulation and hepatic insulin resistance can occur without the development of peripheral insulin resistance (396; 417). For example, in a study examining the time course of hepatic and peripheral insulin resistance, Kraegen et al. (417) reported that rats fed a high-fat diet for 3 days developed hepatic insulin resistance prior to the development of peripheral insulin resistance (417).

The exact mechanism as to how lipid accumulation in the liver leads to insulin resistance remains elusive. In the last decade players such as PKCδ and PKCε, oxidative stress, IKKβ/nucler factor-κB (NF-κB) inflammatory pathway, JNK stress-activated pathway and endoplasmic reticulum (ER) stress have been implicated as FFA induced signals governing hepatic insulin resistance (189). PKC, IKKβ, and JNK have been shown to inhibit insulin signaling in the liver by serine/threonine residue
phosphorylation of the insulin receptor including the IRS complexes (IRS-1/2) (395; 396; 653).

PKC is a protein kinase that directly mediates serine/threonine phosphorylation of the insulin receptor and IRS, thereby impairing insulin signaling (251; 252; 326). Several recent studies have implicated the ‘novel’ PKC isoforms (δ, ε, μ, η, θ) in fat-induced insulin resistance (160). The different isoforms of PKC have been shown to be stimulated by lipid metabolites such as palmitoyl CoA and DAG and to cause insulin resistance in the liver (665). In this way lipid metabolites on their own or in concert with PKC stimulation/activation induce ROS and oxidative stress in the liver (323; 732; 733). Fat infusion as a model of insulin resistance stimulates the membrane translocation of PKCδ which is an indicator of its activation (57; 432). Other PKC isoforms have also been shown to play a role. Acute inhibition of PKCε was recently shown to prevent hepatic steatosis and insulin resistance in rats fed high-fat diet (654), indicating that activation of PKCε may be critical in hepatic insulin resistance caused by prolonged fat elevation. The involvement of PKC in FFA-induced insulin resistance appears to be species- and tissue-specific however the role of PKC in fat-induced hepatic insulin resistance is gaining popularity as a key player.

JNK/stress-activated protein kinase (SAPK) is a protein belonging to the stress-activated kinase group and many studies have implicated JNK in the development of insulin resistance and T2DM (222; 294; 531; 697). For example, JNK activation has been reported in liver, muscle, and fat in animals with dyslipidemia and inhibition of lipid-induced JNK activity resulted in improved insulin sensitivity in mice (294). Additionally in vivo studies in rats using high-fat diet as a model of chronic
hyperlipidemia have shown that chronic elevation of FFA is associated with JNK activation (653; 660), while insulin resistance caused in 3T3-L1 adipocytes by culture in FFA induced JNK activation (222). JNK is capable of phosphorylating IRS-1 at serine 307 (4; 697), a site that has been implicated in whole-body insulin resistance (5; 221; 222; 697; 844).

1.8 Endoplasmic Reticulum Stress in Hepatic Insulin Resistance

The ER is comprised of a membranous network of internalized compartments and is where amino acid polymers are modified, folded, and assembled into secretory or integral proteins (487). A process known as the unfolded protein response (UPR) links the load of newly synthesized proteins with the capacity of the ER to mature them (487). When proteins are not targeted properly to the ER through this UPR response, non-functional proteins build up within the cell and lead to perturbations in normal cellular function—a condition termed ER stress (487). The ER is particularly vulnerable to the occurrence of misfolded proteins because of its contribution in post-translational modification, folding and assembly of newly synthesized proteins. In this way dysfunction of the UPR plays an important role in some diseases especially those involving tissues dedicated to extracellular protein synthesis. T2DM is an example of such a disease, since the demands for constantly varying levels of insulin synthesis make pancreatic β-cells dependent on efficient UPR signaling (487).

Regulation of new protein synthesis in response to changes in ER load is under the control of PKR-like eukaryotic initiation factor-2 (eIF2α) kinase (PERK) (267) which is a ubiquitously expressed trans-membrane protein, but more so in secretory cells (267; 675; 677; 698). During ER stress, PERK responds not to the level of misfolded protein within the ER, but instead to changes in the ER chaperone reserve (266; 267). Because of the
toxicity of accumulated misfolded proteins, cells have evolved chaperone proteins such as immunoglobulin binding protein (BiP) to bind to misfolded proteins to shield them from other molecules (60; 282; 283; 521; 801). BiP is a chaperone protein that binds to the luminal domain of PERK (47; 465) during unstressed conditions, and can bind with folding proteins through interactions with exposed hydrophobic residues (175; 444). When an increase in ER client load is experienced, BiP dissociates from PERK which allows PERK to undergo autophosphorylation which increases the affinity for eIF2α (47; 486) which is responsible for attenuated protein translation and transcription (135; 268; 473; 536). Additionally, BiP overexpression has long been known to suppress the UPR (175; 798).

Many of the genes involved in the UPR are under the control of X-box binding protein (XBP-1) which is upregulated during periods of ER stress (705). During times of ER stress XBP-1 mRNA is spliced and a short intron is removed which generates an active transcription factor (105; 445; 672; 840). The endonuclease responsible for removing the inhibitory intron of XBP-1 mRNA is the ER transmembrane protein, inositiol requirement 1 (IRE1), whose luminal domain shares significant homology with that of PERK, allowing it a similar regulatory interaction with BiP (46; 47; 401; 465; 549). For these reasons, phosphatase activity exists to relieve eIF2α phosphorylation, restoring normal translation initiation activity.

The UPR organizes reductions in protein aggregation and ER stress through upregulation of antioxidant production, protein degradation, chaperone expression, and recruitment of ubiquitin-conjugating enzymes (640; 773). Minimized protein aggregation also results due to targeting of proteins that never attain their correct conformation to act as substrates for cellular protein disposal called ER-associated degradation (ERAD) (464). Such misfolded
proteins are targeted to act as ERAD substrates by the ubiquitin/proteasome pathway (224; 262; 590) and are re-translocated across the ER membrane or targeted to the proteasome where their degradation takes place (370). If these misfolded proteins have a conformation such that they are insoluble, they are resistant to the normal degradation process and they build up to toxic levels within the cell (852). Evidence suggests that some aggregates can actually block or obstruct the proteasome and inhibit its activity (42; 775). Recently autophagy, which targets cytosolic contents to the lysosome for degradation, has been shown to play a role in the clearance of ubiquitinated proteins (370; 453). For example, in one study, an accumulation of ubiquitinated protein (Ub-protein) aggregates were found in the liver of autophagy-deficient animals (414). Thus, when defective proteins are modified by ubiquitination, both the proteasome and autophagy can contribute to their degradation (370).

ER stress is present in the liver; elevated ER stress markers such as Bip and Lys-Asp-Glu-Leu (KDEL) are reported in obese diabetic C57BL/KsJ-db/db mice when compared to 10 week old non-diabetic C57BL6 mice (526). The pancreatic ER kinase (PERK) is an ER membrane protein that phosphorylates eIF2α in response to ER stress. PERK phosphorylation status is thus an indicator of ER stress (676; 677). PERK and eIF2α phosphorylation are increased in mice fed a high fat diet and in ob/ob mice (267; 526; 676). These studies indicate that ER stress in the liver is increased under diabetic conditions (526).

1.9 C-Jun NH₂-Terminal Kinase Activation in Hepatic Insulin Resistance

A different link between oxidative stress and hepatic insulin resistance involves hepatic JNK activation. ROS induce activation of JNK in the liver; it was shown both in vitro (697) and in
vivo (525) that hepatic insulin insensitivity is mediated by redox stress-induced activation of JNK and corresponding serine phosphorylation of IRS-1 (429; 570). The causative role of JNK in hepatic insulin resistance is further exemplified by studies which over-expressed dominant negative JNK in the liver of obese mice. This alteration led to increased IRS-1 tyrosine phosphorylation and decreases in IRS-1 serine phosphorylation (525) and associated improvements in insulin sensitivity, while induction of wild type JNK expression in the liver of normal mice increased insulin resistance (525). JNK is also increased by ER stress (765) and has been shown to be elevated in the liver of obese mice (563). Treatment of liver cells with agents which induce ER stress lead to increased IRS-1 serine phosphorylation which can be blocked with the use SP600215 which is a synthetic JNK inhibitor (563). Hepatic JNK activation is also induced by circulating proinflammatory cytokines such as TNFα (466; 776; 811). The effects of TNFα to induce insulin resistance is significantly reduced in studies employing specific JNK inhibitors (466; 776; 811). These studies indicate that JNK is not only associated with elevated plasma and tissue FFA but is a causal modulator of insulin sensitivity.

1.10 Normal β-Cell Function

Insulin production and secretion takes place in the endocrine pancreas by highly specialized secretory cells called β-cells which are located in islets (652). Within an average human pancreas there are approximately 1 million islets which are comprised of approximately 2500 cells and make up roughly 1% of the total pancreatic mass (652). Within each islet are different cell types such as α, β, and δ cells, however β-cells are by far the most abundant, generally constituting approximately 60% of each islet (652).
Insulin synthesis commences with the transcription of the gene coding for pre-pro-insulin (652). Once the “pre” peptides are cleaved, pro-insulin results where it is then guided to the Golgi apparatus where it is processed (652). During this packaging in the Golgi bodies the pro-hormone is further cleaved by carboxypeptidases that yield C-peptide and insulin in equimolar quantities (652) which are both co-secreted by the β-cell. During the process of insulin secretion the following events occur (652):

1. Glucose transporters-2 (GLUT2) facilitate the diffusion of glucose into the β-cell.
2. Phosphorylation of the enzyme glucokinase functions as a “glucose sensor” and is the rate determining step of the β-cell response.
3. Glucose is oxidized within the cell to increase the intracellular adenosine triphosphate (ATP): adenosine diphosphate (ADP) ratio.
4. Membrane depolarization occurs when ATP-sensitive K⁺ channels close which causes and efflux of Ca²⁺ activating the secretion of insulin granules.
5. Membrane fusion of granules occurs and insulin along with C-peptide is secreted. Insulin circulates unbound to any transporters within the plasma and has a half-life on average, lasting between 5-8 minutes.

In normal individuals glucose induces a biphasic insulin release from the pancreatic β-cell (600). First-phase insulin release occurs when secretory granules near the β-cell plasma membrane are released into the blood by exocytosis (6). The first or acute phase is sharp and reaches a maximum at about 3-5 minutes and lasts approximately 10 minutes. The magnitude of this response is a function of the size of each β-cell, the number of β-cells, the amount of insulin stored per β-cell, and the amount of insulin secreted by each cell (95; 355) and reflects changes in insulin sensitivity (6; 7). The second phase is more blunted and lasts as long as glucose remains elevated (600). Under normal circumstances insulin secretion follows an oscillatory pattern with periods of 105-120 minutes (598). This type of oscillatory insulin secretion is more effective at lowering glucose than chronic insulin infusion (721). Between meals, blood glucose is kept within a narrow range by having the β-cells secrete small quantities of insulin into the blood intermittently. This response is
attributed to the release of granules that lie deeper within the β-cell and ones that contain newly made insulin (355).

1.11 Intracellular Regulators of β-cell Function and Mass

Numerous studies have suggested that signal transduction through the insulin/insulin-like growth factor (IGF)-receptor pathways is involved in the regulation of β-cell mass and function (613). Insulin and IGF-1 are growth factors for β-cells and their receptors use IRS proteins for signal transmission (317). The ability of insulin and IGF-1 to affect IRS is evidenced by culture of INS-1 cells at 16 mM glucose which resulted in a sustained increase in IRS-2 levels compared to culture at 1.6 mM. Mice β-cells lacking either the insulin receptor (IR) (βIRKO) (423) or IGF-1 receptor (βIGF1RKO) (424) have been generated. βIRKO mice lack first-phase insulin secretion in response to glucose but not to arginine and have decreased islet size (423). Islets from βIGF1RKO mice also showed a glucose-specific defect in insulin secretion compared to controls but not defective islet growth. These studies therefore suggest that β-cell IGF-1 receptors are required for β-cell function (424) but dispensable for maintenance of β-cell mass indicating that the regulation of β-cell size, proliferation and apoptosis is redundant and probably not dependent on signal transduction from a single growth factor receptor (423; 424).

Insulin receptor substrates are evolutionarily conserved adapter proteins which are required for many biological processes such as growth control, apoptosis, differentiation and regulation of fuel metabolism (231; 717). Post-receptor signaling mechanisms are required for normal β-cell function (422). β-cell insulin signaling and activity of IRS proteins link various membrane receptors to intracellular signaling pathways (346; 354; 812). Mice deficient for IRS-1 are resistant to insulin and IGF-1 and display increased levels of fasting
plasma insulin; however, these animals do not develop overt hyperglycemia and diabetes
due to islet hyperplasia (22; 734). IRS-1 also stimulates proliferation in rat islets mildly
(513). All these results show that although IRS-1 is required for normal insulin action and
secretion from islets, its loss is not detrimental for the regulation of blood glucose
homeostasis since β-cell mass compensation in the face of insulin resistance is not
drastically impaired (424).

The opposite is true for IRS-2-deficient mice which develop overt diabetes at around 10
weeks of age because of their inability to compensate for the increased demand for insulin
(420). Such mice are born with slightly reduced islet mass and later show loss of β-cell
mass and a reduction of insulin content indicating that IRS-2 is essential for maintenance of
β-cell mass and function (420). IRS-2 KO mice have reduced in vivo and in vitro GSIS
showing that IRS-2 is important for insulin production and secretion (107) which agrees with
studies showing that the IRS-2/PI3K/p70S6K pathway regulates insulin gene transcription
(447). As determined from perifusion experiments, overexpression of IRS-2 in isolated rat
islets increased basal and GSIS (513). Overexpression of IRS-2 (but not of IRS-1)
increases proliferation induced by IGF-1 and glucose (463) while IRS-2 KO mice have
reduced β-cell mass with corresponding increased caspase-3 staining and reduced β-cell
proliferation, confirming the key role of IRS-2 in this process (107). β-cell insufficiency in
IRS-2 KO mice can be rescued by reducing forkhead box 01 (Fox01) (408) or increasing
pancreatic duodenum homeobox-1 (PDX-1) (428) indicating that PDX-1 plays a roll in key
defects in these mice. Results from INS-1 studies indicate that the IRS-2-PI3K-PKB (317;
797; 830) controls β-cell size, proliferation and apoptosis.
Glucokinase has been shown to be involved in glucose sensing and insulin secretion (492) but is now shown to be involved in regulation of β-cell mass (739). Glucokinase and IRS-2 are both required for normal β-cell compensation in response to insulin resistance in terms of hyperplasia (739). Glucokinase is required for normal glucose induced increased expression of IRS-2 in response to increased blood glucose levels (739). Glucokinase heterozygous mice do not have normal β-cell hyperplasia in response to high-fat feeding (739). Proliferation, as evidenced by proliferating cell nuclear antigen staining, is decreased in glucokinase heterozygous mice (739). Glucokinase thus regulates β-cell mass and β-cell function (739).

Activation of cyclic AMP (cAMP) response element binding protein (CREB) increases IRS-2 expression in MIN6 cell lines and in mouse islets (284; 341; 813) which leads to potentiation of the insulin signaling cascade and subsequently enhanced β-cell function (813). CREB can be activated (phosphorylation at serine 133) not only by cAMP-dependent protein kinase A (PKA) but also downstream of a number of other kinases such as PKB/Akt which are implicated in normal β-cell growth (45; 753). β-cell-specific expression of a kinase-dead dominant-negative form of PKBα (rip-kdPKB), reduced islet size and resulted in defective insulin secretion (44). Expression of constitutively active PKBα under the control of the rip promoter (45; 753) resulted in hypertrophy and hyperplasia of islets. Such mice were hyperinsulinemic and resistant to streptozotocin (STZ)-induced diabetes (45; 753).

1.12 Perturbations to β-Cell Function in Type 2 Diabetes Mellitus

During the early development of insulin resistance, insulin release by the β-cell is increased to compensate for the reduction in insulin sensitivity (362). When β-cell function is
assessed, obese subjects without T2DM manifest greater insulin responses to a glucose challenge than age-matched lean subjects (202). The ways in which these subjects experience greater insulin response is due to increased insulin production, storage, and secretion, via increases in β-cell function and mass (586). Thus, normal blood glucose tolerance curves can be achieved in subjects with a wide range of insulin resistance.

Individuals with T2DM manifest β-cell secretory defects and do not adapt to worsening insulin resistance with this compensatory augmentation in β-cell function in the face of increased insulin demand. The major secretory defect associated with T2DM is absence of the first phase insulin release in response to glucose (577). This lessened first-phase insulin response reflects reductions in β-cell function and/or β-cell mass (95; 355; 355; 356; 358; 361; 363; 598). With respect to pulsatile insulin secretion, patients with T2DM have decreased amplitude and lack regularity in insulin pulses after a meal. It has been suggested that these defects contribute to the diminished insulin efficiency observed in T2DM (598). Insulin release in T2DM, however, is maintained when stimulated by other non-glucose secretagogues indicating defects with respect to glucose entry and/or glucose metabolism of the β-cell (165).

β-cell glucose metabolism is under the GLUT2, hexokinase, and glucokinase proteins which are involved in impaired GSIS. The equilibrium exchange value (Km) for GLUT2 is about 20-50 mM and the capacity for maximal transport (Vmax) is 2400 pmol/h/islet (343; 501). In terms of glucose phosphorylation there are two enzymes. Hexokinase has a Km of 1 mM and therefore cannot influence the rate of glucose metabolism at normal glucose ranges which usually range between 4-8 mM (501). Glucokinase however has a Km of ~8 mM and a Vmax of 150 pmol/h/islet and is therefore the rate-limiting step of glucose
metabolism and glucose sensing (501). Defects with respect to these proteins are found in type 2 diabetic humans, as evidenced by intravenous glucose tolerance test studies (355; 360; 362). In humans, spontaneous inactivating mutations of the glucokinase gene have been identified in T2DM (774), while studies have shown that there is a 30-70% reduction in glucokinase mRNA is β-cells of ZDF rats (749). In murine models, haploinsufficiency of β-cell specific glucokinase leads to mild diabetes associated with GSIS (738). Furthermore, reduced expression of β-cell GLUT2 protein is found in all models of reduced GSIS studied thus far including ZDF rats (756), db/db mice (803), ob/ob mice (803), Wistar-Kyoto rats (756), and the Goto-Kakizaki (GK) rat (756).

1.13 Perturbations to β-Cell Mass in Type 2 Diabetes Mellitus

In healthy humans, the β-cell population is in constant flux maintaining a balance between net cell growth and death (201). Cell growth occurs as a result of β-cell hyperplasia and β-cell hypertrophy. β-cell hyperplasia is governed by two factors; the rate of replication from existing cells, and the rate of the neogenesis from stem cells and/or duct cells (515). β-cell hypertrophy, or continued growth without cellular division may also contribute significantly to increased β-cell mass (348; 508). Importantly, compensatory expansion of functional β-cell mass through these mechanisms can prevent the progression to overt hyperglycemia when increasing peripheral insulin resistance leads to an increasing demand for insulin (411). For example, insulin-resistant individuals without T2DM maintain normal glucose concentrations by increasing β-cell mass and related adaptive hyper-secretion of insulin (101; 458). Subjects with T2DM, however have smaller islets compared to non-diabetic obese controls (101; 458) which occurs primarily in response to reduced β-cell mass since α and δ cells are not significantly reduced in mass (89; 261; 504).
It is difficult to assess β-cell mass dynamics in humans because pancreatic tissue is only available from an autopsy (101). β-cell turnover, however, can be readily measured in animal models of T2DM. To better answer these questions various rodent models of T2DM such as the ob/ob mouse, the GK, Otsuka Long-Evans Tokushima Fatty (OLETF), and ZDF rat (16-18; 86; 87) have been used. In 5-7 week-old ZDF rats, before the onset of diabetes, β-cell mass was similar in ZDF and ZF rats and was approximately double that of age-matched lean Zucker animals (586). This closely resembled those changes seen in obese insulin-resistant and diabetic humans matched for body weight (101). At 12 weeks of age, however, β-cell mass in ZDF rats, although twice the value observed in lean animals, represented only 50% of the β-cell mass in the ZF rats (586). Such reductions in β-cell mass were believed, at this time, to be the result of reductions in the net rate of β-cell proliferation (16-18; 55; 254; 520; 632; 856). Additional experiments however, indicated ZDF rats had a greater than average rate of β-cell replication than in the two non-diabetic groups, despite having a significantly lower β-cell mass at 12 weeks than the ZF rats (586). Therefore the mechanism for the decreased β-cell mass was proposed to be the result of increased β-cell apoptosis (127; 200; 586; 735). Indeed, a study by Pick et al., showed increased β-cell death by apoptosis that caused a decrease in β-cell mass which was not related to a reduction in β-cell proliferation rate (647). With respect to human studies of β-cell mass, only a paucity of studies exist. These limited human studies do however indicate such reductions in β-cell mass are due mostly to increased rates of apoptosis (107); when normalized to the β-cell volume, the frequency of apoptosis was 3-fold higher in obese cases of T2DM and 10-fold higher in lean cases of T2DM as compared with their controls (107).
In summary, β-cell decompensation in T2DM is associated with marked reductions in β-cell function and mass expansion. In concert with peripheral insulin resistance and corresponding increased insulin demands, these defects result in the diabetic milieu which only wreaks further havoc on pancreatic β-cells by processes described to follow.

1.14 The Effects of Chronic Hyperglycemia and Hyperlipidemia on β-cell Function

The first evidence that chronic hyperglycemia itself can adversely affect β-cell function came from experiments in T2DM patients. It was shown that glucose concentrations at 115 mg/dl or above caused a loss of first phase insulin secretion (89). Studies in animals provide further evidence that hyperglycemia induces loss of GSIS. 90% pancreatectomy (Px) or chronic infusion of glucose leads to selective loss of GSIS, yet these animals are able to respond to non-glucose secretagogues (440). Female ZDF rats when given a 60% Px are normoglycemic but when given a sugar-rich drink they no longer secrete insulin in response to glucose (440).

Adverse effects of chronic hyperglycemia on β-cell function encompass three distinct phenomena: glucose desensitization, β-cell exhaustion, and glucotoxicity (393). Glucose desensitization refers to the rapid and reversible refractoriness of the β-cell exocytotic machinery that occurs after a short exposure to elevated glucose (393). Glucose desensitization is a physiological adaptive mechanism that occurs even when insulin secretion is inhibited, thus differentiating it from β-cell exhaustion (393). β-cell exhaustion refers to depletion of the readily releasable pool of intracellular insulin following prolonged exposure to a secretagogue (393; 441). The result of this chronic stimulation with glucose or non-glucose secretagogues is that insulin secretion is not possible until a period of rest occurs. An important distinction between β-cell exhaustion and glucose toxicity therefore is
that the exhausted islet has no defects in insulin synthesis, and therefore cell function fully recovers as it rests (393).

The term glucotoxicity describes the slow and progressively irreversible effects of chronic hyperglycemia on pancreatic β-cell function (393). Gradual, time-dependent damage occurs to cellular components of insulin production and, therefore, to insulin content and secretion. At the root of the decline in β-cell function found in the glucotoxic state is abnormal insulin gene expression as well as decreases in insulin content and insulin secretion (393). Such candidates include decreased translational rates in insulin synthesis, suppression of glucokinase gene expression, decreased mitochondrial function, compromised exocytotic mechanisms, and accelerated apoptosis (477; 493). In vitro conditions using a cell line showed glucose toxicity is a continuous rather than a threshold function of glucose concentration, and that the shorter the period of antecedent glucose toxicity, the more likely that full recovery of β-cell function will occur (233). The fact that these associated β-cell defects are reversible up until a certain point in time and then become irreversible thereafter suggests a continuum between β-cell exhaustion and glucotoxicity, the latter becoming predominant after prolonged exposure (233; 518).

The insulin gene is expressed almost exclusively in pancreatic β-cells (594). Metabolic regulation of insulin gene expression enables the β-cell to maintain adequate stores of intracellular insulin to sustain the secretory demand (594). Glucose is the major physiologic regulator of insulin gene expression; it coordinately controls the recruitment of transcription factors such as PDX-1, mammalian homologue of avian MafA/L-Maf (MafA), and the rate of transcription, and the stability of insulin mRNA (594).
Impairment of insulin gene expression after prolonged exposure to elevated glucose levels is associated with diminished activity of PDX-1 (552; 553) and (MafA) (592; 671). A number of kinases are reported to mediate PDX-1 phosphorylation and nuclear translocation, including p38 mitogen-activated protein kinase (MAPK) (474), PI3K (145), and extracellular signal-regulated kinases (ERK) (391). In vivo, PDX-1 expression is also reduced in partially pancreatectomized, hyperglycemic rats (849) and in the diabetic gerbil *Psammomys obesus* (448), and its binding activity is decreased in islets from ZDF rats (269). Subsequent studies have shown that the mechanism involves the loss of mRNA and protein levels of PDX-1, a critical regulator of insulin promoter activity. It was also shown that this abnormality in insulin promoter activity in HIT-T15 cells could be partially corrected by transient transfection of PDX-1 cDNA (272; 552; 553; 671).

MafA expression and binding is also directly activated directly by glucose (853) and is reduced in mouse diabetic models (408). The reduction in MafA binding activity in the glucotoxic insulin-secreting HIT-T15 cell was shown recently to be due to a loss of protein expression without changes in mRNA expression, suggesting that glucose reduces MafA activity through a post-translational mode of action (271). MafA binding to the insulin promoter disappears 20 passages earlier than PDX-1 binding in experiments with glucotoxic HIT-T15 cells which may explain why transfection of PDX-1 cDNA alone was not sufficient for complete restoration of insulin promoter activity (272). Importantly, PDX-1, and MafA do not act in an isolated manner, but interact with each other to induce synergistic activation of insulin transcription (853).
Cultured human islets chronically exposed to high glucose also show increased rates of β-cell apoptosis and a reduced proliferation rate (195; 478). Overexpression of IRS-2 in human islets completely blocked the pro-apoptotic effect of chronic high glucose on human β-cells (513). Moreover, human islets cultured for 3 days with 33.3 mM glucose and simultaneously overexpressing IRS-2 displayed fewer apoptotic β-cells compared to islets that overexpressed IRS-2 after culture under 5.5 mM glucose (513). Overexpression of IRS-2 repressed the rate of β-cell apoptosis below the level observed in control islets cultured at 5.5 mM glucose (513) while overexpression of IRS-1 had no effect (513). This result suggests that IRS-2 could have a central role in human β-cells during the compensatory phase of the development of T2DM. Briaud et al. reported that chronic exposure of INS-1 cells to glucose induces mTOR-dependent Ser/Thr phosphorylation of IRS-2 after only 4 hours which was accompanied by decreased IRS-2 levels after 24 hours, decreased activation of PKB and an increase in apoptosis (72). High levels of IRS-2 might not only prevent glucotoxicity in the face of insulin resistance but could even trigger expansion of β-cell mass by reducing β-cell apoptosis below basal levels (513).

Elevated plasma levels of cholesterol, TG, and FFA and disorders of lipid metabolism contribute to β-cell dysfunction in T2DM (758). Excessive fat in the β-cells has been observed in a number of lipotoxic models, as exemplified by the massive increase in islet fat content which in some studies is shown to precede the development of diabetes in ZDF rats (686). Islet amyloid polypeptide has also been identified as a factor in the deterioration of β-cell function in patients with T2DM (8; 301-303; 777). The degree of islet amyloidosis in patients with T2DM varies in different studies ranging from 46% to 95% depending on age of subjects and method of quantifying amyloid. Initially the rising fat content of the islet is responsible for the compensatory hyperplasia of β-cells. In vitro evidence suggests that a 5-
10 fold increase in islet fat content, which occurs in vivo during the prediabetic phase, such as the ZDF model causes the β-cell hyperplasia and hyperinsulinemia (684; 686; 687). Many studies have documented an initial increase in GSIS following lipid infusion (648) however, prolonged exposure of pancreatic β-cells to FFA increases basal insulin release but inhibits GSIS (500). Deleterious effects of FFA on insulin gene expression in isolated islets are associated with an increased accumulation of intracellular TG, suggesting a role for neutral lipid synthesis in this process (73). Adverse effects of chronic exposure of the β-cell to elevated FFA concentrations include decreased GSIS (648; 854), impaired insulin gene expression (73; 253; 333; 619), and increased cell death (131; 472; 479; 505; 608; 687). Additional studies have shown that the adverse effects of lipid accumulation in β-cells are dependant on whether the lipid is neutral or esterified (655). However, discordant findings have shown increasing TG synthesis by overexpression of the enzyme diacylglycerol-acyltranferase inhibits GSIS but not preproinsulin mRNA levels (382), suggesting that TG accumulation is not directly involved in inhibition of insulin gene expression.

Differences in toxicity of various FFA are also reported. Both palmitate and oleate inhibit insulin secretion, but only palmitate impairs insulin gene expression (517). Palmitate impairment of insulin gene expression appears to be mediated by direct inhibition of insulin promoter activity (383) as palmitate inhibits PDX-1 and MafA binding activities (259) in isolated rat islets. PDX-1 and MafA appear to be affected by palmitate through different mechanisms. Culture of isolated islets in the presence of palmitate prevents the nuclear translocation of PDX-1 that normally occurs upon glucose stimulation, whereas it blocks glucose induction of MafA mRNA expression (259). Importantly, combined, adenovirus-mediated overexpression of PDX-1 and MafA prevents palmitate inhibition of insulin gene
expression in isolated rat islets, consistent with an essential role for these 2 transcription factors in the mechanisms of lipotoxicity (259).

There is disagreement concerning the antecedent roles of glucotoxicity and lipotoxicity in β-cell failure. Some studies indicate prolonged elevations in FFA levels are deleterious to the β-cell, even when blood glucose levels are kept within a normal range (687; 761-763). Others, however, indicate glucotoxicity is the prerequisite for lipotoxicity, at least in β-cells (66; 270; 594; 625; 802; 804). A number of in vivo and in vitro studies have shown that under conditions of elevated glucose levels (i.e. above the normoglycemic level of ~5.6 mM), prolonged exposure to pathological levels of FFA results in accumulation of TG in β-cells and impairment of insulin secretion, insulin gene expression, and cell viability (500). In contrast, other studies of isolated islets show TG accumulation after exposure to exogenous FFA only occurs when glucose concentrations are elevated (73). For example, it has been shown that after 72 hours of culture in the presence of palmitate and low glucose, insulin content or insulin mRNA is not affected. In contrast, when cultured in palmitate and high glucose, both insulin mRNA and insulin content decrease (333). These results clearly support the postulate that hyperglycemia is required for lipotoxicity to occur. Glucotoxicity is associated with decreased protein expression of PDX-1 and MafA, whereas under lipotoxic conditions, the PDX-1 gene is expressed but retained in the cytoplasm and MafA mRNA levels are decreased. It is therefore likely that these conditions, which occur concomitantly in most patients with T2DM, have combined deleterious effects on insulin gene expression (73; 253; 259; 383; 619).

To differentiate in vivo between hyperlipidemia and hyperglycemia as the cause of islet TG accumulation and defective insulin gene expression in ZDF rats, animals have been treated
with either the lipid-lowering drug bezafibrate or the blood glucose-lowering agent phlorizin (270). Islet TG content was decreased and insulin gene expression was preserved by phlorizin but not by bezafibrate treatment, indicating that islet TG accumulation requires hyperglycemia (270). Importantly, phlorizin, but not bezafibrate, prevented the decrease in insulin mRNA levels between 6 and 12 weeks of age indicating that antecedent hyperglycemia, not hyperlipidemia, is associated with increased islet TG content and decreased insulin gene mRNA levels in ZDF rats (270). Similar results have been shown in studies drawing contrasts between high fat fed normoglycemic Wistar rats and high fat fed hyperglycemic GK rats (74). Six weeks of high-fat feeding did not affect GSIS in islets from Wistar rats but decreased the maximal response to glucose in islets from GK rats by approximately 50% (74). The significance of concomitant hyperglycemia was further exemplified by the fact that administration of insulin during the 6 week normalized both blood glucose and plasma FFA levels and completely prevented the decrease in GSIS in islets from high-fat-fed GK animals (74).

1.15 Oxidative Stress and Pancreatic β-cell Dysfunction

Chronic hyperglycemia is a cause of impaired insulin biosynthesis and secretion and once hyperglycemia becomes apparent, β-cell function gradually deteriorates (518; 591; 593-595). This process is defined as glucotoxicity. As is the case with glucose, prolonged exposure of β-cells to elevated levels of FFA leads to the inhibition of insulin gene expression β-cell function as well (687; 758). Also, exposure of islets to palmitate induces generation of ROS (111), and treatment of islets with metformin, which has antioxidant properties, protects from deleterious effects of FFA (568). These two processes are often referred to collectively as glucolipotoxicity (591) as they both share a common mechanism of β-cell impairment and destruction; the production of ROS (591). Because antioxidant
expression levels are very low in pancreatic β-cells compared to other tissues (743). β-cells are prime targets of oxidative stress-induced tissue damage (623).

Oxidative stress specifically suppresses insulin gene transcription in β-cells. Studies utilizing HIT cells (494) or isolated rat islets (369) have shown that insulin mRNA expression was lower in response to oxidative stress. Oxidative stress was also associated with decreased DNA binding activity PDX-1 (369; 494) which is required for β-cell differentiation (108) and the regulation important β-cell genes such as GLUT2, glucokinase and insulin (77). The effect of oxidative stress to decrease nuclear binding of PDX-1 was confirmed with the used of antioxidants. In vitro studies showed the link between elevated glucose and oxidative stress by incubating β-cell derived HIT cells in D-ribose. Insulin gene promoter activity and mRNA expression were suppressed (494). These effects were neutralized by aminoguanidine or NAC implicating the glycation reaction in the development of oxidative stress-mediated reductions in PDX-1 activity (494). In vivo studies indicated that administration of NAC prevented the loss of GSIS in obese diabetic C57BL/KsJ-db/db mice (367). β-cell mass was larger in mice treated with antioxidants and the amount of DNA/PDX-1 co-localization, insulin mRNA, and insulin content were increased in the β-cells of these animals (367). Increased β-cell mass in these animals was due to suppressed rates of apoptosis with unchanged rates of proliferation (367). These studies have since been repeated in other rodent models of obesity and T2DM such as the ZDF rat (735). Lastly treatment of C57BL/KsJ-db/db mice with a different antioxidant, probucol, preserved β-cell mass, GSIS, and glucose tolerance in these mice. Immunostaining for oxidative stress markers such as HNE-modified proteins revealed decreased ROS levels in the islets of probucol treated diabetic animals (245). These data indicate that antioxidant treatment can protect β-cells against glucolipotoxicity in vivo.
1.16 Endoplasmic Reticulum Stress in Pancreatic β-cell Dysfunction

The UPR and ERAD are two safety mechanisms that have evolved to help the ER cope with misfolded proteins. ERAD targets damaged and misfolded proteins for proteasomal degradation by tagging them with ubiquitin (640). Autophagy and the proteasome are then recruited to aggresome-like induced structures (ALIS), but it is autophagy that actually contributes to clearance and delivers the ubiquitinated protein to the lysosome for breakdown (370). ALIS are likely a cytoprotective system, in that they sequester misfolded protein and target it for degradation. If this process is overwhelmed ER stress occurs and various pathways are activated. One such pathway is the suppression of protein synthesis in cells experiencing ER stress. PERK is an ER transmembrane protein that phosphorylates eIF2α which leads to a reduction in protein translation and build up of proteins within the ER or cell (267). This regulation in protein synthesis is important to specialized secretory cells so that they do not overwhelm their protein producing machinery.

It has recently been confirmed in the ZDF model of T2DM that chronic hyperglycemia leads to ER stress and the overwhelming of the UPR. Hyperglycemia in these animals was shown to induce insoluble protein structures or aggregates (370). The authors reported the formation of ubiquitinated protein aggregates in pancreatic β-cells of these rats and speculated that these structures form in response to oxidative stress induced by hyperglycemia as previous data indicates that oxidative stress is a causative factor for the formation of Ub-protein aggregates (726). Kaniuk et al. were able to show that oxidative stress was a strong inducer of Ub-positive aggregates and in turn, high glucose, which is known to promote oxidative stress, also caused an increase in Ub-protein aggregates which was prevented by NAC and taurine (370).
Studies have shown that ER stress is involved in pancreatic β-cell apoptosis (265; 266). ER stress mediated PERK induction leads to the activation of CHOP and downstream caspases (267). The role of ER stress in β-cell apoptosis was reported when the Akita mutation was crossed with CHOP KO mice which resulted in the preservation of β-cell mass and the attenuation of hyperglycemia (560). Indeed it has been shown that in PERK KO mice, mutant β-cells are hypersensitive to agents which induce ER stress such as tunicamycin or thapsigargin (266). These mice lead a normal first few weeks of life but experience a progressive destruction of β-cells after around 4–6 weeks (265; 266).

1.17 The role of the C-Jun-NH₂ Terminal Kinase Pathway in β-cell Dysfunction
When β-cells are subjected to cellular stressors signal transduction pathways such as JNK, p38MAPK and PKC are activated. Recently it has been shown that the JNK pathway is activated by oxidative stress in β-cells (368). Suppression of β-cell JNK activity can protect β-cells from oxidative stress and loss of insulin gene expression (368). When isolated rat islets were exposed to oxidative stress, the JNK, p38 MAPK, and PKC pathways were activated and preceded the loss of insulin gene expression (368). Adenovirus overexpression of dominant negative JNK1 (DN-JNK1) protected insulin gene expression and secretion from oxidative stress while selective inhibition of p38MAPK or PKC did not (368). Suppressive effects of JNK activation on insulin gene expression and secretion correlated with decreased PDX-1 DNA binding suggesting that JNK activation leads to decreased PDX1 binding (368). This postulation has been confirmed in HIT cells in which oxidative stress caused a translocation of nuclear PDX-1 to the cytoplasm (376). Inhibition of PDX-1 translocation from the nucleus to the cytoplasm was prevented with addition of DN-JNK indicating the essential role of JNK in
mediating PDX-1 activity (376). DN-JNK can protect β-cells from the toxic effects of hyperglycemia during transplantation (368), and has been shown to be effective at preventing IL-1β induced β-cell destruction (15). More recent studies examining the mechanism of action whereby palmitate inhibits insulin gene expression indicate increased levels of intracellular ceramide as a causative factor (383). Because ceramide activates JNK in various cell types ceramide-induced JNK activation might inhibit insulin gene transcription via JNK-dependent and -independent pathways.

1.18 Rodent Models of Obesity and T2DM: The Zucker Fatty and Zucker Diabetic Fatty Rat
Many investigators have used this model to study the development, etiology, associated pathologies, possible treatments, and putative mechanisms of its severe genetic obesity. Animals homozygous for the fa allele became noticeably obese by 3 to 5 weeks of age, and by 14 weeks of age their body composition is over 40% lipid (863). The obesity is inherited as a Mendelian recessive trait. The ZF rat is significantly hyperphagic compared to lean littermates as early as 17 days of age (715). Given this, affected animals are hyperlipemic, hypercholesterolemic (864; 865), and develop adipocyte hypertrophy and hyperplasia (345). Obese Zucker rats are hyperinsulinemic, both in the fed state and in response to an OGTT (325; 374; 715; 839). Skeletal muscle of the ZF fa/fa rat is also highly insulin resistant (696), with depressed basal and insulin-stimulated glucose transport (674). In addition, in vivo the ZF rat exhibits severe hepatic as well as peripheral insulin resistance (740). The ZF fa/fa rat is relatively normoglycemic or shows only slightly elevated hyperglycemia (94; 325; 715; 864). Life-long food restriction, while decreasing body weight, results in obese rats whose body composition is approximately 50% lipid as compared to under 20% in lean littermates (128), thus the ZF rat's hyperphagia is not necessary for expression of the obese
syndrome. Since the discovery of the *fa* mutation by Zucker and Zucker (864) the (*fa/fa*) rat has become one of the most widely used rat model of genetic obesity.

Both ZF and ZDF rats are characterized by the *fa/fa* mutation, which produces a truncated, non-functional leptin receptor responsible for their obesity, insulin resistance, and hyperinsulinemia (583), however, only male ZDF rats express the diabetic phenotype (583). In male ZDF rats, glucose intolerance usually develops by 8 weeks and overt hyperglycemia by 10-12 weeks of age (583). Such elevations in fasting and postprandial glycemia are related to defects in insulin signaling in skeletal muscle, fat, liver, and impairments in skeletal muscle GLUT4 (81; 83; 187; 188; 207; 210; 211; 292). Skeletal muscle tissue in ZDF rats also exhibits a shift in muscle fuel use from glucose to FFA as evidenced by increases in peroxisome proliferator activated receptor (PPAR)α and its target genes involved in FFA metabolism (increased acyl CoA oxidase, and CPT-1 mRNA levels) and reductions in GLUT4 and phosphofructo kinase (352). In the liver, male ZDF rats have elevated levels of hepatic glucose production, which may be due to increased glycogenolysis in the fasted state (342), and/or increased glucose cycling and gluconeogenesis (213). The impaired suppression of hepatic glucose output may be related to altered translocation of glucokinase (212) and increases in PKC isoforms (137). Male ZDF rats also show increased lipoprotein profiles compared with lean rats during hyperinsulinemic stages, with substantial increases in both low-density lipoprotein and high-density lipoprotein cholesterol (123) thought to be a result of increased hepatic expression of sterol regulatory element binding proteins (SREBP)-1c, an important transcription factor involved in TG synthesis (133). These defects in peripheral insulin sensitivity are also present in male ZF and female ZDF rats, which for unknown reasons, do not develop T2DM (583).
In addition to altered expression of enzymes involved in lipid metabolism, ZDF islets also show reduced relative glucose stimulated insulin secretion and altered insulin pulsatility (545; 719; 720). This has been suggested to be related to altered glucose sensing (decreased expression of the high Km glucose transporter GLUT2) (344; 554), reduced glucokinase expression (749), alterations of proteins involved in insulin release (voltage dependent Ca\(^{2+}\) and K\(^+\) channels (628; 748; 749), as well as defects in insulin gene transcription (reduced PDX-1 and decreased insulin mRNA expression) (670). These defects bring about changes such that by the prediabetic phase, ZDF rats maintain normoglycemia by a compensatory increase in β-cell function resulting in hyperinsulinemia (583; 749). This compensatory adaptation begins to fail as animals enter the “diabetic phase” defined by drastic increase in β-cell apoptosis, and corresponding decrease in β-cell mass (583; 749). In ZDF rats, defects in β-cell function have been shown to precede the development of hyperglycemia marked by the loss of the adaptive augmentation in β-cell mass, while in ZF rats β-cell mass is maintained (685; 686; 793). These early defects are the results of impairments in glucose sensing characterized by reduction in pancreatic glucokinase and GLUT2 expression, and GSIS (793) as evidenced by IVGTT studies (355; 360; 362).

Many studies have suggested that the decline in β-cell mass and function in the ZDF rat is related to islet steatosis (293; 446; 855). Compared to lean ZDF rats, male diabetic ZDF rats have elevated levels of plasma FFA and TG, and their islets show an approximately 6-fold elevation in islet TG levels by 8 weeks of age, which further increases to a nearly 20-fold elevation at 10 weeks of age. This islet steatosis corresponds with the onset of hyperglycemia (446) as well as increased expression of enzymes involved in lipogenesis.
and lipid esterification (ACC, SREBP-1, glycerol-3-phosphate acyltransferase (GPAT) (293; 365; 855), ceramide and nitric oxide production, and apoptosis (682; 687). Prevention of this islet steatosis by troglitizone (365), or adenoviral transfer of the leptin receptor (682) in prediabetic ZDF rats can prevent the dysfunction of the β-cells. The major implicating factors in islet steatosis and β-cell failure in the ZDF rat is their elevated plasma lipid levels and their defective leptin receptor (446; 762; 855).

This pathophysiology resembles closely that humans as, approximately 35% of obese individuals exhibit continued β-cell compensation to worsening insulin resistance while β-cell function in T2DM is characterized by β-cell defects present during the prediabetic phase (200; 290; 583; 586; 749). Many pharmacologically based treatments that alter various aspects of metabolism have also been shown to be successful in the delaying the development of diabetes in ZDF rats. PPARγ ligands such as the thiazolidinediones have been shown to improve hyperglycemia, hyperinsulinemia, and insulin sensitivity, and help maintain β-cell function (78; 587; 764), although joint PPARγ/α activation, which also lowers plasma FFA, was more effective at improving insulin sensitivity (71). Reduction of hepatic glucose production by Metformin (704) or an inhibitor of fructose-1,6-bisphosphatase (772) prevented development of hyperglycemia. Increasing glucagon-like peptide (GLP-1) action by adenoviral vectors that increase GLP-1 levels in vivo (567) improved glucoregulation. Increasing glycogen synthesis through inhibition of GSK3 improves oral glucose disposal and muscle glucose transport in ZDF rats (129; 289; 618). Interestingly, chronic infusion of a β3-adrenergic receptor agonist normalized hyperglycemia, lowered hyperinsulinemia and FFA, and improved glucose tolerance, presumably through increased thermogenesis, mitochondriogenesis, and increased glucose utilization in adipose tissue (467). Treatment of ZDF rats with AICAR prevented the development of diabetes, prevented the increase of
TG content in pancreatic islets, and attenuated the morphological abnormalities observed in the islets (596; 845). Thus, numerous treatments for T2DM in humans have been shown to delay development of hyperglycemia or improve the metabolic abnormalities observed in the ZDF rat, illustrating their utility as a model for studying the T2DM pathogenesis.

1.19 Exercise Training As a Therapeutic Modality in Type 2 Diabetes Mellitus

Exercise has long been known as a powerful mediator in the progression of T2DM. Results from the Diabetes Prevention Program in the United States demonstrated that lifestyle modification (150 minutes of physical activity per week over a 3 year period) reduced the incidence of T2DM by 58% in individuals at significant risk for developing the disease (184; 322; 412; 460; 461). Very recently a study of diabetic patients with impaired glucose tolerance who exercised at a moderate level for 5-6 years reduced their body weight by 7% and reduced the risk of T2DM by approximately 30-50% (564). Other studies indicate that regular exercise (30 min/day) reduces the development of T2DM by 68% in humans with impaired glucose tolerance (241). Some of these beneficial effects of exercise in humans included reductions in central obesity and increased lean muscle mass (519), increased glucose disposal (63), and increased FFA β-oxidation (62).

Acute physical activity and endurance exercise training leads to augmented insulin-mediated glucose metabolism in healthy individuals and normal rodent models (30; 243; 277; 331). Exercise training in non insulin-resistant subjects also has beneficial effects on enzymes involved in glucose phosphorylation and oxidation (299; 331). The beneficial effects of exercise in the human population predisposed to developing T2DM have been observed over a much shorter time frame (578). For example, in insulin-resistant humans,
6 weeks of exercise training has been shown to increase whole-body insulin-stimulated glucose disposal approximately 3-fold (578).

Importantly benefits of exercise persist well after cessation of physical activity. Results from ZF rat studies suggest that exercise training-mediated improvements in insulin-stimulated glucose uptake, may last up to 7 days after the last exercise bout (292). The long-lasting positive effect of exercise training is verified in other rodent models of obesity that do not have leptin receptor defects such as the OLETF rat (678), where the preventative effects against the development of T2DM lasts for 3 months after the cessation of exercise in this model (678). Continued augmented muscle glucose uptake after the cessation of exercise is verified in humans (827).

1.20 Exercise as a Metabolic Stressor

Interestingly, physical activity is generally considered to be a common form of metabolic stress. For example, stress can be defined as a perturbation to homeostasis that must be met by a physiological adaptive response (666). In this respect, exercise can very fittingly be described as a stressor which differs very little in the “stress” response than other forms of stress such as heat shock or novel environment stress (667). A single bout of moderate to strenuous intensity aerobic exercise stimulates the HPA axis (152) and results in the increased production and secretion of “stress hormones” which include adrenocorticotropic hormone, glucocorticoids (150), catecholamines (249), and glucagon (327). Such changes in the classical stress hormones have pronounced catabolic effects (716). These hormones break down stored fats into FFA and TG that can be used as a substrate during exercise (786; 787). They also increase glucose production in the liver via an upregulation of glycogenolysis and gluconeogenesis (782). Therefore, with exercise as a stressor,
glucocorticoid and catecholamine levels are high providing contractile tissue with substrates necessary for ATP synthesis (782). In these ways glucocorticoids and catecholamines oppose the metabolic effects of insulin.

Glucocorticoids have also been shown to directly inhibit insulin secretion from β-cells (433) and have been reported to reduce β-cell mass in animal models of T2DM (339; 375; 541). Exercise, however, does not have a net effect of exacerbated metabolic control. For example, exercise improves insulin-dependent (800) and non-insulin-dependent glucose uptake (276), improves insulin sensitivity (336; 616), and preserves β-cell mass (405; 596) in animal models of diabetes. Thus, despite being a potent stressor, the effects of exercise stress and non-exercise stressors are clearly different.

Hans Selye is given credit as being the first person to report that the systemic effects of physical exercise differed from those produced by other types of stimuli (666; 667). Such differences are likely related to the fact that with repeated bouts of similar types of exercise (i.e. training), the HPA axis response to an acute exercise bout of similar characteristics and relative intensity becomes attenuated (816). Furthermore, resting levels of these hormones have been found to rise initially but eventually return to pre-training levels during the course of exercise training (103). The net result is that habituation of the hormonal response to stress changes which has an effect systemically such that decreases in insulin production/secretion and action is less pronounced (54; 257; 319), and the glucocorticoid and catecholamine responses are attenuated (150; 218).

A brief statement about the effects of non-exercise forms of stress in obesity and insulin resistance is merited: paradoxically non-exercise forms of stress may confer a protective effect in rodent models of T2DM (364). Chronic repeated immobilization of OLETF rats
decreased body weight, and improved glycemic control and glucose tolerance (364), and prevented 80% of T2DM in this model. In contrast, acute stress transiently increased blood glucose, and short-term (10 days) repeated stress lowered food intake and body weight, without affecting glucose tolerance (364). Importantly, this study suggested that chronic intermittent stress might have different effects than acute stress on T2DM in the OLETF rat. In humans, some studies suggest that the energy mobilizing effects of acute stress are deleterious to short term glycemic control (9; 234; 263; 291; 510), whereas other studies suggest that it need not worsen long term glycemic control (9; 528; 585; 630; 631).

To summarize: despite exercise-induced elevations of stress hormones and their noted diabetogenic actions, exercise training has been shown to be important in the prevention of T2DM (280; 281; 430; 564; 737) Together, these findings suggest the likelihood that exercise in itself, as well as the neurogenic stress component of non-volitional exercise training, may have a protective effect on the development of diabetes, despite elevating HPA-axis activity acutely.

1.21 Exercise Training Increases Glucose Uptake Independently of Insulin

Some of these beneficial effects of exercise are not necessarily related to improvements in protein expression or tyrosine phosphorylation of intermediates in the insulin signaling cascade. Increased glucose uptake may be mediated by simple processes such as increased total lean body mass and increased blood flow to muscle (158). Such increased muscle mass and circulation to these areas allows for glucose to be taken up more effectively simply due to the law of mass action (158). Other mechanisms involve more complex intracellular signaling pathways. For example, glucose transport in skeletal muscle is not only stimulated by insulin but also by insulin independent mechanisms that
are activated by contraction (300; 529; 219; 239), hypoxia (27; 113; 120), nitric oxide (30; 620; 186), and bradykinin (25; 728). Muscle contraction also leads to activation of ERK1/2 and isoforms of MAPK (237). In ZF rats exercise training leads to upregulation of ERK2 protein expression (556) and there is speculation that increases in MAPK signaling may be associated with several exercise-mediated adaptive responses in skeletal muscle (475; 538). Goodyear and co-workers showed that exercise by itself, mimics some of the signaling changes caused by insulin in rat skeletal muscle, including activation of MAPKs, Akt, and P70S6K (237). They also showed that exercise inhibited GSK, while in adipose tissue it was shown that GLUT4 can be stimulated to the membrane independently of PI3K (237). These findings were later reported in skeletal muscle when studies were done with wortmannin, which inhibits PI3K and thus glucose transport by insulin but not by contraction/insulin independent pathways (443; 471; 837). AMPK was soon found to be responsible for this exercised induced GLUT4 translocation to the membrane (503). Using an isolated rat hindquarter preparation Winder et. al showed that perfusion with an AMPK activator (AICAR) increased glucose uptake 200% (427), but this increase in glucose transport was not blocked when wortmannin was added to the study (276). These findings further indicate the role of other kinases during contraction-induced increases in skeletal muscle glucose uptake independent of insulin action.

1.22 Exercise and Insulin Resistance: Rodent Studies

During a single bout of exercise a significant glucose lowering effect is observed in obese rodents (220). Such beneficial effects are present many hours or even days after the last exercise bout (781) and are associated with increased glucose tolerance and insulin sensitivity at peripheral insulin sensitive tissue such as skeletal muscle and fat, but also at the liver (781). It has been suggested that exercise could increase glucose uptake in
skeletal muscle by enhancing the synthesis of GLUT4 at the level of transcription (530). Studies have shown that a single bout of exercise can increase GLUT4 mRNA in untrained (1.4-fold) and trained rats (1.8-fold) and that these effects are sustained for 3 hours after cessation of the exercise bout (530). Similarly a 50% increase in GLUT4 protein and a 200% increase in GLUT4 mRNA have been found in skeletal muscle 16 hours after a single bout of swim exercise (785). Other studies indicate that the acute effect of exercise on glycemia is likely due to the ability of contractile activity to activate skeletal muscle GLUT4 translocation to the cell membrane (80; 81; 173; 288; 404), which has been shown after a single bout of exercise (385; 404). The concept is verified by observations that whole-body glucose disposal during a euglycemic, hyperinsulinemic clamp is enhanced in ZF after a single bout of exercise (100; 220; 237) and is associated with increased insulin-stimulated glucose transport in skeletal muscle (538).

The longer term effect of a single bout of exercise on insulin signaling in insulin resistant skeletal muscle is less definitive. Studies in isolated perfused rat hindquarters demonstrated that the ability of physiological concentrations of insulin to stimulate glucose uptake and glycogen synthesis in muscle is enhanced for several hours after a treadmill run (616). They showed that this effect is restricted to muscles that had performed the work, as judged by glycogen depletion (616). Later it was shown by Cartee et al. that if during the period following exercise, rats were fed a low-carbohydrate diet, contraction-induced increases in insulin sensitivity could be maintained for a longer period time due to a delayed period of glycogen repletion (114). Despite these findings, reports of acute exercise having no effect do exist. For example, it has been shown acutely that one 30 minute bout of moderate to high intensity exercise does not improve glucose tolerance in untrained rats, as assessed by IVGTT (334). Further experimentation is warranted.
Mild exercise training in the form of treadmill running in 25 week-old ZF rats significantly improves glucose disposal during an OGTT and reduces the exaggerated insulin response to a glucose challenge (41). Swim exercise training in younger ZF rats (7 weeks of age) can prevent the deterioration in glucose tolerance and insulin sensitivity seen in older rats of this model (41; 785). Shima et al. determined that exercise training is effective in preventing the development of T2DM in the OLETF rat (680) as their fasting, and 120-minute, plasma insulin levels after an OGTT were significantly lower in the trained OLETF versus sedentary OLETF rats (680). The fact that acute decreases in plasma insulin persisted for several days after cessation of the exercise regime suggested that changes were happening with respect to insulin signaling and glucose entry into the cell (50; 51).

In the ZF rat GLUT4 protein expression is generally not defective (188; 211; 292; 332) however insulin stimulated GLUT4 protein translocation and glucose transport (142; 188; 288) are substantially impaired in isolated skeletal muscle from these obese animals. Several studies have gone on to indicate that exercise training in ZF rats improves whole-body glucose tolerance (140; 644; 646; 708) and insulin action on glucose transport in skeletal muscles and in perfused hind limbs (32; 83; 140; 329; 330; 814) and in isolated incubated skeletal muscles (187; 644; 646; 708). Studies in OLETF, GK, ZF, and ZDF rats also report improvements in whole-body insulin sensitivity after extended exercise training (125; 292; 596; 638; 639; 673; 678; 679; 681; 754). Thus, a well-established adaptive response to exercise training in conditions of insulin resistance is improved glucose tolerance and enhanced skeletal muscle insulin sensitivity (750).

The two adaptations that occur in ZF rats which have been exercise trained are upregulation of GLUT4 total protein expression (32; 83; 187; 211; 292; 644; 646; 673; 708)
and increased GLUT4 translocation (83) to the sarcolemma after insulin stimulation (187). In ZDF rats, treadmill running increases GLUT4 expression compared to untreated ZDF animals (185; 187; 207; 211). Thus, exercise training reverses insulin resistance in the ZF rats, and maintains total GLUT4 expression in red muscle of ZDF rats.

In animal models with normal insulin signaling, exercise training leads to enhancement of specific steps in the insulin signaling cascade including increased mRNA and protein expression of the insulin receptor, IRS-1, PI3K, and MAPK/ERK1 (122; 398; 399). IRS-1 is a well-described substrate of the insulin receptor that, after tyrosine phosphorylation, associates with and activates PI3K (292). ZF and ZDF rats have defects in crucial factors in the insulin signaling cascade. In hind limb muscle from ZF rats IRS-1 protein expression and insulin-stimulated IRS-1 tyrosine phosphorylation are 60% and 38% decreased relative to lean Zucker rats (17). Furthermore the p85 regulatory subunit of PI3K associated with tyrosine phosphorylated IRS-1 in the insulin-stimulated state is only 29% of lean control levels (17). Lastly, IRS-1-associated PI3K activity in muscle immunoprecipitates form these obese animals is only 54% of the level observed in lean animals (17).

Exercise training of ZF rats leads to upregulation of insulin receptor tyrosine phosphorylation (292) and enhanced IRS-1 protein expression (644; 646) with no alteration in the expression of the insulin receptor β subunit (292; 644; 646). The p85 subunit of PI3K (292; 644; 646) as well as Akt/PKB (292) are also unaltered. Recently, however, Hevener et al., showed exercise training, in conjunction with the insulin sensitizing agent, troglitazone, produced normal insulin action in obese ZF rats via normalization of insulin receptor phosphorylation, IRS-1 protein content, and Akt/PKB phosphorylation (292). There are contradicting reports however; in a recent study, (125) 7 weeks of exercise in ZF
rats did not cause any increased insulin action on insulin receptor or IRS-1 tyrosine phosphorylation, PI3K activity or Akt/PKB serine phosphorylation (125). These last data indicate that studies of exercise in obesity and T2DM are less than conclusive.

In summary: In normal rodents moderate intensity exercise training can improve glucose tolerance (43; 334), whole-body insulin sensitivity (335; 336) and insulin action on skeletal muscle glucose transport (287; 626; 627; 694). The protein expression of skeletal muscle GLUT4 plays a major role for insulin stimulation of glucose transport (286; 387) and also plays a role in the increased insulin action on skeletal muscle glucose transport after training (243; 287; 530; 626; 627; 645; 694). Increases in GLUT4 translocation (83) and cell surface GLUT4 content after insulin stimulation (187) have also been shown with endurance training in ZF rats. Skeletal muscle is most often indicated as the primary site for the beneficial adaptive responses of whole-body insulin sensitivity to exercise training in the ZF rat (750).

1.23 What is known In Humans?

The first clues that exercise might enhance the ability of insulin to stimulate muscle glucose utilization were provided by Per Bjorntorp in the early 1970’s (51). He noted that glucose tolerance was better and plasma insulin levels were lower in men who participated in competitive sports when compared to age-matched and weight-matched controls (51). With respect to obese women, it was later discovered that 6 weeks of physical activity also lowered plasma insulin levels (50; 51).

Insulin resistance in T2DM, except for that observed in morbidly obese humans (172), is not generally associated with a decreased skeletal muscle level of GLUT4 protein (859). Insulin stimulation, however, fails to induce normal GLUT4 protein translocation to the
sarcolemma in skeletal muscle from subjects with T2DM (255; 642; 858). Insulin-stimulated insulin receptor, IRS-1, and PI3K tyrosine phosphorylation is decreased in muscle from insulin resistant subjects when compared to insulin sensitive controls (49). Also, insulin-stimulated Akt/PKB kinase activity is reported to be significantly reduced in skeletal muscle from individuals with T2DM (419) although not always (400). In T2DM, insulin stimulated glycogen synthase activity is also reduced (40; 130; 418; 741) while reductions in non-oxidative glucose disposal in these individuals is thought to be secondary to defects in glucose transport (130; 176). Despite these differences, in T2DM no reduction in insulin-simulated MAPK phosphorylation is detected in incubated muscle preparations from T2DM subjects (418). Therefore defects in insulin sensitivity in T2DM are due to defects in insulin signaling proteins and in GLUT4 protein translocation (285).

During a single bout of exercise in men with T2DM a significant glucose lowering effect is observed (315; 436; 522; 786) with a concomitant decrease in plasma insulin (522). Enhanced glucose transport activity seen after an acute exercise bout in T2DM humans persists into the immediate post-exercise period (166; 315; 506) and is associated with enhanced insulin sensitivity immediately after (506) and 20 hours after exercise (166) however this effect is lost approximately 24 hours after an acute exercise bout (144). One underlying cellular mechanism for this glucose lowering effect of acute exercise in insulin resistance and T2DM in humans is an increase in plasma membrane GLUT4 protein in skeletal muscle (385). Protein expression however is not thought to be altered in humans by a single bout of exercise (385).

In humans, compared to Zucker rats, only a paucity of studies have elucidated the effects of exercise training to improve whole-body insulin action and glucose disposal. In humans
endurance training leads to improvements in glucose tolerance (316; 629) and whole-body insulin mediated glucose disposal (161). Exercise training in insulin sensitive humans results in upregulation of insulin-stimulated PI3K activity in both longitudinal (312) and cross sectional studies (407). Contradicting these data however is a recent study showing that 1 week of exercise training for 1 hour per day did not enhance insulin stimulation of PI3K and Akt/PKB in the vastus lateralis of middle-aged men (736).

With respect to the effects of acute exercise on insulin signaling in insulin resistance studies are quite few. Six weeks of exercise training in the offspring of human T2DM subjects is associated with enhancement of insulin-stimulated glucose transport and phosphorylation in red muscle (578). A single bout of cycling in subjects with T2DM has been shown to increase skeletal muscle tyrosine phosphorylation of the insulin receptor and IRS-1 in response to insulin (144). Despite these findings, insulin stimulated IRS-1 association with PI3K was not enhanced by this prior exercise while 24 hours later whole-body insulin stimulated glucose disposal, as assessed by a hyperinsulinemic euglycemic clamp, was not increased relative to the sedentary state (144). Despite these data, the mechanisms by which exercise training can enhance insulin stimulated GLUT4 translocation in insulin resistant muscle is for the most part poorly understood (285).
1.24 Exercise and Maintenance of β-cell Compensation

In both obesity and T2DM, subjects manifest similar impairments in insulin sensitivity and glucose uptake (95-97; 581) thus, differences in peripheral insulin action do not differentiate T2DM from their obese, euglycemic, counterparts. Studies in ZDF rats have shown that loss of β-cell compensation with respect to GSIS (686) and β-cell mass adaptation, are the defining characteristics of T2DM in this model (586). Interestingly, only a paucity of studies exist which clarify the beneficial effects of exercise on the pancreas, per se. Shima and associates (680) utilized the OLETF rat, a model of spontaneous T2DM, and volitional wheel running to assess the effect of exercise training on insulin secretion, insulin resistance and β-cell function. Islet morphology has been shown to be highly correlated with islet function in other models of T2DM (279). It was suggested, therefore, that regular exercise was protective in preserving viable β-cell mass (680). Immunohistochemistry performed on the pancreata from exercise trained and sedentary animals confirmed that exercise minimized the deleterious morphological changes in islet structure (679). Lastly, using the same rodent model as the previous studies, Shima et. al. (681) elucidated some of the pancreatic adaptations, with respect to mass compensation, that were occurring with exercise training. In this particular study the effects of exercise on alterations in the amount of β-cell mass, insulin content and fibrous tissue present in the pancreas were examined in the OLETF rat (681). After a 70% Px to induce hyperglycemia, rats were divided into two subgroups consisting of an exercise group and a sedentary control group. Exercise training resulted in beneficial effects on the pancreas as reflected by an increase in pancreatic volume, accompanied by increases in β-cell mass and insulin content, as well as less islet connective tissue compared with the sedentary non-diabetic sham rats, although
exercise failed to improve the capacity for β-cell proliferation (681). Lastly, exercise has been shown to increase IRS-2 and PDX-1 in ovariectomized female rats (124).

Human studies of the effects of exercise on pancreatic function are rare. Of the paucity of studies that do exist data indicates the capacity for physical training to enhance β-cell function depends on the remaining β-cell secretory capacity (163). One study evaluated the relationship between β-cell secretory capacity and effects of exercise. It assigned individuals who had C-peptide concentrations greater than 1.1 nmol after 1 mg i.v. glucagon as ‘moderate secretors’, and those whose response was lower as ‘low secretors’ (163). The study revealed that following training β-cell secretory capacity was enhanced in diabetic ‘moderate secretors’. However, training did not affect β-cell function of ‘low secretors’ (163). It was postulated that moderately damaged β-cells are more responsive to exercise than severely failing β-cells (163).

Authors commonly suggest that improvements in peripheral insulin sensitivity confer an indirect beneficial effect on β-cells by decreasing insulin demand and minimizing β-cell exhaustion, at the same time minimizing hyperglycemia-mediated loss in β-cell function (596). Studies investigating the mechanism responsible for these observations do not exist.

1.25 Exercise and Inflammation

Cross-sectional studies demonstrate an association between physical inactivity and low-grade systemic inflammation (2; 192; 229; 402; 497; 695; 727; 799; 92). CRP has a role both in the induction of anti-inflammatory cytokines in circulating monocytes and in the suppression of the synthesis of pro-inflammatory cytokines in tissue macrophages (605). A small increase of CRP levels is seen the day after exercise of longer duration (571). However, the findings in two longitudinal studies that regular training induces a reduction in
CRP level (192; 497) suggests that physical activity may suppress systemic low-grade inflammation.

The cytokine response induced by exercise is unique in that it differs from the cytokine response to infection (193; 571; 723). For example, TNFα and IL-1β do not increase with exercise (193; 571), while the cytokines which respond most robustly with exercise are IL-6, and IL-10 which have anti-inflammatory characteristics. Typically, IL-6 is the first cytokine present in the circulation during exercise (193; 571; 572). Plasma-IL-6 increases in an exponential fashion with exercise and is related to exercise intensity, duration, the mass of muscle recruited, one's endurance capacity and declines in the post-exercise period (193; 571-573; 723). IL-6 mRNA is upregulated in contracting skeletal muscle (194; 349; 558; 709; 711) and that the transcriptional rate of the IL-6 gene is markedly enhanced by exercise (378). It has also been demonstrated IL-6 protein is expressed in contracting muscle fibers (295; 575) and is released (711) from skeletal muscle during exercise whereas this is not the case for TNFα (711; 713).

Studies indicate that increased IL-6 during exercise has inhibitory effects on pro-inflammatory cytokines such as TNFα and IL-1β production (662). After exercise, the high circulating levels of IL-6 are followed by an increase in IL-1ra, sTNF-R, and IL-10 (557; 559; 710). The appearance of IL-10 and IL-1ra in the circulation after exercise also contributes to mediating the anti-inflammatory effects of exercise as IL-10 inhibits the production of IL-1α, IL-1β, and TNFα as well as the production of chemokines, including IL-8 and macrophage inflammatory protein-α from LPS-activated human monocytes (516; 602). Thus data indicates that exercise is associated with a change in circulating cytokines that promotes a net anti-inflammatory action which is mediated via IL-6 and IL-10. As is the case for other
hormones, changes in circulating cytokines become relevant when one considers the ratio between a cytokine and its antagonist, such as the IL-1ra/IL-1β ratio (516). It is this net inflammatory status that determines the function of NF-κB to act as either a pro-inflammatory or anti-inflammatory switch. Because exercise is responsible for changes in the ratio between a cytokine and its antagonist it is therefore, possible that physical activity is responsible for altering the profile of the cytokine cascade and the function of NF-κB.

To study the anti-inflammatory effects of exercise low doses of Escherichia coli (E. coli) endotoxin have been administered to humans to induce low grade inflammation. In resting subjects E. Coli induces a 2-3-fold increase in plasma TNFα in contrast to trained subjects who do not show a TNFα response (707). Recent studies support this concept as exercise normalized overexpression of TNFα in TNF-R KO mice (377). In addition, rhIL-6 infusion, which causes an increase in plasma IL-6 mimicking the exercise-induced IL-6 response, inhibited endotoxin-induced increase in plasma TNFα in humans (707).

Taken together, these observations suggested that IL-10 plays an important role in orchestrating the inflammatory reaction mainly via the inhibition of the transcription of their corresponding genes (795; 796). IL-10 also prevents cytokine synthesis by posttranscriptional mechanisms (59). In this way, the anti-inflammatory effects of exercise may offer protection against TNFα-induced insulin resistance. Therefore, IL-6 induces an anti-inflammatory environment by inducing the production of IL-1ra and IL-10, but it also inhibits TNFα production, as suggested by in vitro (199) and animal studies (496; 509).

Surprisingly, in the male ZDF rat, differences in the levels of these cytokines, over time, and when compared to age-matched ZF rats have not been studied, nor has the effect of
volitional exercise to alter pro-inflammatory and anti-inflammatory levels been explored in this model.

1.26 Summary

It has long been known that exercise training is beneficial in the treatment of T2DM and the recognition of its therapeutic usefulness dates back many decades (238; 239; 241; 242). For example, regular exercise (30 min/day), has been shown to reduce the development of T2DM by 68% in humans with impaired glucose tolerance (241). Studies using rodent models of obesity and insulin resistance, such as the ZDF rat, are frequently used to elucidate many of the mechanisms responsible for the deterioration from a prediabetic state to overt T2DM (200; 583; 749; 793; 794). Male ZDF rats develop a phenotype of obesity, insulin resistance, and eventually hyperglycemia due to a leptin receptor mutation, resulting in a phenotype very similar to humans with T2DM (583). In these rodents, glucose intolerance usually develops by age 8 weeks of age, followed by overt hyperglycemia by 10-12 weeks of age (200; 583; 749). In the prediabetic phase, ZDF rats maintain normoglycemia by a compensatory increase in β-cell function resulting in hyperinsulinemia (127; 200; 583; 749). This compensatory adaptation begins to fail as animals enter the “diabetic phase” defined by a dramatic increase in β-cell apoptosis and a corresponding decrease in β-cell mass (200; 583; 586; 749). In obese prediabetic humans, there is a similar period of adaptive β-cell expansion followed by a marked reduction in β-cell mass as the disease progresses (101; 126; 411).

Adaptive responses that underlie the enhanced insulin-stimulated glucose disposal with exercise training are related to an upregulation of GLUT4 protein expression in skeletal muscle tissue (83; 163; 207-209; 238; 239; 241; 242; 673). Increases in GLUT4
translocation (83) and cell surface GLUT4 content after insulin stimulation (187) have also been shown with endurance training in ZF rats. In ZDF rats, treadmill running increases GLUT4 expression compared to untreated ZDF animals (83; 187; 211). Thus, exercise training reverses insulin resistance in the ZF rats, and maintains total GLUT4 expression in red muscle of ZDF rats.

The prevention and treatment of T2DM is not limited to peripheral improvements in insulin sensitivity and glucose tolerance at insulin sensitive tissues. For example, pharmacological interventions that preserve β-cell mass in obese insulin resistant animals, predisposed to developing overt hyperglycemia, result in the staving off of the progression of T2DM (201). In addition agents such as salycilates, which modulate hepatic inflammation, have been shown to be effective to attenuate and treat insulin resistance rodent in models of obesity and T2DM (396).

1.27 Conclusion

- The growing type 2 diabetes epidemic calls for research to help in the management and prevention of the disease. Exercise remains an important option, partly because of the various studies in humans and animal models that demonstrate an attenuation in the progression of diabetes through enhancement of insulin sensitivity and glycemic control.

- Defining features of T2DM include impaired insulin action at the periphery and an insufficient β-cell response to increased insulin demand. There are numerous sites of insulin action throughout the body, namely skeletal muscle, fat, and the liver.
• Unlike what has been learned with respect to the classical sites of peripheral insulin action, mechanisms by which exercise confers protection to the β-cell in terms of function and mass compensation, as well as how exercise may attenuate hepatic insulin resistance remain elusive.

• With these subsequent studies I have focused on the pancreas where insulin is produced and the liver, one of the three major sites of insulin action.
2.1 Study 1: Rationale

Normal β-cells can compensate for insulin resistance by increased insulin secretion and mass, but insufficient compensation leads to the onset of glucose intolerance. The development of T2DM is associated with combined pancreatic β-cell decompensation and insulin resistance. Ensuing chronic hyperglycemia leads to impaired insulin gene expression and loss of normal β-cell phenotype and function.

In rodent models, caloric restriction (545; 545; 550; 550), a reduction in fat mass (217), and increased exercise (125; 292; 596; 681) improve insulin sensitivity and attenuate the development of T2DM. Other studies have shown that improvements in glycemia were not due to reductions in visceral fat pad weight (217), body weight, or food intake (125; 596), which are commonly cited explanations describing the mechanisms behind increased insulin sensitivity with exercise training (125; 201).

Only a paucity of studies exist which clarify the beneficial effects of exercise on the pancreas, per se. Studies in OLETF, GK, ZF, and ZDF rats have shown improvements in whole-body insulin sensitivity and preservation of β-cell mass with exercise training (125; 292; 638; 678-681; 754). Authors commonly suggest that improvements in peripheral insulin sensitivity confer an indirect beneficial effect to β-cells by decreasing insulin demand and minimizing β-cell exhaustion, at the same time minimizing hyperglycemia mediated loss in β-cell function (125; 596). Interestingly, in humans with T2DM, regular exercise has been shown to enhance insulin response to either hyperglycemia or to arginine stimulation (163), suggesting that there may be direct effects of exercise on pancreatic function.
Development of β-cell dysfunction in T2DM is characterized by defects in glucose sensing, insulin secretion and defects in β-cell mass compensation in response to increased insulin demand. Impaired basal insulin secretion and GSIS is characterized by reductions in GLUT2 protein and insulin content. Defective β-cell mass compensation in response to increased insulin demand is also a major predictor of insufficient insulin production and remains one of the defining features of T2DM. Maintained adaptation of β-cell mass to increased insulin demand has recently been shown to be dependent on IRS-2 signaling and downstream Akt/PKB-473-serine phosphorylation (340). Despite the acumen of these recent studies, the potential role of exercise training to maintain β-cell GLUT2 expression and to attenuate the loss of IRS-2 and corresponding Akt/PKB-473-serine phosphorylation remains unknown.

The ER is known to play a pivotal role in β-cells, and ER-stress-dependent apoptosis in islets has been described recently (23; 373). When diabetes occurs, pancreatic β-cells undergo numerous stresses, including oxidative stress. In response to hyperglycemia and corresponding oxidative stress, ER quality control functioning is impaired and as a result, protein misfolding, ER stress, and defective insulin secretion occurs. In a collaborative study we recently showed that hyperglycemia and corresponding oxidative stress lead to ER stress and the formation of cytotoxic ubiquitinated protein structures in pancreatic β-cells (370). This cellular response to ER-stress, called the UPR (663), has been shown to induce serine phosphorylation of IRS proteins in liver and fat via JNK (563) and might therefore also suppress IRS-2 in β-cells.
2.2 Study 1: Purpose

Antioxidant strategies are being considered for the protection of β-cell function during and after the onset of hyperglycemia (622). We wished to examine the possibility that the same beneficial effects of conferred with antioxidants on the β-cell phenotype could occur with the prevention of hyperglycemia with exercise training. The purpose of this study, therefore, was to investigate the effects of a long-term forced exercise training program on β-cell compensation when extended into a period when severe hyperglycemia is manifested in male ZDF rats lacking intervention. We wished to characterize the effects of exercise may have to preserve expression of important signaling molecules determining β-cell compensation to worsening insulin resistance. Because we have previously shown that the UPR and accumulation of ubiquitinated protein aggregates are prevented with the administration of antioxidants we wished to elucidate potential effect of exercise training to attenuate oxidative stress in pancreatic β-cells by looking at this marker.

2.3 Study 1: Hypothesis

We hypothesized that protective effects of exercise training on β-cell function and β-cell mass compensation in male ZDF rats would persist until 19 weeks of age when severe hyperglycemia is manifested in untreated male ZDF rats. More specifically, we hypothesized that beneficial effects of exercise on β-cell compensation would be associated with maintained immunodetectable levels of GLUT2 and Akt/PKB-473-serine phosphorylation and decreased oxidative stress related to attenuated insoluble protein aggregate accumulation.
2.4 Study 2: Rationale

Inflammation is the primary cause of obesity-linked insulin resistance. Chronic elevations in circulating markers of inflammation such as C-reactive protein (CRP) and haptoglobin occur both in obesity and T2DM (151). Cytokines such as TNFα and IL-6 cause insulin resistance in obesity (34), while exposure of cells to TNFα stimulates IRS-1-307 serine phosphorylation (4; 310; 838) resulting in disruption of normal insulin signaling and insulin action (5; 310; 570). A second mechanism that initiates inflammation in obesity and T2DM is oxidative stress which activates inflammatory signaling (79; 215; 838).

Serine/threonine kinases elevated in obesity (805; 857) such as JNK, IKKβ, and PKC isoforms are implicated in the mechanisms of hepatic insulin resistance as they are activated by inflammatory and oxidative stimuli and contribute to inhibition of insulin signaling (4; 222; 563) (24; 57; 104; 432; 653; 654; 697). PKCδ is activated by short-term elevations in plasma FFA (57; 432), while another novel isoform, PKCε, appears to be involved in hepatic insulin resistance caused by high fat feeding (653; 654). A potential pathway may involve FFA-induced activation of PKC, which impairs insulin signaling pathways either directly by phosphorylating serine/threonine sites of insulin receptors and/or IRS-1/2 or indirectly by activating other serine kinases such as JNK and IKKβ. Obesity also causes ER stress which leads to the activation of inflammatory signaling pathways including JNK (526; 562; 563). Therefore JNK has recently emerged as a central metabolic modulator of insulin resistance in obesity (294).

Exercise is beneficial in the prevention and treatment of insulin resistance and T2DM in humans and in rodent models including ZF and ZDF rats (50; 125; 185; 187; 188; 211; 240; 292; 596; 749). Exercise in obesity is associated with altered levels of circulating markers
of oxidative stress and inflammation. For example, a single bout of exercise increases circulating markers of oxidative stress, however lower plasma markers of oxidative stress are found in the trained state. Additionally, a single bout of strenuous exercise increases circulating IL-6 which has an inhibitory effect on TNFα production (662); anti-IL-6 treated or IL-6 KO mice have elevated TNFα (496; 509). Exercise training on the other hand decreases elevated obesity-related systemic markers of inflammation such as CRP (151) while in humans basal circulating levels of IL-6 are lower after increased physical activity (132; 611). To date, however, the potential effect of exercise training to modulate the cellular pathways involved in inflammation and related serine phosphorylation of IRS-1, as it pertains to improved insulin sensitivity in insulin target tissues, remains unknown.

2.5 Study 2: Purpose

The purpose of the study was to identify the signaling pathway whereby exercise inhibits inflammation and improves insulin sensitivity in the liver which is a major target tissue in a model of obesity and T2DM.

2.6 Study 2: Hypothesis

We hypothesized that in the male ZDF rat volitional running would attenuate the development of hyperglycemia and that such improvements in glucoregulation would be associated with decreased circulating plasma markers of oxidative stress and inflammation. More specifically we hypothesized such exercise-mediated improvements in systemic oxidative stress and inflammation would be associated with decreased hepatic oxidative stress and activity of serine/threonine kinases identified as key players in impaired hepatic IRS-1 tyrosine phosphorylation.
LABORATORY METHODS

3.1 Food Intake and Body Mass Measurements

Food intake and body weight were measured each day prior to treatment using a Tanita digital scale model #1144 accurate to 2g (Tanita Corporation of America, Inc. 2625 South Clearbrook Drive Arlington Heights, Illinois). To obtain weekly average food intake values from daily measurements, daily food intakes for animals (excluding the day of fast) were averaged over the week. Total food intake over the course of the study was also determined for each animal by summing the weekly averages. We also calculated food intake spanning the time between treatment (09:00-10:00h) and food removal (16:00-18:00h) on the days of fast (Thursdays). These values for each week were then summated over the weeks of treatment.

3.2 Sample Collection

During the treatment period blood samples were taken via a nick to the nub of the tail. To minimize the stress induced by this method of sampling, a topical anesthetic (Emla™ cream, AstraZeneca, Mississauga, Canada) was applied to the tails 20 minutes before blood sampling. Blood samples were collected into heparinized microvettes (Sarstedt, Montreal, Canada). Samples taken to measure fasting insulin levels (~10µl) were done at the same time as measurement of fasting blood glucose samples and collected plasma was separated from blood samples by centrifugation at 2500 rpm for 1 minute and stored at -20°C. To reduce the chance of infection, a topical germicide (Betadine™ solution, Purdue Pharma, Pickering, Canada) was applied to the tail following blood collection. At the termination of the study animals were decapitated. Blood samples taken at this time were
collected in 1.5ml Eppendorf microtubes (Diamed for insulin, corticosterone, cytokines, MDA, and lipids were collected in chilled microtubes containing a 1:1 mixture of ethylenediammine tetraacetic acid (EDTA) (2.4g/10ml distilled water; Sangon Ltd Canada, Scarborough, ON)/Trasylol (2000 kallikrein IU; Bayer Canada Ltd, Mississauga, ON)-1µl for every 10µl of blood collected. EDTA served as an anticoagulant, while Trasylol is a broad spectrum proteolytic inhibitor that protects plasma against loss of hormone reactivity. The blood and solution were mixed immediately after decapitation and all blood samples were centrifuged at 2500 rpm for 1 minute. Plasma was then aliquoted into new Eppendorf microtubes and stored at -20°C until the hormone assay was performed.

3.3 Pancreas Removal, Preparation and Fixation

Six hours before removal of the pancreas, the animals were injected intraperitoneally with 100 mg/kg BrdU (Sigma Aldrich Canada Ltd., Oakville, ON, Canada), a thymidine analogue incorporated into newly synthesized DNA (200; 406). Within 10 minutes of decapitation the pancreas was removed, blotted, and extraneous fat and lymph nodes were removed (200; 406). The pancreas was then weighed before being placed in Bock’s fixative (75ml distilled water, 25ml 37% formaldehyde (EMD Chemicals, San Diego, California, USA), 5 ml glacial acetic acid (Caledon Laboratory Chemicals, Georgetown, ON, Canada). After fixation, tissue samples were cut into ~20 small pieces (depending on animal’s age and size of pancreas), and randomly placed into tissue cassettes to ensure an equal representation of head and tail segments. The cassettes were then placed in 70% ethanol until time of paraffin embedding at the University Health Network Pathology Research Program Labs, Toronto General Hospital. 4µm slices were cut on an Olympus microtome (Carsen Group, Markham, ON, Canada) from paraffin blocks and mounted onto 25mm x 75mm slides. Slides were stored at room temperature until time of analysis.
3.4 Hepatocyte Homogenate Preparation

Liver samples (150mg) were homogenized by hand-held glass homogenizer in buffer A (50mM Tris-HCl pH 7.5, 10mM EGTA, 2mM EDTA, 1mM NaHCO₃, 5mM MgCl₂, 1mM Na₂VO₄, 1mM NaF, 1µg/ml aprotinin, leupeptin, pepstatin, 0.1mM phenylmethylsulphonyl fluoride (PMSF), 1µM microcystin). The homogenates were centrifuged at 100,000g for 1h at 4°C, and the supernatants were retained as the cytosolic fraction. The pellet was resuspended in buffer B (buffer A + 1% Triton X-100), homogenized by passing through a 23-gauge needle three times, incubated for 15 min on ice, and centrifuged at 100,000 g for 1h at 4°C. The supernatant provided the solubilized membrane fraction. The purity of the cytosolic and membrane fractions was assessed by assaying glucose-6-phosphate dehydrogenase (Sigma, St Louis, MO) and 5'-nucleotidase activities (Sigma, St Louis, MO), respectively. Homogenization of muscle (828) and fat (39) samples was performed as described previously. The results showed that the index of purity of both fractions were >90%.

3.5 Assay Procedures

3.5.1 Plasma Glucose

Plasma glucose concentrations were measured immediately following acquisition of blood samples during the experiments. Blood glucose was measured using a single drop of tail capillary blood (~5µl) with a blood glucose test strip (Ascensia Elite™, Bayer, Toronto, Canada) and glucometer (Ascensia Elite™ XL Blood Glucose Meter, Bayer, Toronto, Canada). On each strip, there are about 10 layers, including a stiff plastic base plate, and other layers containing chemicals or acting as spacers. For instance, there is a layer containing two electrodes (silver or other similar metal). There also is a layer of the immobilized enzyme, glucose oxidase, and another layer containing microcrystalline
potassium ferricyanide, $[K_3\text{Fe(CN)}_6]$. These layers are suitably separated by the spacers to allow a small amount of blood to enter. When the end of a strip is touched to a droplet of blood (usually on a fingertip), the blood flows in by capillary action. The glucose in the blood sample reacts with the glucose oxidase to form gluconic acid, which then reacts with ferricyanide to form ferrocyanide. The electrode oxidizes the ferrocyanide, and this generates a current directly proportional to the glucose concentration (421). Glucose oxidase is obtained from the fungus, Aspergillus niger. It is a dimeric protein with a molecular weight of about 160 kDa. It is strongly specific for beta-glucose (the type of glucose in blood), although 2-deoxy-glucose, D-mannose and D-fructose also can be oxidized, but at a much slower rate. The systematic name is potassium hexacyanoferrate (III), but it is also known as red prussiate or Prussian red, because of its ruby red color. It is a relatively inexpensive chemical and easily obtained (792).

3.5.2 Plasma Corticosterone Assay
Corticosterone was assayed using the ImmuChem™ double antibody I-125 Radioimmunoassay (RIA) supplied from ICN (ICN Biomedicals Inc., Costa Mesa, CA) and specifically designed for use in laboratory mice and rats. All reagents supplied with this double antibody corticosterone RIA kit were in liquid form and ready for use except for corticosterone high and low controls. Such low and high controls had to be reconstituted with 2.0ml of distilled water 30 minutes before use in the assay. Samples were then removed from -20°C storage and allowed to thaw at room temperature, followed by vortexing and centrifugation at 9000 rpm for 1.5 minutes to clear particulates. Six corticosterone standards were provided in concentrations ranging from 0-1000ng/ml. Using this same steroid diluent, 300µl is added in triplicate to tubes to measure blanks or non-
specific binding (NSB) and of the 0 standard. Aliquots of 100µl of each calibrator were pipetted into tubes in triplicate to construct a standard curve. In duplicate, 100µl of each high and low controls is added to each tube. Care was taken to take plasma samples from the middle of the sample to decrease debris and lipid collection. Samples were then prediluted with steroid diluent at ration of 1:200 by adding sample (5µl) into 1ml of diluent (PBS+gelatin+gamma globulins supplied with the kit). These mixtures were then briefly vortexed. Volumes (100µl) of this mixture and controls were then added to the appropriate tubes in duplicate. I-125-corticosterone derivative (200µl) was subsequently distributed to all the tubes followed by addition of the same of amount of rabbit anti-corticosterone antibody was added to all tubes except total count and NSB tubes. A two hour incubation period then followed at room temperature. When the incubation period was over, precipitation solution (500µl) was added to each sample and all tubes except total count tubes. The precipitation solution consisted of a mixture of polyethylene glycol and goat anti-rabbit gamma globulin in TRIS buffer. This reagent (500µl) flocculates the antibody-bound antigen, dividing the bound ligand and free unbound tracer that remained in the supernatant. This bound ligand (I-125 corticosterone is inversely related to the amount of non-radioactive corticosterone or endogenous corticosterone in the sample. The samples were then centrifuged at 2500 rpm for 25 minutes at 4°C and the supernatants were aspirated. Precipitating reagent was added to all tubes followed by vortexing and incubating for 20 minutes. The tubes then were spun at 2000-3000g for 20 min. The supernatant was aspirated and the radioactivity in the pellet was counted for 4 minutes in a gamma counter (Beckman Instruments, Fullerton Ca, USA).

To yield a standard curve, percent bound tracer was plotted against the concentration of corticosterone for all the calibrators. The counts (B) for each of the standards and
unknowns were expressed as a % of the mean counts of the “0=standard” (B₀). The sample values were read directly from the curve. Interassay coefficient of variation was between 6.5%-7.2% and intraassay coefficient of variation was 4.4%-10.3%, as calculated from the reference plasma. The % activity bound for each standard was plotted against the known concentration in order to construct the standard curve. The unknown sample was determined by the interpolation of the standard curve. The coefficient of interassay variation determined on reference plasma was less than 10%. This assay has 100% cross-reactivity with rat and mouse corticosterone, and <0.4% cross-reactivity with other steroid hormones.

\[
\text{% Activity Bound} = \frac{B (\text{Standard or sample})}{B_0} \times 100\%
\]

### 3.5.3 Plasma Insulin Assay

A limited sample volume necessitated a test which was highly sensitive such as the Rat Insulin ELISA kit (Cat# INSKR020, Crystal Chem, Downers Grove, Illinois). Preliminary readings of random samples indicated ZDF samples should be diluted 10X for fed and 5X for fasting samples with diluent 2, while in lean rats 2.5X for fed and 0X for fasting in order to ensure readings off of the high gain area of the curve. For fasted lean samples, insulin concentrations were below the assay’s detection ability. Samples were thawed at room temperature, vortexed, and then cleared of debris and lipids by centrifugation at 9000 rpm for 1.5 minutes while care was taken to take plasma samples from the middle of the sample to decreased debris and lipid collection. While samples were thawing, a 96-well microplate was washed 2 times with 300µl of washing buffer which is supplied with the kits. The plates were then lightly agitated, aspirated, and tapped dry on Kimwipes. 50µl of guinea pig anti-insulin and 45µl of diluent 2 (supplied in kit) were then added to each well. A standard curve was then created by adding 5µl of standards supplied with the kit. Once the samples
had been diluted accordingly 5µl of prediluted sample volume was added to the plate. After the overnight incubation wells were aspirated and washed with wash buffer 3 times. Following the final washing racks were tapped dry on Kimwipes. After the last wash and when the plate was dry, 100µl of anti-guinea pig antibody enzyme conjugate was added to each well and allowed to sit for 3 hours at room temperature after agitating to mix. After 2.5 hours a mixture of horseradish peroxidase-conjugated anti-guinea pig antibody is prepared and then added at 3 hours to the plate where a complex forms between the anti-guinea pig antibody and the guinea pig anti-insulin antibody on the wall of the plate well. After the reaction was complete, aspiration and washing occurs 5X as described above. Just before the 5th wash a mixture of phosphate-citrate buffer, H2O2 and o-phenylenediamine was prepared to yield the enzyme substrate solution. This mixture was made under darkness and kept free from metal as per the manufacturer’s suggestions. Once sufficiently dry, 100µl of this enzyme substrate solution was added to each well which then promptly reacts with the peroxidase complex in a colorimetric reaction which yields a yellow color in direct proportion to the amount of bound conjugate or sample/standard insulin. This reaction was allowed to occur for 30 minutes at room temperature in the complete darkness. To stop this reaction after 30 minutes, 50µl of 1N sulfuric acid was added to each well. Using a spectrophotometer/ELISA plate reader at 490nm and Beer’s law, concentrations were calculated from mean absorbencies of each sample against the standards. To analyze the data, I-SMART version 2.0 (Parkard Instrument Company) was used.

3.5.4 Plasma Triglycerides Assay

In order to measure plasma triglycerides a colorimetric enzymatic kit from Roche Diagnostics GmbH (Mannheim, Germany) was used. This kit is able to correct for free glycerol by a preliminary reaction where lipase and 4-aminophenazone are omitted while
triglycerol was hydrolyzed to glycerol. The production of this oxidation reaction does not react with 4-aminophenazone. Following this step, an enzymatic calorimetric assay reaction is used to determine the quantity of liberated glycerol by enzymatic hydrolysis of triglycerides. The process is broken down into two steps below.

**Step 1.**

Reagents 1 and 2 are prepared. A listing of reagents in each reagent mixture is listed below.

After making the mixtures 1.0ml of reagent 1 working solution was added too all the tubes followed by vortex mixing and room temperature incubation for 5 minutes. It is during this initial reaction that free glycerol is freed from the sample by a conversion from glycerol to glycerol-3-phosphate in a reaction catalyzed by glycerol kinase.

\[
\text{Glycerol kinase} \\
\text{Free glycerol + ATP} \rightarrow \text{Glycerol 3-phosphate} + \text{ADP}
\]

The glycerol-3-phosphate is then converted to dihydroxyacetone phosphate and peroxide by the enzyme glycerol phosphate oxidase.

\[
\text{Glycerol 3-phosphate oxidase} \\
\text{Glycerol 3-phosphate} + \text{O}_2 \rightarrow \text{Dihydroxyacetonephosphate} + \text{H}_2\text{O}_2
\]

Finally, peroxidase converts the peroxide and 4-chlorophenol to an oxidation product that does not react with 4-aminophenazone.

\[
\text{Peroxidase} \\
\text{H}_2\text{O}_2 + 4\text{-chlorophenol} \rightarrow \text{oxidation product} \text{ (does not react with 4-aminophenazone)}
\]

Thus, free glycerol in the sample is eliminated.

**Reagent 1:** Buffer, 0.15mmol/l Tris buffer, pH 7.6, 17.5mmol/l magnesium sulfate, 10mmol/l EDTA, 3.5mmol/l 4-chlorophenol, 6µmol/l potassium hexacyanoferrate (II), 0.15%
sodium cholate, 0.12% hydroxypolyethoxy-n-alkanes, ≥1mmol/l ATP, ≥ 0.4U/ml glycerol kinase, ≥ 5U/ml glycerol phosphate oxidase, ≥0.3U/ml peroxidase.

**Reagent 2:** buffer, 0.15mol/L Tris buffer, pH 7.6, 17.5mmol/l magnesium sulfate, 10mmol/l EDTA, 3.5mmol/l 4-chlorophenol, 6 µmol/l potassium hexacyanoferrate (II), 0.15% sodium cholate, 0.12% hydroxypolyethoxy-n-alkanes, ≥ 6U/ml **Lipase**, and 0.7mmol/l 4-aminophenazone.

**Step 2.**
Following the incubation period of step 1, 1.0ml of reagent 2 is added to each tube followed by vortexing and room temperature incubation for 10 minutes. During this reaction TG in the sample are converted to FFA and glycerol by lipase.

\[
\text{Lipase} \\
\text{Triglyceride} + 3 \text{H}_2\text{O} \rightarrow \text{Glycerol} + \text{fatty acids}
\]

ATP and glycerol are then converted to glycerol-3-phosphate by glycerol kinase

\[
\text{Glycerol Kinase} \\
\text{Glycerol} + \text{ATP} \rightarrow \text{Glycerol-3-phosphate} + \text{ADP}
\]

During the next reaction an enzyme called glycerol phosphate oxidase converts newly formed glycerol-3-phosphate to dihydroxyacetone phosphate and peroxide

\[
\text{Glycerol 3-phosphate oxidase} \\
\text{Glycerol-3-phosphate} + \text{O}_2 \rightarrow \text{Dihydroxyacetone phosphate} + \text{H}_2\text{O}_2
\]

Finally an enzyme called peroxidase converts the peroxide 4-aminophenazone, and 4-chlorophenol in solution to a colored compound called 4-(p-benzoquinone-monoimino)-phenazone

\[
\text{Peroxidase} \\
\text{H}_2\text{O}_2 + \text{4-aminophenazone} + 4\text{-chlorophenol} \rightarrow 4\text{-}(p\text{-benzoquilone-monoimino})\text{-phenazone (4-PBMP)} + 2\text{H}_2\text{O} + \text{HCl}
\]
4-(p-benzoquinone-monoimino)-phenazone can be detected in a spectrophotometer at a wavelength of 500nm. For this reaction, samples were pipetted into small cuvettes and read within 60 minutes of termination or the last reaction. In accordance with this assay, specimens were collected in tubes containing EDTA to prevent coagulation. After thawing of samples, centrifugation was performed at 9000 rpm for 1.5 minutes. When extracting sample, care was used to pipette from the middle of the sample tube. To perform the assay, 20µl of plasma sample was pipetted in duplicate into borosilicate glass test tubes. Reagent blanks included into the assay which had distilled water instead of sample material.

Plasma triglyceride concentrations were calculated using the following equation:

\[
760 \times (A\sample - A\text{ reagent blank}) = \text{Triglyceride Concentration (mg/dL)}
\]

Where: \( A \) = absorbance at 505 nm

The absorbance of 4-PBMP at 550 nm determined the amount of glycerol liberated from triglyceride. The coefficient of interassay variation for this assay was 1.1-1.8% and the intraassay coefficient of variation was 0.8-1.8%.

### 3.5.5 Plasma FFA assay

To measure plasma free fatty acid levels an enzymatic colorimetric kit was used (NEFA C KIT, Wako Chemicals, Richmond, VA, USA). During this reaction acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase (ACS) occurs. During this process acyl-CoA is produced as acylation of coenzyme (CoA) occurs in the presence of acyl-CoA synthetase (ACS).

\[
\text{ACS} \quad \text{FFA} + \text{ATP} + \text{CoA} \rightarrow \text{Acyl-CoA} + \text{AMP} + \text{PPI}
\]
Acyl-CoA produced is unstable and is quickly oxidized by the acyl-CoA oxidase which is present in the mixture. This reaction generates $\text{H}_2\text{O}_2$.

\[ \text{ACOD} \]

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

$\text{H}_2\text{O}_2$, in the presence of peroxidase permits the oxidative condensation of 3-methyl-$N$-ethyl-$N$-(B-hydroxyethyl)-aniline (MEHA) with 4-aminophenazone to form the final reaction product, which is a purple colored adduct.

\[ \text{POD} \]

\[ 2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{MEHA} \rightarrow \text{Final Reaction Product} + 3\text{H}_2\text{O} \]

Reagents 1 and 2 are prepared. A listing of reagents in each reagent mixture is listed below.

**Reagent 1:** 10ml of [phosphate buffer pH 6.9, 3mmol/l magnesium chloride, surfactant and stabilizers] with 3U acyl-coenzyme A synthetase, 30U ascorbate oxidase, 7mg coenzyme A, 30mg adenosine triphosphate, 3mg 4-aminoantipyrine per vial), and gently inverting 20ml of solvent B (1.2mmol/l 3-methyl-$N$-ethyl-$N$-(β-hydroxyethyl)-aniline).

**Reagent 2:** 20ml of [1.2mmol/l 3-methyl-$N$-ethyl-$N$-(β-hydroxyethyl)-aniline] with 132U acyl-coenzyme A oxidase, 150U peroxidase per vial). In addition, a NEFA Standard Solution was included with the kit in aqueous solution (1.0mEq/l oleic acid, surfactant, stabilizers)

The assay protocol supplied with the kit was followed except the amount of reagent was halved to extend the assay capabilities. In accordance with this assay, specimens were collected in tubes containing EDTA to prevent coagulation. After thawing of samples, centrifugation was performed at 9000 rpm for 1.5 minutes. When extracting sample, care was used to pipette from the middle of the sample tube. To perform the assay, 20µl of plasma sample was pipetted in duplicate into borosilicate glass test tubes. To account for
differences in hemolysis or lipemia between plasma samples, specimen blanks were included. Reagent blanks included into the assay which had distilled water instead of sample material. Standards were generated to create a calibration curve by diluting the 1.0mEq/l standard solution to create high (1.97mEq/l), middle (1.00mEq/l), low (0.5 mEq/l), and Blank (0.00mEq/l) standards. For these standards, 50µl of standard was pipetted into the high standard tube, 25µl into the middle standard tube, and 12.5µl of standard and 12.5µl of water into the low standard tube. 25µl of distilled water was accurately pipetted in duplicate into borosilicate glass test tubes to act as a standard and reagent blank, respectively. To each appropriate tube 25µl of standard calibrator or sample is pipetted in duplicate. Following this, 500µl of reagent 1 is added to all tubes and then vortexed to mix and then incubated at 37ºC for 10 minutes. This reaction leads to generation of acyl-CoA from FFA in the plasma sample, catalyzed by the enzyme acyl-CoA synthetase. Following this incubation 2.0ml of reagent 2 is pipetted to all the tubes. As a control specimen blank tubes were created by adding blank specimen blank tubes that have 25µl of sample but do not have any of reagent 1. Blanks in the absence of reagent 1 contain FFA that do not get converted to Acyl-CoA by ACS and can therefore not undergo reaction 2 when reagent 2 is added. Following 1.0ml of reagent 2 to all tubes, the mixtures are vortexed and incubated for 10 minutes at 37ºC. During this time all tubes except blanks lacking reagent 1 undergo a reaction by which acyl-CoA oxidase catalyzes the formation of H₂O₂ using acyl-CoA produced in reaction 1 as a reagent. Following this reaction samples are brought to room temperature, pipetted into cuvettes and then read in a spectrophotometer at 550nm which produces results correct within 1.1%.

The FFA content was calculated from the following equation:

\[ A_{\text{sample}} - A_{\text{specimen blank}} \times C_{\text{Standard}} = C_{\text{Sample}} \]

\[ A_{\text{standard}} \]
Where: A = Absorbance at 550nm; C = FFA concentration (mEq/l)

*This assay has an intraassay coefficient of variation of 1.1-2.7%.

3.5.6 Plasma C-Peptide Assay

In order to measure plasma C-peptide a Rat C-peptide RIA kit from Linco Research Inc. (St. Charles, Missouri) was used. The kit employs an antibody specific for rat C-peptide and the principle is the same as insulin RIA as described above. Except for the I-125-rat-corticosterone which came lyophilized for stability, all the reagents came in liquid form. To form a HPLC purified I-125-I-rat C-peptide the powder was reconstituted with 27.0ml of label hydrating buffer which contains guinea serum as a carrier. It was mixed gently and left for 30 minutes at room temperature. To borosilicate tubes and in duplicate, 150µl of assay buffer (0.05M phosphosaline, 0.025M EDTA, 0.08% Sodium Azide, and 1% Bovine Serum Albumin at a pH of 7.4) is added to make tubes to measure non-specific binding (NSB). To act as the 0ng/ml standard 100µl of assay buffer was added to two borosilicate tubes in duplicate. The assay was performed with the used of 7 standards which created a curve spanning 25-1600pM. The curve was most sensitive between 100pM and 800pM. This kit came with purified rat C-peptide recombinant which was used to test the high and low (in assay buffer) areas of the curve. To the remaining tubes 50µl of assay buffer is added. To these tubes, 50µl of standard or sample is added in duplicated to tubes to generate a curve with 7 points plus low and high quality controls. In accordance with this assay, specimens were collected in tubes containing Trasylol, a protease inhibitor, was added to the specimen before storage to prevent proteolytic degradation. After thawing of samples, centrifugation was performed at 9000 rpm for 1.5 minutes. When extracting sample, care was used to pipette from the middle of the sample tube. After adding 50µl of plasma sample to the appropriate tubes, 50µl of guinea gig anti-rat C-peptide antibody
(diluted in assay buffer) was added to all tubes excluding non specific binding (NSB) and total count (TC) tubes. Following vortexing tubes are incubated at 4°C overnight (20-24 hours). On the following day, 50µl of the reconstituted I-125-rat C-Peptide tracer was then added to all tubes. Tubes were vortexed and allowed to incubate at 4°C overnight (22-24 hours). After the end of the incubation, 500µl of cold (4°C) precipitating reagent was added to all tubes except total count tubes, vortexed, and allowed to incubate 20 minutes at 4°C. This causes precipitation of all antibody bound antigen, therefore dividing the bound ligand from the free unbound tracer that remained in the supernatant. Samples were then centrifuged at ~4°C at 2500 rpm for 25 minutes and the supernatants subsequently aspirated. The precipitating solution is a mixture of 3% polyethylene glycol and goat anti-guinea pig IgG serum within a 0.05% Triton X-100 buffer in 0.05M phosphor-saline, 0.025M EDTA, and 0.08% Sodium Azide. The radioactivity present in the remaining pellet (inversely proportional to the amount of plasma C-Peptide in the sample) was then counted in the gamma counter. The % activity bound was calculated in the same manner as corticosterone RIA. The % activity bound for each standard was plotted against the known concentration to obtain standard curve. The concentrations of the unknown samples were determined by interpolation with a coefficient of inter-assay variation determined on reference plasma less than 10%. This assay has 100% cross-reactivity with rat and mouse C-peptide and non-detectable cross-reactivity with rat insulin, glucagon, somatostatin, amylin, and pancreatic polypeptide. The assay protocol supplied with the kit was followed except the amount of reagent added to each tube was halved to extend the assay capabilities.
3.5.7 Plasma IL-6 Assay

To measure rat IL-6 a commercially available ELISA kit and protocol from R & D Biosystems (R & D Systems Inc., Minneapolis, MN., USA) was used. In accordance with this assay, specimens were collected in tubes containing EDTA to prevent coagulation. After thawing of samples, centrifugation was performed at 9000 rpm for 1.5 minutes. When extracting sample, care was used to pipette from the middle of the sample tube. Before commencing the assay, all reagents were brought to room temperature before use. Rat IL-6 controls were supplied in powdered form and needed to be reconstituted with 1.0ml of deionized water. Wash buffer, also supplied in the kit, was then prepared by adding 25ml of wash buffer concentrate was added to 600ml of deionized water. IL-6 standard also came in powdered form and needed to be reconstituted with 1.0ml of calibrator diluent which produced a stock solution of 4000 pg/ml from which all standards were made. Following this step, 50µl of Assay Diluent RD1-54 was added to each well followed by 50µl of Standard, Control or sample per well. All wells were then mixed by tapping of the plate frame for 1 minute. The plate was then covered with the adhesive strip provided and incubated for 2 hours at room temperature. Following incubation each well was aspirated and washed with wash buffer. This process was repeated 4 times for a total of 5 washes. The plate was then inverted and blotted against clean paper towels followed by the addition of 100µl of rat IL-6 Conjugate to each well. The plate was then mixed again and covered with a new adhesive strip and incubated for 2 hours at room temperature. Following incubation the wash step was repeated as described above. 100µl of Substrate Solution was then added to each well followed by a 30 minute incubation at room temperature. The plate and substrate solutions were protected from light at all times as suggested by the
protocol insert. Following those 30 minutes 100µl of stop solution was added to each well. The plate was then read at 450nm within 30 minutes of adding the stop solution.

### 3.5.8 Plasma Haptoglobin Assay

Plasma haptoglobin was determined using a commercially available ELISA kit and protocol (Life Diagnostics, West Chester, PA, USA). In accordance with this assay, specimens were collected in tubes containing EDTA to prevent coagulation. After thawing of samples, centrifugation was performed at 9000 rpm for 1.5 minutes. When extracting sample, care was used to pipette from the middle of the sample tube. Before commencing the assay, all reagents were brought to room temperature before use. The kit came equipped with 1 x 12ml stabilized Hemoglobin, 1 x 12ml Hemoglobin diluent, 1 x 0.5ml Haptoglobin Calibrator (2mg/ml), 1 x 11ml Chromogen reagent, 1 x 8ml Substrate containing stabilized hydrogen peroxide and 1 x 12ml PBS. The assay was performed according the kit instructions with no modifications. In short: prior to commencing the assay equal volumes of hemoglobin and hemoglobin diluent were mixed and Reagent 1 and reagent 2 were prepared. The chromogen and chromogen substrate were mixed in a ration of 9:5. Firstly, 7.5µl of each prepared calibrator (0-2mg/ml) along with test specimens were transferred to blank microplate in duplicate. Following this step 100µl of reagent 1 was added to each microwell and the plate was tapped to ensure mixing of calibrators/specimens and hemoglobin. Following mixing 140µl of reagent 2 was added to each microwell and incubated for 5 minutes at room temperature. The plate was then read immediately at 630nm.

### 3.5.9 Plasma Malondialdehyde Assay

Plasma malondialdehyde levels are often assayed as an indicator of lipid peroxidation (337). For this assay lipid peroxidation was measured by the thiobarbituric acid (TBA)-
malondialdehyde assay and reported as TBA-reactive substances (TBARS) (337). Plasma samples were incubated with 2-thiobarbituric acid (TBA) and orthophosphoric acid in the presence of butylated hydroxyl toluene (BHT) for 45 minutes at 95°C, cooled to 25°C, and then extracted with n-butanol. Absorbance of the butanol phase was measured at 535nm. Results were calculated using an extinction coefficient of 1.56 x 105m⁻¹ (615). Before commencing the assay plasma was thawed completely. Reagents were then prepared in the following order. 11mg of 10mM BHT was dissolved in 5ml of ethanol and then stored in a Falcon conical centrifuge tube at 0-4°C until use. Next 2ml of 6N HCL was dissolved in 118ml of distilled water and stored at room temperature until use. A solution of 1% orthophosphoric acid in 0.1N HCL was prepared by adding 0.470ml of 85% orthophosphoric acid dissolved in 39.63ml of 0.1N HCL. This solution was stored in a Falcon conical until use. Lastly a solution of 0.6% TBA was prepared by dissolving 72mg TBA in 12ml 0.1N HCL. This solution was stored at 0-4°C in a Falcon conical until use. To commence the assay 100µl of thawed plasma was added to 10ml disposable borosilicate tubes. 10µl of BHT was then added to each tube and then vortexed. Following vortexing 700µl of orthophosphoric acid was added to each tube and then the tubes were re-vortexed. Following mixing 200µl of TBA reagent (200µl for the blank) was added to the tubes and then vortexed. The tubes containing this mixture were then incubated at 90°C for 45 minutes in a hot water bath. When finished the tubes were immediately put on ice to cool the samples to room temperature. Once cooled, 1ml of n-butanol and 2ml of chloroform were added to each tube and then mixed by vortexing. Following vortexing the samples were centrifuged at 2000 rpm for 10 minutes and then the n-butanol supernatant was aspirated. The lower fraction was then read at 535nm.

**Calculations**  
10X nmols of TBARS/ ml of plasma/0.156  
Where ‘X’ is the optical density (OD) reading of the sample.
3.5.10 Protein Carbonyl Assay

Protein carbonyls are a plasma marker of protein oxidation which react with 2,4-dinitrophenylhydrazine (DNPH) to form protein-hydrazone which is detectable by this assay. To perform quantification of protein oxidation, this assay was done on liver tissue which was homogenized at 4 °C in a solution containing HEPES (10mmol/l; pH 7.4), NaCl (137mmol/l), KCl (4.6mmol/l), KH₂PO₄ (1.1mmol/l), MgSO₄ (0.6mmol/l), ethylenediaminetetraacetic acid (1.1mmol/l), leupeptin (0.5µg/ml), pepstatin (0.7µg/ml), phenylmethylsulfonyl fluoride (40µg/ml), and aprotinin (0.5µg/ml) to solubilize protein and cell debris was removed by centrifugation. A Bradford assay was performed to determine protein concentrations in these samples. Oxidative protein damage, assessed by the formation of carbonyl groups, was measured as described by Levine et al (454). In order to remove nucleic acids, samples were incubated with 10% streptomycin sulfate until a final concentration of 1% streptomycin sulfate was reached. The test tubes were left at room temperature for 15 minutes and then centrifuged at 6000 x g for 10 minutes in a tabletop centrifuge. The supernatant was separated from the pellet and discarded. One mg of protein was precipitated by addition of 20% trichloroacetic acid (TCA) and centrifuged (8500g) for 3 minutes, after which the supernatant was discarded. Protein pellets were incubated with and without DNPH (10mM in 2M HCl) and were allowed to stand at room temperature for 1 hour, during which time the mixture was vortexed every 15 minutes. Following incubation, protein was re-precipitated using 20% TCA and the pellet was obtained by centrifugation (8500g) for 3 min. The pellet was washed 3 times with ethanol-ethyl acetate (1:1) to remove free DNPH, allowing the samples to stand for 10 minutes each time before discarding the supernatant. The pellet was then re-dissolved in guanidine solution (6M with 20mM potassium phosphate, adjusted to pH 2.3 with trifluoroacetic acid)
for 1 hour at 37°C. Insoluble material was removed by centrifugation (8500 x g) for 3 minutes. Carbonyl content was calculated from the sample absorbance at 365 nm compared to their complementary HCl-treated blanks, using a molar absorption coefficient of 22000M⁻¹cm⁻¹.

3.6 Western Blot

3.6.1 Liver

The protein concentration in all samples was determined by the detergent-compatible modified Lowry microassay (BioRad), using serum albumin as the standard.

<table>
<thead>
<tr>
<th>Table 1. Preparation of Diluted Albumin (BSA) Standards</th>
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<td>Dilution Scheme for Test Tube and Microplate Procedure (Working Range = 1–1500 µg/ml)</td>
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3.6.2 Protein Quantification and Western Blotting

The protein concentration in all samples was determined by the detergent-compatible modified Lowry microassay (BioRad), using serum albumin as the standard. Fifty µg of protein in all samples were mixed with equal volumes of 3 x sample-loading buffer (6.86 M urea, 4.29% sodium dodecyl sulphate (SDS), 300 mM dithiothreitol, 43 mM Tris-HCl pH 6.8) and left at room temperature for 30 min. The mixture was then vortexed and subjected to SDS-PAGE (10% polyacrylamide). Following electrophoretic separation, protein was transferred to polyvinylidene fluoride membranes. The membranes were then incubated for 1 hour at room temperature in Tris-buffered saline-Tween (TBST) containing 5% non-fat dried milk, pH 7.4. After the blocking step, membranes were washed in rinsing solution (TBST, pH 7.4) and then incubated overnight with the primary antibody dissolved in the blocking solution. The antibody concentrations used were anti-JNK (1:1000), anti-pJNK-Thr183-Tyr185 (1:250), anti-IkBα (1:1500) (Santa Cruz Biotechnology, Inc., Santa Cruz CA, USA), anti-PKCδ/ε (1:000) (Sigma, Saint Louis, Missouri, USA), anti-actin (1:3000) (Sigma, Saint Louis, Missouri, USA), anti-IRS-1 (1:500) and anti-pIRS-1-Ser307 (1:500) (Upstate Biotechnology, Lake Placid, NY, USA). After three washes with TBST (20 min each), membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Baie d’Urfe, Quebec) at 1:4000 dilution. The membranes were then washed several times with TBST and developed using ECL (Zymed Laboratories, San Francisco, CA).

3.7 Immunohistochemistry

3.7.1 Double Staining for Insulin / Bromodeoxyuridine

Paraffin sections were dewaxed with xylene and hydrated through graded alcohols and brought to water. Following hydration, endogenous peroxidase activity was blocked with
3% H₂O₂ and washed in water. Antigen unmasking was then carried out by enzyme digestion using pepsin (Sigma labs) and then washed in PBS. Following antigen retrieval, blocking of avidin/biotin activity was performed with the avidin/biotin blocking kit (Vector labs, Burlington, ON). Slides were then washed with PBS, blocked with normal goat serum (Vector labs), drained, and then incubated with rabbit anti-insulin IgG (Dako, Mississauga, ON) primary antibody at 1:100 for 1 hour at room temperature, in a moist chamber. Slides were then washed with PBS and incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (Vector labs) at 1:500 for 1 hour at room temperature, washed with PBS and then incubated with peroxidase-conjugated ultrastreptavidin labeling reagent (Vector Labs). Colour development for insulin was done with freshly prepared diaminobenzidine (DAB) (Dako), which yields a brown colour. The slides were washed in water, blocked again with 3% H₂O₂, washed in water again, and then rinsed in PBS, followed by incubated with normal horse serum. The slides were then drained and incubated with mouse anti-BrdU primary antibody (Invitrogen, Burlington, ON) at 1:1000 overnight at room temperature, in a moist chamber. The slides were washed with PBS and incubated with the secondary antibody, biotinylated horse anti-mouse IgG (Vector Labs) at a concentration of 1:500 for 1 hour at room temperature, were washed with PBS and then incubated with peroxidase-conjugated ultrastreptavidin labeling reagent (Vector Labs.) Colour development for β-cells incorporating BrdU (BrdU⁺ cells) was done with freshly prepared nickel DAB, which yields a dark blue/black colour. Finally, the sections were counterstained with Mayer’s hematoxylin. Sections were then washed in water, dehydrated in graded alcohol, cleared with xylene and mounted.
3.7.2 Double Immunohistochemical Fluorescent Staining for Insulin/GLUT2, Insulin / Akt/PKB, Insulin / FK2, and Insulin/DAPI/TUNEL: Multiple-Labeling Immunofluorescent Microscopy

Paraffin sections stained during fluorescent studies were dewaxed and hydrated exactly as described above. Antigen unmasking/epitope retrieval was performed by irradiating hydrated sections with microwaves while in a 10mmol/l solution of sodium citrate buffer, pH 6.0. The temperature of citrate buffer was kept above 95°C for 20 minutes in this fashion and then allowed to cool at room temperature until the solution and sections were approximately 20°C. Following antigen unmasking, slides stained for insulin/Akt/PKB and insulin/FK2 were washed in PBST, and then blocked for 3 hours at room temperature in a solution of 1% bovine serum albumin (BSA) and 5% donkey serum in 0.2% saponin phosphate buffered saline (PBS). Slides stained for GLUT2 were handled similarly, with the exception that blocking solution did not contain saponin. Sections were then immunostained simultaneously in antibody mixtures of either guinea pig anti-insulin IgG (Dako) (1:150) and rabbit derived anti-GLUT2 1:1000 polyclonal antibody (Chemicon), or guinea pig anti-insulin IgG (Dako) (1:150) and a mouse derived anti-phospho-Akt-Ser473 (1:200) monoclonal antibody (Cell Signaling Technology), overnight at 4°C. Staining for ALIS was performed with a antibody mixture of guinea pig anti-insulin IgG (Dako) (1:150) and a mouse derived anti-FK2 (1:200) monoclonal antibody (Biomol), overnight at 4°C. On the following day slides were washed copiously in PBST and then incubated in a secondary antibody mixture that contained donkey anti–species-specific IgG conjugated to fluorescein isothiocyanate (FITC, 1:200), or cyanine-3 (CY3, 1:200); both from Jackson ImmunoResearch). Following secondary antibody/fluorophore conjugate incubation, sections were counterstained in 1ug/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 minutes, washed with PBS and 3 quick successive baths of double distilled water (ddH₂O) followed
by mounting with a water-based mounting medium (AQUA POLYMount™, Polysciences). Controls for primary antibody specificity (anti-insulin IgG) were performed in spleen sections which were harvested, fixed, and processed identically. Secondary antibody specificity was performed by processing pancreatic sections identically to that explained above while omitting the overnight primary antibody incubation. For determination of apoptotic β-cells, a TUNEL-TMR-red kit (Roche Diagnostics) was used to fluorescently mark cells with fragmented DNA strands. In these sections, pancreatic tissue was cleared of wax in 3 successive baths of xylene and rehydrated in graded ethanol as described above. Sections were then digested with Proteinase K at a concentration of 20µg/ml in 10mM Tris/HCL for 15 minutes at 37°C. Slides were then washed in TBS, incubated with TUNEL TMR-red for 1 hour at 37°C, washed in TBS and 1 hour at room temperature in a solution of 3% BSA, 1% TritonX-100 in TBS. Following blocking, slides were incubated overnight at 4°C with primary insulin antibody (same as above, 1:150). On the following day, slides were washed in TBST (0.1% Tween) and then incubated for one hour at room temperature with FITC conjugated donkey anti-guinea pig (1:200). Slides were then washed 5 times for 5 minutes in TBST and then stained with DAPI as described above, followed by mounting of coverslips. All slides stained for fluorescent studies were kept in a -20°C freezer until analysis (1-5 days later).

3.8 Microscopy and Digital Image Processing

β-cell mass was determined from the insulin antibody-stained sections by scanning and capturing tiled images of the entire cross-sectional area of the pancreas for each animal. Such images contained on average, between 350 and 500 islets which were all used in subsequent calculations. Digitally, these images could be further increased in magnification to 400X without a significant loss in image quality. For each animal, at a
magnification of 20X, the relative cross-sectional area of insulin stained β-cells was divided by the cross-sectional area of all the pancreatic tissue (200; 406) over the area occupied by all ~20 sections of each slide. Tissue areas were objectively quantified, using one common preset positive pixel count algorithm, available with APERIO SCANSCOPE™ software (Vista, California, USA). Quantitative imaging of GLUT2 and Akt/PKB was performed by capturing monochromatic images of a minimum of 200 sequential fields of view for each pancreatic section. This process of tiling and image gathering was performed for each of the wavelengths corresponding to the fluorophores under study and worked out to analysis of a minimum of 20 random islets per animal at a magnification of 40X (minimum of 160 islets per treatment condition). Images corresponding to a particular islet and filter set were then merged and pseudocolored using IMAGE PRO PLUS™ software (Media Cybernetics). A “masking” feature available in Image Pro Plus allowed for the accurate quantification of staining which was specific to β-cells alone. For identification of ALIS, a minimum of 60 random islets per treatment condition were imaged at 40X and pseudocolored as described above. Sections were imaged using an upright Olympus BX50 microscope equipped with Olympus UPlanSApo 10x/0.40, UPlanSApo 20x/0.75, and Olympus UPlanFI 40x/0.75 lenses and automated tiling platform. Illumination was provided by a 100 Watt HBO Mercury energy source and was filtered with DAPI 377/50 nm, FITC, 482/35nm, and TRITC (CY3) 543/22nm filter sets. Images were captured using a Photometrics COOLSNAP™ HQ2 (Roper Biosciences) camera and computer running Media Cybernetics IN VIVO™ software (Bethesda, MD). All images were saved in a lossless TIFF format.
3.9 Image/Data Analysis

3.9.1 Measurement of β-cell Mass and β-cell Replication

β-cell mass per animal was estimated as the product of the total cross-sectional area of β-cells/total tissue and the weight of the isolated pancreas before fixation (200; 406). β-cell mass was determined from the insulin antibody-stained sections by scanning and capturing tiled images of the entire cross-sectional area of the pancreas for each animal. Such images contained on average, between 350 and 500 islets which were all used in subsequent calculations. Digitally, these images could be further increased in magnification to 400X without a significant loss in image quality. For each animal, at a magnification of 20X, the relative cross-sectional area of insulin stained β-cells was divided by the cross-sectional area of all the pancreatic tissue (200; 406) over the area occupied by all ~20 sections of each slide. Tissue areas were objectively quantified, using one common preset positive pixel count algorithm, available with APERIO SCANSCOPE™ software (Vista, California, USA). β-cell mass per animal was estimated as the product of the total cross-sectional area of β-cells/total tissue and the weight of the isolated pancreas before fixation (200; 406).

Measurement of β-cell replication was performed by capturing no less than 30 random images of areas of interest outlined from the large tiled images of the entire pancreatic cross-sectional area. BrdU+ nuclei were determined at 40X magnification in an experimentally blinded fashion by two individuals (406). No less than 3000 β-cells per animal (a minimum 24,000 per treatment group) were counted and classified as BrdU+ or negative, and only those β-cells with a clearly defined nucleus were measured.
3.9.2 Mean β-Cell Size Measurements, Islet Size Measurements, and Indicators of Neogenesis

Mean β-cell size or β-cell hypertrophy was calculated by scanning islet areas which were used for β-cell replication studies (see above). For these calculations, the relative cross-sectional area of insulin stained tissue was divided by the number nuclei counted within that same insulin stained area to yield mean β-cell size. Only those β-cells with a clearly defined nucleus were measured. Using the data generated from scanning entire slide areas used for our β-cell mass studies (see above) the number of islets for each animal (calculated as average number per total slide area) was determined. With this same data set, we also determined the relative sizes, or cross-sectional surface areas of individual islets, by converting pixel values to micrometers squared and tabulating for each animal to yield islet distribution characteristics. Using the same insulin-stained sections that were used for islet β-cell mass measurements, the total number of β-cells as singles and clusters as doublets, triplets, quadruplets, and up to five cell diameters (very small islets) were counted for each animal. Single and clustered β-cells, regardless of their location, show no signs of replication and therefore were considered neogenic and may aggregate to form new islets (340). Neogenic areas were measured by adding the areas represented by these small neogenic clusters which were less than 6 cells. The possibility of measuring islets twice was eliminated by association of a particular islet with an object number assigned by computers during the scanning process.

3.10 Western Blot Analysis

A Kodak cassette was loaded with membranes which were anchored in place using acetate film and tape. Film was exposed for between 5-300 seconds depending on the strength of ELC reaction. Negative optical densities were then analyzed using a computerized image
analysis system using Imaging Research Software (Imaging Research, St. Catherines, ON, Canada).

3.11 Statistical Analysis

For all measurements made over time, a two-way (treatment group x time interval) repeated measures analysis of variance (ANOVA) was used. To examine the effects of treatment at specific times, the ANOVA was collapsed, decomposing the ANOVA, allowing one to test differences between groups. For parameters measured at euthanasia, a one-way (treatment) ANOVA was used. Duncan’s post-hoc analysis was used to determine differences between relevant mean values. All values are reported as a mean ± SEM and were obtained using STATISTICA\textsuperscript{TM} software (v.6.0, Tulsa, OK) with $P \leq 0.05$ as the limit for statistical significance.
Exercise improves glucose tolerance in obese rodent models and humans; however effects with respect to mechanisms of β-cell compensation remain unexplained. We examined exercise effects during the progression of hyperglycemia in male ZDF rats until 19-weeks of age. At 6-weeks old, rats were assigned to: 1) basal-euthanized for baseline values; 2) exercise-swam individually for 1h/day, 5d/week; and 3) controls (n=8-10/group). Thirteen weeks of exercise resulted in maintenance of fasted hyperinsulinemia and prevented increases in fed and fasted glucose (P<0.05) compared with sham-exercised and sedentary controls (P<0.05). β-cell function calculations indicate prolonged β-cell adaptation in exercised animals alone. During an IPGTT exercised rats had lower 2h-glucose (P<0.05) versus controls. Area-under-the-curve analyses from baseline for IPGTT glucose and insulin indicate improved glucose tolerance with exercise was associated with increased insulin production and/or secretion. β-cell mass increased in exercised versus basal animals however, mass expansion was absent at 19 weeks in controls (P<0.05). Hypertrophy and replication contributed to expansion of β-cell mass; exercised animals had increased β-cell size and BrdU incorporation rates versus controls (P<0.05). The relative area of GLUT2 and Akt/PKB was significantly elevated in exercised versus sedentary controls (P<0.05). Lastly, we show formation of ubiquitinated protein aggregates, a response to cellular/oxidative stress, occurred in non-exercised 19 week-old ZDF rats but not in lean, 6 week-old basal, or exercised rats. In conclusion, improved β-cell
compensation through increased \(\beta\)-cell function and mass occurs in exercised but not sedentary ZDF rats and may be, at least in part, responsible for improved glucoregulation.

4.2 Aims

Obesity is the most common metabolic disease in North America (438) and along with its co-morbidities is currently responsible for approximately 15% of the annual health care expenditure (438). One such co-morbidity is T2DM, which develops in approximately 35-40% of the obese adult population (438). The cause of T2DM is unknown but is predominantly associated with obesity (438), a progressive decline in peripheral insulin sensitivity and corresponding decrements in glucose disposal in skeletal muscle, fat, and the liver (157). T2DM, however, is not defined solely by decreased hepatic and peripheral insulin action; \(\beta\)-cell defects with respect to insulin production and/or secretion must also be present, and in fact, are responsible for the discrepancy between insulin resistant obese non-diabetic and type 2 diabetic patients. Studies in obese insulin resistant but non-diabetic humans have revealed that the initial physiological response to increased body weight, and its associated increase in insulin demand, is a compensatory period marked by expansion of \(\beta\)-cell mass (64; 65; 101; 102; 410) and \(\beta\)-cell function (359), which together allow for the increased production and/or secretion of insulin. These adaptations continue uninterrupted in obese insulin resistant hyperinsulinemic individuals. However, in subjects with T2DM this adaptive period of \(\beta\)-cell compensation is short-lived and may fail altogether (809). For example, humans with T2DM have smaller islets and show a 40-60% reduction in \(\beta\)-cell mass compared to non-diabetic hyperinsulinemic controls (809). Why these defects occur in T2DM and not in obesity and insulin resistance alone is an issue that has not been explored sufficiently.
Due to the scarcity of available human pancreata, studies using rodent models of obesity and insulin resistance, such as the ZDF rat, are frequently used to elucidate mechanisms responsible for the deterioration from a prediabetic state to T2DM (127; 200; 586; 749). Male ZF rats develop a phenotype of obesity, insulin resistance, and hyperphagia due to a leptin receptor mutation, and diabetic ZDF rats also eventually develop hyperglycemia resulting in a phenotype very similar to humans with T2DM (127). In these rodents, glucose intolerance usually develops by age 8 weeks of age (586; 749), followed by overt hyperglycemia by 10-12 weeks of age. It begins with β-cell hyperplasia and hyperinsulinemia, which compensates for the progressively increasing insulin resistance and maintains normoglycemia. After 10 weeks, β-cell apoptosis first begins to exceed the rate of replication, and the β-cell mass declines. The level of insulin secretion is no longer sufficient to overcome insulin resistance and maintain normoglycemia and diabetes begins (127; 200; 586; 749).

As in humans, the prediabetic phase in ZDF rats is characterized by the maintenance of normoglycemia by a compensatory increase in β-cell function resulting in hyperinsulinemia (101; 102; 127; 749). This initial compensatory adaptation reflects what occurs in obese insulin resistant subjects. However, as is the case for people with T2DM, this compensatory adaptation begins to fail as animals enter the “diabetic phase” defined by reductions in β-cell function and dramatic increases in β-cell apoptosis and a corresponding decrease in β-cell mass (586). Much work has addressed the β-cell defects responsible for the inability to maintain adaptive hyperinsulinemia and thus euglycemia (101; 102; 127; 154-156; 202; 409; 410; 586; 596; 749). Indeed, studies in ZDF rats and in humans have elucidated that reductions in β-cell function correlate strongly with decreased levels of GLUT2 mRNA and protein levels (269; 270; 545; 546; 548; 735). Such reductions in GLUT2 levels have since
been confirmed in ZDF rats (545; 548; 793), and other models of T2DM including the GK (546) rat and db/db mouse (390). In addition, recent studies have demonstrated a role for molecular candidates/markers in the control of the relationship between net β-cell growth and net β-cell death. One such study indicates a role of the insulin/IGF-1 signaling pathway in the adaptive β-cell response to insulin resistance, particularly IRS-2, and its downstream intermediate, Akt/PKB (340). Thus, normal β-cell glucose entry through the action of GLUT2 and corresponding β-cell function, as well as the preservation of β-cell insulin signaling to promote β-cell mass expansion, are required to maintain adaptive hyperinsulinemia during worsening insulin sensitivity.

In humans, stress, nutrition, body composition, and physical activity influence the development of T2DM. In rodent models such as the Zucker rat, caloric restriction (545), a reduction in fat mass (216; 217), and increased exercise (125; 292; 596; 680; 681) improve insulin sensitivity and attenuate the development of hyperglycemia. These interventions, which increase insulin action or sensitivity, are thought to result in a decreased demand for insulin and thus attenuate β-cell exhaustion and increases in β-cell apoptosis. Different exercise modalities, including forced-treadmill running and swimming are associated with the partial maintenance of β-cell mass expansion in OLETF (681) and ZDF rats (406; 596). Exercise is also associated with increases in β-cell function. For example, ZDF animals that either run on treadmills (596) or individually swim (406) manifest a protracted period of adaptive hyperinsulinemia such that euglycemia is maintained. Furthermore, in humans with T2DM, regular exercise enhances insulin response to either hyperglycemia or to arginine stimulation (163), suggesting that there may be direct effects of exercise on pancreatic β-cell function. If insulin sensitivity is increased and hyperglycemia reduced, this would by itself improve β-cell function. Together
these findings indicate that regular physical activity exerts protective effects on pancreatic β-cells. However only a paucity of such studies currently exists and the direct effects of exercise per se on pancreatic β-cell adaptations to worsening insulin resistance remain unclear.

Exercise is an acute stressor eliciting a hormonal response that is common to other types of stressors. For example, acute exercise leads to a rise in plasma glucocorticoids (150), catecholamines (249), and glucagon (327), all of which exacerbate insulin resistance. Despite aggravating insulin resistance, it is possible that periodic elevations in stress hormones, such as glucocorticoids, may exert protective effects on the β-cell. In support of this, glucocorticoid exposure stimulates β-cell expansion in rodents (275; 386; 457; 779; 780). Also, the diabetogenic effects of streptozotocin (STZ), alloxan, and partial Px can be reduced by pre-treatment with glucocorticoids or mild foot shock (275; 313; 314; 372; 439; 635; 806). Moreover, frequent periodic stress in OLETF rats improves glycemic control and glucose tolerance, preventing 80% of T2DM in this model (364). It is interesting, therefore, that although glucocorticoids cause insulin resistance and increased hepatic glucose production that aggravates metabolic control in diabetes, they may confer some protective effect on the development of the disease by altering β-cell function in the prediabetic animal.

Therefore the purpose of this study is to investigate the effects of long-term forced exercise training on pancreatic β-cell adaptations with respect to β-cell function and maintenance of β-cell mass expansion. We are also concerned with investigating the non-exercise stress on pancreatic function and the development of T2DM in the male ZDF rat. Specifically we aimed to test the hypothesis that protective effects of exercise training in vivo on β-cell
function and β-cell mass compensation are associated with maintained immunodetectable levels of GLUT2 and Akt/PKB protein. We also examined the role of Akt/PKB in maintaining adaptive β-cell proliferation and ameliorating loss of β-cell mass. We show that episodic stress which increases circulating glucocorticoids, if elevated intermittently through either exercise or stress associated with forced exercise, may have protective effects on β-cell mass, and the development of hyperglycemia in this animal model of T2DM. Additionally we reveal that 13 weeks of frequent swim training prevents hyperglycemia up until at least 19 weeks of age and is associated with protective effects on β-cell function and compensatory mass expansion. This partial maintenance of β-cell compensation was associated with increased immunodetectable levels of GLUT2 and Akt/PKB molecules.

4.3 Research Design and Methods

4.3.1 Animals

Male ZDF rats (ZDF/Gmi-fa/fa) were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) at 5 weeks of age with initial body weights of 150-175g. Rats were singly housed in opaque micro-isolation cages, handled daily, and kept at a constant temperature of 22-23°C in humidity-controlled rooms on a standard 12h (07:00-19:00) light/dark cycle. The animals were fed water and PURINA™ 5001 chow ad libitum throughout the experiment. All experiments were approved by the Animal Care Committee of the Faculty of Medicine at the University of Toronto in accordance with regulations set forth by the Canadian Council for Animal Care.

4.3.2 Experimental Design

Thirty-five male ZDF rats and 10 lean Zucker rats were obtained at 5 weeks of age. Of the 35 ZDF rats, animals were randomly divided into 4 groups. All groups, including the lean
animals, underwent the same 1-week acclimatization period. At 6 weeks of age, one group of ZDF animals was euthanized by decapitation to serve as a basal (B) baseline control (n=8), while the remaining 3 ZDF groups: exercise (E), sham (S), and sedentary controls (C) were incorporated into the long term study for 13 weeks, after which time they were euthanized at 19 weeks of age (n=9/group). Zucker lean animals (L; n=10) served as a sedentary lean control group.

4.4 Treatment Protocols

Each day, animals were transported to a treatment room, where exercise animals were forced to swim. The exercise group individually swam in cylindrical tanks with a diameter and height of 60cm and 100cm, respectively, in water at a depth of 30-45cm, once per day between 09:30 and 11:30h, 5d/week for 1 hour, as described previously (406). During swimming, rats wore elastic chest bands to which attachable weights could be added. Rats commenced exercising without any additional weight for the first week; however, during the second week of treatment, exercised rats had 3% body weight added. To maintain a constant training intensity sufficient to cause exhaustion by the end of one hour, this weight was increased by 1% each week reaching a maximum of 13% body weight by week 12 of the study. Swum animals were required to swim vigorously in order to remain afloat and did so for the duration of the hour. To minimize stress associated with cold or hot water exposure, water temperature was monitored and maintained at ~35°C. At the end of the treatment period, weights were removed and rats were towel dried and left for ~1h in a heated room to minimize the effects of cold exposure. In an attempt to separate the effects of exercise and the stress associated with the exercise environment, sham rats were individually placed in identical swimming tanks and wore the same chest bands, but sat in shallow water (~5cm) at the same temperature, duration, and frequency as exercised rats.
This sham treatment provided the closest similarity to the swim environment as possible and has previously been shown by us to cause a transient increase in glucocorticoid levels, similar to what is observed during swimming exercise (406). Sedentary control ZDF and lean animals were subjected to the same sampling and handling procedures as exercise and sham animals, except they remained in their cages without food and water for the duration of the treatment hour. After 13 weeks, exercise, sham, and sedentary control animals (ZDF and lean groups) were euthanized between 09:00-12:00h by decapitation.

4.5 Food Intake, Body Weight, and Postprandial Glucose Sampling

Food intake and body weight were measured each day prior to treatment. To obtain weekly average food intake values from daily measurements, daily food intake for animals (excluding the day of fast) were averaged over the week. To measure postprandial blood glucose concentration, blood was sampled each morning (09:00h) by “tail nick” using a 27G needle and analyzed using a blood glucose monitor (ASCENDIA ELITE™ XL Blood Glucose Meter, Bayer, Toronto, Canada). To obtain weekly blood glucose values from daily measurements, daily blood glucose values (excluding the day of fast) were averaged over the week.

4.6 Fasting Blood Glucose and Fasting Plasma Insulin Levels

To measure postprandial blood glucose concentration, blood was sampled each week on Monday morning (09:00h-1000h) by “tail nick” using a 27G needle. Once per week, the rats were fasted for 16-18h after which blood samples were taken via a nick to the nub of the tail (see above). To minimize the stress induced by this method of sampling, a topical anesthetic (EMLA™ cream, AstraZeneca, Mississauga, Canada) was applied to the tails 20 minutes before blood sampling. Samples taken to measure fasting insulin levels (~10µl)
were done at the same time as measurement of fasting blood glucose samples and collected into heparinized microvettes (Sarstedt, Montreal, Canada). Plasma was separated from blood samples by centrifugation at 2500rpm for 1 minute and stored at -20°C. To reduce the chance of infection, a topical germicide (BETADINE™ solution, Purdue Pharma, Pickering, Canada) was applied to the tail following blood collection.

4.7 Intraperitoneal Glucose Tolerance Test (IPGTT) with Respective Insulin Levels

All groups were subjected to an IPGTT 4 days prior to euthanasia. For basal animals, this test was performed at 6 weeks of age, while exercise, sham, and control animals received an IPGTT after 13 weeks of treatment (i.e. 19 weeks of age). Prior to glucose injection, rats were fasted overnight for 16-18 hours. During the normal treatment time (i.e. between 09:30-11:30h), rats were administered an intra-peritoneal injection of 50% dextrose (Abbott Laboratories Limited, Montreal, Canada) at a dose of 2g/kg body weight. Blood glucose and insulin levels were measured via tail nick (see above) at 30 minute intervals starting at T=0, just prior to injection, for 2h.

4.8 Resting Hormone Measurements Made at Euthanasia

Basal animals were euthanized by decapitation at 6 weeks of age, while exercise, sham, and control animals were euthanized at 19 weeks of age, also by decapitation. For animals sampled over the 13 weeks of treatment, euthanasia occurred approximately 24 hours after their last treatment session. Trunk blood was collected in 1.5ml tubes containing EDTA and trasylol, for all hormones. Immediately after decapitation, all blood samples were centrifuged at 2500rpm for 1 minute with transferred plasma stored at -20°C. Postprandial insulin, glucose, C-peptide, lipids, and corticosterone were measured from samples taken at this time.
4.9 Analytical Procedures and Methods: For a Detailed Description see Materials and Methods Chapter 3

Trunk blood glucose was measured using a single drop of blood obtained at the time of decapitation with a blood glucose test strip (ASCENSIA ELITE™, Bayer, Toronto, Canada) and glucometer (ASCENSIA ELITE™ XL Blood Glucose Meter, Bayer, Toronto, Canada). Plasma insulin levels were determined using a rat insulin Elisa assay kit (Crystal Chem Inc, Illinois, USA). Plasma corticosterone and C-peptide levels were measured by commercially available radioimmunoassay (RIA) kits (Medicorp Inc., Montreal Canada). Plasma free fatty acids (FFA) and triglycerides (TG) were determined by an enzymatic colorimetric method (ACS-ACOD; Wako Chemicals, Richmond, VA).

4.10 Results

4.10.1 Weekly Body Mass and Food intake (Figure 1)

At the commencement of the study, body mass was not different between the 3 ZDF treatment groups and increased equally in all three groups over the study ($P<0.05$, 1A). Lean Zucker rats weighed significantly less than the 3 ZDF groups at all points during the duration of the study. Daily food intake increased in all treatment groups ($P<0.05$, 1B) and was similar between exercise and sedentary controls over the duration of the study. Sham-treated animals ate less than sedentary controls at weeks 3-5 and less than exercised rats for weeks 2-4 and weeks 10 and 11 of the study. There were no differences in food intake between exercised and sedentary control rats over the last 2 months of the study. For the entire period of the study, lean animals ate significantly less than exercised, sham-treated, or sedentary control rats ($P<0.05$).
4.10.2 Glucose Measurements (Figure 2)

Daily fed blood glucose (BG) measurements for each animal were summated, averaged over the 4 week days, and plotted over the course of the study (2A). Pre-intervention fed glucose values were similar between exercised and control groups but were both elevated when compared to sham-treated rats, at this time ($P<0.05$, 2A). All three groups displayed elevated fed glucose concentrations when compared to lean ZDF rats at this time ($P<0.05$). At week 7 of the intervention period, fed glucose increased sharply in control and sham rats and remained elevated with respect to exercised rats for the duration of the study ($P<0.05$). In contrast to sedentary control and sham-treated ZDF rats, exercised animals did not show a marked increased in fed glucose throughout the duration of the study, although they were significantly higher than the lean controls ($P<0.05$). Relative to exercised animals, sham animals became hyperglycemic after the 8th week of the study ($P<0.05$) while sedentary control rats had elevated glucose levels relative to exercised animals after the 2nd week of the study ($P<0.05$). With respect to fasted BG (2B), glucose concentrations were not different between the 3 ZDF treatment groups at the start of the study, however all showed elevated concentrations relative to the lean animals for the entire duration of the study (all $P<0.05$). Similar to those data seen in our fed glucose measurements, fasted glucose levels increased similarly in sham-treated and sedentary control animals over the duration of the study ($P<0.05$). Fasted glucose was lower in exercised rats than in the sham-treated and controls rats for the final 2 and 3 weeks of the study (both $P<0.05$). This same observation was seen at euthanasia (Table 1).

4.10.3 Insulin (Figure 3A, Table 1), HOMA-IR (Figure 3B), β-cell Function (Figure 3C), and C-peptide (Table 1)

Decreased β-cell function and associated hypoinsulinemia is the hallmark of T2DM, we therefore examined changes in fasting (baseline) and glucose stimulated (fed) insulin
secretion. Initially, the three pre-diabetic ZDF groups showed similar fasting insulin, and were hyperinsulinemic compared to the lean rats which had insulin levels too low for detection by our assay (3A). In sham and sedentary controls adaptive hyperinsulinemia increased gradually until the 8th (sham) and 9th (sedentary control) week of intervention, then dropped drastically. As expected, declining insulinemia coincided closely with the sharp increase in fed and fasting glucose. Interestingly, performance of HOMA-IR (3B) calculations indicate no differences between the treatment groups, which when viewed in conjunction with fasting insulin and glucose data, suggest increased insulin delivery and not insulin action (3B). In contrast to sham and sedentary control rats, exercised rats displayed an adaptive hyperinsulinemia, which resulted in fasting insulin values which were significantly higher than sham and sedentary controls by week 11 of intervention and for the remaining 3 weeks ($P<0.05$). This maintenance of compensatory basal hyperinsulinemia in relation to fasting glucose levels can be represented by dividing fasting insulin by fasting glucose levels (3C). As seen with fasting insulinemia values, increases in β-cell function ceased and began to decline in sham-treated and sedentary control animals towards the end of the study period. In contrast, exercised animals continued to show adaptive increases in β-cell function with time and function remained higher relative to sham and control rats after and including week 11 of intervention period ($P<0.05$). This maintenance of compensatory hyperinsulinemia in exercised but not in sham-treated and sedentary control rats is further exemplified with fed insulin measurements at the time of euthanasia (Table 1). Lastly, as a more accurate measure of insulin secretion, plasma C-peptide (Table 1) concentrations were elevated in all ZDF rats relative to lean rats ($P<0.05$). At the time of euthanasia (19 weeks of age) exercised animals had significantly higher plasma C-peptide levels than either basal, sham, or sedentary control animals (Table 1, $P<0.05$),
while basal animals were not different from sham or sedentary controls, which did not have adaptive increase in β-cell insulin secretion/function ($P<0.05$).

### 4.10.4 IPGTT Plasma Glucose (Figure 4, Table 1) and IPGTT Plasma Insulin Levels (Table 1)

An IPGTT was performed in basal animals (6 weeks of age) and after 13 weeks of intervention in lean controls, and ZDF control, exercise, and sham-treated rats at 19 weeks of age. As expected, all obese ZDF groups showed impaired glucose tolerance compared to lean animals at 19 weeks of age ($P<0.05$). Prior to glucose injection, sham and control rats had elevated fasting glucose when compared to exercised and 6 week-old basal rats ($P<0.05$) which were not different. Throughout the 120 minutes following glucose bolus, glucose excursions in shams and controls were similar, but glycemia was reduced at all time points in exercised rats relative to sedentary controls ($P<0.05$) and at all time points, excluding t=30, when compared to shams ($P<0.05$). As an overall indicator of the post-load excursion, $\Delta$ glucose values at each time point (t=0 or baseline glucose level subtracted from actual glucose values) were summated for each animal (Table 1). At 19 weeks of age, all ZDF groups showed similar glucose excursions and were significantly less glucose tolerant than 6 week-old basal ZDF animals or 19 week-old lean ZDF, as expected ($P<0.05$). Similar calculations were performed on insulin data (Table 1), revealing increased β-cell responsiveness to a glucose stimulus in all 19 week old ZDF rats when compared to basal animals at 6 weeks of age ($P<0.05$), as expected. At 19 weeks of age however, exercised animals displayed increased insulin secretion over this 2 hour time period versus sham-treated and sedentary control animals ($P<0.05$).
4.10.5 Lipid and Corticosterone Measurements Made at Euthanasia (Table 1)

Obesity and T2DM are linked to increased circulating plasma lipids and free corticosterone therefore we measured these parameters at the time of euthanasia. We found that at 19 weeks of age, plasma TG and FFA were elevated in the 3 obese ZDF groups when compared to basal and lean animals; however, there were not differences between exercise, sham-treated, and sedentary control animals (Table 1). Corticosterone measurements made at euthanasia indicate that large increases in resting corticosterone levels occur over time in sedentary control ZDF rats when compared to 6 week-old basal animals and lean Zucker rats ($P<0.05$). Both exercise training and sham-treatment were effective to normalize this age/obesity associated increase in circulating corticosterone levels compared to sedentary control ZDF animals ($P<0.05$).

4.10.6 Bright-Field Immunohistochemical $\beta$-Cell Studies: Morphology, Mass, Proliferation, Neogenesis, Hypertrophy, and Size Distribution (Figure 5)

As expected (406; 586) we found that over the course of the study $\beta$-cell morphology deteriorated markedly in sedentary control and sham treated ZDF animals (5A). Thirteen weeks of swim training, in contrast, which drastically attenuated rapid $\beta$-cell decompensation, was responsible for partially preserving $\beta$-cell morphology that resembled 6 week-old basal and lean animals. Deleterious changes in islet morphology seen in 13-week old sham-treated and sedentary control animals are characterized by significant fibrosis, less intense insulin staining, and weaker nuclear counterstaining performed with hematoxylin. At the time of euthanasia, pancreatic sections were analyzed to determine changes occurring with respect to $\beta$-cell mass. At 19 weeks of age, after 13 weeks of intervention, compensatory expansion of $\beta$-cell mass in response to worsening insulin sensitivity and obesity was seen in sham and exercised rats compared to 6-week old basal
animals ($P<0.05$). This compensatory mass expansion was not seen in sedentary control animals, which had a significantly lower $\beta$-cell mass than either sham or exercise-treated animals (both $P<0.05$, 5B). $\beta$-cell proliferation, as assessed by BrdU incorporation into replicating cells, was increased in all obese ZDF rats versus lean controls ($P<0.05$) and overtime, $\beta$-cell proliferation dropped off significantly in all 13-week obese ZDF treatment groups compared to 6 week-old basal animals ($P<0.05$). However, at the termination of the study, $\beta$-cell proliferation was higher in exercised animals compared to the other non-exercised ZDF groups (both $P<0.05$, 5C). At 19 weeks of age, $\beta$-cell proliferation decreased by approximately 75% in sedentary control animals compared to basal rats whereas exercised rats had higher proliferation than sedentary controls (100% greater), and were only reduced by 50% compared to basal rats ($P<0.05$). By dividing islet areas by the number of cells occupying those areas (data generated from proliferation studies) it is able to measure $\beta$-cell hypertrophy. All ZDF rats, including basal animals had $\beta$-cells with ~2-fold higher mean areas than the lean phenotype ($P<0.05$, 5D). After 13 weeks of treatment, only sham-treated and exercised rats demonstrated $\beta$-cell hypertrophy when compared to basal animals ($P<0.05$) and had further increases in mean $\beta$-cell areas compared to sedentary controls (both $P<0.05$). When looking at indicators of neogenesis both exercise and sham-exercise treatment resulted in an increased number of $\beta$-cell clusters ($P<0.05$, 5E).

In efforts to better understand the contributors to changes in $\beta$-cell mass seen in our study we determined the number of islets/$\beta$-cell clusters that fell within a certain size range spanning $750\mu m^2$ to $50000\mu m^2$, where mean $\beta$-cell size/area was based on preceding hypertrophy measurements (5F). As expected, $\beta$-cell clusters were more numerous in each of the 19-week old ZDF treatment groups relative to 6-week old basal animals and 19-
week old lean ZDF rats ($P<0.05$). Exercise and sham-exercise treatment resulted in a greater number of β-cell clusters for each size range (excluding the 5000-10000 range) when compared to sedentary control animals ($P<0.05$).

4.10.7 Fluorescent Immunohistochemical β-Cell Studies: Morphology, Insulin Intensity, GLUT2, AKT/PKB, ALIS formation (FK2) and Death (Figure 6)

At euthanasia, β-cell morphology (6A) and β-cell insulin content, as assessed by intensity of fluorescence, in pancreata of exercised animals was similar to 6-week old ZDF rats and 19-week old lean animals (6A). With respect to GLUT2 detected by immunohistochemistry, there were pronounced differences in the levels of GLUT2 (Figure 6B). At 6 weeks of age (basal animals) detectable GLUT2 was decreased relative to lean controls which were 13 weeks older ($P<0.05$, Figure 6B). Drastic decreases in GLUT2 also occurred in sham-treated and sedentary control rats at 19 weeks of age compared to lean rats ($P<0.05$, 6B). Exercise training attenuated the reduction of GLUT2 over time ($P<0.05$).

Because the Akt/PKB pathway has been implicated in normal β-cell survival and compensatory mass adaptation we performed immunohistochemical staining to identify and locate Akt/PKB. Staining for Akt/PKB shows decreased detection in 19-week old ZDF rats, including lean rats, relative to 6-week old basal animals ($P<0.05$, 6C). Between the 4 groups of animals, detectable Akt/PKB was maintained at lean levels in exercised rats, such that levels were higher than in sham-treated and sedentary control rats ($P<0.05$). At termination of the experiment Akt/PKB levels were not different between exercised and lean-non diabetic rats.

Diabetes is thought to alter the balance of protein quality control mechanisms within the β-cell and trigger the unfolded protein response (278). Through activation of ER associated
degradation, an increase in protein ubiquitination of misfolded proteins might subsequently be expected. Oxidative stress associated with hyperglycemia is known to induce protein ubiquitination and their accumulation in cytosolic structures known as ALIS (370). To investigate protein ubiquitination in a diabetic model, pancreatic sections were isolated from 19-week ZDF rats. Pancreatic sections from each animal were stained with the mAb FK2, which recognizes both mono- and poly-ubiquitinated proteins (Ub-proteins), but not free ubiquitin (214). In parallel, tissues were stained for insulin to identify pancreatic β-cell islets. As expected, sedentary control animals demonstrated many ALIS in pancreatic β-cell, indicative of oxidative stress caused by hyperglycemia (370). Closer analysis revealed that these Ub-protein aggregates were present in the cytosol of both β-cells and the surrounding acinar cells. In contrast, the signal for Ub-proteins was diffusely cytosolic in both of the non-diabetic control rats. In contrast, the presence of ALIS was not detected in basal, lean, or exercised animals. Sham-exercised animals were characterized by the presence of a paucity of ALIS, while sedentary control animals demonstrated many ALIS; the numbers of such structures were many more than any of the other groups incorporated into the study (6D). Spleen sections from the ZDF rat and the two non-diabetic control rats were also isolated and examined by immunofluorescence using the FK2 mAb. In three different spleen sections, we did not observe Ub-protein aggregates in ZDF rats, suggesting their formation is not a systemic response to hyperglycemia (data not shown). These studies demonstrate that pancreatic β-cells are exposed to less oxidative stress when exercised.

4.11 Discussion
This study clearly demonstrates that 13 weeks of swim-exercise training results in the maintenance of euglycemia through attenuation of the loss of β-cell mass, which was
related to increased β-cell proliferation, increased budding of new insulin positive clusters (neogenesis), and augmented β-cell hypertrophy. Furthermore, the reduction of hyperglycemia with regular exercise is characterized by attenuated loss of β-cell function and higher detectable levels of insulin staining intensity, GLUT2 and Akt/PKB protein. Additionally, we show for the first time that exercise is associated with reductions in the protein-ubiquitination-degradation pathway and subsequent ALIS formation.

Exercise maintained fed and fasting euglycemia with glucose values. We did not cannulate animals and perform a hyperinsulinemic-euglycemic clamp as the recovery period following surgery would have likely nullified the training effect. We were also concerned that chronically cannulated animals would be susceptible to infection. Other studies in ZF and ZDF rats indicate drastic deterioration in insulin sensitivity occurs overtime (292; 596). A well accepted non-invasive, indirect measure of insulin sensitivity (HOMA-IR) can be obtained from the product of fasting insulin and fasting glucose. We performed such calculations with fasted data and observed deteriorations in all groups. Our finding of unimproved insulin sensitivity with exercise does not agree with what has been reported previously in ZDF (596) and ZF animals (125; 292). However such studies employed forced treadmill running paradigms and were terminated between 11 and 14 weeks of age well before gross obesity and severe hyperglycemia occurs. Current findings do agree, however, with recent studies in ZF rats (329) in which exercise training did not improve insulin sensitivity. Although we did not measure muscle GLUT4 protein in the current study, swimming (406) and running (596) studies performed in ZDF rats also indicate no change in red gastrocnemius (406; 596) or white gastrocnemius (406) GLUT4 protein expression, which may explain why we did not observe improvements in insulin sensitivity in these animals. In contrast to their unexercised counterparts, swim animals maintained
compensatory increases in β-cell adaptation. Exercised animals also showed sustained adaptive hyperinsulinemia for the duration of the study, in contrast to sham-treated and sedentary controls which were unable to manufacture and/or secrete sufficient insulin to keep glucose levels below hyperglycemic values. Thus, exercise treatment in ZDF animals is effective in attenuating the loss of compensatory hyperinsulinemia which normally occurs with worsening insulin resistance and obesity in ZF rats, and in obese non-diabetic humans. In vivo basal β-cell function, described as a quotient of weekly fasted insulin divided by weekly fasted glucose, gives a rudimentary indication of the β-cell’s response to a basal glucose stimulus. This index increased in a compensatory fashion in ZDF rats during the prediabetic phase. However, as animals entered the diabetic phase, β-cell function dropped off considerably in sham-treated and sedentary rats, but not in exercised rats.

Glucose tolerance was shown to deteriorate considerably over time in all 13 week old ZDF animals, regardless of treatment. Interestingly, although all groups manifested impaired glucose tolerance at termination of the study, exercised rats displayed significant improvements at 2 hour post-glucose load. In accordance with calculations of insulin sensitivity, exercise, was not associated with an attenuated glucose excursion during the IPGTT test. Glucose area under the curve (AUC) was not different between the 3, 19 week-old groups which may be related to the fact that swim exercise (406) and treadmill running (596) are, at times, not associated with augmented red skeletal muscle total GLUT4 protein. Our present findings that exercised animals recover from their glucose excursions after glucose bolus likely reflects increased total insulin release during this time relative to the other groups.
Further evidence to support our findings of maintained β-cell compensation in exercised ZDF rats is provided by fed insulin concentrations as well as circulating C-peptide levels also measured at euthanasia. Exercised animals displayed elevated fed insulin levels in comparison to non-exercised animals. C-peptide is a better indication of insulin secretion rather than plasma insulin concentrations because the former is not metabolized by the liver (163). Thus, these data indicate adequate β-cell function following an overnight feeding stimulus in exercised rats, but inadequate responses in sedentary and sham-treated rats. These findings are associated with exercise-induced improvements in β-cell morphology in ZDF rats (596) and are in line with human studies suggesting that exercise increases the insulin response to either hyperglycemia or arginine (163). Indeed, a strong positive correlation exists between the maintenance of normal islet morphology and β-cell function (279). In addition to demonstrating that regular exercise training is associated with sustained β-cell compensation and function, we also show that exercise is associated with attenuation of loss of normal β-cell mass. We demonstrate that by 6 weeks of age, during their prediabetic phase, basal animals already manifested increased β-cell proliferation compared to lean animals. Furthermore, we show that daily swim exercise partially maintains this adaptive proliferation which in contrast was clearly decreased in sham-treated and sedentary control animals at 19 weeks of age. This is in agreement with our previous study at 12 weeks of age in ZDF rats (406). We show that neogenesis and β-cell hypertrophy is increased in exercised rats compared to sedentary control rats. Proliferation, neogenesis, and hypertrophy data support our findings of increased β-cell mass which has previously been shown in ZDF rats with exercise training (406), and with forced running (596). However with the current study we extended the exercise duration by a minimum of 6 weeks compared to other studies (406; 596). Interestingly, we also confirm our previous
findings that chronic intermittent stress associated with either sham-exercise treatment (406) or restraint stress (37) results in sustained β-cell mass compensation in ZDF rats. These findings of increased β-cell mass in sham-treated ZDF rats are likely the result of observed increases proliferation, neogenesis and β-cell hypertrophy which occur in rodents, including ZDF rats (205) and guinea pigs (780), with repeated exposure to glucocorticoids. The role of glucocorticoids to influence pancreatic compensation and function is difficult to reconcile with observations that glucocorticoids directly inhibit insulin secretion and β-cell function in normal mouse islets (433) and induce insulin resistance at peripheral insulin target tissues (52; 53).

We did not measure fat pad mass for this study, as we were most concerned with the timely removal of the pancreas prior to auto-digestion. We recognize that favorable changes in body composition, such as reductions in visceral fat pad mass, may have occurred in exercised and sham-treated rats. A reduction in resting corticosterone levels with exercise does indicate that hypothalamic-pituitary-adrenal axis adaptation and/or glucocorticoid activity in a tissue specific manner have been altered. Furthermore, reduced basal corticosterone levels with exercise in conjunction with our reported lack of differences with respect to insulin sensitivity suggests that changes in skeletal muscle, fat, and liver corticosterone metabolism may be taking place.

Recently the role of β-cell insulin signaling and downstream Akt/PKB activation (45; 225; 706; 753; 825), as well as the, maintenance of GLUT2 protein (344; 742) expression have been implicated in the role of maintaining β-cell mass compensation and β-cell function respectively. In ZF rats, Akt/PKB has been implicated in regulating duct-derived neogenesis and β-cell proliferation, cell size, differentiation and survival (3; 45; 167; 225; 706; 753;
Normal Akt/PKB signaling has also been shown to be critically important for controlling β-cell mass relative to metabolic homeostasis (462; 614). To the best of our knowledge, we show for the first time that exercise is associated with phospho-Akt-473 activation and the partial maintenance of immunodetectable levels of β-cell GLUT2 protein expression. Our findings of increased Akt/PKB activation in β-cells of exercised animals offers an explanation for increased mean β-cell hypertrophy as Akt/PKB activity correlates with protein synthesis and β-cell hypertrophy (119). It does not, however, directly explain increases in the rates of β-cell proliferation in this group as mitogenesis is under the control of the ERK 1/2 pathway (75). The fact that Akt/PKB and ERK1/2 are both under the control of IRS-2 tyrosine phosphorylation suggests that increased signaling via this insulin signaling intermediate is taking place. Our immunohistochemical experiments indicating GLUT2 is partially maintained in exercised animals is likely related to increased β-cell function. Exercise prevented fed or fasted hyperglycemia, which induces oxidative stress and causes a subsequent loss of GLUT2 (270). Further supporting this concept are studies indicating that prevention of hyperglycemia with agents that improve glucose clearance attenuates the loss of GLUT2. Additionally studies that employed antioxidants such as NAC have resulted in the maintenance of adaptive insulin content (367). We feel that the prevention of hyperglycemia in these exercised animals combined with the fact that exercise can increase pancreatic antioxidant defenses (141) may have resulted in decreased loss of GLUT2 protein expression in response to chronic hyperglycemia observed in sham-treated and sedentary control rats. Previously GLUT2 reductions were attenuated by caloric restriction in male ZDF rats (545) however, preservation of GLUT2 with exercise was not related to decreased food intake in our exercised animals. Despite an ongoing debate about the cause and effect relationship between hyperglycemia and β-cell
dysfunction, considerable evidence indicates that antecedent elevated plasma glucose levels are an important factor for the loss of β-cell phenotype in ZDF rats (270) and in humans (147; 180). We show that lost of β-cell GLUT2 is not secondary to β-cell damage. GLUT2 levels in basal ZDF rats were significantly reduced relative to lean rats prior to significant reductions in β-cell mass, marked deteriorations in normal islet morphology, and adaptive increases in proliferation in basal rats at this time, indicating an existing defect may be exacerbated by chronic hyperglycemia. We propose that GLUT2 loss in 19 week-old sedentary ZDF animals occurs subsequently to maintained hyperglycemia, likely as a result of glucotoxicity and increased oxidative stress. Further support that oxidative stress is reduced in β-cells of exercised animals is the presence of ALIS in pancreatic sections of hyperglycemic ZDF animals, but not in the islets of exercised animals where hyperglycemia was prevented. Glucose toxicity is associated with the formation of reactive oxygen species (621; 622) which have been shown to induce ALIS formation in a number of different cell types (726). Oxidative stress damages long-lived proteins, and these are subsequently targeted to ALIS for ubiquitination (370). When the production of misfolded proteins exceeds its degradation, the proteins often aggregate leading to intracellular accumulation. This type of chronic ER stress can result in apoptotic cell death. (98). We have previously shown that insoluble proteins (ALIS) are present in the islets of 19-week old ZDF rats but not in young ZDF rats or lean phenotypes (370). Additional in vitro studies with β-cell lines confirmed a dose response relationship between increasing hyperglycemia and ALIS formation (370). Furthermore, we showed that formation of ALIS formation could be prevented by administering known antioxidants such as NAC and taurine which have both been shown to decrease oxidative stress in pancreatic β-cells (370). We confirm the presence of ALIS in β-cells of aged male ZDF rats exposed to
chronic hyperglycemia and also show that swim exercise leads to nearly a complete prevention of ALIS formation and is theorized to be due to prevention of hyperglycemia and subsequent oxidative stress. In sham-treated rats, we saw a minimal effect to prevent ALIS formation despite marked hyperglycemia in this group suggesting that severity of hyperglycemia alone is not solely responsible for the induction of ALIS formation in male ZDF rats. It is possible that the duration of islet exposure in vivo to hyperglycemia may contribute to the formation of ALIS.

Although we cannot exculpate the role of intracellular lipid metabolites and lipotoxicity to induce oxidative stress, we report no differences between the ZDF groups with respect to plasma TG or FFA. We acknowledge the possibility that reduced oxidative stress in exercised β-cells (reduced protein ubiquitination) could be related to a reduction in lipid accumulation. However, because de novo lipid synthesis in pancreatic β-cells has been shown to be dependent on circulating plasma lipid levels and in direct relation to elevated glucose levels (537) we speculated that reductions in β-cell oxidative stress are related to maintenance of euglycemia in exercised animals. It is possible that circulating lipids were different between the groups at some time over the course of the study however, in a different study conducted by our group (406) as well as that conducted by Pold et al. (406; 596) lipids were not different at 12 and 13 weeks of age respectively. Due to the way in which we removed and fixed entire pancreas specimens we could not measure islet lipids or the effects of exercise to alter de novo lipogenesis; two recent studies however did (392; 435). Kibenge et al. showed that swim exercise had no effects on islet FFA oxidation (392), while Lamontagne et al. showed running did not alter islet fatty acid oxidation, or islet expression of key transcription factors and enzymes of lipid metabolism (435). For these reasons we conclude that decreased protein ubiquitination, shown to be prevented with
decreased oxidative stress (370) during exercise is the result of maintained euglycemia and subsequent attenuation of oxidative stress. This theory is supported by our findings of reduced oxidative stress-induced protein ubiquitination in pancreatic β-cells studied. Present findings of less oxidative stress in β-cells of ZDF rats that underwent habitual intermittent exercise not only lends support to our findings of decreased loss of GLUT2 protein and β-cell function in these exercise rats, but also is likely related to partial maintenance of β-cell mass in the current study.

We attempted to measure apoptosis by TUNEL and cleaved caspase 3 staining as well as using nuclear stains such as DAPI and propidium iodide which can be used to identify intensified fluorescence associated with DNA fragmentation (659). With the current study however, extensive background staining of cleaved caspase 3 made it impossible to definitively identify positive staining; therefore, we are not confident with these experiments. With respect to TUNEL staining, TUNEL positive cells in pancreatic islets could not be identified with any frequency that validates quantification. In addition, simple nuclear staining with DAPI and propidium iodide did not stain clearly in the nucleus in the older ZDF islets at any variation in concentration or duration of dye staining. We believe that this may be related to our difficulty in locating TUNEL positive nuclei in the islets of older animals despite clear evidence showing increased apoptosis in paraffin embedded tissues in sections from male ZDF rats (340; 586). We speculate that exercised animals had decreased apoptosis and or necrosis because of reduced hyperglycemia and therefore oxidative stress. Preserved IRS-2/Akt/PKB-serine signaling in exercised animals, which has profound anti-apoptotic effects in pancreatic β-cells (167), further lends support to our findings of decreased β-cell mass compensation in exercised rats.
In summary, we show for the first time that swim training in male ZDF rats is associated with preserved β-cell compensation with respect to insulin secretion and β-cell mass up until 19 weeks of age, an age when ZDF rats are expected to be severely hypoinsulinemic. Our findings of maintained adaptive hyperinsulinemia in exercised animals is associated with partial maintenance of β-cell GLUT2 protein, proper β-cell insulin signaling, and prevention of the formation of ALIS, which is linked to β-cell growth and survival. These studies reveal novel mechanisms for the protective role of exercise on β-cell compensation during the development of T2DM.
Figure 1: Body weight (A) and food intake (B) were measured daily. Average body weight and food intake per rat per day is presented for the weeks during the intervention period. Body weight and food intake measurements were made within a 3 hour period of lights on (07:00) and prior to treatment. ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) lean (n=10). Data are presented as means ± SEM. ||, P<0.05, lean vs. exercise; ¶, P<0.05, lean vs. sham; #, P<0.05, lean vs. control; **, P<0.05, exercise vs. sham; ††, P<0.05, exercise vs. control; §§, P<0.05, sham vs. controls.
Figure 2: Postprandial glucose (A) and fasting glucose (B) measurements were made daily within 3 hours of lights on (07:00), prior to the administration of treatment. Average postprandial glucose per rat per day is presented for the weeks during the intervention period. Fasting glucose was measured weekly after an 18 hour fast. ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) lean (n=10). Data are presented as means ± SEM. ||, P<0.05, lean vs. exercise; ¶, P<0.05, lean vs. sham; #, P<0.05, lean vs. control; **, P<0.05, exercise vs. sham; ††, P<0.05, exercise vs. control; §§, P<0.05, sham vs. controls.
Figure 3A: Fasting Insulin

**Figure 3:** Fasting plasma insulin was measured weekly after an 18 hour fast (A). Weekly HOMA-IR (B) was calculated: Fasting insulin and fasting glucose values were made within 3 hours or lights on (07:00) and prior to treatment. Fasting insulin and fasting glucose values were made within 3 hours or lights on (07:00) and prior to treatment. ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) leans (n=10). Data are presented as means ± SEM. ||; P<0.05, lean vs. exercise, ¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§; P<0.05, sham vs. controls.
Figure 3C: β-Cell Function

Figure 3: Weekly β-cell function (C) was calculated: Fasting insulin (ng/ml) / fasting glucose (mmol/l). Fasting insulin and fasting glucose values were made within 3 hours or lights on (07:00) and prior to treatment. ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) leans (n=10). Data are presented as means ± SEM. ||; P<0.05, lean vs. exercise, ¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§; P<0.05, sham vs. controls.
Figure 4: Intraperitoneal Glucose Tolerance Test

Figure 4: Four days prior to euthanasia and, after an 18 hour fast, an intraperitoneal glucose tolerance test (IPGTT) was performed. Plasma was sampled every 30 minutes over a 2 hour period. ◊, basal (B) group (n=8); ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) leans (n=10). Data are presented as means ± SEM. *; P<0.05, basal vs. sham, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. control, §§; P<0.05, lean vs. basal, ¶; P<0.05, lean vs. exercise, ¶¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§§; P<0.05, sham vs. controls.
Figure 5: β-cell morphology (A). Representative pancreatic sections at 6 weeks of age (B), and after 13 weeks of treatment period (E, S, C, and L). Insulin stained cells appear reddish-brown while non-insulin stained tissue appears purple. β-cell mass (B). % β-cell proliferation/BrdU+ incorporation (C). Examples of BrdU+ nuclei are identified with pointers. β-cell hypertrophy (D), β-cell neogenesis (Figure E). ⊙, basal (B) group (n=8); ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) leans (n=10). Data are presented as means ± SEM. *, P<0.05, basal vs. sham, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. control, §; P<0.05, lean vs. basal, ||; P<0.05, lean vs. exercise, ¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§; P<0.05, sham vs. controls.
Figure 5: Pancreatic clusters at 6 weeks of age (B), and after 13 weeks of treatment period (E, S, C, and L). Mean islet size distribution (F). ◊, basal (B) group (n=8); ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) leans (n=10). Data are presented as means ± SEM. *; P<0.05, basal vs. sham, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. control, §; P<0.05, lean vs. basal, ||; P<0.05, lean vs. exercise, ¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§; P<0.05, sham vs. controls.
Figure 6-Fluorescent Studies: β-cell insulin (FITC) intensity (A). Immunodetectable β-cell GLUT2 (CY3) and relative GLUT2 pixel area (B). Immunodetectable β-cell Akt/PKB (CY3) and relative Akt/PKB pixel area (C). ◊, basal (B) group (n=8); ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) leans (n=10). Data are presented as means ± SEM. *; P<0.05, basal vs. sham, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. control, §; P<0.05, lean vs. basal, ||; P<0.05, lean vs. exercise, ¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§; P<0.05, sham vs. controls.
Figure 6D: β-Cell ALIS Formation

Figure 6-Fluorescent Studies: β-cell insulin (FITC) intensity, β-cell ALIS (CY3) formation (D). ◆, basal (B) group (n=8); ■, exercise (E) group (n=9); ▲, sham (S) group (n=9); ●, untreated (C) group (n=9); □, untreated (L) lean group (n=10). Data are presented as means ± SEM. *; P<0.05, basal vs. sham, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. control, §; P<0.05, lean vs. basal, ||; P<0.05, lean vs. exercise, ¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§; P<0.05, sham vs. controls.

Table 1: Data are means ± SEM. Data are presented as means ± SEM. *; P<0.05, basal vs. sham, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. control, §; P<0.05, lean vs. basal, ||; P<0.05, lean vs. exercise, ¶; P<0.05, lean vs. sham, #; P<0.05, lean vs. control, **; P<0.05, exercise vs. sham, ††; P<0.05, exercise vs. control, §§; P<0.05, sham vs. controls.
5.1 Abstract

Classical stress activated systems and oxidative stress are involved in insulin resistance which along with β-cell failure is a hallmark feature of T2DM. Exercise, which decreases systemic markers of inflammation improves insulin resistance. The purpose of the study was to identify the signaling pathway whereby exercise inhibits inflammation and improves insulin sensitivity in the liver which is a major target tissue in a model of obesity and T2DM. 4 groups of Zucker/fa/fa animals (3 groups of ZDF and 1 group ZF) were obtained at 5 weeks of age and were divided into basal (B, n=8), exercise (E, n=9), and sedentary controls (C, n=8). A group of obese but non-hyperglycemic ZF rats were incorporated as controls (F, n=8). B animals were euthanized at 6 weeks of age while the remaining 3 groups 10 weeks later. On average, E animals ran 5250m/day which improved insulin sensitivity, and maintained fed and fasted glucoregulation and glucose tolerance. In agreement with improved insulin sensitivity, as assessed by HOMA-IR, improved glucoregulation was also seen during an IPGTT. 10 weeks of running decreased whole-body markers of inflammation and oxidative stress in the blood and in the liver. Exercise lowered circulating IL-6, haptoglobin, and MDA levels and protein oxidation in the liver. Exercise reduced phosphorylated JNK (pJNK) indicating decreased JNK activity. In accordance with decreased hepatic pJNK, serine-307 phosphorylated IRS-1 was reduced in E rats. IκBα, however, was not different between any of the groups. In summary, we show that volitional running attenuates development of hyperglycemia in ZDF rats through
improvements in glycemia and in association with decreases in circulating markers of inflammation and oxidative stress, and decreases in hepatic JNK activation, and serine phosphorylation of IRS-1.

5.2 Aims

Type 2 diabetes mellitus (T2DM) and obesity are now the most common metabolic diseases in North America (860; 861). The exact cause of T2DM is unknown, but is characterized by a progressive decline in peripheral and hepatic insulin sensitivity and β-cell adaptation which eventually leads to insulin insufficiency and hyperglycemia (438). A hallmark feature of obesity and T2DM, is hepatic insulin resistance.

Chronic elevations in circulating markers of inflammation such as CRP and haptoglobin occur both in obesity and T2DM (151). Obesity causes endoplasmic reticulum stress and leads to the activation of inflammatory signaling pathways including JNK contributing to insulin resistance (526; 562; 563). A second mechanism that initiates inflammation in obesity and T2DM is oxidative stress which activates inflammatory signaling (79; 215; 838).

Inflammation is the primary cause of obesity-linked insulin resistance. Cytokines such as TNFα and IL-6 have been shown to cause insulin resistance in obesity (34). The inhibition of signaling downstream of the insulin receptor is a primary mechanism through which inflammatory signaling leads to insulin resistance (805). Exposure of cells to TNFα stimulates serine phosphorylation-307 of IRS-1 (4; 310; 838) resulting in disruption of normal insulin signaling and insulin action (5; 310; 570). Serine/threonine kinases elevated in obesity (805) such as JNK, IKKβ and the protein kinase C (PKC) isoforms (805; 857) are activated by inflammatory and oxidative stimuli and contribute to inhibition of insulin signaling (4; 222; 563). Modulation of hepatic JNK in rodents affects glucose metabolism
and JNK is stimulated by oxidative stress, increased FFA and other adipocytes-derived products such as cytokines (56; 159; 380; 388; 498). JNK has recently emerged as a central metabolic modulator of insulin resistance in obesity (294).

Other serine kinases such as PKC, and IKKβ are implicated in the mechanisms of hepatic insulin resistance (24; 57; 104; 432; 653; 654; 697). PKCδ is activated by short-term elevations in plasma FFA (57; 432), while another novel isoform, PKCζ, appears to be involved in hepatic insulin resistance caused by high fat feeding (653; 654). A potential pathway may involve FFA-induced activation of PKC, which impairs insulin signaling pathway either directly by phosphorylating serine/threonine sites of insulin receptor and/or IRS-1/2 or indirectly by activating other serine kinases such as JNK and IKKβ. Rodent models of obesity and insulin resistance such as ZF, ZDF rats, and ob/ob, and db/db, mice, that lack functional leptin signaling or production, manifest increased systemic inflammation and impaired insulin signaling (187; 749).

Exercise is beneficial in the prevention and treatment of insulin resistance and T2DM in humans and in rodent models including ZF and ZDF rats (50; 125; 185; 187; 188; 211; 240; 292; 596; 749). Exercise training decreases elevated obesity-related systemic markers of inflammation such as CRP and IL-6 (151). Swim training of rats has also been shown to blunt the pro-inflammatory cytokine response to lipopolysaccharide (136) and to decrease CRP (13) while in humans basal circulating IL-6 and CRP are lower after increased physical activity (132; 611). IL-6 which increases during and shortly after exercise has an inhibitory effect on TNFα production (662); anti-IL-6 treated or IL-6 KO mice have elevated TNFα (496; 509). To date, however, the potential effect of exercise training to modulate the cellular pathways involved in inflammation and related serine phosphorylation of IRS-1 as it
pertains to improved insulin sensitivity in insulin target tissues remains unknown. The purpose of the study was to identify the signaling pathway whereby exercise inhibits inflammation and improves insulin sensitivity in the liver which is a major target tissue in a model of obesity and T2DM.

5.3 Research Design and Treatment Protocols

5.3.1 Animals

Male ZDF/ZF rats (ZDF/Gmi-fa/fa; ZF/fa/?) were obtained from Charles River Laboratories (Saint-Constant, Quebec, Canada) at 5 weeks of age with initial body weights of 150-175g. Rats were singly housed in opaque micro-isolation cages, and kept at a constant temperature of 22-23°C in humidity-controlled rooms on a standard 12h (07:00-19:00) light/dark cycle. The animals were fed water and PURINA™ 5001 chow ad libitum throughout the experiment. All experiments were approved by the Animal Care Committee of the Faculty of Medicine at the University of Toronto in accordance with regulations set forth by the Canadian Council for Animal Care.

5.3.2 Experimental Design

Twenty-five male ZDF rats and 8 ZF rats were obtained at 5 weeks of age. All rats underwent the same 1-week acclimatization period. Of the 25 ZDF rats, animals were randomly divided into 3 groups. At 6 weeks of age, one group of ZDF animals was euthanized by decapitation to serve as a basal (B) baseline control (n=8), while the remaining 2 ZDF groups: exercise (E, n=9), and sedentary controls (S, n=8) were incorporated into the long term study for 10 weeks, after which time they were euthanized at 16 weeks of age (n=9/group). ZF animals (F; n=8) served as a sedentary fatty non-diabetic control group.
5.3.2 Volitional Exercise Training: Wheel Running
Male ZDF rats incorporated into the running portion of the experiment were housed in rodent activity wheel and cage assemblies which allowed animals to exercise voluntarily (Harvard Apparatus, Holliston, Massachusetts). Assemblies included a stainless steel activity wheel with low-friction Teflon bearings which allowed for quiet, smooth operation. Wheel hub supports had a cut away to allow food hoppers and water bottle to fit adjacent to the entire assembly which sat on top of common polycarbonate rat cages. The cage floor space adjacent to the activity wheels met National Institutes of Health floor space requirements for a single rodent. The dimensions of the wheel were 34.5cm x 9cm and had a radius of 17.25cm. Each wheel assembly was fitted with a magnetic switch and LCD counter which allows for the recording of an animals activity. To program the computers we used the radius of the wheel and the formula:

\[
\text{Circumference} = 2\pi r = 2 \times 3.14159 \times 17.24 = 108\text{cm} = 1.08\text{m}
\]

Once programmed, the computers produced data in km/h for velocity and km for distance ran. Sedentary control ZDF and ZF animals were subjected to the same sampling and handling procedures as exercised animals. After 10 weeks, all animals were euthanized between 09:00-12:00h by decapitation.

5.3.3 Run Distance, Food Intake and Body Weight Measurements
Each morning total distance ran, average and maximum velocity, and time elapsed were recorded. Food intake (excluding the day of fast) and body weight were measured each week on Thursday prior to food removal for the overnight fast. Cage changes were also performed at this time to ensure that no food remained in the substrate bedding.
5.3.4 Intraperitoneal Glucose Tolerance Test (IPGTT) with Respective Insulin Levels

All groups were subjected to an IPGTT 3 days prior to euthanasia. For basal animals, this test was performed at ~6 weeks of age, while exercised, sedentary fatty and sedentary control animals received an IPGTT between ~16 weeks of age. Commencing each Thursday afternoon, rats were fasted overnight for 15-18 hours and were then administered an intra-peritoneal injection of 50% dextrose (Abbott Laboratories Limited, Montreal, Canada) at a dose of 2g/kg body weight between 0900h and 1200h. Blood glucose and insulin levels were measured via tail nick (see above) at 30 minute intervals starting at T=0, just prior to injection, for T=120 minutes.

5.3.5 Resting Hormone Measurements Made at Euthanasia

For animals sampled over the 10 weeks of treatment, euthanasia occurred within 5 hours after the last bout of volitional running. Trunk blood was collected in 1.5ml tubes containing EDTA and trasylol, for all hormones. Immediately after decapitation, all blood samples were centrifuged at 2500rpm for 1 minute with transferred plasma stored at -20°C. Trunk blood glucose was measured using a single drop of blood obtained at the time of decapitation with a blood glucose test strip (ASCENSIA ELITE™, Bayer, Toronto, Canada) and glucometer (ASCENSIA ELITE™ XL Blood Glucose Meter, Bayer, Toronto, Canada). Plasma insulin levels were determined using a rat insulin Elisa assay kit (Crystal Chem Inc, Illinois, USA). Plasma corticosterone and C-peptide levels were measured by commercially available radioimmunoassay (RIA) kits (Medicorp Inc., Montreal Canada). Plasma free fatty acids (FFA) and triglycerides (TG) were determined by an enzymatic colorimetric method (ACS-ACOD; Wako Chemicals, Richmond, VA). Measurement of liver protein carbonyls was performed on cytosolic fractions using a 2,4-dinitrophenylhydrazine (DNPH)-reliant carbonyl assay kit (Cayman Chemicals, Ann Arbor, Michigan, USA). To
measure IL-6 a quantitative sandwich enzyme technique assay was used (Rat IL-6 Quantikine Immunoassay, R&D Systems, Minneapolis, MN, USA). Plasma haptoglobin was determined using a commercially available ELISA kit and protocol (Life Diagnostics, West Chester, PA, USA). To measure plasma malondialdehyde a thiobarbituric acid (TBA)-malondialdehyde assay was used (337).

5.4 Analytical Procedures and Methods: For a Detailed Description see Materials and Methods Chapter 3

5.5 Results

5.5.1 Weekly Run Distance, Body Mass and Food intake (Figure 1)

Figure 1A depicts a steady increase in the distance ran by exercised rats until the 6th week of intervention, after which the mean distance began to decrease. At the commencement of the study, body mass was not different between the 3 ZDF groups and increased equally in both exercised and sedentary ZDF rats until the 5th week of treatment ($P<0.05$, 1B). By the end of the study exercised animals weighed only 6% less than sedentary animals although this was significant ($P<0.05$, 1B). ZF animals weighed more than either exercised or sedentary ZDF animals for the entire duration of the study as expected ($P<0.05$, 1B). Fat pad mass increased over time in ZDF rats but did not differ between exercised and sedentary ZDF animals (Table 1). Food intake and caloric restriction are important factors determining the progression of hyperglycemia in this model. We measured weekly food intake to determine if differences in this parameter could be responsible for improvements with exercise. Food intake in ZF animals was greater than in sedentary or exercised ZDF animals at all time points of the study excluding the first week ($P<0.05$, 1C). Importantly, food intake between sedentary and exercised ZDF animals did not differ at any point during the study.
5.5.2 Glucose Measurements (Figure 2)

A change in the glycemic profile is an indication of a change in insulin sensitivity and/or change in insulin production. We therefore measured glucose on a biweekly basis and plotted this data over the course of the study (Figures 2A & 2B). Pre-intervention fed glucose values were similar between all groups incorporated into the study. By the 4th week of the study sedentary ZDF animals showed increased glucose relative to exercised animals \((P<0.05, \text{2A})\). Although there were weeks during the remainder of the study where exercised and sedentary ZDF rats did not differ (i.e. 8 & 9), by the last two weeks of study exercised animals had fed glucose values which were approximately 1/3rd of that seen in sedentary ZDF animals \((P<0.05, \text{2A})\). ZF rats maintained glucose levels \((P<0.05, \text{2A})\) below ZDF animals due to adaptive hyperinsulinemia as expected. After the first week of volitional running, exercised animals had lower fasting glucose than either ZF or sedentary ZDF rats \((P<0.05, \text{2B})\) however by the last 2 weeks of study glucose values between these two groups did not differ. The effect of volitional wheel running to maintain fasting glucose below levels seen in sedentary ZDF animals was observed over the last 6 weeks of the study, and by the end of the study fasted glucose in exercised animals was approximately 70% less than in sedentary ZDF animals \((P<0.05, \text{2B})\).

5.5.3 Insulin (Figure 2C, Table 1), HOMA-IR (Figure 2D) and C-peptide (Table 1)

Figure 2C shows changes in measured fasting insulin values over the course of the study. As expected, ZF animals displayed adaptive hyperinsulinemia relative to the ZDF groups during the very first week of study which continued over the entire treatment period \((P<0.05, \text{2C})\). Interestingly sedentary ZDF animals showed \(\beta\)-cell compensation and adaptive hyperinsulinemia relative to exercised animals at week 6 and 8 of the intervention
period ($P<0.05$, 2C), however this effect was lost by the 10th week. Similarly, at euthanasia (fed insulin values), exercised rats and ZF animals showed increased insulin relative to sedentary control ZDF rats ($P<0.05$, Table 1). As an index of insulin sensitivity, we performed HOMA-IR calculations which showed improved insulin sensitivity in ran animals ($P<0.05$, 2D). Observation of C-peptide values at the time of euthanasia shows an interesting effect of exercise. At this time, C-peptide levels were lower in both ZDF groups ($P<0.05$, Table 1) but were not different between sedentary and exercised ZDF animals despite pronounced differences in fed insulin levels at this time (Table 1).

5.5.4 IPGTT Plasma Glucose (Figure 3, Table 1) and IPGTT Plasma Insulin Levels (Table 1)

An IPGTT was performed in basal animals (6 weeks of age) and after 10 weeks of intervention in all other groups. Over time and as expected, glucose tolerance decreased drastically in sedentary ZDF rats compared to basal animals at 6 weeks of age ($P<0.05$, Figure 3A). Prior to glucose injection, sedentary ZDF rats had elevated fasting glucose relative to exercised ZDF and sedentary ZF animals ($P<0.05$, Figure 3A). Two hours following glucose injection exercised and ZF animals recovered from their hyperglycemia excursion while sedentary ZDF animals did not ($P<0.05$, Figure 3A). Glucose values were lower in exercised rats than age-matched sedentary counterparts at all time points during the IPGTT excluding T=60 minutes ($P<0.05$, Figure 3A). Examination of insulin values reveals that fasting insulin was elevated in ZF animals compared to other groups at all time points during the IPGTT ($P<0.05$, Figure 3B). Plasma insulin levels did not differ between exercised and sedentary ZDF animals until after 90 minutes into the assessment and remained lower in exercised animals vs. sedentary ZDF animals until completion of the IPGTT ($P<0.05$, Figure 3B). Observation of insulin production and/or secretion in sedentary
animals combined with glucose values during this test reveals insufficient insulin to allow proper recovery from a glucose excursion. Basal, older ZF, and exercised animals, however, were not impaired in this fashion ($P<0.05$, Figure 3B).

5.5.5 Western Blot Data: pJNK, IκBα, PKCε, PKCδ, and Serine Phosphorylated IRS-1 (Figure 4)

Actin expression was not significantly different between any of the groups for any of the protein loadings. PKC activation, as indicated by membrane translocation, is also responsible for serine phosphorylation of IRS and related impairment in insulin signaling in vitro (252). More specifically, the isoforms PKCδ/ε have been shown to be altered in hepatic insulin resistance (57; 432; 654). We found that there was a tendency for a lower PKCε membrane/cytosolic ratio in basal animals vs. older more obese groups (Figure 4A). In contrast to PKCε we found that PKCδ activation was lower in exercised and sedentary ZDF rats vs. fatty rats ($P<0.05$, Figure 4B). Interestingly we also found that exercised rats had lower PKCδ activation than their much younger 6 week-old ZDF counterparts ($P<0.05$, Figure 4B). JNK activation has been implicated in the serine phosphorylation of IRS-1 and hepatic insulin resistance. We therefore measured pJNK and total JNK. Total JNK protein expression was not different between either of the groups (Figure 4C). Exercise prevented the increase in phosphorylation of JNK activation when compared to sedentary ZF and ZDF animals ($P<0.05$, Figure 4C). Activation of NFκB and disassociation/degradation of its inhibitor IκBα by IKKβ have also been implicated in the pathogenesis of hepatic insulin resistance. Measurement of IκBα indicates that exercise treatment was not associated with reduced IKKβ activity when compared to aged-matched sedentary ZF and ZDF counterparts ($P<0.05$, Figure 4D). Because JNK can serine phosphorylate IRS-1, we next measured serine-307 phosphorylated and total IRS-1. Total IRS-1 protein was not
significantly changed (Figure 4E) but serine-307 phosphorylated IRS-1 was elevated in sedentary ZDF animals vs. all other groups (P<0.05, Figure 4E). When corrected for total IRS-1 protein levels serine-307 phosphorylated IRS-1 was elevated in sedentary ZDF rats vs. younger basal animals, exercised ZDF animals, and age-matched ZF animals (P<0.05, Figure 4E).

5.5.6 Measurements Made at Euthanasia (Table 1)

At the time of euthanasia we sampled mixed trunk blood and measured a number of factors involved in glucose homeostasis, inflammation, and oxidative stress. Haptoglobin is a protein made in hepatocytes and adipocytes in response to systemic inflammation. Haptoglobin was elevated in sedentary ZF and ZDF animals when compared to 6 week-old basal animals but was normalized in exercised ZDF rats (P<0.05, Figure 5A). Interestingly, haptoglobin was elevated in ZF rats when compared to sedentary age-matched ZDF rats (P<0.05, Figure 5A) and may be indicative of increased visceral fat pad mass these animals vs. the ZDF groups (P<0.05, Table 1). Because we wanted to ascertain if exercise exerted an effect to lower circulating IL-6 in these animals we performed an ELISA to measure this cytokine/myokine. Compared to basal animals, only ZF and exercised animals has lower circulating IL-6 (P<0.05, Figure 5B). Levels of this cytokine/myokine were not different between ZF and exercised rats or between sedentary ZDF and ZF animals. Therefore exercise prevented the increase in IL-6 (P<0.05, Figure 5B). MDA is a widely accepted marker of oxidative stress. We measured the levels of this marker in the plasma of terminated animals and found that MDA levels were increased in ZF and sedentary control ZDF rats but not in exercised ZDF rats (P<0.05, Figure 5C). At the termination of the study sedentary ZDF rats had augmented plasma MDA levels compared to basal and ZF rats (P<0.05, Figure 5C). Hepatic protein carbonyl formation was reduced
in exercised ZDF and sedentary ZF animals when compared to sedentary control ZDF animals (P<0.05, Figure 5D). Importantly exercise prevented the increase in this marker of protein oxidation (P<0.05, Figure 5D) relative to basal animals. FFA were increased in ZF and sedentary ZDF rats relative to basal animals (P<0.05, Figure 5B), but were normalized in exercised rats. We also found that sedentary ZDF rats had increased plasma FFA vs. ZF animals (P<0.05, Table 1). Plasma TG increased over time in all 16 week-old animals relative to basal but where higher in sedentary ZDF rats vs. both ZF rats and exercised ZDF animals (P<0.05, Table 1). Interestingly, triglyceridemia was not different between ZF and exercised ZDF animals at the termination of the study. Lastly, elevated circulating levels of glucocorticoids are associated with visceral adiposity and diabetes in rodent and man. We measured free corticosterone and found that over time basal secretion of this hormone is greatly increased in ZDF animals lacking intervention, while exercise training is effective to attenuate this increase and does so independently of reductions in epididymal fat pad mass (P<0.05, Table 1).

5.6 Discussion
This study demonstrates that 10 weeks of volitional running maintains euglycemia and insulin sensitivity, as assessed by HOMA-IR, in male ZDF rats. Maintenance of glucoregulation is associated with reduction in systemic and hepatic oxidative stress markers, hepatic JNK activation, and serine phosphorylation of IRS1.

In the present study volitional running ~5km/day maintained insulin sensitivity and retained preserved glucose tolerance in the male ZDF rat as has been shown before with forced running (596). With respect to body mass, there were differences between sedentary and exercised ZDF rats throughout and by the end of the study. We did not measure body
composition with the use of DEXA or MRI; however we did not find differences between these two groups with respect to epididymal fat pad mass. It is important to note that changes in the metabolic profiles of exercised animals in this study were not related to food intake. Caloric restriction has been shown to be an effective treatment to improve insulin sensitivity and glycemia in obese individuals with and without T2DM (564; 769; 808). Furthermore in rodents, caloric restriction attenuates or prevents the development of hyperglycemia in spontaneous models of T2DM (37; 330). Plasma lipid data indicated that exercised animals had lower circulating levels of FFAs and TG when compared to sedentary ZDF rats. These improved plasma lipid profiles may be related to the fact that habitual running likely increased muscle lipid uptake as shown (661). Although we did not observe differences in epididymal fat pad mass between sedentary and exercised ZDF animals, we cannot discount the possibility of reductions in total visceral, or in subcutaneous fat in exercised animals.

Importantly, from 8-10 weeks of intervention, exercised animals responded to worsening obesity and insulin demand with increased insulin production and/or secretion. In contrast, in sedentary animals, β-cell decompensation was evident. This concept of β-cell failure in untreated male ZDF rats is thought to be critical to the development of hyperglycemia (594; 596; 597; 599; 749; 757). Pold et al. showed that ZDF rats have both hepatic and peripheral insulin resistance that is improved with exercise as evidenced by improved rates of glucose appearance and glucose clearance (596). These studies outline the mechanism of maintenance of euglycemia with forced exercise. Results from our current study of volitional running confirm these results in ZDF rats (596) as well as in running studies using ZF rats (125; 292). Evidence that insulin sensitivity is maintained is provided by observation of fasting glucose and insulin values and HOMA-IR calculations. By week 8 of
the intervention period sedentary ZDF animals had pronounced fasting hyperglycemia and hypoinsulinemia compared to exercised animals. This maintenance of normoglycemia with less insulin indicates that exercised animals are less insulin resistant. Importantly, because fasting glucose levels mainly reflect hepatic gluconeogenesis and glycogenolysis we assume that exercised rats had improved basal hepatic insulin sensitivity. Glucose tolerance deteriorated considerably over time in 16 week-old ZDF animals lacking intervention but was maintained in animals which ran daily. Just prior to euthanasia exercised animals had 2 hour post-load glucose values which were not different from 6 week-old basal animals, showing a normal glucose tolerance curve. Furthermore, 2-hour post-load glucose values in exercised ZDF rats were not different from 16 week-old ZF animals which compensate for gross insulin resistance with adaptive hyperinsulinemia (125; 292). The notion of improved insulin sensitivity is supported by the fact that exercised animals secreted less insulin than sedentary rats over the last 30 minutes of the IPGTT. Our present findings of protective effects with volitional exercise are of importance because beneficial effects of various types of non-exercise stressors have been shown to improve glucoregulation in ZDF animals (37; 406) as well as OLETF (364).

In obesity TNFα is elevated in plasma and adipose tissue (193; 389; 311; 669). TNFα is a pro-inflammatory cytokine that induces the production of other pro-inflammatory cytokines such as IL-6 and the acute phase reactants CRP and haptoglobin (579). We measured haptoglobin with this study because CRP is not an ideal measurement of the liver’s response to inflammatory status in rats. We measured IL-6 and not TNFα because TNFα is difficult to detect in plasma and because IL-6 is increased in obesity and T2DM and is induced by acute exercise. Increased IL-6 is a potent inducer of AMPK activity and stimulator of lypolysis (381), as well as a player in substrate utilization and FFA β-oxidation.
We found that circulating basal levels of IL-6 is decreased by volitional exercise which is of interest because elevated circulating levels of systemic markers of inflammation such as IL-6 do, in part, contribute to hepatic insulin resistance in obesity and T2DM. The response of IL-6 and the role it plays in exercise and insulin resistance, however, is a contentious issue. This is highlighted by the fact that we actually found increased plasma IL-6 levels in 6 week-old ZDF basal animals which were not severely obese or insulin resistance. It may be that differing reports with respect to the effect of exercise on IL-6 levels may reflect altered levels of production from muscle as a myokine and/or from adipose tissue/macrophage consortiums which produce IL-6 namely in response to TNFα. We were not able to measure tissue specific expression of IL-6 and TNFα which would have addressed these possible changes occurring with exercise. Thus, the exact role of IL-6 in obesity and its induction in exercise is poorly understood. Decreased haptoglobin values seen in exercised rats is likely related to the decreased hepatic and systemic oxidative stress (protein carbonyls and MDA levels respectively) and lower markers of whole body inflammatory status seen in exercised rats versus sedentary controls.

It is well known that exercise increases tyrosine phosphorylation of insulin signaling intermediates as well as membrane GLUT4 translocation which both relate to improved glucose tolerance and insulin sensitivity (83; 125; 161; 162; 211; 292; 673). Improved hepatic insulin signaling is also due to decreased serine phosphorylation of IRS complexes. In response to stimuli such as ER stress, oxidative stress, cytokines, and FFA, JNK is activated where it associates with and phosphorylates IRS-1 on ser-307 impairing insulin action (4; 222; 563). Oxidative stress in hepatocytes is increased by the presence of long chain lipid metabolites such as diacylglycerol (DAG) and through the action of PKC (323; 732; 733), and in response to TNFα signaling with its receptor. In rats, oxidative stress and
JNK activation is further augmented by increased circulating FFA leading to release of adipocyte-derived products, such as cytokines, (56; 159; 380; 388; 498). Removal of inflammatory mediators or pathway intermediates such as JNK, TNFα, IKKβ protects against insulin resistance in obese rodent models and in humans with drugs that target these pathways (294; 311; 318; 766; 846). Further evidence to support the role of JNK phosphorylation in inducing insulin hepatic insulin resistance is as follows. In obesity JNK activity is elevated in liver, muscle, and fat tissues and loss of JNK prevents the development of T2DM in both genetic and dietary mouse models of obesity (294). In order to understand the mechanisms by which exercise improves glucoregulation, we prepared liver homogenate and immunoblotted for players known to govern hepatic insulin sensitivity. Long chain fatty acid metabolites such as DAG increase oxidative stress and activation of PKC isoforms in hepatocytes while JNK activation either in conjunction with PKCδ/ε activation or on its own has been shown to serine phosphorylate IRS-1 and inhibit hepatic insulin signaling (24; 104). In order to ascertain the effect of exercise to prevent activation of PKCδ/ε we immunoblotted for both of these PKC isoforms and measured their degree of membrane translocation which indicates activation. PKCε elevation appears to be involved in hepatic insulin resistance in response to chronic FFA elevation characteristic of the ZF and ZDF models (653; 654). Although we observed a tendency for increased PKCε activation in sedentary ZDF animals vs. younger basal rats, no significant effects or exercise or time were found. Because PKC isoforms are induced predominantly in response to long chain fatty acid metabolites such as DAG, and since we did not see any differences between exercised and sedentary ZDF animals we did not measure hepatic fat content. PKCδ, conversely, was shown to be reduced in exercised and sedentary ZDF animals when compared to ZF animals. This is likely related to the increased portal fat
accumulation seen in ZF animals which is known to be a potent inducer of hepatic PKCδ activation. Interestingly, PKCδ activation in hepatocytes occurs primarily in response to short-term FFA infusion (57; 432). We measured phosphorylated JNK and IκBα (whose decreased levels indicated increased IKKβ activity) as both JNK and IKKβ are involved in the mechanism of fat-induced hepatic insulin resistance (24; 57; 104; 432; 653; 654; 697).

Liver NFκB activation is important for IL-6 production (23). Since NFκB is a transcription factor for pro-inflammatory cytokines including IL-6, and because we saw reduced IL-6 in exercised animals, we expected that IκBα would be maintained or even elevated in the liver of exercised animals. IL-6 is downstream of NFκB however we did not see changes in IL-6. Levels of IL-6 seen in this study may be reflective of what is happening at the muscle opposed to the liver however. Exercise did not modulate IκBα in this study; however, liver may not be a predominant source of IL-6. In support of increased hepatic inflammation we show that haptoglobin, which is produced by liver cells upon NFκB translocation to the nucleus is increased in sedentary ZDF animals when compared to animals which were exercised or did not show marked hepatic insulin insensitivity (basal animals). However we did not directly measure NFκB activation. We show that JNK phosphorylation parallels increased accumulation of adipose tissue seen in sedentary ZF and untreated sedentary ZDF animals. Volitional wheel running effectively maintained JNK below levels seen in sedentary ZDF rats and similar to young ZDF animals. The mechanisms of how JNK is activated remain a contentious issue but oxidative stress as well as ER stress are potent activators of JNK (525; 697). Hepatic JNK activation is also induced by circulating pro-inflammatory cytokines such as TNFα (466; 776; 811). In order to examine the possibility that decreases in JNK activation in exercised animals were related to changes in hepatic oxidative stress we measured hepatic protein carbonyl content which indicates protein
oxidation (454). Protein carbonyl formation production is reduced in by volitional running. In order to assess systematic markers of oxidative stress we chose the MDA assay which measures whole-body lipid peroxidation in response to increased free radicals and reactive oxygen species. Our finding of reduced hepatic protein carbonyl formation is supported by plasma MDA levels reduced in exercised animals. As mentioned, JNK activation is capable of inducing serine phosphorylation of IRS-1. When we immunoblotted for IRS-1 we found that IRS-1 serine phosphorylation was drastically reduced in exercised animals when compared to sedentary ZDF rats such that it was similar in expression to less insulin resistant basal animals. These results taken together, therefore, indicate that JNK activation and/or increased production of pro-inflammatory cytokines are responsible for increased serine IRS-1 phosphorylation. PKC isoform activation in our study does not appear to reflect the pattern of serine phosphorylation which is consistent with previous reports.

In summary, we show for the first time that volitional wheel running effectively attenuates loss of glucoregulation in male ZDF rats until 16 weeks of age. Such improvements in fed and fasting glycemia are related to decreases in whole-body markers of inflammation and oxidative stress. Such findings of decreased serine phosphorylation of IRS-1 with volitional exercise are associated with decreased JNK activation.
Figure 1A: Mean Weekly Run Distance

Figure 1B: Body Mass

Figure 1: Run distance (A), Body mass (B) and food intake (C) were measured weekly. Average body weight is presented for the weeks during the intervention period. Body weight measurements were made within a 3 hour period of lights on (07:00) and prior to treatment. □, fatty (F) group (n=8); ■, exercise (E) group (n=9); ●, untreated sedentary control (S) group (n=8). Data are presented as means ± SEM. *; P<0.05, basal vs. fatty, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. sedentary, §; P<0.05, fatty vs. exercise, ||; P<0.05, fatty vs. sedentary, ¶; P<0.05, exercise vs. sedentary.
Figure 1C: Food Intake

Food intake (C) were measured weekly. Average food intake is presented for the weeks during the intervention period. Food intake measurements were made within a 3 hour period of lights on (07:00) and prior to treatment. ○, fatty (F) group (n=8); ■, exercise (E) group (n=9); ●, untreated sedentary control (S) group (n=8). Data are presented as means ± SEM. *; P<0.05, basal vs. fatty, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. sedentary, §; P<0.05, fatty vs. exercise, ¶; P<0.05, fatty vs. sedentary, ||; P<0.05, exercise vs. sedentary.
Figure 2A: Postprandial Glucose

Weeks of Intervention

Postprandial Glucose (mM)

0.0
2.0
4.0
6.0
8.0
10.0
12.0
14.0
16.0
18.0
20.0
22.0

Figure 2B: Fasting Glucose

Weeks of Intervention

Figure 2: Postprandial glucose (A) and fasting glucose (B) measurements were made daily within 3 hours of lights on (07:00), prior to the administration of treatment. Average postprandial glucose per rat per day is presented for the weeks during the intervention period. Fasting glucose was measured weekly after an 18 hour fast. □, fatty (F) group (n=8); ■, exercise (E) group (n=9); ●, untreated sedentary control (S) group (n=8). Data are presented as means ± SEM. *; P<0.05, basal vs. fatty, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. sedentary, §; P<0.05, fatty vs. exercise, ||; P<0.05, fatty vs. sedentary, ¶; P<0.05, exercise vs. sedentary.
Figure 2: Fasting insulin (C) measurements and were made daily within 3 hours of lights on (07:00), prior to the administration of treatment. Average postprandial glucose per rat per day is presented for the weeks during the intervention period. Fasting glucose was measured weekly after an 18 hour fast. HOMA-IR (D), fatty (F) group (n=8); ■, exercise (E) group (n=9); ●, untreated sedentary control (S) group (n=8). Data are presented as means ± SEM. *; P<0.05, basal vs. fatty, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. sedentary, §; P<0.05, fatty vs. exercise, ||; P<0.05, fatty vs. sedentary, ¶; P<0.05, exercise vs. sedentary.
Figure 3: Intraperitoneal Glucose Tolerance Test

3A) Glucose

Figure 3: Four days prior to euthanasia and, after an 15-18 hour fast, an intraperitoneal glucose tolerance test (IPGTT) was performed. Plasma was sampled every 30 minutes over a 2 hour period. Glucose (A) and Insulin (B) are shown over this 2 hour post glucose load period. □, fatty (F) group (n=8); ■, exercise (E) group (n=9); ●, untreated sedentary control (S) group (n=8). Data are presented as means ± SEM. *, †; P<0.05, basal vs. fatty, ‡; P<0.05, basal vs. exercise, §; P<0.05, basal vs. sedentary, ¶; P<0.05, fatty vs. exercise, ||; P<0.05, fatty vs. sedentary, ¶; P<0.05, exercise vs. sedentary.
Figure 4: Immunoblot data for total PKCε (A), PKCδ (B), Phosphorylated JNK/Total JNK (C), IkBa (D), Serine-307 phosphorylated IRS-1/Total IRS-1 (E). Basal (B) group (n=8); Fatty (F) group (n=8); Exercise (E) group (n=9); Untreated sedentary control (S) group (n=8). Data are presented as means ± SEM. *: P<0.05, basal vs. fatty; †: P<0.05, basal vs. exercise; ‡: P<0.05, fatty vs. exercise; §: P<0.05, basal vs. sedentary; ¶: P<0.05, fatty vs. exercise; ‖: P<0.05, fatty vs. sedentary; ||: P<0.05, exercise vs. sedentary.
Figure 5: Plasma Haptoglobin (A), Plasma IL-6 (B), Plasma MDA (C), Hepatic Protein Carbonyls (D). Basal (B) group (n=8); Fatty (F) group (n=8); Exercise (E) group (n=9); Untreated sedentary control (S) group (n=8). Data are presented as means ± SEM. * P<0.05, basal vs. fatty, † P<0.05, basal vs. exercise, ‡ P<0.05, basal vs. sedentary, § P<0.05, fatty vs. exercise, ¶ P<0.05, fatty vs. sedentary, ¶ ¶ P<0.05, exercise vs. sedentary.
### Table 1

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<th>Exercise (F, n=9)</th>
<th>Control (C, n=8)</th>
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<td>Free Fatty Acids (mEq/L)</td>
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<td>10.7±1.1†§</td>
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<td>16.6±2.04‡</td>
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<td>Insulin (ng/ml)</td>
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<td>33.4±2.05†‡‡‡</td>
<td>26.1±1.87†‡</td>
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<td>4946.2±326.14‡§</td>
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<td>Corticosterone (ng/ml)</td>
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<td>129±43.7±‡</td>
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<td>Epididymal Fat Pad Mass/Body Mass (g/kg)</td>
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<td>14.2±0.37§</td>
<td>5.5±0.37§</td>
<td>10.5±1.56§</td>
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**Table 1:** Data are means ± SEM. Data are presented as means ± SEM. *; P<0.05, basal vs. fatty, †; P<0.05, basal vs. exercise, ‡; P<0.05, basal vs. sedentary, §; P<0.05, fatty vs. exercise, ¶; P<0.05, fatty vs. sedentary, ‖; P<0.05, exercise vs. sedentary.
6.1 Food Intake and Body Weight

Caloric restriction has been shown to improve insulin sensitivity and glycemia in obese individuals with and without T2DM (564; 769; 808). Furthermore in rodents, caloric restriction attenuates or prevents the development of hyperglycemia in spontaneous models of T2DM (37; 330). Therefore, it is important to note that improved metabolic profiles of swim-exercised animals in this study were not related to changes in food intake or body weight. Plasma lipid data indicate that ran animals had lower circulating levels of FFA and TG when compared to sedentary ZDF rats. These improved plasma lipid profiles may be partially responsible for the beneficial effects of exercise seen in this study as plasma FFA are inversely correlated with insulin sensitivity (56). Also elevated plasma FFA may have been partially responsible for lessened JNK phosphorylation and related IRS-1 serine phosphorylation seen in this study. These improved plasma lipid profiles may be related to the fact that habitual running increases the lipid content of muscle despite concomitant increase in muscle insulin sensitivity (661). Although we did not observe differences in epididymal fat pad mass between sedentary and ran ZDF animals, we cannot discount the possibility of reductions in total visceral, or in subcutaneous fat in exercised animals. A limitation of this study is that we did not measure body composition with the use of dual X-ray absorptiometry (DEXA) or magnetic resonance imaging (MRI).

6.2 Maintenance of Euglycemia With Exercise

Studies in ZF and ZDF rats indicate drastic deterioration in insulin sensitivity occurs over time (292; 596). In this study, swim exercise maintained fed and fasting euglycemia through adaptive hyperinsulinemia. We cannot ascertain whether true changes in insulin
sensitivity occurred in this study as we did not cannulate animals and perform a hyperinsulinemic-euglycemic clamp. We were concerned the recovery period following surgery would have likely nullified the training effect and that chronically cannulated animals would be susceptible to infection. A well-accepted non-invasive, indirect measure of insulin sensitivity (HOMA-IR) can be obtained from the product of fasting insulin and fasting glucose. We performed such calculations with fasted data from swim and run studies and observed deteriorations in unexercised groups. Current findings of unimproved indices of insulin sensitivity in swum animals agree with observations in ZF rats (329) however contradict what has been found with other studies of various types of exercise in Zucker rats (125; 292; 596). Possible reasons for the present discordance in findings may be due to the fact that we extended the treatment period until 19 weeks of age when untreated animals were severely obese and hyperglycemic. The aforementioned studies reporting improved insulin sensitivity with exercise employed forced treadmill running paradigms which were terminated between 11 and 14 weeks of age, well before the time when gross obesity and severe hyperglycemia occurs in this model. Unchanged insulin sensitivity as assessed by HOMA-IR in swum animals may also be related to differences in exercise stimulus between swimming and running.

Results from our volitional run study do indicate improved insulin sensitivity and confirm results in ZDF rats (596) as well as in running studies using ZF rats (125; 292). Evidence that insulin sensitivity is maintained is provided by combined observations of fasting glucose and insulin values, and with the use of HOMA-IR. For example, by week 8 of the intervention period sedentary ZDF animals had pronounced fasting hyperglycemia and hypoinsulinemia. This maintenance of normoglycemia with less insulin indicates that exercised animals require less insulin for glucose disposal. Inferences regarding improved
insulin sensitivity in ran animals are supported by our HOMA-IR calculations. Importantly, because fasting glucose levels mainly reflect hepatic gluconeogenesis and glycogenolysis we assume that exercised rats had improved basal hepatic insulin sensitivity. Enhanced peripheral insulin-stimulated glucose disposal with exercise training may be related to an up-regulation of GLUT4 protein expression, (32; 82; 83; 162; 211), GLUT4 translocation (82; 83), and cell surface GLUT4 content after insulin stimulation (187). We did not measure GLUT4 with the current studies however our results of improved glucoregulation are in agreement with results from other exercise studies with rodents (32; 82; 83; 162; 211). Other factors are also likely involved in improving insulin sensitivity such as increased white gastrocnemius glycogen content with exercise. Indeed, greater insulin sensitivity is associated with increased muscle glycogen synthesis in humans (144; 578; 612). Others have also shown that skeletal muscle AMPK levels increase with training and are associated with improved insulin sensitivity and a delay in the development of T2DM in male ZDF rats (596). The effects of swim exercise are less well-understood. For example, swimming (406) and running (596) studies also performed in ZDF rats indicate no change in red gastrocnemius (406; 596) or white gastrocnemius (406) GLUT4 protein expression. This may explain why we did not observe improvements in insulin sensitivity in swum animals; the stimulus may not have been sufficient.

Chronically elevated glucocorticoids are associated with obesity and T2DM in animal models (10; 19-21; 31; 33; 118; 148; 149; 375; 416; 433; 451; 523; 540; 716). Studies have since elucidated that that glucocorticoids are implicated in the development of insulin resistance (6; 10; 85; 609; 784; 815). A study by Akerblom and colleagues (10) demonstrated that cortisone impairs glucose tolerance in rats. They found that administration of cortisone acetate to rats for 28 days caused a diabetic blood glucose
response to an OGTT and typical hyperinsulinemia of steroid diabetes. A more recent study by Willi and colleagues found that, in humans, the synthetic glucocorticoid dexamethasone drastically impairs glucose tolerance and that troglitazone, an insulin sensitiser, opposes the metabolic effects of dexamethasone (815). Patients with dexamethasone showed a 75% rise in glucose peak with fasting and insulin values increased by 2.3 and 4.4-fold above the baseline. Furthermore insulin resistance was also induced by dexamethasone with a 34% reduction in maximal glucose disposal rates (815). These above studies indicate that glucocorticoids and insulin have antagonistic effects. In this way, the glucocorticoids are responsible for causing metabolic derangements both centrally, as pertaining to the liver, and fat, and peripherally, both straining pancreatic β-cells indirectly. In the present studies we found that daily swimming and volitional running lowered basal circulating glucocorticoids by 58% and 75% respectively. These results of decreased basal corticosterone are in agreement with studies performed in rodents and man (150; 152; 177; 178; 722). Despite or present findings of decreased circulating corticosterone with exercise it is difficult to speculate about what may be happening at a tissue level; the effects of glucocorticoids on their target tissues is governed not simply by the absolute levels of plasma hormone but by the ratio of free glucocorticoid to carrier protein (corticosterone binding globulin) and by the tissue-specific expression of 11βHSD1 which activates glucocorticoids intracellularly.

Taken together, it is likely that a number of peripheral adaptations occur with exercise that improve insulin sensitivity, which may in turn cause a reduced reliance on increased pancreatic function and sustained hyperinsulinemia.
6.3 Sustained Adaptive β-cell Function in Exercised Animals

T2DM is not defined solely by decreased hepatic and peripheral insulin action. β-cell defects with respect to insulin production and/or secretion must also be present, and in fact, are responsible for the discrepancy between insulin resistant obese non-diabetic and type 2 diabetic patients. The prediabetic phase in ZDF rats, like in humans, is characterized by the maintenance of normoglycemia by a compensatory increase in β-cell function which results in hyperinsulinemia (101; 102; 127; 749). As is the case for people with T2DM, this compensatory adaptation begins to fail as ZDF animals enter the “diabetic phase” defined by a reductions in β-cell function and dramatic increases in β-cell apoptosis and a corresponding decrease in β-cell mass (586). In obese Zucker rats, caloric restriction (545), a reduction in fat mass (216; 217), and increased exercise (125; 292; 596; 680; 681) improve insulin sensitivity and attenuate the development of hyperglycemia. These interventions, which increase insulin action or sensitivity, are thought to result in a decreased demand for insulin and thus attenuate β-cell exhaustion and increases in β-cell apoptosis. Exercise is in itself also associated with increases in β-cell function. For example, ZDF animals that either run on treadmills (596) or individually swim (406) manifest a protracted period of adaptive hyperinsulininemia such that euglycemia is maintained. Furthermore, in humans with T2DM, regular exercise enhances insulin response to either hyperglycemia or to arginine stimulation (163), suggesting that there may be direct effects of exercise on pancreatic β-cell function.

Pancreatic decompensation in untrained male ZDF rats is confirmed with the present study. By 19 weeks of age, control animals had become overtly hyperglycemic with respect to postprandial and fasted glucose concentrations and at the same time showed reduced insulin levels relative to exercised rats. Swim-exercised animals showed
sustained adaptive hyperinsulinemia for the duration of the study, in contrast to sham-treated and sedentary controls which were unable to manufacture and/or secrete sufficient insulin to keep glucose levels below hyperglycemic values. These data indicate that exercised animals are able to compensate for insulin resistance by increasing insulin production and/or secretion in response to a sustained glucose stimulus. In vivo basal β-cell function, described as a quotient of weekly fasted insulin divided by weekly fasted glucose, gives a rudimentary indication of the β-cell’s response to a basal glucose stimulus. This index increased in a compensatory fashion in ZDF rats during the prediabetic phase, however, as animals entered the diabetic phase, β-cell function dropped off considerably in sham-treated and sedentary rats. Based on this ratio, exercised animals had greater β-cell function than controls, offering a plausible explanation for the maintenance of postprandial and fasted glucose values below the human diabetic criteria of greater than 11.1mmol/l and greater than 7.1mmol/l, respectively.

Glucose tolerance was shown to deteriorate considerably over time in all 19 week-old ZDF animals in the swim study, regardless of treatment. Interestingly, although all groups manifested impaired glucose tolerance at termination of the study, exercised rats displayed significant improvements 2 hours post-glucose load. In accordance with calculations of insulin sensitivity, exercise, was not associated with an attenuated glucose excursion during the IPGTT test. Our present findings that exercised animals recover from their glucose excursions after glucose bolus likely reflects increased total insulin release during this time relative to the other groups. In volitional run studies, glucose tolerance deteriorated considerably over time in 16 week-old ZDF animals lacking intervention but was maintained in animals which ran daily. Just prior to euthanasia, exercised animals had
2-hour post-load glucose values which were not different from 6 week-old basal animals, showing a relatively normal glucose tolerance curve. Furthermore, 2-hour post-load glucose values in exercised ZDF rats were not different from 16 week-old ZF animals which compensate for gross insulin resistance with adaptive hyperinsulinemia (125; 292).

A possible explanation for the improvements in β-cell function seen with exercise may be related to the prevention of hyperglycemia. Chronic hyperglycemia has been shown to adversely affect β-cells by down regulating insulin gene and protein expression (594), and also by impairing GSIS (591; 594). *In vitro* studies of the HIT-T15 β-cell line show chronic exposure to elevated glucose concentrations, similar to what was seen in untreated ZDF rats in this study, causes defective insulin gene expression accompanied by marked decreases in insulin content and abnormal insulin secretion (624). Isolated islets chronically exposed to high glucose concentrations also reveal decreased insulin gene expression and insulin secretion (76; 180; 489). These data outlining β-cell vulnerability to chronic hyperglycemia offer at least a partial explanation as to why improved insulin availability was seen in swim exercised animals in this study and why evidence of decreased β-cell function did not exist in volitionally run animals.

In agreement with the improved glycemia and enhanced β-cell function in swum animals we found that swim training is associated with the maintenance of normal islet morphology relative to sedentary control animals. These results confirm recent observations that exercise protects β-cell morphology in ZDF rats (596). A strong positive correlation exists between the maintenance of normal islet morphology and β-cell function (279). Islets from control rats had significant fibrosis and less intense staining for insulin compared with the other groups after 13 weeks of treatment. In
exercised animals, islets were dense and circular with insulin staining characteristics that were similar to that in basal animals. In fact, islet morphology between 19-week-old exercised and 6-week-old basal animals was not noticeably different.

With the present swim study we showed that immunodetectable GLUT2 levels decreased significantly over time in male ZDF rats which agrees with literature as reductions in GLUT2 levels are reported in ZDF rats (545; 548; 793), and other models of T2DM including the GK (546) rat and the db/db mouse (390). Perhaps more importantly, our immunohistochemical experiments indicate that GLUT2 is partially maintained with exercise. Studies have shown reductions in β-cell function correlate strongly with decreased levels of GLUT2 mRNA and protein levels (269; 270; 545; 546; 548; 735). Previously GLUT2 reductions were attenuated by caloric restriction in male ZDF rats (545) however, preservation of GLUT2 with exercise was not related to decreased food intake in our exercised animals. Thus this attenuated loss of GLUT2 in exercised animals is one possible mediator of improved increased β-cell function.

A definitive role of GLUT2 in β-cell function is evasive however. For example, in it has been shown that in male ZDF rats with no severe impairment in GSIS, β-cells may still expresses GLUT2 in 50% of their β-cells (756) and have β-cells full of glycogen (490). Furthermore, normal glucose entry into the cell despite decreased GLUT2 expression is verified in db/db mice (490) which also have islets that are full of glycogen. For these reasons, prudence is merited when making assumptions about the role of GLUT2 in β-cell function.
In exercised animals, reductions in GLUT2 may occur in response to prevention of hyperglycemia. For example, when islets from normal mice are placed in STZ-treated mice or transplanted into db/db mice, the expression of GLUT2 is down-regulated (803). These reports are contradicted by other data indicating that exposure to chronic hyperglycemia is not associated with reductions in GLUT2 in isolated islets (803), in male ZDF rats (554; 756), and in normal rats made hyperglycemic for 7 days with Px (803). For these reasons the role of attenuated hyperglycemia to prevent losses of GLUT2 is unclear.

Other studies indicate that with exercise chronic hyperglycemia may decrease β-cell function via defects in the glucose sensing machinery of the pancreatic β-cell. For example in GK rats, reduction in β-cell glucose transport is insufficient to explain the reduction in GSIS, suggesting that molecular sites distal to GLUT2 are involved the functional derangement of β-cells (546; 547; 601). It has also been shown that in vivo (456; 492) and in vitro using MIN6N8 cells (397) glucose levels alter the cellular content of glucokinase and also the activity of this enzyme (121). Glucosamine has been shown to inhibit glucokinase in vitro and to result in diminished GSIS but not arginine-stimulated insulin secretion in islets from normal rats (28). These results indicate that chronic hyperglycemia leads to defects in glucose sensing machinery of pancreatic β-cells. Despite an ongoing debate about the cause and effect relationship between hyperglycemia and β-cell dysfunction, considerable evidence indicates that antecedent elevated plasma glucose levels are an important factor for the loss of β-cell phenotype in ZDF rats (270) and in humans (147; 180). With the present study measurement of glucokinase would have allowed for a more detailed and informative discussion on this topic. Despite the findings mentioned in the preceding section, the effects of exercise to attenuate the loss of β-cell GLUT2 levels may be related to the fact that exercise prevented the development of hyperglycemia in this
current study. However, this area is an ongoing topic of controversy. For these reasons
discussion regarding disparities with respect to glucose entry and glucose sensing in β-cells
of different treatment groups in these current studies is based on speculation.

Some studies suggest that loss of β-cell GLUT2 may be secondary to lipid accumulation
within the islets. Evidence to support this comes from studies with male ZDF rats where
wild-type leptin receptor expression was produced by recombinant adenovirus infusion
(793). Recovered leptin signaling in these studies lead to a 17-fold increase in GLUT2 and
normalization of GSIS, supporting the concept that normal leptin signaling and prevention
of lipid accumulation is important in the preservation of GSIS in these animals (793).

FFA activate the expression of uncoupling protein-2 (UCP2) in β-cells, resulting in
mitochondrial uncoupling. Because GSIS depends upon ATP generation from glucose
metabolism, such uncoupling is predicted to impair insulin secretion (74). UCP2
expression is increased in islets after high-fat feeding in rodents (74; 117) or exposure
to FFA in vitro (434) and islets isolated from UCP2 KO animals are protected from
lipotoxicity (351). Deletion of UCP2 restores β-cell function in ob/ob mice, animals fed a
high-fat diet (351; 729; 850), and protects mouse islets form lipotoxicity (350), whereas
UCP2 overexpression leads to impaired GSIS (434; 833). Consistent with this model is
the observation that islets overexpressing hormone-sensitive lipase, which is induced by
glucose in β-cells (823), have increased UCP2 expression and are lipotoxic (824).

Whether lipid accumulation directly affects β-cell function, or whether it serves as a
precursor for other active molecules such as DAG or phospholipids is not known,
however several candidates have been proposed, including the ATP-sensitive
potassium channel, PKC, and UCP2 which are believed to be modulated by these lipid
metabolites (604). We did not measure β-cell lipid content or enzymes regulating de
novo lipid synthesis, nor did we measure oxidative stress or UCP2 expression in β-cells of these animals but reduced islet toxicity and corresponding induction of UCP2 levels may partially be responsible for increased β-cell function seen in these animals. Reduced islet lipid content and corresponding lipotoxicity is a possible outcome of volitional exercise in this model that should not be discounted.

With a volitional running model we reproduced data indicating increased β-cell function and subsequent attenuation of hyperglycemia in this model of T2DM. Importantly, from 8-10 weeks of intervention, exercised animals responded to worsening obesity and insulin demand with increased insulin production and/or secretion. In contrast, in sedentary animals, β-cell decompensation was evident. Exercised rats in our swim studies were not characterized by reduced plasma lipids, however animals which volitionally ran did show improved hyperlipidemia, possibly conferring protective β-cell effects. Perhaps more importantly, the combined effect of reduced plasma glucose and lipids were observed in this study and therefore may have interacted in concert to diminish deleterious effects on β-cell function. When glucose concentration increases, FFA partitioning is switched to lipogenesis, a switch that entails coordinated changes in metabolic signaling (603), lipogenic gene expression (625), and changes in AMPK activity. Glucose negatively regulates AMPK activity in β-cells (637; 650), and AMPK inhibition at high glucose levels results in increased MCoA levels, diminution of FFA oxidation, and stimulation of lipogenesis (603; 637). These process are exemplified by the fact that agents that activate AMPK, such as AICAR, leptin, metformin, and troglitazone, prevent lipotoxicity in several experimental models (603; 637). Prevention of FFA-induced β-cell death by AICAR in INS-1 cells was also recently reported by El-Assaad et al. (182). Activation of AMPK with AICAR decreases TG content, and increases
FFA oxidation. AMPK activation has been shown to be increased in skeletal muscle of ZDF rats (596) but the effects of exercise to modulate AMPK in pancreatic β-cells is unknown at this time.

SREBPs are expressed ubiquitously and play an essential role in regulation of lipid homeostasis and directly activate the expression of more than 30 genes dedicated to the biosynthesis of cholesterol, FFA, TG and phospholipids (304). SREBP-1c over-activation has been associated with β-cell lipotoxicity in many animal models of T2DM, such as ob/ob, db/db, IRS-2-/-, and STZ-induced diabetic rats (365; 459; 495; 617; 688-691; 730; 747; 755; 762; 763; 807; 831; 832; 842). In ZDF rats overexpression of SREBP-1c has also been found (759; 762). Because the isoform SREBP-1c transactivates genes involved in FFA synthesis (204) it is a prime candidate as a player in lipotoxicity theories. Glucose also regulates SREBP-1c gene transcription (274); a strong correlation between chronic high-glucose treatment and SREBP-1c activation in INS-1 cells and rat islets exists (789). Both high glucose treatment and SREBP-1c activation in INS-1 cells resulted in lipid accumulation, impaired GSIS, apoptosis including upregulation of lipogenic and pro-apoptotic genes and downregulation of IRS-2, Bcl-xl and PDX-1 (789). Chronic high glucose treatment alone causes glucolipotoxicity INS-1 cells (790). These lipotoxic effects of high glucose were largely prevented by induction of a dominant-negative mutant of SREBP-1c, suggesting SREBP-1c is a major factor responsible for β-cell glucolipotoxicity (789). With this, many studies indicate chronic hyperglycemia alone is sufficient to cause β-cell dysfunction and death. The role of exercise to modulate these aforementioned key players is an exciting area for future study.
Further evidence to support our findings of maintained β-cell compensation in exercised ZDF rats is provided by fed insulin concentrations as well as circulating C-peptide levels also measured at euthanasia. C-peptide is a better indication of insulin secretion rather than plasma insulin concentrations because the former is not metabolized by the liver (163). Thus, these data indicate adequate β-cell function following an overnight feeding stimulus in exercised rats, but inadequate responses in sedentary and sham-treated rats.

Our findings of improved glucose tolerance and β-cell function with exercise may be related to the fact that exercised animals manifested improvements in basal plasma glucocorticoid concentrations mentioned previously. *In vitro* studies have shown that direct administration of glucocorticoids to the pancreas either by incubation or by perfusion can lead to inhibition of GSIS (34; 433; 524), with no change in basal insulin secretion (433). An additional study by Jeong and colleagues (338; 339) incubated isolated rat pancreatic islets in concentrations of 1, 10, and 100 nmol/L dexamethasone for 1 to 6 hours. They found that dexamethasone inhibited first-phase insulin secretion in a dose-dependent manner (338; 339). Further evidence for the role that glucocorticoids play in inhibiting insulin secretion was found by Davani and colleagues (148; 149), who inhibited islet 11βHSD1 activity with carbenoxolone (148; 149). An almost complete and dose-dependent reversal in the inhibition of insulin release was caused by 11βHSD1 inhibition. To ascertain the effects of increased glucocorticoids *in vivo*, Delaunay and colleagues developed transgenic mice that selectively overexpressed glucocorticoid receptors in pancreatic β-cells (164). These mice demonstrated reduced glucose tolerance even though both the transgenic and control mice had identical fasting blood glucose concentrations (164). Overall, these recent studies show that glucocorticoids directly inhibit GSIS *in vitro* (524) as well as *in vivo*.
In line with these findings it is tenable that improvements in β-cell function in exercised animals were related to this decreased inhibitory effect of elevated corticosterone found in sedentary ZDF rats.

Lastly, if insulin sensitivity is increased and hyperglycemia reduced, as is the case with exercise, this would by itself improve β-cell function. Treatments that periodically lower insulin secretion in the face of increased insulin resistance have been effective at attenuating or even preventing T2DM in both humans (97) and in male ZDF rats (14). Because brief periods of β-cell rest protract the progression of T2DM in ZDF rats, we speculate that brief and intermittent inhibition of insulin secretion which occurs during exercise (319) may attenuate pancreatic β-cell decompensation. Restoration of GSIS can be obtained following normalization of blood glucose levels for short periods of time (752). Supporting that GSIS can be restored in vivo: partial restoration of GSIS occurs in response to 20h of insulin infusion in hyperglycemic patients with T2DM (768). Normalization of blood glucose levels by insulin treatment also leads to recovery of GSIS (442). Together these findings indicate that regular physical activity exerts protective effects on pancreatic β-cell function and response to glucose stimulus possibly by inhibiting insulin secretion during exercise and providing a period of β-cell rest.

### 6.4 β-cell Mass is Partially Maintained in Exercised Rats

In addition to demonstrating that exercise training is associated with sustained β-cell compensation and function, we confirm with the present swim studies attenuated loss of normal β-cell mass. Our immunohistochemical analysis of the pancreas showed that in exercised animals there was a significant attenuation in the reduction of β-cell mass when compared with age-matched controls, similar to observations made in insulin-resistant
OLETF rats which undergo volitional wheel running (681) and male ZDF rats treated with treadmill training (596). However, with the current study we extended the exercise duration by a minimum of 6 weeks compared to other studies (406; 596).

We demonstrate that by 6 weeks of age, during their prediabetic phase, basal animals already manifest increased β-cell proliferation compared to lean animals. Furthermore, we show that swim exercise partially maintains this adaptive proliferation which in contrast was clearly decreased in sham-treated and sedentary control animals at 19 weeks of age. This is in agreement with our previous study at 12 weeks of age in ZDF rats (406). With the present study we probed further into the mechanisms of mass compensation with exercise in these animals. We show that neogenesis and β-cell hypertrophy is increased in exercised rats compared to sedentary control rats which supports our findings of increased β-cell mass.

In addition to inducing functional changes, chronic hyperglycemia can also decrease β-cell mass by inducing apoptosis and also exacerbate the consequences of lipotoxicity (174; 586). Oxidative stress leads to a down-regulation of Bad (anti-apoptotic) and increases the activity of Bax and Bid (proapoptotic) genes as cleaved Bid leads to the induction of caspases-3 and -9 (437). A balance between members of this family, therefore, is what determines whether β-cell mitochondria become permeabilised or remain intact. The involvement of these pathways has been shown in vitro in isolated islets (195). Increased apoptosis was found when islets were cultured in 16.7 mM glucose as compared with islets cultured in 5.5 mM glucose or 11 mM mannitol + 5 mM glucose (195). The antiapoptotic gene Bcl-2 was unaffected by these conditions whereas Bcl-xL was reduced, and the proapoptotic genes Bad, Bid, and Bik were overexpressed in the islets maintained in high
glucose concentrations (195). As was the case with defects in β-cell function, hyperglycemia is also proposed as the prerequisite for hyperlipidemia-induced β-cell lipotoxicity (66; 270; 594; 625; 802). These findings are consistent with the clinical observation that the majority of hyperlipidemic individuals are not diabetic. In this way, these data indicate that partial preservation of β-cell mass with exercise training may be related to prevention of hyperglycemia in this study.

Interestingly, we also confirm our previous findings that chronic intermittent stress associated with either sham-exercise treatment (406) or restraint stress (37) results in sustained β-cell mass compensation in ZDF rats. Despite aggravating insulin resistance, it is possible that periodic elevations in stress hormones, such as glucocorticoids, may exert protective effects on the β-cell. In support of this, glucocorticoid exposure stimulates β-cell expansion in rodents (275; 386; 457; 779; 780). Also, the diabetogenic effects of STZ, alloxan, and partial Px can be reduced by pre-treatment with glucocorticoids or mild foot shock (275; 313; 314; 372; 439; 635; 806). It is interesting, therefore, that although glucocorticoids cause insulin resistance and increased hepatic glucose production that aggravates metabolic control in diabetes, they may confer some protective effect on the development of the disease by altering β-cell mass compensation in the prediabetic animal. The role of glucocorticoids to influence pancreatic compensation and function is difficult to reconcile with observations that glucocorticoids directly inhibit insulin secretion and β-cell function in normal mouse islets (433) and induce insulin resistance at peripheral insulin target tissues (52; 53). A reduction in resting corticosterone levels with exercise does indicate that HPA-axis adaptation and/or glucocorticoid activity in a tissue-specific manner have been altered.
Recently the role of β-cell insulin signaling and downstream IRS-2/Akt/PKB activation (45; 225; 706; 753; 825), as well as maintenance of GLUT2 protein (344; 742) expression have been implicated in the role of maintaining β-cell mass compensation and β-cell function respectively. In ZF rats, serine phosphorylation of Akt/PKB has been implicated in regulating duct-derived neogenesis and β-cell proliferation, cell size, differentiation and survival (3; 45; 167; 225; 706; 753; 797). Normal Akt/PKB signaling has also been shown to be critically important for controlling β-cell mass relative to metabolic homeostasis (462; 614). To the best of our knowledge, we show for the first time that exercise is associated with phospho-Akt-473 activation in β-cells. Our findings of increased Akt/PKB activation in β-cells of exercised animals offers a possible explanation for increased mean β-cell hypertrophy as Akt/PKB activity correlates with protein synthesis and β-cell hypertrophy (119). It does not, however, directly explain increases in the rates of β-cell proliferation in this group as mitogenesis is under the control of the ERK 1/2 pathway (75). The fact that Akt/PKB and ERK1/2 are both under the control of IRS-2 tyrosine phosphorylation suggests that increased signaling via this insulin signaling intermediate is taking place. Normal β-cell glucose entry through the action of GLUT2 as well as the preservation of β-cell insulin signaling to promote β-cell mass expansion, are required to maintain adaptive hyperinsulinemia during worsening insulin sensitivity.

6.5 Oxidative Stress, ER Stress, and Pancreatic ALIS Formation

Pro-oxidants and markers for oxidative tissue damage, such as 8-hydroxy-deoxyguanine, 4-hydroxy-2-nonenal proteins, and oxidation of DNA bases, have been reported to be elevated in serum, plasma, white blood cells, and pancreas biopsies of patients with T2DM (230; 244; 535; 610; 649; 692). Compared with non-diabetic control subjects, these markers are at times 5-fold above normal (230). Within the β-cell, the
major antioxidant enzymes are catalase, glutathione peroxidase (GPx), and the two O$_2^-$ dismutases (SOD) (621). It has been demonstrated that pancreatic islets contain relatively small amounts of the antioxidant enzymes CuZn-SOD, Mn-SOD, catalase, and GPx (248). The pathophysiological implication of the islet's intrinsically low level of antioxidant enzyme expression and activity is that the $\beta$-cell is at greater risk of oxidative damage than other tissues with higher levels of antioxidant protection (624).

Both hyperglycemia and hyperlipidemia are responsible for increasing the production of free radicals which are known to accelerate the pathogenesis of T2DM as evidenced by the glucose and lipid toxicity theories (232). Multiple biochemical pathways and mechanisms of action for glucose toxicity have been suggested. These include glucose autoxidation, PKC activation, methylglyoxal formation and glycation, hexosamine metabolism, sorbitol formation, and oxidative phosphorylation (232). All these pathways have in common the formation of ROS. For this reason oxidative mechanisms have become increasingly implicated in the development of T2DM (232).

Oxidative stress is a well-documented cause of $\beta$-cell decompensation in T2DM (622); over time, the accumulation of ROS leads to defective insulin secretion (621). In vivo ZDF rat studies have shown that progressive worsening of hyperglycemia, glucose tolerance, insulin secretion, PDX-1 binding to the insulin promoter, and insulin gene expression are associated with the increase of oxidative stress markers in untreated rats (796). Additional in vivo experiments with the ZDF rat substantiated the findings of defective insulin gene expression in the glucotoxic state (269). Development of hyperglycemia in this animal model is accompanied by loss of insulin gene expression, islet PDX-1 mRNA and PDX-1 DNA binding activity (269). DNA binding activity and gene expression of PDX-1 and insulin
were reserved and glycemic control was improved when animals were treated with troglitazone (269), aminoguanidine (735), phlorizin (270), or NAC (735).

In addition to causing chronic oxidative stress and a reduction in secretory capacity, hyperglycemia-induced oxidative stress damages long-lived proteins leading to increases in accumulation of ubiquitinated protein aggregates, the UPR, and ER stress (759; 789). The ER is particularly vulnerable to the occurrence of misfolded proteins because of its contribution in post-translational modification, folding and assembly of newly synthesized proteins. A highly developed ER is present in pancreatic β-cells due to their involvement in insulin and digestive enzyme secretion. The UPR and ERAD are two safety mechanisms that have evolved to help the ER cope with misfolded proteins. ERAD targets damaged and misfolded proteins for proteasomal degradation by tagging them with ubiquitin. Accumulations of insoluble ubiquitinated proteins are referred to as aggresomes and ALIS (370) which are storage compartments for ubiquitinated proteins and, like the Ub-protein aggregates observed in β-cells, they are found in the cytoplasm (370). ALIS contain ubiquitinated protein (22) and they are formed in a number of different cell types (106; 726). ALIS have also been shown to be regulated by the autophagy pathway (726).

Studies show that different cell types deal with protein misfolding in a variety of ways, and this likely reflects differences in protein misfolding rates, rates of degradation and the type of protein degradation systems in operation. Evidence for this comes largely from the observation that Ub-protein aggregates were found in both acinar cells and β-cells, suggesting a common mechanism for sequestering misfolded proteins in various cell types (370) (See Appendix). Pancreatic tissue is made up of highly secretory cell types such as α-cells, β-cells, δ-cells, and acinar cells, and it may be that their secretory abilities
result in an increase in the protein misfolding rate under stress. When diabetes occurs, pancreatic β-cells undergo numerous stresses, including oxidative stress, protein misfolding and damage, and loss of ER quality control functioning resulting in defective insulin secretion. Previous data suggests that oxidative stress is a causative factor for the formation of Ub-protein aggregates (726) and in pancreatic β-cells high glucose, which is known to promote oxidative stress, also causes an increase in Ub-protein aggregates (370). Chronic oxidative stress can also cause β-cell death via apoptosis either through ER-mediated or direct caspase activation (437). Recent studies show that Ub-protein aggregate formation occurs in hyperglycemic male ZDF rats (370). Additional in vitro studies with β-cell lines confirmed a dose-response relationship between increasing hyperglycemia and ALIS formation (370). Formation of ALIS formation could be prevented by administering NAC and taurine which have both been shown to decrease oxidative stress in pancreatic β-cells (370). Furthermore when high glucose-treated INS-1 β-cells were allowed to recover at a basal glucose level, ALIS, or Ub-protein aggregates were no longer evident. These same studies showed that regulation or clearance of these Ub-protein aggregates or ALIS was by autophagy and not the proteasome (370).

With our swim studies we confirm the presence of ALIS in β-cells of aged male ZDF rats exposed to chronic hyperglycemia, but go on to show swim exercise leads to nearly a complete prevention of ALIS formation. Because ALIS are induced by hyperglycemia and oxidative stress and are prevented by anti-oxidants, we propose that prevention of hyperglycemia and subsequent oxidative stress in exercised animals is the reason for decreased insoluble protein aggregation in the β-cells of these animals. In sham-treated rats, we saw a minimal effect to prevent ALIS formation despite marked hyperglycemia in this group suggesting that severity of hyperglycemia alone is not solely responsible for the
induction of ALIS formation in male ZDF rats. We acknowledge the possibility that reduced oxidative stress in exercised β-cells (reduced protein ubiquitination) could be related to a reduction in lipid accumulation. Although we cannot exculpate the role of intracellular lipid metabolites and lipotoxicity to induce oxidative stress, we report no differences between the ZDF groups with respect to plasma TG or FFA in these swim studies. However, because de novo lipid synthesis in pancreatic β-cells has been shown to be dependent on circulating plasma lipid levels and in direct relation to elevated glucose levels (537) we speculated that reductions in β-cell oxidative stress are related to maintenance of euglycemia in exercised animals. It is possible that circulating lipids were different between the groups at some time over the course of the study, however, in a different study conducted by our group (406) as well as those conducted by Pold et al. (406; 596) lipids were not different at 12 and 13 weeks of age respectively. Due to the way in which we removed and fixed entire pancreas specimens we could not measure islet lipids or the effects of exercise to alter de novo lipogenesis; two recent studies however did (392; 435). Kibenge et al. showed that swim exercise had no effects on islet FFA oxidation (392), while Lamontagne et al. showed running did not alter islet FFA oxidation, or islet expression of key transcription factors and enzymes of lipid metabolism (435). For these reasons we speculate that decreased protein ubiquitination, shown to be prevented with decreased oxidative stress (370) during exercise is the result of maintained euglycemia and subsequent attenuation of oxidative stress. Present findings of less oxidative stress in β-cells of ZDF rats that underwent habitual intermittent exercise not only lends support to our findings of decreased loss of GLUT2 protein and β-cell function in these exercised rats, but also is likely related to partial maintenance of β-cell mass in the current study.
Swim exercise may confer a protective effect on β-cells with respect to minimizing ALIS formation for the following reasons: It may be that periods of β-cell rest experienced during the daily exercise treatments provided β-cells with sufficient time to recover from glucose insensitivity and/or β-cell exhaustion. Also, decreased rates of insulin production and secretion may reduce the effect of defects or perturbations in protein synthesis and folding machinery which are present in T2DM. Decreased demand for protein production and less reliance on the UPR occurring with secretory inhibition during and acutely after exercise may have given β-cells in exercised animals sufficient time to clear these insoluble protein aggregates. This notion is supported by studies by Kaniuk et al. which showed that when high glucose treated INS-1 β-cells were allowed to recover at a basal glucose level, ALIS, or Ub-protein aggregates were no longer evident. Since in these studies ALIS were shown to be cleared by autophagy, it is possible that exercise altered the β-cells capacity to degrade these structures. More detailed discussion, however, is not possible as studies concerning the effect of exercise to regulate β-cell autophagy do not exist.

Exercise also increases the expression of anti-oxidant enzymes in β-cells (141) which have been shown to be protective with respect to β-cell function and mass preservation during the progression for impaired glucose tolerance to overt diabetes. Because induction of ALIS requires oxidative stress induced by hyperglycemia and can be prevented by anti-oxidant administration it is possible that exercise prevents the development of ALIS through conferred protection from oxidative stress through increased anti-oxidant status (141). A more simple, and more-likely, explanation however, is that exercise through improvements in glucoregulation, prevented oxidative stress in pancreatic β-cells simply by preventing hyperglycemia. This concept is supported by the fact that in prior studies of ALIS formation
and clearance showed ALIS formation could be prevented with removal of a hyperglycemic stimulus (370).

To investigate β-cell death, we attempted to measure apoptosis by TUNEL and cleaved caspase-3 staining as well as using DAPI and propidium iodide which can be used to identify intensified fluorescence associated with DNA fragmentation (659). With the current study extensive background staining of cleaved caspase-3 made it impossible to definitively identify positive staining. With respect to TUNEL staining, TUNEL positive cells in pancreatic islets could not be identified with any frequency that validates quantification. In addition, simple nuclear staining with DAPI and propidium iodide did not stain clearly in the nucleus in the older ZDF islets at any variation in concentration or duration of dye staining. We believe that this may be related to our difficulty in locating TUNEL positive nuclei in the islets of older animals despite clear evidence showing increased apoptosis in paraffin embedded tissues in sections from male ZDF rats (340; 586).

To summarize: in the present swim studies, decreased ALIS formation in exercised animals provides an indirect measure of β-cell oxidative stress. We speculate that swum animals had decreased apoptosis and/or necrosis because of reduced hyperglycemia and therefore oxidative stress. It is also likely that preserved IRS-2/Akt/PKB-473 signaling in exercised animals had anti-apoptotic effects in pancreatic β-cells (167) which would agree with our findings of decreased loss of β-cell mass compensation in exercised rats. The exact mechanisms of how exercise training attenuates the loss of β-cell mass remains to be elucidated, but we believe that by improving insulin sensitivity peripherally there may be less strain on β-cell function and subsequent maintenance of normoglycemia.
6.6 Reduced Systemic Markers of Inflammation

Chronic elevations in circulating markers of inflammation such as CRP, haptoglobin, and TNFα occur both in obesity and T2DM (151). Like TNFα, IL-6 secretion is increased by the adipose tissues of obese subjects (115; 206; 512; 751). TNFα and IL-1β stimulate the production of IL-6 and a linear relationship between plasma levels of TNFα and IL-6 is reported (579).

The function of IL-6 remains elusive as it has both pro-inflammatory and anti-inflammatory functions. For example, IL-6 exerts inhibitory effects on TNFα and IL-1β production (744; 745). Levels of TNFα are markedly elevated in anti-IL-6-treated mice and in IL-6 KO mice (496; 509), indicating that circulating IL-6 is involved in the regulation of TNFα levels. In addition, recombinant human (rh) IL-6 infusion inhibits the endotoxin-induced increase in
circulating levels of TNFα in healthy humans (707). The anti-inflammatory effects of IL-6 are also demonstrated by the fact that IL-6 stimulates the production of IL-1ra and IL-10 (710).

IL-6 seems to have primarily an anti-inflammatory effect. However; the role of IL-6 in insulin resistance and glucoregulation is unclear, to say the least. Studies of IL-6 action in humans have shown patients with T2DM given rhIL-6 respond with decreased insulin levels comparable with that in age and body mass index-matched healthy controls, indicating that IL-6 enhances insulin sensitivity (580). Similarly IL-6 also enhances lipolysis (84; 534; 569; 580; 718) and lipid β-oxidation. In one study IL-6 increased fat oxidation (palmitate) in L6 myotubes comparably to AICAR, a compound known to increase lipid oxidation (580). In accordance, Wallenius et al. (788) demonstrated that IL-6 KO mice developed mature-onset obesity, insulin resistance and impaired glucose tolerance. In addition, when mice were treated with IL-6, there was a significant decrease in body fat and insulin resistance in the IL-6 KO but not in the wild-type mice. Studies show administered physiological concentrations of rhIL-6 to healthy and type 2 diabetic subjects (580; 771) is a potent modulator of fat metabolism in humans. These effects are mediated through the activity of AMPK activity which stimulates a variety of processes that increase ATP generation, including FFA β-oxidation and glucose transport in skeletal muscle (110). Incubation of skeletal muscle cells with IL-6 increases the phosphorylation of AMPK (an indicator of its activation) which results in an inhibition of its target molecule, ACC. In addition, AMPK activity is very low in IL-6 KO mice. These data suggest that activation of AMPK is, at least in part, dependent on the presence of normal IL-6 signaling (381). These studies suggest IL-6 plays a role as an insulin sensitizing hormone. Other studies however show that circulating IL-6 levels may be (35; 36; 588) associated with insulin resistance. For example individuals with elevated levels of IL-6 and IL-1β are at increased risk of
developing T2DM (702; 703). *In vitro* studies demonstrate that IL-6 can induce insulin resistance in isolated 3T3-L1 adipocytes (634) and in mice (394). IL-6 has also been shown to increase hepatic glucose production and secretion of insulin, glucagon and cortisol, and to inhibit insulin signal transduction in hepatocytes (847). The exact role of IL-6 during insulin resistance is the subject of fervent discussion and research.

Swim training of rats has been shown to blunt the pro-inflammatory cytokine response to lipopolysaccharide (136) and to decrease CRP (13) while in humans basal circulating IL-6 and CRP are lower after increased physical activity (132; 611). We have confirmed these findings with our present run studies. We found that circulating basal levels of IL-6 is decreased by volitional exercise which is of interest because elevated circulating levels of systemic markers of inflammation such as IL-6 do, in part, contribute to hepatic insulin resistance in obesity and T2DM (847). Typically, IL-6 is the first cytokine present in the circulation during exercise (193; 571; 572). The level of circulating IL-6 increases in an exponential fashion (up to 100-fold) in response to exercise and declines in the post-exercise period (193; 571-573; 723). Moderate intensity to strenuous exercise is associated with a 20-fold increase in plasma IL-6 levels during the exercise bout (662). In our moderate intensity run studies animals ran between 3-7 hours per night and an increase in IL-6 may have conferred beneficial effects with respect to fat metabolism at skeletal muscle. Such an effect in our studies is tenable as unleptinized rats have severe impairments in AMPK activity and corresponding fat metabolism (758; 760; 763). We unfortunately did not measure IL-6 levels before and in response to exercise therefore we cannot verify this response in volitionally run male ZDF rats.
IL-6 which increases during and shortly after exercise has an inhibitory effect on TNFα production (662) and a stimulatory effect on anti-inflammatory IL10 (710). Recent studies support this concept as exercise normalized overexpression of TNFα in TNF-R KO mice (377). IL-10 also prevents cytokine synthesis by post-transcriptional mechanisms (59). Exercise however, is likely to suppress TNFα also via IL-6-independent pathways, as demonstrated by the finding of a modest decrease of TNFα after exercise in IL-6 KO mice (377). High levels of epinephrine are provoked by exercise, and epinephrine infusion has been shown to blunt the appearance of TNFα in response to endotoxin in vivo (770). Because epinephrine infusion induces only a small increase in IL-6 (712), the mechanism whereby epinephrine inhibits TNFα production is not clear. However, it appears that epinephrine and IL-6 inhibit TNFα via independent mechanisms. Therefore, IL-6 stimulates an anti-inflammatory environment by inducing the production of IL-1ra and IL-10, but it also inhibits TNFα production, as suggested by in vitro (199) and animal studies (496; 509). In this way, the anti-inflammatory effects of exercise may offer protection against TNFα-induced insulin resistance. Because exercise is responsible for changes in the ratio between a cytokine and its antagonist it is therefore, possible that physical activity is responsible for altering the profile of the cytokine cascade and the function of NF-κB. It is this net inflammatory status that determines the function of NF-κB to act as either a pro-inflammatory or anti-inflammatory switch.

With the present run studies we did not detect reduced hepatic levels of IκBα which indirectly suggests that NFκB translocation to the nucleus was unaltered. However; we did not measure activated NFκB (p65 NFκB) and therefore we cannot rule out the possibility of reduced nuclear translocation of this transcription factor. Other results from our studies
suggest that NFκB activation and induction of hepatic proinflammatory activities were reduced; findings of reduced haptoglobin and IL-6 are in agreement with this statement.

In response to stimuli such as ER stress, oxidative stress, cytokines, and FFA, JNK is activated where it associates with and phosphorylates IRS-1 on ser-307 impairing insulin action (4; 222; 563). Decreased JNK activation and subsequent IRS-1 serine phosphorylation may be related to these findings as increased circulating IL-6 and haptoglobin are known to correlate with increased TNFα in plasma. Because TNFα functions to activate JNK in hepatocytes and to phosphorylate IRS-1 at serine residue 307 (466; 776; 811) it may be that exercised-mediated reductions in JNK phosphorylation and serine phosphorylation of IRS-1 are in part explained by these reduced levels of plasma proinflammatory molecules. It may be that differing reports with respect to the effect of exercise on IL-6 levels may reflect altered levels of production from muscle as a myokine and/or from adipose tissue/macrophage consortiums which produce IL-6 namely in response to TNFα. We were not able to measure tissue-specific expression of IL-6 and TNFα which would have addressed these possible changes occurring with exercise.

The deleterious effects of increased circulating pro-inflammatory cytokines are not limited to their effects on peripheral insulin action (298). Recently it was discovered that TNFα, and other proinflammatory cytokines such as IL-1β have cytotoxic effects on β-cells (181; 484; 511; 783) and can inhibit insulin synthesis and release, in addition to decreasing β-cell viability (636). Such cytokines cause β-cell dysfunction and β-cell apoptosis due to activation of NF-κB, resulting in increased nitric oxide production and oxidative stress (139; 179; 483; 606). Studies indicate that exposure of human donor islets to high glucose levels resulted in increased production and release of IL-1β, followed by DNA fragmentation and
β-cell dysfunction. Further treatment of the islets with phlorizin normalized plasma glucose and prevented β-cell expression of IL-1β and decreased the oxidative stress experienced by the β-cells. These findings indicate the inflammatory process involved in glucotoxicity (478). Interestingly, studies in rodent models of T2DM, show that ZDF β-cells are more susceptible to cytokine-mediated cytotoxicity than β-cells from lean ZDF rats (683). We did not measure NFκB in pancreatic β-cells in this study but our indirect measures of decreased β-cell oxidative stress in pancreatic β-cells and our findings of decreased plasma MDA and plasma markers of systemic inflammation in run studies possibly suggests β-cells of swum animals were protected by a combined reduction in oxidative/proinflammatory cytokined-induced β-cell death.

6.7 Evidence of Reduced Hepatic Oxidative Stress

In obesity, JNK activity is elevated in liver, muscle, and fat tissues and loss of JNK prevents the development of T2DM in both genetic and dietary mouse models of obesity (294). The mechanisms of how JNK is activated remains a contentious issue, but oxidative stress is a potent activator of JNK (525; 697). Our findings of reduced hepatic JNK activation with volitional exercise is of importance because removal of inflammatory mediators or pathway intermediates such as JNK, TNFα, IKKβ protects against insulin resistance in obese rodent models and in humans (294; 311; 318; 766; 846). Since NF-κB is a transcription factor for pro-inflammatory cytokines including IL-6, and because we saw reduced IL-6 in exercised animals, we expected that IκBα would be maintained or even elevated in the liver of exercised animals. Exercise did not modulate IκBα in this study; however, liver may not be a predominant source of IL-6. In support of increased hepatic inflammation we show that haptoglobin, which is produced by liver cells upon NF-κB translocation to the nucleus is
increased in sedentary ZDF animals when compared to animals which were exercised or did not show marked hepatic insulin insensitivity (basal animals).

In order to examine the possibility that decreases in JNK activation in exercised animals were related to changes in hepatic oxidative stress we measured hepatic protein carbonyl content which indicates protein oxidation (454). We report that protein carbonyl formation is reduced in by volitional running. In order to assess systematic markers of oxidative stress we chose the MDA assay which measures whole-body lipid peroxidation in response to increased free radicals and ROS. Our finding of reduced hepatic protein carbonyl formation is supported by plasma MDA levels reduced in exercised animals. As mentioned, JNK activation by itself is capable of inducing serine phosphorylation of IRS-1. When we immunoblotted for IRS-1 we found that IRS-1 serine phosphorylation was reduced in exercised animals when compared to sedentary ZDF rats such that it was similar in expression to less insulin-resistant basal animals. PKC isoform activation in our study does not play a role in IRS-1-307 serine phosphorylation as no detectable differences were found between exercised and sedentary ZDF rats. Taken together, these results indicate that JNK activation is responsible for increased serine IRS-1 phosphorylation.
Pancreatic β-cell Dysfunction and Hepatic Insulin Resistance in T2DM: A Common Link Between Oxidative Stress, ER Stress and JNK Pathways?

Oxidative, ER stress and JNK signaling are involved both in β-cell dysfunction and hepatic insulin resistance in T2DM. Glucose stimulates protein synthesis in pancreatic β-cells through dephosphorylation of eIF2α (236) which when activated leads to the induction of chaperone proteins such as CHOP and BiP. Hyperglycemia has been shown to cause ER stress (759; 789); chronic high glucose treatment in INS-1 cells led to pronounced induction of the ER stress marker genes, BiP and CHOP10 (789). In diabetes, ER stress is particularly prevalent in pancreatic β-cells which attempt to compensate for increased insulin resistance with increased insulin production (370). As a consequence the ER is often overwhelmed and an overall increase in protein misfolding occurs (526) and the resultant ER stress can cause apoptotic cell death (370). It has recently been confirmed in the ZDF model of T2DM that chronic hyperglycemia leads to ER stress and the
overwhelming of the UPR (370). Studies have confirmed that ER stress is involved in pancreatic β-cell apoptosis (265; 266). ER stress-mediated PERK induction leads to the activation of CHOP and downstream caspases (267). Studies with the Akita mouse have shown this relationship. The Akita mouse develops diabetes due to an accumulation of misfolded protein caused by a missense mutation (560). The role of ER stress in β-cell apoptosis was reported when the Akita mutation was crossed with CHOP KO mice which resulted in the preservation of β-cell mass and the attenuation of hyperglycemia (560). In PERK KO mice, mutant β-cells are hypersensitive to agents which induce ER stress such as tunicamycin or thapsigargin (266). In this way, chronic high glucose may induce ER stress through overloading protein synthesis and could be the underlying mechanism in β-cell glucolipotoxicity (789). This may be relevant to tissues known to be sensitive to redox perturbation, such as the pancreatic β-cell.

ER stress is present in the liver; elevated ER stress markers such as Bip and KDEL are reported in obese diabetic C57BL/KsJ-db/db mice when compared to 10 week old non-diabetic C57BL6 mice (526), while PERK and eIF2α phosphorylation are increased in mice fed a high fat diet and in ob/ob mice (267; 526; 676). These studies indicate that ER stress in the liver is increased under diabetic conditions (526).

JNK is increased by ER stress (765) and has been shown to be elevated in the liver of obese mice (563). Treatment of liver cells with agents which induce ER stress lead to increased IRS-1 serine phosphorylation which can be blocked with the use SP600125 which is a synthetic JNK inhibitor (563). Other studies indicating that ER stress leads to JNK activation involved the used of XBP-1 heterozygous mice (563). In such mice a reduced hypoglycemic response to an insulin tolerance test revealed the presence of
insulin resistance in these rodents. The liver of XBP-1 -/+ also showed increased PERK phosphorylation and a significant increase in JNK activity (563). As a result IRS-1 serine phosphorylation was also increased compared to wild type controls (563).

The link between ER stress in the pancreas and the liver may be oxidative stress as evidenced by the presence of JNK activation and impaired insulin signaling in both of these tissues (368). Suppression of β-cell JNK activity can protect β-cells from oxidative stress and loss of insulin gene expression (368). DN-JNK can protect β-cells from the toxic effects of hyperglycemia during transplantation (368), and has been shown to be effective at preventing IL-1β induced β-cell destruction (15). As we have seen, oxidative stress also induces JNK phosphorylation in the liver. More importantly, it was shown both in vitro (697) and in vivo (525) that hepatic insulin insensitivity is mediated by redox stress-mediated activation of JNK. In vitro studies show ROS are able to activate serine kinases such as JNK, leading to phosphorylation of critical sites such as serine 307 of IRS-1 which attenuates insulin signaling and action (429; 570). With the present studies we show with indirect (ALIS formations in β-cells) and direct (plasma MDA and hepatic protein carbonyl formation) indicators of oxidative stress are reduced by exercise. These decreases in oxidative stress were associated with decreased β-cell ER stress and hepatic JNK phosphorylation. Since hyperglycemic conditions are associated with ER stress and JNK activation in both the pancreas and liver, we propose that a common link between improved β-cell compensation and hepatic insulin signaling occurring with exercise may be related to a reduction of ER stress in these tissues. Could it be that if we had measured JNK activity in the pancreatic β-cell and ER stress in the liver we may have found an effect of exercise to confer similar protective effects?
6.9 Conclusion

With these studies we confirm that male ZDF rats exhibit decreased pancreatic β-cell compensation in the face of worsening insulin resistance. Such decreases in insulin secretion stem from perturbations to β-cell function and mass. These studies demonstrate that two different models of exercise maintain euglycemia in male ZDF rats. Ameliorations in glycemia with exercise are related to attenuated loss of normal pancreatic and hepatic function.

In summary, we show for the first time that swim training in male ZDF rats preserves β-cell compensation with respect to insulin secretion and β-cell mass. These beneficial effects persist until 19 weeks of age when ZDF rats are expected to be severely hypoinsulinemic. Attenuated loss of β-cell mass was related to increased β-cell proliferation, increased budding of new insulin positive clusters (neogenesis), and augmented β-cell hypertrophy.
Our findings of maintained adaptive hyperinsulinemia in exercised animals are associated with partial maintenance of β-cell GLUT2 protein and proper β-cell insulin signaling. Additionally, we show for the first time that exercise is associated with reductions in the protein-ubiquitination-degradation pathway and subsequent ALIS formation. These studies reveal novel mechanisms of the protective role of exercise on β-cell compensation during the development of T2DM. Importantly, results from the studies described above suggest there are some direct effects of chronic exercise training on the β-cell itself.

To address hepatic changes occurring with exercise we also examined the liver. We show for the first time that volitional wheel running effectively attenuates loss of glucoregulation in male ZDF rats until 16 weeks of age. Such improvements in fed and fasting glycemia are related to decreases in whole-body markers of inflammation and oxidative stress. Findings of decreased serine phosphorylation of IRS-1 with volitional exercise are associated with decreased JNK activation. Our present findings of protective effects with volitional exercise are of importance because beneficial effects of various types of non-exercise stressors have been shown to improve glucoregulation in ZDF animals (37; 406) as well as OLETF rats (364).

6.10 Importance of Contributions to the Current State of Knowledge

Obesity and T2DM are reaching pandemic proportions-prevention remains the best option for curbing this threat to the global population’s health. Exercise remains an attractive modality as it attenuates or even prevents the progression of T2DM in animal models and humans. From a treatment perspective, there are currently a number of pharmacological agents that increase insulin sensitivity at skeletal muscle, fat, and the liver, and promote insulin secretion. Agents that promote insulin sensitivity, such troglitazone, and
rosiglitazone, cause liver toxicity and may be contraindicated for those individuals with other liver complications. Salicylates were at one time thought to be a viable treatment option of the insulin resistant population, however studies indicate that the dosage required to confer protective effects would likely cause additional, if not worse complications. Agents which promote insulin secretion such as the sulfonylureas only offer a temporary solution to insulin insensitivity and may even accelerate the progression to exogenous insulin dependency. Exercise as a treatment option has been shown to be just as protective as the PPARγ activators in terms of increasing insulin sensitivity and is not associated with deleterious effects on the liver. Additionally regular physical activity promotes decreases in adipose tissue and increases in muscle mass which together address the cause of the disease and not simply its effects. Lastly, with the present study, evidence that regular aerobic exercise promotes \( \beta \)-cell adaptation to increased insulin demand is provided. Together these beneficial effects of exercise make it a very attractive prevention and treatment strategy in T2DM.

6.11 Limitations of the Study

Limitations were recognized and discussed briefly throughout the manuscript portion (chapters 4 and 5) and the discussion section (chapter 6) of this thesis. Below is a point–form list of the major limitations of the undertaken studies.

- Collection of frozen pancreatic tissue would have allowed for the quantification of key players which regulate lipid metabolism, and glucose utilization of the \( \beta \)-cell itself. Such players include ACC, AMPK, MCoA, SREBP among others. Inferences made about protein content of \( \beta \)-cell GLUT2 and IRS-2 signaling (serine phosphorylation of Akt/PKB at residue 473) would be much stronger with a quantitative measurement such as
western blotting or RT-PCR. Measurement of JNK in the pancreatic β-cells would have been novel and important to substantiate many of our proposed mechanisms.

- Frozen pancreatic tissue would have also allowed for measurement of anti-oxidant status and direct markers of oxidative stress in the pancreas such as HNE and 8-hydroxy-deoxyguanaine.

- In attempts to characterize how β-cell improvements with exercise may translate to altered ex vivo β-cell function islet isolation and GSIS studies in vitro would have been invaluable. In this way one would be able to perform mRNA studies on isolated islet tissue and address changes in aforementioned players mechanistically. Apoptosis, which was too difficult to measure in our fixed tissue, would have easily been measured in this fashion.

- A hyperinsulinemic euglycemic clamp would have provided valuable information about changes which occurred in insulin sensitivity and the rates of glucose appearance and disposal; the HOMA-IR is only a crude indication of insulin sensitivity.

- This study would have been greatly improved by studying hepatic IKKβ activity and p65 NF-κB expression. To definitively characterize changes in inflammation occurring with volitional exercise the present study should have included studies measuring tissue specific expression of TNFα, and IL-6. This would have been a relatively simple, although expensive experiment to perform.

- Measurement of more specific markers of ER stress in the pancreas and liver would have strengthened many of the ideas presented in this thesis. With our pancreatic studies frozen tissue would have allowed for RT-PCR quantification of ER chaperone
proteins, and markers of ER stress such as PERK and CHOP induction, which would have provided mechanistic information with respect to β-cell adaptations stimulated by exercise.

• These studies were performed in a rat model of T2DM and not in the human population and should also be mentioned. The leptin receptor mutation in ZF and ZDF rats, alters many aspects of fat metabolism, HPA function, and β-cell physiology while most humans are not leptin receptor deficient. Rodents and humans share many highly conserved molecular functions although caution is always merited when trying to extrapolate results to the human population. With this, we acknowledge that this data should be interpreted with some caution.

6.12 Future Directions

To date a pressing question remains: why is it that approximately 2/3rds of the obese insulin-resistant population maintain sufficient β-cell compensation during the progression of increased insulin demand when the remaining 1/3rd cannot and ultimately go on to develop hyperglycemia? Because obese normoglycemic and hyperglycemic (T2DM) individuals share many similarities with respect to peripheral insulin action, the answer most likely lies in the pancreas. Decreased GSIS and fewer β-cell numbers are a characteristic of T2DM. At a molecular level however, what differentiates β-cells from patients with T2DM from those with adaptive hyperinsulinemia? Similar to what is known (or not known) in humans, differences with respect to the β-cells of ZF and ZDF rats remains a mystery. A very simple and important study would be to first discern the differences between the islets of these two animals which share an identical (that we know of) genotype but drastically different phenotype. Islet isolation studies and quantification of key players in β-cell
function and viability could potentially lead to answers regarding the differences between β-cells which adapt and those which don’t. Identification of such differences may also lead to a focused area for therapeutic/pharmacological interventions to target.

Exercise remains an extremely attractive tool to prevent and treat obesity, insulin resistance and T2DM. Studies are just beginning to examine the effects of exercise to preserve β-cell function during the development of impaired glucose tolerance and T2DM. β-cells have an extremely low antioxidant status making them very vulnerable to oxidative stress either induced by glucose or FFA, but most likely both as described by glucolipotoxicity theory. Defects in β-cell adaptation to increased insulin demand have been shown to be due to loss of glucose sensing machinery such as glucokinase and GLUT2 which arise mainly due to defects in PDX-1 and MafA. Oxidative stress also changes the expression of proapoptotic:antiapoptotic genes which among other signaling pathways leads to increased β-cell apoptosis. ER stress and JNK activation are implicated in these processes. Therefore, it would be merited to continue with studies on the effects of volitional exercise in attenuating β-cell decompensation during the progression of T2DM. Similar study designs with islet isolation techniques would allow for investigation pertaining to improvements in GSIS and β-cell viability associated with exercise. Following in vitro GSIS studies, real time RT-PCR could be employed to quantify the gene expression of known players in β-cell function and viability. Among these: glucokinase, GLUT2, insulin, antioxidants such as glutathione peroxidase, PDX-1 and MafA. Obtaining definitive apoptosis data with these studies would be of prime importance.

As indicated throughout the discussion of this thesis, ER stress and JNK activation in pancreatic β-cells could be the link between obesity, insulin resistance, and β-cell
decompensation. Hideaki Kaneto’s group is pushing this idea forward and it would be very interesting to apply what is being learned and to measure markers of ER stress such as KDEL, BiP, and PERK in exercised animals. Measurement of downstream JNK signaling and associated perturbations in normal β-cell insulin signaling would similarly be worthwhile.

Studies of classical stress kinase signaling in the liver are becoming more common. We have shown with our recent studies that JNK activation and related serine phosphorylation of IRS-1 is reduced in response to an exercise training regime. Chronic exposure to elevated saturated FFA or hyperglycemia induces ER stress in the liver and adipose cells from obese rodents (371) which are characterized by PERK activation, eIF2α phosphorylation, and BiP induction (563). SREBP has been implicated in this process. Furthermore, the mechanism of obesity-related JNK activation appears to involve ER stress through IRE1 activation (765). Activated IRE1 recruits the scaffolding protein TRAF2 to the ER membrane which triggers a MAPK cascade leading to JNK activation (532; 533; 765). Thus IRE1 offers a plausible mechanistic link between obesity and peripheral insulin resistance. With this, because of what has been learned with respect to oxidative stress, either in response to chronic hyperglycemia or hyperlipidemia and accumulated intracellular fatty acid metabolites, it is logical to focus on hepatic ER stress signaling. To date the effects of exercise to attenuate ER stress in the liver of obese models of T2DM has not been studied.
7.1 Abstract

Diabetes-induced oxidative stress can lead to protein misfolding and degradation by the ubiquitin-proteasome system. This study examined protein ubiquitination in pancreatic sections from ZDF rats. We observed large aggregates of Ub-proteins in insulin-expressing β-cells and surrounding acinar cells. The formation of these aggregates was also observed in INS1 832/13 β-cells after exposure to high glucose (30mmol/l) for 8–72 h, allowing us to further characterize this phenotype. Oxidative stress induced by aminotriazole (ATZ) was sufficient to stimulate Ub-protein aggregate formation. Furthermore, the addition of the antioxidants NAC and taurine resulted in a significant decrease in formation of Ub-protein aggregates in high glucose. Puromycin, which induces defective ribosomal product (DRiP) formation was sufficient to induce Ub-protein aggregates in INS1 832/13 cells. However, cycloheximide (which blocks translation) did not impair Ub-protein aggregate formation at high glucose levels, suggesting that long-lived proteins are targeted to these structures. Clearance of Ub-protein aggregates was observed during recovery in normal medium (11mmol/l glucose). Despite the fact that 20S proteasome was localized to Ub-protein aggregates, epoxomicin treatment did not affect clearance, indicating that the proteasome does not degrade proteins localized to these structures. The autophagy inhibitor 3MA blocked aggregate clearance during recovery and was sufficient to induce their formation in normal medium. Together, these findings demonstrate that diabetes-induced oxidative
stress induces ubiquitination and storage of proteins into cytoplasmic aggregates that do not colocalize with insulin. Autophagy, not the proteasome, plays a key role in regulating their formation and degradation. To our knowledge, this is the first demonstration that autophagy acts as a defense to cellular damage incurred during diabetes.

7.2 Aims

T2DM is a disease instigated by an insulin deficiency or resistance that results in a chronic hyperglycemic state (693). A chronic excess of glucose causes multiple etiologies and ultimately results in kidney failure, macrovascular disease and neuropathies. Prolonged hyperglycemia associated with diabetes, leads to deterioration of the structure and function of organs, and this glucose toxicity is especially evident in pancreatic islets (622). Pancreatic islets contain β-cells that when subjected to chronic excess glucose, secrete less and less insulin that eventually results in a loss of function. In a normal situation, β-cells can overcome insulin insufficiencies by increasing insulin secretion. However once a hyperglycemic state is reached, β-cell function increasingly deteriorates and cells are unable to detect glucose signals (622). It is this loss of β-cell function that defines the disease. Many biochemical pathways contribute to the formation of reactive oxygen species. Over time, the accumulation of these reactive oxygen species will lead to chronic oxidative stress and defective insulin secretion (621). In addition to causing chronic oxidative stress, hyperglycemia can also lead to a reduction in insulin secretory capacity, lipid accumulation and ER stress (759; 789). A very important function of the ER is to maintain a tightly regulated quality control system and monitor protein maturation. In diabetes, the ER becomes overwhelmed and an overall increase in protein misfolding occurs (526). In the β-cell in particular, the combination of oxidative stress and the constant folding of secretory proteins such as insulin, can flood and devastate the quality control
capabilities of the ER. Protein misfolding and inefficient quality control systems as a result of oxidative stress can lead to cell toxicity and death (223), and insulin-secreting β-cells are particularly susceptible. Two mechanisms are employed by the ER to deal with misfolded protein. The first one, the unfolded protein response or UPR sets in motion many cellular survival strategies to minimize protein aggregation such as, upregulation of antioxidant production, increasing protein degradation, upregulation of chaperone expression and in some cases, protease synthesis and recruitment of ubiquitin-conjugating enzymes and caspases (640; 773). The second mechanism, the ERAD pathway also responds to the presence of misfolded proteins by retrotranslocating them across the ER membrane where they encounter ubiquitin-proteasomal degradation. The ERAD and UPR surveillance mechanisms can specifically recognize misfolded proteins and these two systems are intricately linked, in that induction of the UPR results in increased ERAD capacity (98). When the overproduction of misfolded protein exceeds their degradation, and quality control is compromised, the proteins will self-associate and aggregate, leading to intracellular accumulation and tissue deposition (98; 112). Protein aggregating diseases include Huntington’s and Alzheimer’s (246) and recently, diabetes has been compared to these conformational diseases since islet amyloidosis is thought to contribute to the progression and development of diabetes (278). The ubiquitin-proteasome pathway has traditionally thought to be the foremost defense mechanism in removing protein aggregates in the cell, and certainly, aggregates found in neurodegenerative diseases stain positive for ubiquitin, implying recognition by the ubiquitin-proteasomal system. However, recent evidence suggests that these aggregates are blocking or obstructing the proteasomal chamber and somehow causing their inhibition (42; 775). Recently, the autophagy pathway has been considered as a novel participant in clearing toxic protein aggregates
from the cell (453). In the autophagy pathway, targeted proteins or organelles are recognized and packaged into autophagosomes that eventually fuse with lysosomes and the contents are degraded (453). In one study, an accumulation of ubiquitinated protein aggregates were found in the livers of animals that were deficient in Atg7, a protein involved in autophagosome formation (414). These animals were found to have a fully functional proteasome, suggesting autophagy is responsible for removal of these ubiquitinated misfolded protein aggregates. In another study, cultured cells expressing GFP-huntingtin formed huntingtin aggregates within the cell that colocalized with LC3, a component of the autophagosome (48). Interestingly, another ubiquitin binding protein called p62 (or sequestosome1) also colocalized to these aggregates and is thought to play a role in recruiting both proteasomal and autophagic components (48; 664). Thus it can be summarized that when defective proteins are modified by ubiquitin in the cell, several studies have demonstrated a role for both the proteasome and the autophagy system in contributing to clearing ubiquitinated aggregates. However, what is not known is if both degradative systems act together synergistically or if there are certain cellular signals or environmental cues that cause one degradative mechanism to contribute more than the other. In a normal cell, 30% of newly synthesized proteins fail to become properly folded by chaperones and these misfolded or truncated proteins, (DRiPs), are degraded by the ubiquitin-proteasomal system (223). Sequestering misfolded proteins into cytoprotective storage compartments such as aggresomes is one way the cell can deal with on overload of misfolding protein (223; 347). Aggresomes recruit proteasomes, chaperones and ubiquitination enzymes that are thought to help clear the protein aggregates. This large ubiquitinated structure is found at the microtubule organizing centre (MTOC) and its formation is dependent of microtubules and actin. Recent studies have demonstrated the
existence of another protein storage compartment that is also thought to contain misfolded proteins. Formed in a number of different cell types (including macrophages, dendritic cells, and epithelial cells), ALIS are induced by a number of different stresses including PAMPs (Pathogen Associated Molecular Patterns), heat, oxidative stress and starvation (106; 726). ALIS (or DALIS), as they are known in dendritic cells) are transient structures that do not localize to the MTOC and their formation is independent of microtubules or actin (449; 450; 726). It has been shown that newly synthesized proteins are targeted to ALIS when a cell undergoes stress, the exception being when cells are grown under starvation conditions, where long-lived protein are exclusively targeted to ALIS (449; 450; 726).

Previous studies have suggested that changes in the ubiquitin-proteasome system accompany type II diabetes (278). In this present study, we visualized subcellular protein ubiquitination events that occur during diabetes using a rat model. Using \textit{in vivo} and \textit{in vitro} studies, we show that ubiquitinated protein (Ub-protein) aggregates form in response to high glucose. Since the proteasome plays a central role in the ERAD pathway, we investigate the contribution the proteasome has in regulating high glucose induced Ub-protein aggregates. A role for oxidative stress in forming these aggregates is also observed. Furthermore, we extend this study and compare the regulation and clearance of these Ub-protein aggregates induced under high glucose to known and established misfolded protein storage components observed in cells undergoing stress. These findings shed light on the outcome of misfolded protein in diabetes, and we attempt to provide a potential mechanism of action involved in formation and more importantly, clearance of these Ub-protein aggregates.
7.3 Materials and Methods

7.3.1 Cell Culture

INS1 832/13 β-cells were obtained from Dr. Christopher Newgard (297) and were cultured in RPMI 1640 with 10% fetal bovine serum, 10mmol/l HEPES, 1mmol/l pyruvate, L-glutamine, 50µmol/l β-mercaptoethanol, and antibiotics and seeded in 24-well plates on glass coverslips 16–24 h before use. Cells were grown at 37°C in 5% CO₂. In high glucose studies, the medium was supplemented to 30 mmol/l glucose. Cells were transfected with GFP-LC3 (353) using FuGene 6. LCB is a protein marker of autophagosomes. Media and reagents for cell culture were from Life Technologies (Grand Island, NY). HeLa cells and neuroendocrine PC12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin and 50µg/ml streptomycin. ES cells were grown in DMEM containing 20% FCS, 0.1mM non-essential amino acids solution, 1µM 2-mercaptoethanol, 2mM glutamine, 1000U/ml leukaemia inhibitory factor (LIF), 5U/ml penicillin and 50µg/ml streptomycin. HeLa cells at subconfluency were transfected with the indicated cDNAs using FuGENE 6 reagent (Roche Molecular Biochemicals). Cells were analysed at 18 h after transfection. Mock transfection was performed using the empty pCI-neo expression vector. For immunoblotting, proteins were separated by SDS–PAGE (12 or 15% gel) and transferred to a polyvinylidene difluoride membrane.

7.3.2 Pharmacological Agents

The following agents (all from Sigma-Aldrich except where noted) were used at the indicated concentration: cycloheximide (20µg/ml), nocodazole (5µmol/l), cytochalasin D (10µmol/l), 3-amino-1,2,4-triazole (ATZ) (1mmol/l), 3-methyladenine (3MA) (10 mmol/l), puromycin (5µg/ml), epoxomicin (1µmol/l; Biomol), NAC (1mmol/l), taurine (1mmol/l), and human insulin (100nmol/l; Eli Lilly). INS1 832/13 β-cells (297) were fixed in 2.5%
paraformaldehyde in PBS pH 7.2 for 15 min at 37°C. Fixed cells were stained as previously described (106). The rabbit 20S proteasome antibody was from Biomol. Samples were analyzed using a Leica DMIRE2 fluorescence microscope (x63 and x100 objectives) and OpenLab software. Images were imported into Adobe Photoshop and assembled in Adobe Illustrator for labeling.

### 7.3.3 Animals

Male ZDF rats (ZDF/Crl-Leprfa) and male Zucker lean rats (+/?) were obtained from Charles River Laboratories at 5 weeks of age with initial weights of 90-135g. The animals were fed water and Purina 5001 chow ad libitum throughout the experiment. All experiments were approved by the Animal Care Committee of the University of Toronto in accordance with regulations set forth by the Canadian Council for Animal Care.

### 7.3.4 Pancreas Removal and Fixation

Within 10 min of killing, the pancreas was removed and blotted, and extraneous fat and lymph nodes were removed. The pancreas was then weighed before being placed in Bock’s fixative. After fixation, tissue samples were cut and placed into tissue cassettes that were placed in 70% ethanol until paraffin embedding. 4µm slices were cut on an Olympus microtome (Carsen Group, Markham, ON, Canada) from paraffin blocks and mounted onto 25x75mm slides.

### 7.3.5 Frozen Tissue Removal and Fixation

All frozen tissues were mounted using OCT mounting compound and were cut at 10µm using a cryostat. Coronal brain sections containing the hippocampus and pituitary sections containing the anterior, intermediate, and posterior lobes were cut (10µm). Tissue sections were thaw-mounted onto VistaVision HistoBond adhesive slides.
7.3.6 Immunofluorescence and Microscopy

After sectioning, frozen tissues were placed in 4% formaldehyde in PBS for 5 min at 4°C. Slides were washed in PBS, put in methanol for 10 min at 20°C, and allowed to air dry for 3 h at room temperature. They were subjected to graded ethanol rehydration (100–50%) and brought to water. For paraffin-embedded pancreata, sections were mounted onto slides and dried for staining. Glass-mounted sections were cleared from paraffin with xylene and rehydrated by sequential washings with graded ethanol solutions (95–70%). All slides were subjected to permeabilization for 3 h, and sections were incubated overnight at 4°C with the primary antibodies. The slides were washed with PBS, followed by the specific secondary antibodies for 2 h. The following antibodies were used for immunofluorescence: 1) mouse monoclonal antibody (mAb) FK2 (Biomol), 2) rabbit polyclonal antibody against insulin (Santa Cruz Biotechnology, Santa Cruz, CA), and 3) a rabbit polyclonal antibody against glucagon (Novocastra). The nuclei were stained using DAPI. INS1 832/13 β-cells were fixed in 2.5% paraformaldehyde in PBS pH 7.2 for 15 min at 37°C. The rabbit 20S proteasome antibody was from Biomol. Samples were analyzed using a Leica DMIRE2 fluorescence microscope (63X and 100X objectives) and OpenLab software. Images were imported into Adobe Photoshop and assembled in Adobe Illustrator for labeling.

7.3.7 Enumeration of Ub-protein Aggregates

A Leica DMIRE2 fluorescence microscope was used to visually quantify Ub-protein aggregates. In the tissue sections, Ub-protein aggregates were counted in random fields of cells stained for Ub-proteins with the FK2 antibody and insulin to visualize pancreatic islets and for the nucleus using DAPI. At least 100 cells were counted for each experiment. The average ±SD for at least three experiments is presented in each experiment.
7.4 Results

7.4.1 INS-1 β-cells form Ub-protein Aggregates in Response to High Glucose

To further characterize Ub-protein aggregates that form during diabetes, we examined protein ubiquitination in INS-1 cells, a pancreatic β-cell line (297). As shown in Figure 2, Ub-protein aggregates morphologically similar to those observed in pancreatic β-cells in vivo were observed in INS-1 cells when grown in 30mM glucose. This glucose concentration is similar to that observed in vivo during T2DM (101) and is sufficient to induce insulin secretion in INS-1 β-cells in vitro (789). Ub-protein aggregates were not observed when cells were grown at 11mM glucose, a physiologically normal concentration (297; 789). The appearance of Ub protein aggregates in INS-1 cells increased with the duration of high glucose treatment. Ub-protein aggregates appeared as early as 8 h and were maximal in number at approximately 48 h (Figure 1A and 1B). In these experiments, cells were co-stained with a polyclonal antibody to insulin. As shown in Figure 1A, insulin-containing secretory granules were observed in INS-1 cells, consistent with previous findings (26). The Ub protein aggregates and insulin did not colocalize at any time point that we examined, suggesting that misfolded insulin is not targeted to these structures (Figure 1A arrows). Formation and retention of Ub-protein aggregates was dependent on the continuous presence of high glucose. When cells treated with 30 mM glucose for 48 h were returned to normal media (11mM glucose), the number of Ub-aggregates decreased in number (Figure 1A and 1B). After 24 h recovery, only 15% of cells were found to contain Ub-protein aggregates (Figure 1B). Thus, high glucose induces the formation of Ub-protein aggregates in insulin secreting INS-1 β-cells and these structures are subject to endogenous clearance mechanisms if returned to normal glucose levels.
7.4.2 Oxidative Stress MediatesUb-protein Aggregate Formation in INS-1 β-cells in Response to High Glucose

Glucose toxicity is associated with the formation of reactive oxygen species (622). Oxidative stress has been shown to induce ALIS in a number of different cell types (450). The possibility that oxidative stress could cause Ub-protein aggregates to form in β-cells was explored. INS-1 cells grown in low glucose media (11mM) were treated with aminotriazole (ATZ), a drug that causes oxidative stress by inhibiting catalase (488). Cells were then fixed and stained for Ub-proteins using the FK2 mAb. As shown in Figure 2, treatment with ATZ for 10 h caused the formation of Ub-protein aggregates in approximately 35% of cells, compared to 5% in the untreated cells. This demonstrates that oxidative stress is sufficient to induce Ub-aggregate formation in INS-1 cells. A number of clinical studies have investigated the therapeutic benefits of antioxidants and suggested that they protect β-cells from hyperglycemia and oxidative stress (621). In our experiments, we observed a significant decrease in the percentage of cells with Ub-protein aggregates when two different antioxidants were included with ATZ treatment (Figure 2). These included NAC which raises cellular glutathione levels (848) and taurine, a semi-essential amino acid which has the ability to scavenge the reactive oxygen species hypochlorite (320). These antioxidants have been used in ZDF rats to prevent glucose toxicity (735). Because hyperglycemia can result in an increase in oxidative stress, we hypothesized that adding antioxidants would also effect formation of Ub-protein aggregates during high glucose treatment. Indeed, the addition of either NAC or taurine to high glucose media resulted in a significant decrease in Ub-protein aggregates after 10 h, compared to high glucose treatment alone (Figure 2). Taken together, these data demonstrate that diabetes-induced oxidative stress plays an important role in the formation of Ub-protein aggregates in β-cells.
7.4.3 Formation and Clearance of Ub-protein Aggregates is Regulated by Autophagy in INS-1 β-cells.

Next, we examined the mechanisms that regulate Ub-protein aggregate formation and clearance in INS-1 cells. In the first set of experiments, we treated cells to high glucose for 24 h, allowing Ub-protein aggregates to form, and then returned the cells to low glucose media to allow recovery and clearance of the aggregates. As shown in Figure 3A, recovery led to the rapid clearance of Ub-protein aggregates; after 10 h recovery, there was approximately a 50% drop in the number of cells containing these structures. In contrast, maintaining cells in the high glucose media (30 mM) led to a further accumulation of Ub-protein aggregates. Next we allowed cells to recover in the presence of epoxomicin, a potent and specific inhibitor of the proteasome (502). Surprisingly, cells treated with the proteasome inhibitor displayed clearance of Ub-protein aggregates that was comparable to the control. Thus, despite recruitment of the proteasome to Ub-protein aggregates during high glucose treatment, our data suggest that this protease complex does not play a significant role in their clearance. We also treated control cells with epoxomicin, without prior high glucose treatment, and did not observe Ub-protein aggregate formation (Figure 3B). Thus, proteasome inhibition is not sufficient to induce Ub-protein aggregate formation. This suggests that proteasome inhibition does not underlie the accumulation of Ub-proteins during high glucose treatment. Autophagy controls the degradation of misfolded proteins, cytoplasmic aggregates, and entire organelles by mediating their delivery to lysosomes (453). Recent studies have demonstrated a role for autophagy in the degradation of ubiquitinated proteins, including those localized to ALIS (203; 726; 841). To investigate a role for autophagy in the clearance of Ub-protein aggregates during diabetes, INS-1 cells were allowed to recover from high glucose treatment in the presence of 3-methyl adenine (3MA), an autophagy inhibitor. Surprisingly, we observed an increase in Ub-protein
aggregates in cells treated with 3MA that was even higher than when cells were grown in high glucose alone (Figure 3A). Consistent with this observation, treatment of control cells with 3MA, without prior high glucose treatment, was sufficient to induce the rapid formation of Ub-protein aggregates (Figure 3B). These findings demonstrate that autophagy plays a critical role in mediating clearance of Ub-protein aggregates and regulating their formation under normoglycemic conditions.

7.4.4 Pancreatic Tissue Isolated from the Obese Zucker Diabetic Fatty Rat Exhibit Ub-protein Aggregates

Diabetes is thought to alter the balance of protein quality control mechanisms within the β-cell and trigger the unfolded protein response (278). Through activation of ER associated degradation, an increase in protein ubiquitination of misfolded proteins might subsequently be expected. To investigate protein ubiquitination in a diabetic model, pancreatic sections were isolated from 19-week ZDF rats. These animals lack both copies of the Leptin gene, which regulates satiety control, and as a consequence rapidly gain weight and develop type II diabetes (127; 584; 731). As controls for non-diabetic animals, 6-week old ZDF rats and the 19-week heterozygote (Gmi-fa/fa) were also examined. Pancreatic sections from each animal were stained with the mAb FK2, which recognizes both mono- and poly-ubiquitinated proteins (Ub-proteins), but not free ubiquitin (214). In parallel, tissues were stained for insulin to identify pancreatic β-cell islets. Sections were then analyzed by epifluorescence or confocal microscopy. As shown in Figure 4A, all pancreatic tissues contained a small number of infiltrating immune cells that stain non-specifically with secondary antibodies (Figure 4A, see arrow heads). Surprisingly, large Ub-protein aggregates were observed in the pancreas of the 19-week diabetic ZDF rats (Figure 4A, see arrows). Closer analysis revealed that these Ub-protein aggregates were present in the
cytosol of both β-cells and the surrounding acinar cells (Figure 4A, see inset). In contrast, the signal for Ub-proteins was diffusely cytosolic in both of the non-diabetic control rats. The number of Ub-protein aggregates in β-cells and acinar cells was enumerated in each group of rats. Whereas in the control rats, the number of aggregates was low, the 19-week diabetic ZDF rats showed a significant increase in the number of these structures in both β-cells and acinar cells (Figure 4B). Spleen sections from the ZDF rat and the two non-diabetic control rats were also isolated and examined by immunofluorescence using the FK2 mAb. In three different spleen sections, we did not observe Ub-protein aggregates in ZDF rats, suggesting their formation is not a systemic response to hyperglycemia.

7.5 Discussion

The current study demonstrates the first examination of Ub-protein aggregate formation in an animal model of T2DM. Under normal conditions, misfolded proteins are recognized and degraded. Here we show that misfolded protein accumulates during diabetes and is sequestered into defined Ub-protein aggregates within the cytoplasm. Although diabetes is thought to induce cellular stress in a number of different organs, we observed these Ub-protein aggregates in pancreatic tissue samples and not in spleen tissue samples taken from the same diabetic animal, suggesting these Ub-protein aggregates may be specific to this tissue. The difference in accumulation of Ub-protein aggregates between tissues may be attributable to organ sensitivity towards diabetes or differences in vascularization. Previous studies have shown that different cell types deal with protein misfolding in a variety of ways, and this likely reflects differences in protein misfolding rates, rates of degradation and the type of protein degradation systems in operation. In the paper by Szeto et al., numerous cell types were tested for their ability to form Ub-protein aggregates under stress. It was shown that some cell types, such as primary human skin fibroblast do
not form Ub-protein aggregates in response to most forms of cellular stress in vitro. It is possible that spleen tissue is not subjected to the same amount of stress, or a different type of stress as compared to pancreatic tissue during diabetes, or it may be that spleen cells are more efficient at removing misfolded protein during stress. That said, it is important to note that the pancreas is especially sensitive to changes in blood glucose and hyperglycemia, much more so that other organs and responds quickly by secreting cytokines, insulin, glucagons and pancreatic enzymes. Pancreatic tissue is made up of highly secretory cell types such as α-cells, β-cells, δ-cells, and acinar cells, and it may be that their secretory abilities result in an increase in the protein misfolding rate under stress. Evidence for this comes largely from the observation that Ub-protein aggregates were found in both acinar cells and beta cells, suggesting a common mechanism for sequestering misfolded proteins in various cell types. Previous data suggests that oxidative stress is a causative factor for the formation of Ub-protein aggregates (726). In parallel, oxidative stress is also a well-documented cause of the progression and development of diabetes. Notably, the field of antioxidant therapy in treating progressive diabetes is emerging (622). Antioxidant strategies are being considered for the protection of beta cell function after the onset of hyperglycemia (622). Here we show that oxidative stress is also a strong inducer of Ub-positive aggregates and in turn, high glucose, which is known to promote oxidative stress, also causes an increase in Ub-protein aggregates. It is possible that high glucose is stressing the cell in other, unrelated ways, but the addition of antioxidants and their subsequent prevention of Ub protein aggregates, strongly suggests that what we are seeing during high glucose stress is the result of oxidative stress. In addition to the microscopic visualization of the Ub-protein aggregates observed by immunofluorescence, we attempted to further characterize the Ub-protein aggregates that
were formed in INS-1 cells after stimulation with high glucose. Aggresomes are storage compartments for ubiquitinated proteins and, like the Ub-protein aggregates observed in β-cells, they are found in the cytoplasm. Recently, we and others have studied another type of Ub-protein storage compartment, termed ALIS, whose formation is the consequence of cellular stresses including microbial stress, oxidative stress and heat (106; 153; 449; 450; 589; 724; 726). ALIS contain ubiquitinated protein that originates from DRiPs (22) and they are formed in a number of different cell types (106; 726). ALIS have also been shown to be regulated by the autophagy pathway (726). Considering all these characteristics of ALIS and their visual similarity to the Ub-protein structures exhibited in high glucose stimulated β-cells, we propose that these are the same structure (henceforth, Ub-protein aggregates will be referred to as ALIS). The ubiquitinated-protein cargo in these aggregates during diabetes is unknown, although it was demonstrated that insulin is not present. So that under glucose stress, when insulin is being secreted out of the β-cells at a high rate, and there is an increased tendency for misfolded secretory insulin, this is not being targeted to ALIS. Could high glucose and starvation be linked to the same signaling pathways that target long-lived proteins into these cytoplasmic aggregates? We observed that when high glucose treated INS-1 β-cells were allowed to recover at a basal glucose level, ALIS, or Ub-protein aggregates were no longer evident. Since the recovery period abolished ALIS formation, the cells must have a way of regulating or clearing these structures. Certainly, there is evidence in the literature that Ub-protein aggregates “choke” or suffocate the proteasomal machinery, clogging it up with misfolded protein and it cannot efficiently degrade the aggregates, therefore secondary “backup” autophagic machinery is recruited (42; 775). We have shown that the autophagy pathway is a major participant in regulation and clearance of these Ub-protein aggregates or ALIS. A recent study using atg7 knockout
mice that are deficient in autophagy, observed accumulation of ubiquitinated proteins even though proteasomal activity was normal, suggesting that some proteins are selectively targeted and subsequently degraded by autophagy (414). Together with our data, one can now appreciate a central role for autophagy in development, immunity, and cellular stress responses, including responses to hyperglycemia during diabetes. The ER is particularly vulnerable to the occurrence of misfolded proteins because of its contribution in post-translational modification, folding and assembly of newly synthesized proteins. A highly developed ER is present in pancreatic cells due to their involvement in insulin and digestive enzyme secretion. The UPR and ERAD are two safety mechanisms that have evolved to help the ER cope with misfolded proteins (Figure 5A). ERAD targets damaged and misfolded proteins for proteasomal degradation by tagging them with ubiquitin. When diabetes occurs, pancreatic β-cells undergo numerous stresses, including oxidative stress, protein misfolding and damage, loss of ER quality control functioning and as a result, defective insulin secretion. In our model, the data suggests that under high glucose stress, secretory insulin originating from the ER is not targeted to ALIS, rather preformed, long-lived proteins are targeted to ALIS for ubiquitination (Figure 5B). From here, autophagy and the proteasome are recruited to ALIS, but it is autophagy that actually contributes to clearance and delivers the ubiquitinated protein to the lysosome for breakdown. As with ERAD, ALIS are likely a cytoprotective system, in that they sequester misfolded protein and target it for degradation. The demonstration that ALIS are observed in 1) a diabetic rat model in vivo, 2) in pancreatic β-cell culture under stress mimicking diabetes, combined with the fact that these structures are regulated by autophagy may have future potential clinical consequences, in that factors that promote autophagy may likely protect β-cell from death during hyperglycemia.
Figure 1. INS-1 Beta cells subjected to high glucose levels form Ub-protein aggregates that do not colocalize with insulin granules *in vitro*. A Representative microscopic images of beta cells costained for Ub-protein, using the mAb FK2, and insulin after high glucose treatment. Ub-protein aggregates are indicated with arrows. The Ub-protein aggregates are no longer observed after recovery (48 hour 30mM glucose treatment followed by 24 hours of 11mM glucose treatment) with lower amounts of glucose. B Enumeration of Ub-protein from A. Asterisks, significantly different from cells grown in normal, unsupplemented media at the same time (11mM glucose), P<0.05.
Figure 2. Oxidative stress can induce Ub-protein aggregates and the phenotype in response to high glucose and oxidative stress can be reduced when antioxidants are added. β-cells were treated with ATZ or high glucose for 10 hours in the presence or absence of two antioxidants (NAC and taurine) as indicated. The cells were stained for Ub-proteins with the FK2 mAb and the number of cells with Ub-protein aggregates was enumerated by microscopic analysis. Asterisks, significantly different from cells not treated with antioxidants, P<0.05.
Figure 3A: Ub-Protein Regulation by Autophagy

Figure 3B: Ub-Protein Are ALIS

Figure 3. Formation of Ub-protein aggregates is regulated by autophagy in INS-1 β- cells. A Ub-protein aggregate formation in beta cells grown in the presence of high glucose (30mM) for 24 h. At the 24 h point, the high glucose media was washed off and 3MA (closed triangles), epoxomicin (open circles), normal media (closed circles) and high glucose media (closed squares) was added and the cells were allowed to grow for the indicated times. The cells were fixed, stained for Ub-proteins with the FK2 mAb and the number of Ub-protein aggregates was enumerated. B Shows Ub-protein aggregate formation in beta cells grown in the presence of high glucose (30mM Glc; closed squares), 3MA (closed triangles), epoxomicin (open circles) and normal media (closed circles) for the indicated times. The cells were fixed, stained for Ub-proteins with the FK2 mAb and the number of Ub-protein aggregates was enumerated.
Figure 4. Zucker Diabetic Fatty rat pancreatic tissue sections exhibit Ub-protein aggregates in vivo. A Representative microscopic images of pancreatic sections isolated from 19 week Zucker diabetic fatty rats. Tissue sections from each rat were stained and examined using immunofluorescence to see whether Ub-protein aggregates were formed in vivo under diabetic stress. Pancreatic sections were stained using an antibody against insulin to identify Beta cell islets and mAb FK2 (identifies mono and polyubiquitin) was used to examine Ub-protein aggregates. Ub-protein aggregates were observed in pancreatic beta cells and in acinar cells in the tissue section of the diabetic rat. B Enumeration and quantitative analysis of Ub-protein aggregates in acinar cells and beta cells from pancreatic sections from A. Asterisks, significantly different from tissue taken 6 week ZDF rats and tissue taken from 19 week ZDF rats, P<0.05.
Figure 5. A Hypothetical model of the role of UPR and ERAD in diabetes. Pancreatic beta cells are extremely susceptible to ER stress in a hyperglycemic state. The quality control mechanisms of the ER begin to breakdown in insulin-secreting beta cells under severe glucose stress and misfolded protein begins to accumulate. Two pathways, the UPR and ERAD are working at full capacity to try and properly degrade misfolded protein, however these systems can become inundated. The physical characteristics of unfolded and misfolded proteins cause self-aggregation and the result is protein aggregates within the cytoplasm. These misfolded proteins are retrotranslocated to the cytoplasm and ubiquitinated. From here, it is thought that the proteasome is recruited and proteasomal degradation occurs. B Hypothetical model of Ub-protein aggregate regulation by autophagy. During high glucose stress or diabetes, Ub-protein aggregates are observed in the cytosol of beta cells. In contrast to the ubiquitinated aggregates arriving from the ERAD system (see above), the formation of these aggregates are thought to be a result of long-lived proteins within the cytosol being selectively targeted to these structures under stress. The autophagy pathway negatively regulates these Ub-protein aggregates. While proteasomes appear to be localized to Ub-protein aggregates, it is unclear whether they participate in clearance of Ub-protein aggregates. Inhibition of autophagy by 3MA results in enhanced Ub-protein aggregate formation during high glucose stress. During starvation, autophagy also mediates degradation of long-lived proteins in the cytosol.


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