Protective effect of peroxiredoxin 2 on oxidative stress induced β-cell toxicity in the pancreatic β-cell line MIN6

by

Fang Zhao

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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2011

Abstract

Type 1 and type 2 diabetes are characterized by an excessive loss of insulin producing β-cells. β-cells are particularly susceptible to increased oxidative stress induced apoptosis due to low expression of major antioxidants. Peroxiredoxin-2 (PRDX2) belongs to a group of antioxidants with antiapoptotic roles. Preliminary data indicate PRDX2 is expressed in the β-cells. Endogenous PRDX2 in the β-cell line MIN6 is found to decrease under oxidative stress conditions. I hypothesize that PRDX2 has a role in protecting β-cells against oxidative stress induced apoptosis. Overexpression or knockdown strategies were used to examine the role of PRDX2 in insulin-secreting MIN6 cells treated with various stimuli (cytokines, palmitate, streptozotocin) to induce apoptosis. Results showed that PRDX2 overexpression decreased oxidative stress induced apoptosis markers and cell death indicators, whereas knockdown of PRDX2 exaggerated oxidative stress induced toxicity. These findings suggest that PRDX2 plays a protective role in pancreatic β-cells under oxidative stress conditions.
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<tbody>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>IDDM</td>
<td>Insulin dependent diabetes mellitus (type 1 diabetes)</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>NIDDM</td>
<td>Non-insulin dependent diabetes mellitus (type 2 diabetes)</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>Vm</td>
<td>Membrane potential</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Cyclin Ds</td>
<td>D-type cyclins</td>
</tr>
<tr>
<td>Cdk4</td>
<td>Cyclin dependent kinase 4</td>
</tr>
<tr>
<td>Cdk6</td>
<td>Cyclin dependent kinase 6</td>
</tr>
<tr>
<td>GIP</td>
<td>Gastric inhibitory peptide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NF κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Mitochondrial superoxide dismutase</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterfied fatty acid</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol trisphosphate</td>
</tr>
<tr>
<td>Acyl-CoA</td>
<td>Acyl coenzyme A</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>NOD mice</td>
<td>Non-obese diabetic mice</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling protein-1</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>PRDX</td>
<td>Peroxiredoxin</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>MIN6</td>
<td>Mouse Pancreatic β-cell line</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum free medium</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>AP</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered Saline with Tween 20</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>ASK-1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 Diabetes mellitus overview

Diabetes mellitus is classified as a pandemic chronic disease by the World Health Organization. It affects more than 220 million people worldwide [130]. Each year, diabetes mellitus claims the lives of more than 1 million people and the number of deaths is expected to double by 2030 [130].

Insulin is a hormone secreted from pancreatic β-cells which is central to the maintenance of glucose homeostasis. It facilitates the glucose uptake in the liver cells, muscle cells and adipose. Diabetic hyperglycemia is a result of either the insufficient insulin secretion from pancreatic β-cells and/or defective insulin actions [114]. The two main types of diabetes mellitus are type 1 and type 2. Type 1 diabetes (T1DM), also known as insulin dependent diabetes mellitus (IDDM), juvenile, or early onset diabetes, is an autoimmune disorder which affects 10-15% of all diabetic cases [130]. T1DM is characterized by the autoimmune destruction of the islet β-cells and insulitis. It is caused by the infiltration of the body’s own immune cells, mainly T-lymphocytes, to the pancreatic islets leading to the release of pro-inflammatory cytokines and ensuing insulitis [33]. This subsequently induces the programmed destruction of the pancreatic insulin secreting β-cells and drastically decreased endogenous insulin secretion. Type 2 diabetes mellitus (T2DM), also known as non-insulin dependent diabetes mellitus (NIDDM) is characterized by reduced response to insulin action (referred to as insulin resistance) in tissues such as muscle and fat cells, as well as the pancreatic islets themselves, resulting in glucose intolerance and impaired glucose stimulated insulin
secretion (GSIS). T2DM affects 90% of the total number of diabetic cases [130]. The occurrence of insulin resistance in glucose impaired individuals is initially compensated by an increase in β-cell mass via proliferation and hyperinsulinemia. However, during the onset of T2DM this compensatory mechanism is eventually replaced predominantly by a loss of β-cell mass through apoptosis, leading to insufficient insulin secretion and hyperglycemia. Obesity, usually associated with insulin resistance, is the leading risk factor for T2DM. Over 85% of all patients with T2DM are overweight. [1]

1.2 Islets of Langerhans

Pancreatic islets are sub-organs in the pancreas which contain clusters of heterogeneous endocrine cell types. Typical islets contain glucagon producing α-cells, insulin secreting β-cells, somatostatin producing δ-cells, pancreatic polypeptide producing PP-cells and ε-cells which secrete ghrelin. δ-cells, ε-cells and PP-cells occupy only a small percentage of total islet cell mass from 3-5% (δ-cells) to <1% (ε-cells). In rodent islet, β-cells are the most abundant cell type (65 to 80%), α-cells make up for 15 to 20% [45]. In healthy human adults, the islets of Langerhans only account for 1-2% of the total weight of the pancreas, however these islet cells are able to produce sufficient endocrine hormones to regulate and maintain systemic glucose homeostasis. In rodents, the islets are usually spherical in shape with α- and δ-cells in the periphery, located in the mantle surrounding the β-cells located at the core of the islet. In contrast, human islets display more irregular morphology, which is exemplified by that all cell types are randomly distributed within the islet. The ratio of islet cell types also differs in humans with α-cells at 33-46% and β-cells at 48-59% of the total islet mass. δ-cells and PP-cells
remain at the same percentage as in rodents [20]. Due to the arrangement of rodent islets, the $\beta$-cells are highly coupled to each other electrically via gap junctions, this allows the $\beta$-cells to function as one unit in response to elevated levels of glucose and subsequently release insulin through synchronized membrane potential ($V_m$) and $Ca^{2+}$ oscillations [11]. This phenomenon is reduced in human islets, where only clusters of $\beta$-cells within an islet are able to be coupled to each other. $\alpha$-cells as well as $\delta$-cells and other cells types within the islet are not coupled and function independently. The cells in the islets of Langerhans are also innervated by the sympathetic and parasympathetic nervous systems which indicate that the islet cells are modulated by many different levels not only nutrient and paracrine/autocrine signals.

1.3 Regulation $\beta$-cell mass

Abnormal regulation of $\beta$-cell mass is one of the most critical aspects in the pathogenesis of diabetes. In the late stage of T1DM, $\beta$-cells are nearly absent. The $\beta$-cells are also severely diminished in T2DM, particularly when the disease is advanced. The size of $\beta$-cell mass is affected by a combination of $\beta$-cell proliferation, apoptosis and neogenesis [73]. The regulation of $\beta$-cell mass is a dynamic process, it increases under conditions in which the body’s demand for insulin is increased due to metabolic changes, such as during the neonatal period, or in obese individuals and during pregnancy [55;96;97]. The peak of $\beta$-cell replication is during the neonatal and infant stage with the post-natal $\beta$-cell mass expansion. The ability for $\beta$-cells to regenerate declines drastically after the infant stage [87].
In obese individuals, \( \beta \)-cell mass increases in response to the increased insulin demand due to peripheral insulin resistance. If the increase of \( \beta \)-cell mass cannot compensate for the increased demand, glucose intolerance develops which may eventually lead to T2DM [102]. The reduction of \( \beta \)-cell growth in T2DM could be due to impaired development of the fetal endocrine pancreas, postnatal obesity or the destruction and loss of \( \beta \)-cells by glucolipotoxicity [45]. Similar to obesity, during pregnancy, \( \beta \)-cell mass is increased to compensate for the increased demand for insulin. However, if the increase is unable to meet the demand, the pregnant woman may suffer from gestational diabetes which affects 4-14% of all pregnant women in the US [72]. In T1DM, \( \beta \)-cells are lost through autoimmune induced apoptosis and destruction. There is still debate on how pancreatic \( \beta \)-cells regenerate themselves [38;92;115]. But it is clear that \( \beta \)-cells are able to replicate and regenerate in some manner. The balance between \( \beta \)-cell regeneration and apoptosis determines \( \beta \)-cell mass and therefore the loss of balance between the regeneration and apoptosis contributes to the development of diabetes [80].

### 1.4 \( \beta \)-cell proliferation

In the adult pancreas, growth factors play an important role in the growth and regeneration of \( \beta \)-cell mass. A few examples of growth factors which can affect \( \beta \)-cell growth are insulin, glucose, and GLP-1 [31].

#### 1.4.1 Effect of Insulin on \( \beta \)-cell proliferation
It has been widely reported that peripheral insulin resistance leads to hyperinsulinemia and an amplification of β-cell mass. In a study by Otani K. et al., they found that β-cell specific insulin receptor knockout mice exhibited decreased β-cell mass in adults and the development of diabetes [98]. However, mice double heterozygous for the insulin receptor and insulin receptor substrate 1 alleles (IR/IRS-1 mice), express only half of the amount of the IR/IRS-1 proteins compared with wild type (WT), exhibit severe insulin resistance and hyperinsulinemia, as well as β-cell hyperplasia and increased β-cell mass [19]. β-cell mass was also increased in acute euglycemic-hyperinsulinemic infused rats [99]. Alternatively, hyperinsulinemia as a result of ectopic transplantation of insulinoma cells in rats led to a significant decrease in pancreatic β-cell mass. This loss of β-cell mass was not due to autoimmune induced apoptosis [13]. These studies indicate that insulin action can modulate β-cell proliferation and apoptosis.

1.4.2 Effects of glucose on β-cell proliferation

Circulating glucose has been known influence β-cell proliferation and survival. Bernard C. et al. have reported that rats with glucose infusion to simulate hyperglycemia (22mM) for only 24 hours showed a 50% increase in the number of β-cells. The number of β-cells returned to normal 7 days after discontinuation of the glucose infusion which was associated with increased β-cell apoptosis [12]. Furthermore, a longer infusion of glucose leads to sustained β-cell mass hypertrophy even when infusion is stopped [16;126]. It is possible that glucose acts through autocrine activation of insulin signaling proteins to induce β-cell proliferation [5]. In addition, glucose has been found to stimulate β-cell survival and proliferation through inhibition of a constitutive apoptotic
pathway [63]. However, chronic hyperglycemia can be detrimental to β-cell function and survival as mentioned in section 1.5.2.

1.4.3 Effect of GLP-1 on β-cell proliferation

Glucagon like peptide-1 (GLP-1) is an incretin hormone secreted from intestinal ileal L-cells in response to nutrient ingestion. GLP-1 promotes β-cell replication, neogenesis, and function [18]. GLP-1R activation has been found to increase β-cell proliferation and mass [39], 7 day GLP-1 treatment of db/db mice delays the onset of diabetes [128]. Even in mice that have already developed diabetes, GLP-1 treatment reduces circulating blood glucose levels and increases β-cell mass through neogenesis of insulin producing cells from PDX-1 expressing ductal precursor cells [119]. The ability of GLP-1 to induce β-cell neogenesis was also confirmed with in vitro studies with rat and human duct cell lines. Rat pancreatic ductal (ARIP) cells treated with GLP-1 were able to differentiate into insulin secreting cells in a time and dose dependant manner and acinar (AR42J) cells were able to differentiate into pancreatic hormone producing cells with treatment of GLP-1 and its analog exendin-4 [65;138].

1.4.4 Other inducers of β-cell proliferation

Like other cell types, replication of β-cell is accomplished through the successful passage through the different phases of the cell cycle. D-type cyclins (cyclin Ds) initiate entry into the cell cycle for the β-cells through forming complexes with cyclin dependant kinases (Cdk4 and Cdk6). The involvement of cyclin Ds and Cdk4 was first established
in 1999 separately by Rane and Tsutsui [106;127]. It was found that Cdk4 knock-out mice exhibited an insulin deficient diabetic phenotype, however, when endogenous Cdk4 expression was returned, β-cell proliferation increased and the mice returned to normoglycemia [84]. Knock-in mice (Cdk4R24C mice) with a mutant form of Cdk4 that is resistant to inhibition, showed islet hyperplasia as a result of increased β-cell mass [92]. Cdk4R24C cDNA transfected human islets show increased β-cell proliferation [32] and cyclin D1 and Cdk4 overexpression in both rat and human islets enhanced proliferation. This demonstrates that Cdk4 and cyclin Ds play a major role in β-cell cell cycle regulation and proliferation.

It has also been found that GLP-1 and its receptor agonist exendin-4 as well as gastric inhibitory polypeptide (GIP), all of which have been well established in their roles in inducing β-cell proliferation, can all induce cyclin D1 expression in β-cells [49;69]. Inhibition of the phosphatidylinositol 3-kinases (PI3K) pathway reduced the effect of GLP-1 on β-cell proliferation as well as cyclin D1 expression.

1.5 β-cell apoptosis

Apoptosis, as well as proliferation of β-cells, is important for the maintaining β-cell mass and glucose homeostasis. In fact, diminished β-cell function and increased β-cell apoptosis not compensated by adequate regeneration or neogenesis is fundamental for the pathogenesis of both T1DM and T2DM [40]. β-cell apoptosis is caused by a variety of factors including autoimmune proinflammatory cytokines in T1DM [68], chronic hyperglycemia and hyperlipidemia in T2DM [68;124] and secondary factors such
as oxidative stress [40], endoplasmic reticulum (ER) stress [4] and mitochondrial dysfunction [118],

1.5.1 Effect of proinflammatory cytokines on β-cell apoptosis

In the progression of T1DM, proinflammatory cytokines including interleukin (IL)-1β, interferon (IFN) γ and tumour necrosis factor (TNF) α are released in the islet by infiltrating T-lymphocytes. These cytokines are directly responsible for mediating β-cell apoptosis through a combination of production of reactive oxygen species (ROS) in the mitochondria by altering the action of the electron transport chain (ETC) [121], creation of nitric oxide (NO) via activation of inducible nitric oxide synthase (iNOS) through upregulation of nuclear factor-κB (NF-κB) [83] and the activation of c-Jun N-terminal kinase (JNK/SAPK) pathway and/or the FAS pathway [43]. Free oxygen radical scavengers such as dimethylthiourea and citiolone have been reported to prevent inflammatory cytokine induced toxicity of β-cells [120]. The cytokines themselves could also activate stress related kinases which will up regulate the expression of other proinflammatory cytokines such as TNF-α, IL-6 and monocyte chemoattractant protein-1 (MCP-1) forming a positive feedback loop and further damage the cells via production of ROS [2]. Proinflammatory cytokines has been known to induce the upregulation of iNOS to produce NO and the subsequent translocation of NF-κB to the nucleus [22]. NF-κB is a transcription factor for NADPH oxidase, which in turn generates even more superoxides and increases oxidative stress for the β-cells. IL-1β has been found to be able to induce iNOS alone but its effect is greatly amplified in the presence of IFN-γ and/or TNF-α [56]. However, it has been found that when iNOS gene expression or activity is
absent, the cytokines’ harmful effect upon the β-cells is prevented [76]. Overexpression of mitochondrial isoform of superoxide dismutase (MnSOD) is also able to prevent the cytokine induced upregulation of iNOS and NF-κB activation [6], however overexpression of MnSOD seem to increase β-cell apoptosis.

1.5.2 Effect of glucotoxicity on β-cell apoptosis

Glucotoxicity is a term used to refer to the deleterious effect in tissues of chronic exposure to elevated levels of circulating glucose. Even though glucose infusion has been found to be associated with β-cell proliferation and an increase in β-cell mass (section 1.4.2), chronic high glucose concentrations have also been associated with β-cell apoptosis in both in vitro and in vivo models of T2DM [37]. Glucotoxicity has been found to be related to the cytotoxic cytokine IL-1β from β-cells [15]. In vitro exposure of human islets to high concentrations of glucose led to an increase in the production of IL-1β and subsequent activation of NF-κB and FAS pathways as well as DNA fragmentation resulting in β-cell toxicity [81]. Chronic hyperglycemia has been theorized as one of the major causes of the loss of β-cell mass through β-cell apoptosis during the pathogenesis of T2DM. Glucose induced toxicity in T2DM is exacerbated in the presence of hyperlipidemia, specifically elevated levels of long chain non-esterfied fatty acids (NEFA) [53].

1.5.3 Effect of lipotoxicity on β-cell apoptosis
Lipotoxicity is the pathologic changes in organs and tissues resultant from elevated fat levels in circulation or in tissues. T2DM is often preceded by metabolic syndrome, a combination of risk factors such as dyslipidemia, hypertension and obesity. Elevated levels of long chain saturated NEFA have been shown to decrease insulin secretion, induce β-cell dysfunction and destruction [101;139]. NEFA such as palmitic acid are highly toxic to islet β-cells, however, monounsaturated and short chain saturated NEFA are well tolerated and could even ameliorate the toxic effects of long chain saturated NEFA [34].

NEFA acts on the β-cell through lipid binding G-protein coupled receptors (GPCR) that are expressed in the β-cells, GPCR40 has been found to be involved in GSIS through the activation of the phospholipase C (PLC) pathway and inositol trisphosphate (IP3) mediated release of Ca\textsuperscript{2+} ions from the ER [111] which contributes to the exocytosis and release of insulin.

Under normal conditions, fatty acids could also be used as an energy source by the β-cells. They are broken down mainly through β-oxidation into acyl-CoAs in the mitochondria and peroxisomes [50]. In the mitochondria, β-oxidation of NEFA is coupled with the production of ATP. The NADH and FADH\textsubscript{2} produced as a result of β-oxidation as well as the subsequent processing of acyl-CoAs in the TCA cycle can produce 106 mol of ATP per mol of palmitic acid. Short chain NEFA are usually directly processed in the mitochondria while long chains are almost exclusively processed in the peroxisome first [50]. The goal of β-oxidation in the peroxisome is to reduce the length of the long chain NEFA which are poor substrates for mitochondrial β-oxidation [51]. The
shorter acyl-CoAs released by peroxisomes are then transported into the mitochondria for further β-oxidation and the generation of ATP.

Production of ROS in the mitochondria can be attributed mostly to the electron transport system [44;121]. Other than inducing activation of the electron transport chain by fatty acid metabolism, it has been found that long chain saturated NEFA may directly interact with respiratory chain proteins and increase the production of oxygen radicals [112].

### 1.5.4 Effect of glucolipotoxicity on β-cell apoptosis

Glucolipotoxicity refers to the combined, deleterious effects of elevated circulating glucose and fatty acid levels on pancreatic β-cell function and survival [100]. This combined damage from elevated glucose levels and fatty acid stems from the ability of glucose to affect fatty acid metabolism, promoting increased synthesis of cellular lipids. The combination of excessive intracellular fatty acids and glucose leads to decreased insulin secretion, impaired insulin gene expression, and β-cell death by apoptosis. In adult rodent models, it has been shown that infusion of glucose and lipids could result in β-cell dysfunction and insulin resistance in as little as 72 hours [47]. However, the relevance of glucolipotoxicity in humans is still unclear. Several studies have shown the inhibitory effect of chronically elevated fatty acid levels [14;23;24;75]. This suggests that high glucose and high lipid levels may contribute to β-cell failure in T2DM in addition to decreased β-cell function observed after the onset of diabetes.
1.5.5 Effect of oxidative stress on β-cell apoptosis

Oxidative stress is the process of release of free radicals which cause cellular damage. Oxidative stress plays a major role in the destruction of insulin producing β-cells during the onset of T1DM and T2DM [8;108]. Increasing evidence suggest that the formation of ROS such as peroxides, hydroxyl radicals, superoxide anion and reactive nitrogen species (RNS) contribute to β-cell dysfunction and apoptosis [41;48]. Studies have shown that elevated markers of oxidation, such as hydroperoxides [52;94] and oxidative DNA damage [104;105;110] have been found in the pancreatic islets of patients with T2DM. Oxidative damage to the pancreatic islets has also been found to be higher in non-obese diabetic (NOD) mice, a T1DM mouse model, compared to their diabetic resistant controls [60].

Under normoglycemic conditions, glucose is metabolized by the β-cells through the TCA cycle. Electron donors such as NADH are created by passing electrons through four complexes (complex I, II, III and IV) of the electron transport chain (ETC) in the mitochondria creating a voltage gradient across the mitochondrial membrane which produces ATP via ATP synthase.

Under chronic hyperglycemic conditions such as during diabetes mellitus, excessive amounts of glucose are oxidized by the TCA cycle which in turn produces excessive amount of electron donors (NADH and FADH₂). These electron donors are forced into the ETC, blocking the transfer of electrons to complex III. These surplus electrons are picked up by oxygen molecules which create superoxides [17].

Superoxides are usually converted by MnSOD into H₂O₂. H₂O₂ produced in this fashion is then usually reduced to H₂O and O₂ by catalases or glutathione peroxidases.
However, production of excessive superoxides during diabetes can have severe detrimental effects. These reactive radicals contribute to the development of complications in diabetes such as retinopathy, neuropathy and nephropathy. It has been shown that a reduction of these free radicals by inhibition of the ETC through complex II as well as upregulation of the MnSOD and uncoupling protein-1 (UCP-1) can protect against the development of secondary complications in bovine aortic endothelial cells [93]. These superoxides, in addition to the instigation of diabetic complications, lead to β-cell dysfunction and cell death due to the limited ability for β-cells to process ROS from lack of major antioxidants like SOD and catalase. [50]

This oxidative destruction of β-cell mass disrupts the fine balance of β-cell growth and death that is required to maintain glucose homeostasis and is a major contributor to the onset of both T1DM and T2DM [107].

Figure 1 illustrates the production of ROS in the β-cell by various sources.
Figure 1: Oxidative stress induced β-cell apoptosis

Cytokines are secreted by T-lymphocytes and by β-cells under glucolipotoxicity. These cytokines cause altered electron transport chain action in the mitochondria and induces the activation of iNOS and NF-κB, both of which increase ROS levels in the β-cell. Elevated levels of ROS will lead to β-cell toxicity and apoptosis unless prevented by antioxidant molecules such as peroxiredoxins.

1.5.6 Other Apoptosis inducers

Streptozotocin (STZ) is a member of a group of drugs known as alkylnitrosourea. This chemical is commonly used to induce β-cell injury in both in vitro and in vivo models of diabetes. Although it has been generally accepted that STZ directly alkylate DNA causing strand breaks and induction of apoptosis [125], it has been theorized that creation of free radicals plays a major role in STZ induced DNA damage and
cytotoxicity. It has been found that STZ enhances the induction of oxygen radicals in pancreatic β-cells [95] and stimulates the creation of H₂O₂ and DNA fragmentation in isolated rat islets [122]. DNA damage done by STZ has been shown to be preventable by N-monomethyl-arginine, an inhibitor iNOS, as well as nicotinamide, a free radical scavenger [9]. Furthermore, a recent study has reported that the diabetogenic effect of STZ can be prevented through the overexpression of metallothionein, an antioxidant [26].

1.6 Peroxiredoxins

Peroxiredoxins (PRDX) are a family of thioredoxin dependant peroxide reductases which are found to be ubiquitously expressed in most animal and plant cells. This family of peroxidases is highly conserved throughout the evolutionary process [90]. They have been found in archea, prokaryotes as well as eukaryotes which indicate their ancient origins [109]. The first member of this family was identified in yeast in 1988. There has been extensive research on this family and currently there are six members found to be expressed in mammalian cells.

PRDX are small proteins which may form homo and hetero dimers or oligomers. The conserved cysteine residue in the N-terminus allows for the peroxidase activity resulting in disulfide bond formation intramolecularly or with other PRDX. The oxidized PRDX is regenerated via thioredoxin. Mammalian PRDX have been known to be linked to cytokine induced H₂O₂ production which has been shown to mediate signaling cascades involved with proliferation, differentiation and apoptosis [131].

Due to the highly conserved nature of their structure, PRDX could be split into three categories based on their reactive cysteine residues (Cys): typical 2-Cys, atypical 2-
Cys, and 1-Cys [132]. All PRDX have a reactive cys residue at the N-terminus, however, 2-Cys have an additional reactive cys residue in the C-terminus as well. Furthermore, the 2-Cys can be subdivided into typical and atypical groups. This categorization depends on the position of the cys residue in the C-terminus. Catalytic cys residues in N-terminus have been highly conserved in all species in which PRDX are found, suggesting that the 1-Cys developed first and is the more ancestral form. PRDX1, PRDX2, PRDX3 and PRDX4 are typical 2-Cys and PRDX5 atypical 2-Cys. PRDX6 is the 1-Cys with only one active cysteine residue [109]. There is a structural region called the “thioredoxin fold” which is highly conserved through all PRDX. The increase in function of PRDX from a purely antioxidant role to signaling through modulation of hydrogen peroxide could be explained through the emergence of the different evolutionary isoforms.

PRDXs are also described as soluble factors with immune regulatory roles. PRDX1 and PRDX2 in mammals were originally identified in the cytosolic fraction of red blood cells. These proteins were initially found to enhance the cytotoxic effect of the natural killer cells. Thus they were named natural killer cell enhancing factor A and B [117]. It was thought that since PRDX1 and PRDX2 are not found in the membrane of the red blood cells, so they were only released after the red blood cell has undergone lysis to bind with an unknown receptor on the NK cells.

PRDX4, unlike PRDX1 and PRDX2, has a hydrophobic N-terminal signal sequence which targets it to the endoplasmic reticulum (ER). This attribute separates PRDX4 from all other mammalian PRDXs as it allows PRDX4 to be actively secreted and functional in the extracellular space [85]. Other than being an antioxidant in the extracellular space, it also acts as a cytokine and enhances the function of NF-κB as well.
as up regulating NF-κB expression dependant genes such as intracellular adhesion molecule-1 (ICAM-1) and inducible nitric oxide synthase (iNOS) in astrocyte cell cultures [58]. Recently, transgenic mice expressing high levels of PRDX4 in the pancreas has been found to be more resistant to high dose STZ induced diabetes and β-cell death [36].

Different members of PRDXs have been found to be distributed in various organelles throughout the cell. PRDX1, 2 and 6 are cytosolic proteins, PRDX3 is only expressed in mitochondria, PRDX4 is localized in the ER and extracellular space, and PRDX5 exist in different forms throughout the mammalian cell in the cytosol, mitochondria and the nucleus.

Mammalian PRDXs are highly expressed in the cytosol. An individual red blood cell could contain up to 15 million molecules of PRDX2 [79], PRDX are one of the most abundantly expressed proteins in E. Coli and they are 0.1%-0.8% of all soluble proteins in mammalian cells [78]. In most cells, PRDX have multiple antioxidant functions. If the plasma membrane’s integrity is compromised due to infections or physical damage, the PRDXs are released into the extracellular space. For example, PRDX1 is released from apoptotic cells during systemic sclerosis, and is secreted by lung cancer cells in vitro and in vivo [25]. The clinical significance of PRDXs in the extracellular space is currently not well known. Recent studies have found that tumour associated macrophage changes phenotype to regulatory or wound healing macrophages as the tumour grows and progresses. These macrophages have been found to inhibit immune response to the tumour as well as contribute to angiogenesis which promotes tumour growth. PRDX2 in mouse is capable of setting off this change in phenotype of macrophages. It is possible
the tumour cells secrets PRDXs to change the surrounding environment to be more acclimatable for the growth and migration of the tumour cells [62].

Induction of oxidative, ferric, and β-mercaptoethanol stress have been found to induce PRDX expression. Recently, it has been found that also H₂O₂, okadaic acid, tissue plasminogen activator, butylated hydroxyanisole, and even ionizing radiation could induce upregulation of PRDXs [42]. Currently, PRDXs have been investigated in regards to a broad physiological and pathological field. It has been found that its expression is related closely to certain types of diseases such as various forms of cancer and neurological diseases such as Alzheimer’s and Parkinson’s disease.

It has been found that PRDXs have atypical expression in various types of cancers including thyroid tumours, oral, lung, bladder, esophageal, and breast. In breast cancer, all six PRDXs have abnormal expression but in oral cancers only PRDX1 expression is altered [136]. Since ROS have long been considered to have potential carcinogenic properties as well as to promote metastasis of tumour cells and that oxidative stress is able to induce PRDX expression, so it is possible that upregulation of PRDX expression is correlated with tumourigenesis, therefore PRDX have considered to be biomarkers for cancers [27,67] and demonstrated anti-tumour properties [21,28,89].

PRDX expression also have a strong correlation with certain neurological disorders such as Alzheimer’s [82], Parkinson’s [64], Amyotrophic lateral sclerosis and stroke [61] as well as lung diseases such as sarcoidosis [71,113]. In addition, they can be a possible central regulator of circadian rhythms [7]. This suggests that PRDXs have a multiple roles in the survival of the organism other than just the reduction and clearance of ROS.
Recent studies have suggested that PRDX3 and PRDX4 have the ability to prevent pancreatic β-cell apoptosis and diabetes [36;131]. However, little is known about whether PRDX2 has a role in β-cell apoptosis and survival.

Figure 2: Model for peroxiredoxin function

ROS such as H₂O₂ are reduced to H₂O by the cysteine residues on peroxiredoxins to form disulfide bonds. The peroxidatic cysteine residue of the reduced peroxiredoxin reduces the peroxide, and is in turn converted to cysteine sulfenic acid (SOH). The resolving cysteine residue (SH) of another peroxiredoxin then forms a disulfide bond with the cysteine sulfenic acid, releasing H₂O. This intermolecular disulfide bond is in turn reduced by Thioredoxin [10]. Thioredoxin reductase uses NADPH to reduce the oxidized form of thioredoxin.

1.6.1 Peroxiredoxin 2

PRDX2 is a typical 2-Cys. It has many aliases due to its structure as well as its many functions. PRDX2 has a hollow cylinder shape that provided the name Torin prior
to being named PRDX2 [59]. Many other names were given to PRDX2 such as Calpromotin [88], thio-specific antioxidant, thioredoxin specific peroxidase, protector protein [77] and natural killer enhancing factor B [116]. PRDX2 is an obligate homodimer in a head to tail orientation. PRDX2’s catalytic activity depends on two highly conserved cysteine residues. Cys 51, the peroxidatic cysteine, reacts with H$_2$O$_2$ to form a sulfenic acid which then reacts with cys 172 to form a disulfide bond. Arg 127 lowers the pKa by providing a positive charge to cysteine 51 which enhances its reactivity with H$_2$O$_2$ and is therefore instrumental to facilitate catalysis. Thioredoxin reduces the disulfide bond which is in turn reduced by thioredoxin reductase with NADPH [59;79]. Figure 2 illustrates a model of oxidation and reduction in typical 2-Cys PRDX function.

It has been found that in hamsters fed a high fructose diet to induce fatty livers, hyperinsulinemia and hyperlipidemia develop elevated levels of PRDX2 in the liver, suggesting that PRDX2 may be linked to the onset of metabolic syndrome [137].

1.7 Rationale

Loss of β-cell mass brought on partially due to excessive β-cell toxicity and apoptosis is a cause of both T1DM and T2DM. Reactive oxygen and nitrogen species have been found to be elevated in mice and patients with both T1DM and T2DM [60;110]. Reactive oxidative and nitrosative damage is a major contributor of β-cell dysfunction and cell death due to low levels of major antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalases in the β-cells [74].
Peroxiredoxins are a family of highly conserved thioredoxin dependant peroxide reductases. They have been found to be ubiquitously expressed in almost all organisms and cell types including β-cells. PRDX1 and PRDX2 have been found to be highly expressed in the islet cells but not in the exocrine pancreas [8]. PRDX1 and PRDX2 expressions in the β-cell are correlated with oxidative stress inducers [8]. PRDX3 and 4 has already found to have protective effects on the β-cell against cytokine and STZ induced β-cell death [36;131].

Cytokine induced β-cell apoptosis is partially through activation of the NF-κB/iNOS pathway. Recently PRDX2 has also been found to be related to NF-κB expression in granulosa cells [134] as well as being able to inhibit neuronal apoptosis through ASK-1 pathway [64]. This may suggest that PRDX2 is related to oxidative stress induced dysfunction and death in the β-cell.

Previous unpublished results by the Wang lab revealed that the GLP-1 analog exendin-4 treatment upregulates PRDX2 levels in β-cells. This raises the possibility that PRDX2 may be related to the modulation of β-cell death and survival pathways. My preliminary studies showed that PRDX2 expression decreases during induced β-cell death by proinflammatory cytokines (IL-1 β, TNF α, IFN γ), palmitic acid and streptozotocin, further suggesting that PRDX2 may regulate β-cell apoptosis and/or survival.

1.8 Hypothesis

It is my hypothesis that PRDX2 is important for the survival of pancreatic β-cells during periods of oxidative stress. I postulate, elevation of PRDX2 expression can protect β-cells from oxidative stress induced apoptosis induced by oxidative stress stimulating
agents and reduction of PRDX2 may induce β-cell apoptosis conditions which cause oxidative stress. To test this hypothesis, the insulin producing β-cell line MIN6 will be used as a model. Elevation of PRDX2 in the clonal β-cells will be achieved by transient transfection of the expression vector encoding PRDX2. Ablation of PRDX2 in the β-cells will be conducted by knocked down strategy using siRNA. These cells transfected with or without the plasmids or siRNA constructs will then be challenged with various stimuli to induce oxidative stress associated toxicity and apoptosis in these β-cells, partially through the induction of oxidative stress including proinflammatory cytokines, palmitic acid and STZ. The cell death and apoptosis will be measured to determine the role of PRDX2 in modulating β-cell toxicity and apoptosis using various methods, including nuclear staining based on criterion of nuclei condensation and fragmentation, Western blot analysis using anti-caspase-3 antibody, and flow cytometry assisted cell sorting (FACS) analysis in the β-cells stained with propidium iodide.

**1.9 Objective**

1. To examine PRDX2 expression in pancreatic islets and β-cells.
2. To quantify apoptosis of the β-cell line MIN6 induced by pro-inflammatory cytokine cocktail, palmitic acid and STZ.
3. To determine if overexpression of PRDX2 in β-cells can prevent oxidative stress induced β-cell apoptosis.
4. To determine if knockdown of PRDX2 in β-cells increases β-cell apoptosis under normal and oxidative stress conditions.
Chapter 2: Materials and Methods

2.1 Chemical and reagents

Roswell Park Memorial Institute (RPMI) 1640 medium, TRIzol reagent, Lipofectamine 2000, trypsin-EDTA and OPTI-MEM were purchased from Invitrogen (Burlington, ON, Canada). Fetal Bovine Serum (FBS), β-mercaptoethanol, Palmitic acid, Bovine serum albumin (BSA), fatty acid-free BSA, Streptozotocin, poly-L-lysine and Triton X 100 were purchased from Sigma Aldrich (Oakville, ON, Canada). pCMVsport6-PRDX2 vector was purchased from Open Biosystems (Huntsville, AL, USA). siGENOME smartpool siRNA was purchased from Dharmacon RNAi technologies (Chicago, IL, USA). Scrambled siRNA was purchased from Ambion (Austin, TX, USA). AffinityScript one-step RTPCR kit was purchased from Stratagene (Mississauga, ON, Canada). IL-1β, TNF-α and IFN-γ were purchased from R&D Systems (Minneapolis, MN, USA). PRDX2 antibody (mouse monoclonal) and GAPDH antibody (mouse monoclonal) were purchased from ABCAM (Cambridge, MA, USA). Caspase-3 antibody (rabbit, polyclonal) was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Bradford Assay reagent was purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). Enhanced chemiluminescence Plus reagent was purchased from GE Global Research (Niskayuna, NY, USA). Paraformaldehyde was purchased from BioShop Canada Inc. (Burlington, ON, Canada). All other additional chemicals and reagents were purchased from either BioShop Canada Inc. (Burlington, ON, Canada) or Sigma Aldrich (Oakville, ON, Canada).

2.2 Equipment
Thermocycler used for RTPCR was purchased from Eppendorf Canada (Mississauga, ON, Canada). Mini-PROTEAN Tetra Cell used for SDS-PAGE, Trans-Blot SD Semi-Dry Transfer Cell and PVDF membrane was purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). Fluorescent microscope used to visualize immunostaining was purchased from Nikon Instruments (Melville, NY, USA). FACS-Calibur flow cytometer was purchased from Becton-Dickinson Biosciences (Mississauga, ON, Canada).

2.3 Animals

Rats were housed in a pathogen free animal facility (Vivarium SMH). Rats were maintained on a 12 light / 12 dark cycle. Mice had free access to water and pellets. All animal protocols were approved by the animal care committee at SMH and in accordance with national guidelines from the Canadian council of animal care.

2.4 Isolation of Pancreatic Islets

Islets were isolated from the pancreas of 7-8 week old male Sprague–Dawley rats (weight 150–200 g), obtained from Charles River Canada (Montreal, QC, Canada), by perfusion of the pancreas through the common bile duct with 10 mL of a collagenase solution (10 mg/100 g body weight) and incubation of the excised pancreas with shaking at 37°C. The pancreatic cells were washed, filtered through 355 µm mesh, and separated on a density gradient created by resuspending the pellet in histopaque-1077 (Sigma, St. Louis, MO) and layering on serum-free media (low-glucose (LG)-RPMI 1640 described below without serum). Islets were collected from the interphase and further purified from contaminating single cell types by sedimentation. Isolated islets were cultured in LG-
RPMI 1640 (7.5% FBS, 1% penicillin/streptomycin, 0.25% HEPES, and 2.5 mM glucose) at 37°C and 5% CO₂.

2.5 Cell culture

The mouse insulinoma cell line MIN6 was a generous gift from Dr. Rozakis Adcock’s lab. MIN6 cells exhibit normal glucose metabolism and glucose stimulated insulin secretion [66]. MIN6 cells (passage 50–70) were maintained in RPMI 1640 medium containing FBS (10% v/v), 100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulphate, 55 mg/500 mL sodium pyruvate, 1.14 g/500 mL HEPES, and 1.7 µL/500 mL β-mercaptoethanol at 37°C in an atmosphere of humidified air (95%) and CO₂ (5%). In studies involving serum-starvation, serum was replaced by 0.1% BSA in serum free RPMI 1640 (SFM).

2.6 Treatment of oxidative stress agents

Palmitic acid (PA) was dissolved in serum free RPMI1640 medium (0.4mM containing 1% fatty acid-free BSA (FA-free BSA)). This mixture was shaken (225RPM at 37°C for 3 hours) to allow binding of PA with BSA. MIN6 cells were seeded at 80% confluency in 12 well plates (BD, Mississauga, ON, Canada) the day previous to treatment. 1% FA free BSA was also shaken at 225RPM at 37°C for 3 hours to act as control. The MIN6 cells were then serum starved for 1 hour and treated with indicated concentrations of PA in SFM with 1% FA-free BSA for 24 hours. For 0mM PA, the MIN6 cells were treated with 1% FA-free BSA in SFM.
A cytokine cocktail (10 ng/mL IL-1β, 50 ng/mL TNF-α and 50 ng/mL IFN-γ) was prepared in serum free RPMI 1640 medium. MIN6 cells seeded at 80% confluency in 12 well plates the night before were serum starved for 1 hour then treated with the cytokine cocktail for 0, 16 or 24 hours.

STZ was dissolved in serum free RPMI 1640 medium. MIN6 cells seeded at 80% confluency in 12 well plates the night before were serum starved for 1 hour then treated with indicated amounts of STZ for 4 hours.

2.7 Overexpression and knockdown of peroxiredoxin 2

Expression vector encoding PRDX2 (pCMVSPORT6-PRDX2, Open biosystems) or pEGFP (Clontech laboratories Inc., Mountain View, CA, USA) were transiently transfected with lipofectamine 2000 (Invitrogen) in MIN6 cells seeded at 80% confluency to elevate PRDX2 expression or GFP respectively. The cells were allowed to grow in complete RPMI 1640 medium for 24 hours post transfection before the treatment with stress agents.

siGENOME smartpool (Dharmacon) targeting mouse PRDX2 or scrambled siRNA (Ambion) were transiently transfected in 80% confluent MIN6 cells with lipofectamine 2000. The cells were allowed to grow in complete medium for 24 hours post transfection before treatment with stress agents.

For overexpression experiments, MIN6 cells were seeded in 12 well plates at 70% confluence and allowed to attach to the plates the night before transfection. On the day of transfection 1.6µg per well of either pCMVSPORT6-PRDX2 or pEGFP were diluted in 100µL per well of Opti-MEM, 4µL per well of lipofectamine 2000 were diluted in 100µL per well of Opti-MEM. The cells seeded the day before were washed with SFM twice.
800µL of Opti-MEM was finally placed in the wells. After the 20 minute incubation, 200 µL per well of the DNA-lipofectamine 2000 mixture was added to each of the wells. The DNA-lipofectamine mixture was allowed to incubate with the cells for 6 hours under normal tissue culture conditions, i.e. humidified at 37°C with 5% CO₂. Then the mixture media was discarded and replaced with complete media containing 10% FBS.

For siRNA experiments, 40 pmol per mL of siRNA and 2µL per mL of lipofectamine 2000 were used instead.

2.8 RT-PCR

Total RNA was extracted using the TRIzol reagent according to the manufacturer's instructions. RTPCR was performed using AffinityScript one-step RTPCR kit according to the manufacturer’s protocol.

The primers used were

PRDX2 fwd: 5’ ATCCCTCTGCTTGCTGATGT 3’
PRDX2 rev: 5’ TTGACTGTGATCTGGCGAAG 3’
GAPDH fwd: 5’ TGCCACTCAGAAGACTGTGG 3’
GAPDH rev: 5’ TTCAGCTCTGGGATGACCTT 3’

The DNA was first denatured at 95°C then annealed at 60°C and extended at 72°C. This was repeated for 30 cycles before a final extension at 72°C.

2.9 Western blot analysis

Cells and tissues were lysed using RIPA lysis buffer. Protein concentration of samples was measured using the Bradford assay with varying concentrations of BSA as a
standard. Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) gels were made (5% stacking and 15% separating). 20µg of protein per sample was denatured with 1X SDS buffer with bromophenol blue at 99°C for 5 minutes then loaded in a SDS-PAGE gel Prestained Protein Molecular Weight Marker (Fermentas Canada, Burlington, ON, Canada) was used to estimate the molecular weight of the protein bands.

The proteins were then transferred from the gel onto the PVDF membrane using the Trans-Blot SD Semi-Dry Transfer Cell.

The membrane was washed with Tris-Buffered Saline with Tween 20 (TBST), blocked with 5% milk in TBST. The membrane was washed again in TBST before being incubated with primary antibodies in TBST with 3% BSA targeting PRDX2 (1:1000, 22kD), GAPDH (1:20,000, 37 kD) or cleaved caspase-3 (1:1000, 17-19 kD) at 4°C overnight. The membranes were washed with TBST then incubated with their respective HRP conjugated secondary antibodies in TBST with 5% milk (anti-mouse 1:5000; anti-rabbit 1:3000) for 1 hour at room temperature. They were then washed with TBST before being subjected to enhanced chemiluminescence with ECL plus then visualized with Clonex BioFlex MSI Film (Clonex Corporation, Markham, ON, Canada).

The films were then scanned and the images were quantified using ImageJ based on intensity and width of the bands.

2.10 Immunocytochemistry

Glass cover slips were placed in 12 well plates and coated with poly-L-lysine (1:20 in PBS) for 1 hour. MIN6 cells were seeded at 60% confluency on the cover slips overnight. The cells were then transfected with pCMVspport6-PRDX2 or pEGFP using lipofectamine 2000 according to manufacturer’s protocol. 24 hours post transfection, the
cells were serum starved for 1 hour and treated with 0.4mM PA, cytokine cocktail, or 1mg/mL STZ for the indicated amounts of time. The coverslips were washed with PBS and fixed with 4% paraformaldehyde for 1 hour at room temperature. The cells were permeabilized and blocked with 5% BSA and 0.1% Triton X 100 in PBS respectively for 1 hour. Primary antibody targeting PRDX2 (1:250 in PBS with 5% BSA) was incubated overnight at 4°C. Primary antibody was removed and the coverslips were washed with PBS. Secondary antibody conjugated with Alexa 555 (1:500 in PBS with 5% BSA) was incubated for 1 hour at room temperature followed by incubation with DAPI (1:5000). The coverslips were then mounted on a glass microscope slide with DAKO fluorescence mounting medium and allowed to dry overnight in a cool, dark space before visualization with Nikon Fluorescence microscope (Eclipse TE 200).

2.11 Nuclear staining for the detection of apoptosis

Apoptosis of stained MIN6 cells was identified by DAPI-nuclear staining using typical morphologic criteria of chromatin condensation and fragmentation. After visualization of immunocytochemistry stained cells, the number of apoptotic cells was counted and was divided by the total number of transfected cells to determine the percentage of apoptotic transfected cells. 350-450 transfected cells were counted for each condition.

2.12 Flow cytometry analysis

Flow cytometry analysis of MIN6 cells overexpressing PRDX2 and treated with palmitic acid, cytokines or STZ was performed using propidium iodide (PI). MIN6 cells were seeded in 24 well plates at 70% confluency and allowed to attach overnight. The
cells were than transfected with vectors for PRDX2 or GFP whose expression is driven by CMV promoters. The cells were than treated with 0.4mM palmitic acid or proinflammatory cytokine cocktail for 24 hours or STZ for 4 hours. The supernatants were discarded. The cells were then washed with PBS, trypsinized, collected, centrifuged and resuspended in a solution containing 1µg/mL PI in PBS. The cells were incubated in the PI solution for 30 minutes before they were subjected to FACS. Cell-associated fluorescence was measured using a FACS-Calibur flow cytometer (Becton-Dickinson Biosciences), using a blue argon laser of 488 nm at 15 mW, and the results were analysed using FCS Express version 3 (De Novo Software). Fluorescence in the FL2 channel (log red fluorescence, long pass 650 nm filter) for PI were acquired and recorded, using logarithmic scales, for a minimum of 10000 cells per sample. The FACS machine was standardized using unstained and PI positive MIN6 cells.

2.13 Statistical analysis

Histograms are expressed as the mean +/- SEM. Statistical significance was determined with unpaired 2-tailed student's t-test. A p-value of less than 0.05 was considered statistically significant.
Chapter 3: Results

3.1 Peroxiredoxin 2 is expressed in α- and β-cell lines, pancreatic islets and the rat pancreatic tissues

PRDX2 has been reported in previous studies to be ubiquitously expressed in all tissues. [86] To examine whether or not PRDX2 is expressed in the used islet cell lines and in the pancreas, RT-PCR and Western blot were performed on α- and β-cell lines as well as rat pancreatic islets and whole pancreas. Both RT-PCR (Fig. 3A) and Western blot (Fig. 3B) analysis showed that PRDX2 mRNA as well as protein are expressed in isolated islets and α- and β-cell lines.
Figure 3: PRDX2 is expressed in α- and β cell lines and pancreatic islets

(A) RT-PCR performed on mRNA extracted from INS-1, MIN6, α-TC6, In-R1-G9 and mouse Islet. (B) Western blot probing for PRDX2 (1:1000) and GAPDH (1:20,000) was performed on protein extracted from α-TC6, MIN6, pancreatic islet and whole pancreas.

3.2 Apoptosis induced by palmitic acid, cytokines and streptozotocin reduces endogenous peroxiredoxin 2 levels

Palmitic acid (PA) has been known to be detrimental to pancreatic β-cells causing suppressed insulin secretion and β-cell dysfunction and eventual cell death [54]. PA induces lipotoxicity and subsequent β-cell apoptosis through ER stress and calcium depletion, which has recently been found to be related to the production of reactive
oxygen species (ROS) in the mitochondria [46;91]. As PRDX2 is an antioxidant, in order to determine whether the expression level of PRDX2 is altered upon treatment with PA., Western blot analysis was performed in the MIN6 cells treated with increasing concentrations of PA. The results show that as β-cell apoptosis increases, PRDX2 expression in these β-cells decrease in PA treated cells (Fig. 4).

Figure 4: Palmitic acid treatment is associated with decreased PRDX2 expression levels in β-cells

(A) Western blot performed on proteins extracted from MIN6 cells treated with 0, 0.2, 0.4 mM of palmitic acid with 1% BSA in serum free medium for 24 hours probing for PRDX2 (1:1000), GAPDH (1:20,000), and cleaved caspase 3 (1:1000). (B) Histogram represents quantitative analysis of relative PRDX2 expression over GAPDH in MIN6 cells with treatment of increasing concentrations of palmitic acid. (C) Relative cleaved caspase 3 expression in MIN6 cells increases with increasing concentrations of palmitic acid. n=3, *p<0.05
In T1DM, β-cell apoptosis involves a signaling cascade initiated by cytokines such as IL-1β, TNF-α and IFN-γ. Specifically, IL-1β and IFN-γ and/or TNF-α induces oxidative and nitrosative stress through the upregulation of iNOS and the production of nitric oxide (NO) [29]. Also recent studies have revealed that IL-1β is a major contributor in the loss of β-cell mass in type 2 diabetes [35]. Our results showed that treatment of MIN6 cells with the cytokine cocktail is similar to treatment with PA. PRDX2 expression was found to be inversely correlated with cytokine cocktail induced β-cells apoptosis. Endogenous PRDX2 expression is reduced with increased exposure time to cytokines (Fig. 5).
Figure 5: Cytokine cocktail treatment is associated with decreased PRDX2 expression levels in β-cells

(A) Western blot performed on proteins extracted from MIN6 cells treated with 10ng/mL IL-1 β, 50ng/mL TNFα and 50ng/mL IFNγ in serum free medium for 0, 16 and 24 hours. 
(B) Histogram represents quantitative analysis of relative PRDX2 expression over GAPDH in MIN6 cells with treatment of cytokine cocktail for an increasing period of time. (C) Relative cleaved caspase 3 expression in MIN6 cells increases with increasing temporal treatment of cytokine cocktail. n=3, *p<0.05

Streptozotocin (STZ) is a member of a group of drugs called alkyl nitrosoureas which enters the β-cells through the glucose transporter 2 [133]. STZ induces β-cell apoptosis via the generation of NO and directly damages DNA. It has been reported that in β-cells treated with STZ, endogenous PRDX2 expression is altered [8]. Therefore we
investigated PRDX2 expression in MIN6 cells in response to increasing concentrations of STZ. The Western blot analysis using PRDX2 antibody showed that similar to PA and cytokine treatment, STZ treatment dose dependently reduced endogenous PRDX2 expression, which was associated with increased pro-casepase-3 levels, suggesting that PRDX2 expression is reduced in the process of STZ-induced beta-cell apoptosis (Fig. 6).

**Figure 6: Streptozotocin treatment is associated with decreased PRDX2 expression levels in β-cells**

(A) Western blot performed on proteins extracted from MIN6 cells treated with 0, 0.5, 1 mg/mL of STZ in serum free medium for 4 hours. (B) Histogram represents quantitative analysis of relative PRDX2 expression over GAPDH in MIN6 cells with treatment of increasing concentrations of STZ. (C) Relative cleaved caspase 3 expression in MIN6 cells increases with increasing concentrations of STZ. n=3, *p<0.05
3.3 Overexpression of peroxiredoxin 2 can protect against palmitic acid, cytokines and streptozotocin induced β-cell toxicity.

There is a possible inverse correlation between endogenous PRDX2 expression and β-cell apoptosis brought on by oxidative stress (fig 4-6). It is possible that PRDX2 may play a role in the process of β-cell apoptosis. To elucidate this relationship, vectors coding for PRDX2 or GFP driven by Cytomegalovirus (CMV) promoters were transiently transfected in MIN6 cells. These cells were then subjected to either treatment of PA (0.4mM) for 24 hours, a proinflammatory cytokine cocktail for 24 hours or STZ (1mg/mL) for 4 hours.

Western blot shows that cleaved caspase 3 expression in cells overexpressing PRDX2 treated with PA (Fig. 7) was significantly reduced compared to its GFP control. Also immunocytochemistry of MIN6 cells with PRDX2 overexpression showed fewer numbers of cells with nuclear condensation/fragmentation, and indicator of apoptosis, following PA treatment (Fig. 8). Furthermore, Fluorescence assisted cell sorting (FACS) of PA treated cells overexpressing PRDX2 also revealed that PRDX2 overexpression decreased the number of propidium iodide (PI) positive, an indicator of cell death, cells (Fig. 9). These results show that PRDX2 overexpression is able to attenuate PA induced toxicity in the β-cells.

Western blot results showed that cleaved caspase 3 expression in MIN6 cells overexpressing PRDX2 is significantly reduced following cytokine cocktail treatment compared with GFP controls (Fig. 10). In addition, immunocytochemistry of cytokine treated MIN6 cells revealed that PRDX2 overexpression significantly reduced the
number of transfected cells with nuclear fragmentation and condensation (Fig. 11). FACS performed on cytokine treated MIN6 cells demonstrated that PRDX2 overexpression can significantly reduce the number of PI positive cells (Fig. 14). These results suggest that PRDX2 overexpression can attenuate cytokine induced β-cell toxicity.

Western blot showed that PRDX2 overexpression in MIN6 cells could significantly reduce STZ induced cleaved caspase-3 expression (Fig. 12). STZ treated MIN6 cells overexpressing PRDX2 subjected to immunocytochemistry showed decreased nuclear fragmentation and condensation compared to its controls (Fig. 13). FACS performed on MIN6 cells overexpressing PRDX2 following STZ treatment showed significant decrease in PI positive cells (Fig. 14). These results show that PRDX2 overexpression can protect β-cells from STZ induced toxicity.
**Figure 7: Overexpression of PRDX2 attenuates palmitic acid induced cleaved caspase-3 expression**

(A) MIN6 cells were transiently transfected with pCMV-sport6-PRDX2 or pEGFP. 24 hours post transfection, the cells were treated with 0.4mM palmitic acid with 1% Fatty acid free BSA in serum free medium for 24 hours. Protein harvested from these cells was subjected to western blot probing for PRDX2 (1:1000), cleaved caspase 3 (1:1000), and GAPDH (1:20,000). (B) Quantitative analysis of average cleaved caspase 3 expression with SEM, n=4, *p<0.05
Figure 8: Overexpression of PRDX2 attenuates palmitic acid induced nuclear fragmentation and condensation

(A) MIN6 cells were grown on glass cover slips, then transfected and treated with PA similar to Fig. 7. The cells were then subjected to immunocytochemistry, probing for PRDX2 (1:250). The nuclei were stained with DAPI (1:5000). Nuclei of stained red (PRDX2 overexpression) and green (GFP transfected) cells were assessed. Abnormal nuclear morphology was used as an indicator of apoptosis. White arrows are pointing to representative transfected apoptotic cells. (B) Histogram quantifying transfected cells with abnormal nuclei. n=3, *p<0.05
Figure 9: Overexpression of PRDX2 attenuates palmitic acid induced β-cell death

(A) MIN6 cells were transfected and treated similarly to Fig. 7, 8. The cells were collected and treated with 1µg/mL PI for 30 minutes at room temperature and subjected to FACS. Fluorescence in the FL-2 channel was acquired and recorded for 10000 events.

(B) Relative number of PI positive cells compared with BSA control with SEM, n=4, * p<0.05, ** p<0.01
Figure 10: Overexpression of PRDX2 attenuates cytokine cocktail induced cleaved caspase 3 expression

(A) MIN6 cells were transfected with pCMV-sport6-PRDX2 or pEGFP were treated with 10ng/mL IL-1β, 50ng/mL TNFα and 50ng/mL IFNγ (Cyto) in serum free medium for 24 hours. Protein harvested from these cells was subjected to western blot analysis. (B) Histogram representing quantitative analysis of average cleaved caspase 3 expression with SEM, n=3, *p<0.05
Figure 11: Overexpression of PRDX2 attenuates cytokine cocktail induced nuclear fragmentation and condensation

(A) MIN6 cells were grown on glass cover slips, then transfected and treated similar to Fig. 10. The cells were then subjected to immunocytochemistry similarly to Fig. 8. White arrows are pointing to representative transfected apoptotic cells. (B) Histogram quantifying transfected cells with abnormal nuclei. n=3, *p<0.05
Figure 12: Overexpression of PRDX2 attenuates streptozotocin induced cleaved caspase 3 expression

(A) MIN6 cells were transfected with pCMV-sport6-PRDX2 or pEGFP were treated with 1mg/mL STZ in serum free medium for 4 hours. Protein harvested from these cells was subjected to western blot analysis. (B) Quantitative analysis of average cleaved caspase 3 expression with SEM, n=3, *p<0.05
Figure 13: Overexpression of PRDX2 attenuates cytokine cocktail induced nuclear fragmentation and condensation

(A) MIN6 cells were grown on glass cover slips, then transfected and treated similarly to Fig. 12. The cells were then subjected to immunocytochemistry similar to Fig. 8. White arrows are pointing to representative transfected apoptotic cells. (B) Histogram quantifying transfected cells with abnormal nuclei. n=3, *p<0.05
Figure 14: Overexpression of PRDX2 attenuates cytokine and STZ induced β-cell death

(A) MIN6 cells were transiently transfected with pCMV-sport6-PRDX2 or pEGFP. 24 hours post transfection, the cells were treated with a cytokine cocktail for 24 hours or STZ for 4 hours. The cells were then collected and treated with 1µg/mL PI for 30 minutes at room temperature and subjected to FACS. Fluorescence in the FL-2 channel was acquired and recorded. (B) Relative number of PI positive cells compared with BSA control with SEM, n=4, ** p<0.01
3.4 Knockdown of peroxiredoxin 2 exaggerated palmitic acid; cytokines, and streptozotocin induced β-cell death.

PRDX2 overexpression appears to be able to inhibit oxidative and nitrosative stress induced β-cell toxicity and apoptosis/death as seen in figures 8-14. To verify that PRDX2 is responsible for this reduction in apoptosis/death induced by PA, cytokines and STZ, siRNA targeting mouse PRDX2 or scrambled sequence were transiently transfected into MIN6 cells. The transfected cells were then serum starved for 1 hour and treated with PA for 16 hours, a cytokine cocktail for 16 hours or STZ for 3 hours. Western blot analysis shows that cells transfected with siRNA targeting PRDX2 had increased cleaved caspase 3 expression under all conditions indicating increased β-cell toxicity and apoptosis. (Fig. 15, 16)
Figure 15: Knockdown of PRDX2 exaggerates palmitic acid induced cleaved caspase 3 expression

(A) MIN6 cells were transiently transfected with siGENOME smart-pool siRNA targeting PRDX2 or scrambled sequence as control for 24 hours. The cells were then subjected to 0.4mM Palmitic acid in 1% Fatty acid free BSA or 1% Fatty acid free BSA only as control. Western blot were performed on the cell probing for PRDX2 (1:1000), cleaved caspase 3 (1:1000) and GAPDH (1:20,000). (B) Histogram representing relative average cleaved caspase 3 for palmitic acid treated MIN6 cells n=3 *p<0.05 **p<0.01
Figure 16: Knockdown of PRDX2 exaggerates cytokine cocktail and streptozotocin induced cleaved caspase 3 expression

(A) MIN6 cells were transfected similar to Fig. 15. The cells were then subjected to cytokine cocktail (10ng/mL IL-1β, 50ng/mL TNFα and 50ng/mL IFNγ) or 1mg/mL STZ with serum free medium as control. The protein from these cells were then collected and subjected to western blot probing for PRDX2 (1:1000), cleaved caspase 3 (1:1000) and GAPDH (1:20,000). (B) Histogram representing relative average cleaved caspase 3 for Cytokine cocktail and STZ treated MIN6 cells n=3, *p<0.05, **p<0.01
Chapter 4: Discussion

Pancreatic β-cell dysfunction and excessive loss of pancreatic β-cells are the primary causes T1DM and T2DM [129]. Studies have shown that ROS are a major contributor to increased β-cell apoptosis. Multiple different inducers of β-cell apoptosis, such as cytotoxic cytokines in T1DM, glucolipotoxicity in T2DM and diabetes inducing chemicals like streptozotocin, all partake in the production of various ROS or RNS. They act partially through oxidative stress to induce β-cell dysfunction and apoptosis and contributes to the loss of β-cell mass.

Diabetic mice as well as patients with diabetes have been found to have elevated production of ROS and decreased antioxidant function in the β-cells and islets [60;110]. β-cells exhibit low levels of glutathione peroxidase, catalase and superoxide dismutase even under normal glucose conditions. Due to lower expression of major antioxidants within the β-cells [79], pancreatic β-cells are particularly vulnerable to the detrimental effects of ROS mediated cellular damage and death compared with other cell types.

PRDX are a family of antioxidants which are expressed ubiquitously. PRDX expression in β-cells has been found to correlate with oxidative stress [8]. In this study I sought to investigate whether PRDX2 is able to prevent β-cell damage and apoptosis caused by oxidative stress inducing agents that are relevant to T1DM and T2DM.

Previously, the group of Bast et al. have detected the expression of PRDX1 and PRDX2 in mouse pancreatic β-cells. Consistent with their results, we found that PRDX2 is expressed in α- and β-cell lines, pancreatic islets and whole pancreas using RT-PCR and western blot (Fig. 3).
The objective of this study was to examine, if any, the protective effect of PRDX2 on β-cell toxicity induced by oxidative stress agents. To establish a relationship between PRDX2 and ROS induced β-cell toxicity, several β-cell oxidative stress inducers were used including proinflammatory cytokines, palmitic acid and STZ to treat the β-cell line MIN6. As shown, treatment with all three inducers led to an increase in β-cell apoptosis and death as determined by increased cleaved caspase 3 expression levels in a dose or time dependant fashion. It is also found that this increase in toxicity is correlated with a decrease in endogenous PRDX2 expression (Fig. 4, 5, 6). This correlation suggests that there is potentially a cause and effect relationship between PRDX2 expression and β-cell toxicity and apoptosis which implies that PRDX2 could be an important factor for the survival of pancreatic β-cells during periods of oxidative stress. This data actually contradicts previous findings by Bast et al., who found that endogenous PRDX2 expression increased following STZ treatment both on the RNA transcript as well as the protein level in their study [8]. This conflict could be due to the differences between our experimental protocols. Bast et al. allowed the β-cells to recover for 24 hours after STZ treatment before they were harvested whereas the experiments performed in this study was designed to examine the effect of STZ treatment on endogenous PRDX2 levels, therefore the cells were harvested immediately after treatment with STZ. Our results, when taken together, illustrate a potential temporal element in the expression and regulation of PRDX2 during oxidative stress. They reflect the possibility that PRDX2 levels are decreased by the onset of oxidative stress, however if the β-cell survives, PRDX2 expression is up regulated to increase the chance of survival of the β-cell through increased ROS clearance and possibly prepares the β-cell for future oxidative stress.
I rationalized if PRDX2 can indeed protect β-cells from oxidative stress, overexpression of PRDX2 in β-cells should be able to prevent or ameliorate the damage induced by oxidative stress and reduce the number of β-cell apoptosis or death under oxidative stress conditions. To test this, PRDX2 or GFP was transiently overexpressed in MIN6 cells by transfection of a vector encoding PRDX2. Following transfection, the cells were then treated with proinflammatory cytokines, palmitic acid or STZ. Using western blot analysis, immunocytochemistry, as well as FACS, it was found that the cells which were transfected to elevate PRDX2 expression had significantly decreased apoptosis and/or cell death when compared with cells which were transfected with GFP only (Fig. 7-14.)

Although the immunocytochemistry of apoptotic cells overexpressing GFP or PRDX2 are not significant when treated with PA (Fig 8), it is showed that there is a trend towards decreased number of apoptotic cells for the PRDX2 transfected group. Further repetition of this experiment may yield significant results as in figures 11 and 14.

The western blot in figure 12 shows that when treated with STZ, PRDX2 expression actually increased in the GFP transfected group. This could be due to the fact that the transfection itself is damaging to the β-cells, thus as proposed before, PRDX2 expression is up regulated as a coping mechanism. This inconsistency does not appear in figures 7 and 10 because the β-cells were treated for 24 hours with cytokines or PA as compared to 4 hours only with STZ in figure 12. Due to this difference in treatment time, the STZ treated cells were allowed to incubate in medium containing 10% FBS for 19 hours longer than the other 2 groups after transfection. This protocol reflects the
experiments performed by Bast et al. and the results were similarly different from those in figure 6.

There has been no previous literature directly linking PRDX2 to the prevention of β-cell death through physiologically relevant methods, and as such, the mechanisms involved in its beneficial effects are unclear. However, it has been shown in a recent study that PRDX3 is able to prevent cytokine (IL-1β, TNF-α and IFN-γ) induced apoptosis in β-cells through the inhibition of iNOS [131]. iNOS activation has been well documented to be related to the activation of NF-κB [3]. In a recent study, Yang S. et al. reported that PRDX2 is able to prevent apoptosis in granulosa cells through regulation of the IκB and NF-κB pathway, [134], and it has been shown that PRDX2 has an inhibitory effect on the Ras-ERK-NF-κB pathway since deletion of PRDX2 leads to Ras-ERK-NF-κB activation [57]. It would be of interest to further investigate whether or not PRDX2 has a direct role in the regulation of the NF-κB pathway in β-cells.

Palmitic acid does not use the NF-κB/iNOS pathway to induce apoptosis in β-cells but through the induction of oxidative ER stress [30]. It is unclear how PRDX2 is able to inhibit palmitic acid induced apoptosis. It could be due to the clearance of ROS produced in the ETC by PRDX2 to reduce the amount of stress placed on the ER which is the driving force behind glucomlipotoxicity of β-cells. PRDX4, which is only expressed in the ER, has been found to protect cells from ER stress induced apoptosis through the removal of H₂O₂ [123]. It is possible that PRDX2 is able to protect β-cells through a similar mechanism.

It is conceivable that if PRDX2 indeed plays a role in the prevention of proinflammatory cytokines and palmitic acid induced β-cell apoptosis or death, ablation
of PRDX2 should be able to abolish this protective effect. I thus performed experiments to knockdown PRDX2 expression using transient transfection of siRNA in MIN6 cells. These cells were then treated with the oxidative stress agents including proinflammatory cytokines, palmitic acid and STZ. Immunoblot of protein harvested from the treated cells showed that cleaved caspase 3 expression is increased in cells with PRDX2 knockdown compared to scrambled siRNA controls, (Fig 15, 16) suggesting that a loss of PRDX2 in β-cells increases and the numbers of β-cell apoptosis induced by cytokines, palmitic acid and STZ. The β-cells with PRDX2 knockdown displayed increased β-cell apoptosis even at basal levels. These results support the data in the overexpression experiments, and suggest that PRDX2 is responsible for the protection of β-cells against oxidative stress induced toxicity and apoptosis.

4.1 Conclusions

This study has shown that PRDX2 is expressed in the pancreatic β-cells and that overexpression of PRDX2 in the pancreatic β-cell line MIN6 is able to decrease β-cell apoptosis induced by oxidative stress stimulating agents such as proinflammatory cytokines, palmitic acid, and streptozotocin. These results suggest that PRDX2 plays a protective role in the pancreatic β-cells and it is able to enhance β-cell survival when threatened with increased oxidative stress. To verify this assumption, PRDX2 was knocked down in MIN6 cells. As shown, the protective effect seen in the overexpression experiments was abolished under oxidative stress conditions. The ablation of PRDX2 also increased the β-cell apoptosis even under basal conditions. These results suggest that
PRDX2 is important for the survival of pancreatic β-cells during periods of oxidative stress.

4.2 Future directions

With the current findings, PRDX2 is shown to attenuate toxicity in the β-cells. The toxicity has been assumed to be from ROS induced stress. However, the amount of oxidative stress brought on by these oxidative stress inducers has not been measured. Therefore, one of the key experiments which should be performed is the measurement of the production of ROS in the β-cells following treatment with oxidative stress inducers. This could be accomplished through kits which use fluorogenic probes to detect oxidative stress within target cells using fluorescence microscopy [103]. This could better elucidate the relationship between PRDX2 expression and regulation with the oxidative stress experienced by the β-cells.

To further verify the overexpression and knockdown data, it would be of interest to see if a rescue experiment involving overexpression of PRDX2 following knockdown would be able to reduce the toxicity seen in the siRNA experiments.

PRDX3 and PRDX4 have been shown to be able to prevent β-cell apoptosis through inhibiting the induction of iNOS and NF-kB in T1DM models. [36;131] A recent study also showed that PRDX2 is able to inhibit granulosa cell apoptosis through modulation of the NF-kB pathway [134]. This raises the possibility that PRDX2 could act through the same pathway to protect β-cells from dysfunction and apoptosis. It would be interesting to establish a signaling pathway, if any, in which PRDX2 can influence β-cell survival and death.
Several studies in the past have linked the ability for PRDX1 to prevent apoptosis with the apoptosis signal-regulating kinase (ASK-1) pathway. [42;70] A study by Hu X. et al., published in 2011, found that PRDX2 is able to prevent apoptosis in dopaminergic neurons in a Parkinson’s disease model through the ASK-1 pathway. [64] This opens up the possibility that PRDX2 could potentially be acting through the ASK-1 pathway to protect β-cells against apoptosis which are not caused by oxidative stress which would explain figure 7, 10 and 12, where basal cleaved caspase-3 activity is reduced in PRDX2 overexpressed cells compared with control and figure 15-16 where basal cleaved caspase-3 expression is increased in PRDX2 knockdown cells. ASK-1 activity is highly related to thioredoxins [135] which raises the possibility that PRDX2 could affect ASK-1 activity through thioredoxin. It would be of interest to elucidate if there are any relationships between PRDX2 and the ASK-1 pathway in β-cells.

An in vivo model of PRDX2 overexpression localized to the β-cells with a tet-on and Cre/loxP recombinant system could clarify the potential role of PRDX2 have during the onset of T1DM and T2DM, and whether overexpression of PRDX2 could prevent or reverse the loss of β-cell mass through amelioration of oxidative damage induced β-cell apoptosis.
Chapter 5: Reference List


