MECHANISMS OF HIGH GLUCOSE-INDUCED DECREASE IN β-CELL FUNCTION

By

Christine Tang

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physiology
University of Toronto

© Copyright by Christine Tang (2010)
Title: Mechanisms of high glucose-induced decrease in β-cell function
Degree: PhD
Year of Convocation: 2010
Full Name: Christine Tang
Department: Physiology, University of Toronto

General Abstract
Chronic hyperglycemia, a hallmark of type 2 diabetes, can decrease β-cell function and mass (β-cell glucotoxicity); however, the mechanisms are incompletely understood. The objective was to examine the mechanisms of β-cell glucotoxicity using in vivo and ex vivo models. The hypothesis is that oxidative stress plays a causal role in high glucose-induced β-cell dysfunction in vivo via pathways that involve endoplasmic reticulum (ER) stress and JNK. The model of β-cell glucotoxicity was achieved by prolonged i.v. glucose infusion (to achieve hyperglycemia).

In Study 1, 48h glucose infusion increased total and mitochondrial superoxide levels in islets, and impaired β-cell function in vivo and ex vivo. Co-infusion of the superoxide dismutase mimetic Tempol decreased total and mitochondrial superoxide, and prevented high glucose-induced β-cell dysfunction in vivo and ex vivo. These results suggest that increased superoxide generation plays a role in β-cell glucotoxicity.

In Study 2, 48h glucose infusion increased activation of the unfolded protein response (XBP-1 mRNA splicing and phospho-eIF2α levels). This was partially prevented by Tempol. Co-infusion of the chemical chaperone 4-phenylbutyrate with glucose decreased spliced XBP-1 levels, and prevented high glucose-induced β-cell dysfunction in vivo and ex vivo. Co-infusion of 4-phenylbutyrate also decreased total and mitochondrial superoxide induced by high glucose. These results suggest that 1) ER stress plays a causal role in high glucose-induced β-cell dysfunction, and 2) there is a link between oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo.
In Study 3, JNK inhibition using the inhibitor SP600125 in rats or JNK-1 null mice prevented high glucose-induced β-cell dysfunction \textit{ex vivo} and \textit{in vivo}. SP600125 prevented high-glucose-induced β-cell dysfunction without decreasing total and mitochondrial superoxide levels. Both Tempol and 4-phenylbutyrate prevented JNK activation induced by high glucose. These results suggest a role of JNK activation in high glucose-induced β-cell dysfunction downstream of increased superoxide generation and ER stress \textit{in vivo}.

Together, the results suggest that 1) oxidative stress, ER stress and JNK activation are causally involved in β-cell glucotoxicity, and 2) High glucose-induced oxidative stress and ER stress are linked, and both impair β-cell dysfunction via JNK activation \textit{in vivo}.
Acknowledgements

First and foremost, I would like to give a big THANK YOU to my supervisor Dr. Adria Giacca for all your guidance, advices and support throughout my studies. I would like to thank you for giving me the opportunity to experience research firsthand as an inexperienced summer student, and later inspiring me to pursue my doctoral studies. Your patience and understanding have allowed me to grow both as a graduate student and as a person. You are not only my mentor, but also my good friend. THANK YOU for everything!

I am extremely grateful to my supervisory committee members Drs. Catharine Whiteside and Minna Woo, and to our collaborators Drs. Allen Volchuk and Michael Wheeler. Your advices, inputs and criticisms were critical in bringing my projects to fruition. Thank you for all your time and patience during my research years. Your help is greatly appreciated!

A special thanks to our amazing technician Loretta Lam, not only for her technical support throughout the years, but also for her encouragement and advice. Thank you for your continuous support despite the fact that I have made you angry on numerous occasions. You truly are my mom in the lab.

To Danna Breen, June Guo, Khajag Koulajian, Andrei Oprescu, Edward Park, Sandra Pereira, Cristina Dirlea, and Jiwan Dhaliwall, my colleagues and true friends. Thank you for all your support throughout the years! All of you have made these few years in the lab very memorable and fun. Your friendship will be cherished forever.

I would also like to thank my parents, and my sisters Jenny and Margaret, for your continuous and unconditional love, encouragement and support over my years.
Last but not least, I would like to thank the love of my life and best friend Steven, for always being there for me. Your advice and support, both in and out of the lab, are greatly appreciated. I cannot thank you more for “planting the seed”. I don’t know how you do it, but you always have a way to make me achieve more than I think possible. THANK YOU for all your encouragement!
# Table of Contents

1. **Introduction** ................................................................................................................................. 1

1.1. **Diabetes Mellitus** ....................................................................................................................... 1

1.1.1. Type 1 Diabetes Mellitus ............................................................................................................. 1

1.1.2. Type 2 Diabetes Mellitus ............................................................................................................. 2

1.2. **Hyperglycemia and Type 2 Diabetes** .......................................................................................... 2

1.2.1. Hyperglycemia and Insulin Resistance ....................................................................................... 3

1.3. **Hyperglycemia and β-Cell Dysfunction** .................................................................................... 6

1.3.1. Acute Effects of Glucose on Insulin Secretion ............................................................................. 6

1.3.2. Insulin Secretory Abnormalities in Individuals with Type 2 Diabetes ......................................... 9

1.3.3. Chronic Effect of High Glucose on β-Cell Function and Mass .................................................. 9

1.3.4. Level of Impairment of β-Cell Function by Chronic Glucose Exposure ................................. 10

1.3.4.1 Insulin Gene Transcription ....................................................................................................... 10

1.3.4.2. Insulin Biosynthesis ............................................................................................................... 12

1.3.4.3. ATP Production .................................................................................................................... 13

1.3.4.4. Late Stages of Insulin Secretion ............................................................................................... 15

1.3.4.5. β-Cell Mass .......................................................................................................................... 15

1.4. **β-Cell Glucotoxicity, Lipotoxicity, and Glucolipotoxicity** ......................................................... 16

1.4.1. Obesity and β-Cell Lipotoxicity ................................................................................................. 17

1.4.2. β-Cell Glucolipotoxicity ........................................................................................................... 18

1.5. **Reactive Oxygen Species and Oxidative Stress** ........................................................................ 21

1.5.1. Reactive Oxygen Species Derived from Chronic Glucose Exposure ........................................ 21

1.5.2. Sites of Reactive Oxygen Species Generation ............................................................................ 21

1.5.3. Oxidative Stress, Type 2 diabetes and β-Cell dysfunction ......................................................... 24

1.5.4. Evidence for a Role of Oxidative Stress in β-Cell Glucotoxicity and Lipotoxicity .................... 25

1.5.5. Cellular Sites of Oxidative Stress-Induced Impairment of β-Cell Function by Glucotoxicity .......... 26

1.5.5.1. Glucose Oxidation and Uncoupling Protein 2 ........................................................................ 26
1.5.5.2. Insulin Gene Transcription and Insulin Biosynthesis ........................................... 27

1.5.6. Downstream signaling mechanisms of oxidative stress-induced impairment of β-cell function by glucotoxicity ............................................. 28

1.5.6.1. C-jun N-terminal Kinase .................................................................................. 28

1.5.6.2 Nuclear Factor kappa B (NFκB) ........................................................................ 30

1.5.6.3. β-Cell Insulin Resistance ............................................................................... 30

1.5.6.4. Endoplasmic Reticulum (ER) stress ................................................................. 31

1.5.7. Oxidative Stress and β-Cell Lipotoxicity ............................................................... 32

1.6. Summary ................................................................................................................. 34

1.7. Rationale and Significance of the Studies ................................................................. 36

1.8. General Hypothesis ................................................................................................. 38

1.9. Specific Aims ............................................................................................................ 38

2. General Methods ......................................................................................................... 39

2.1. Procedures .............................................................................................................. 39

2.1.1. Experimental Animal Model and Surgical Procedures ......................................... 39

2.1.1.1. Animals .......................................................................................................... 39

2.1.1.2. Surgery .......................................................................................................... 39

2.1.2. Infusion Period .................................................................................................... 40

2.1.3. Islet Isolation ....................................................................................................... 40

2.1.4. Evaluation of β-Cell Function Ex vivo ................................................................ 41

2.1.5. Islet ROS, total and mitochondrial superoxide .................................................... 42

2.1.5.1. Dihydro-dichlorofluorescein diacetate ............................................................. 42

2.1.5.2. Hydroethidine and MitoSOX ............................................................................ 42

2.1.6. Western Blotting .................................................................................................. 43

2.1.7. Two-step hyperglycemic clamp ........................................................................... 44

2.1.8. Evaluation of GSIS and β-cell Function In vivo .................................................... 45

2.2. Laboratory Methods ................................................................................................. 45

2.2.1. Plasma Glucose .................................................................................................... 45

2.2.2. Plasma Insulin Assay .......................................................................................... 46

2.2.3. Plasma C-peptide assay ...................................................................................... 47
2.3. Calculations ........................................................................................................... 48
  2.3.1. Insulin Clearance ......................................................................................... 48
  2.3.2. Insulin Sensitivity Index (M/I) ................................................................. 48
  2.3.3. Disposition Index ........................................................................................ 48

3. Study 1 .................................................................................................................. 50
  3.1. Abstract .......................................................................................................... 51
  3.2. Introduction .................................................................................................... 52

3.3. Materials and Methods ..................................................................................... 54
  3.3.1. Animals and Surgery .................................................................................. 54
  3.3.2. Ex vivo Studies ............................................................................................ 54
    3.3.2.1. 48h Infusions .......................................................................................... 54
    3.3.2.1.1. Groups and Treatments ..................................................................... 54
    3.3.2.1.2. Pancreatic Islet Isolation and Ex vivo Evaluation of GSIS ............... 55
    3.3.2.1.3. Islet ROS, total and mitochondrial superoxide ................................... 55
    3.3.2.1.4. β-Cell Apoptosis .............................................................................. 55
    3.3.2.2. 96h Infusions .......................................................................................... 56
  3.3.3. In vivo Studies ............................................................................................... 56
    3.3.3.1. Groups and Treatments ....................................................................... 56
    3.3.3.2. Two-step hyperglycemic clamp ............................................................. 56
  3.3.4. Islet Morphology .......................................................................................... 57
  3.3.5. Plasma Assays ............................................................................................. 57
  3.3.6. Calculations .................................................................................................. 57
  3.3.7. Statistics ...................................................................................................... 58

3.4. Results ............................................................................................................... 59
  3.4.1. 48h infusions ............................................................................................... 59
    3.4.1.1. Glucose Stimulated Insulin Secretion in Freshly Isolated Islets .......... 59
    3.4.1.2. Reactive Oxygen Species in Islets ....................................................... 60
    3.4.1.3. Total and Mitochondrial Superoxide Levels in Islets ......................... 60
    3.4.1.4. β-Cell Apoptosis ................................................................................ 60
    3.4.1.5. Islet Morphology ................................................................................ 61
    3.4.1.6. Two-Step Hyperglycemic Clamp ........................................................ 61
3.4.2. 96h Infusions .................................................................62

3.5. Discussion ...........................................................................64

4. Study 2 ......................................................................................80

4.1. Abstract ................................................................................81

4.3. Material and Methods ............................................................83

4.3.1. Animals and Surgery ..........................................................83

4.3.2. 48h Infusion Period ...............................................................83

4.3.2.1. 48h Infusion of Tempol ......................................................83

4.3.2.2. 48h Infusion of PBA ..........................................................84

4.3.2.3. Pancreatic Islet Isolation and Ex vivo Evaluation of glucose stimulated Insulin Secretion (GSIS) ................................................84

4.3.2.4. RNA Isolation and Real Time Polymerase Chain Reaction ................................................84

4.3.2.5. Measurement of XBP-1 mRNA Splicing ................................85

4.3.2.6. Western Blot Analysis .........................................................85

4.3.2.7. Total and Mitochondrial Superoxide .....................................85

4.3.2.8. Immunohistochemistry ......................................................85

4.3.2.9. Two-Step Hyperglycemic Clamp ..........................................86

4.3.3. Ninety-six Hours Infusions ...................................................86

4.3.4. Plasma Assays .....................................................................87

4.3.5. Calculations .........................................................................87

4.3.6. Statistics .............................................................................87

4.4. Results .....................................................................................88

4.4.1. 48h Infusions .......................................................................88

4.4.1.1. Hyperglycemia, Oxidative Stress and Activation of the Unfolded Protein Response ........88

4.4.1.2. Effect of Chemical Chaperone 4-Phenylbutyrate on High glucose-induced β-Cell Dysfunction ex vivo .........................................................89

4.4.1.3. 4-Phenylbutyrate and Activation of the Unfolded Protein Response .................................90

4.4.1.4. Total and Mitochondrial Superoxide Levels in Islets ........................................................90

4.4.1.5. Two-Step Hyperglycemic Clamp ........................................91

4.4.1.6. Interleukin-1β mRNA expression ..........................................92

4.4.2. 96h Infusions .......................................................................92
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4EBP-1</td>
<td>Eukaryotic translation initiation factor 4E binding protein 1</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>Bip</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCF-DA</td>
<td>Dichlorofluorescein Diacetate</td>
</tr>
<tr>
<td>DI</td>
<td>Disposition Index</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic initiation factor 2 alpha</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>eIF4G</td>
<td>Eukaryotic initiation factor 4G</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERO1</td>
<td>Endoplasmic reticulum oxidoreductin 1</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead transcription factor 1</td>
</tr>
<tr>
<td>GFAT</td>
<td>Glucosamine-fructose-6-phosphate aminotransferase</td>
</tr>
<tr>
<td>Ginf</td>
<td>Glucose infusion rate</td>
</tr>
<tr>
<td>GLUT2</td>
<td>Glucose transporter 2</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose stimulated insulin secretion</td>
</tr>
<tr>
<td>H-TAU</td>
<td>High dose taurine</td>
</tr>
<tr>
<td>HG</td>
<td>High glucose</td>
</tr>
<tr>
<td>IAPP</td>
<td>Islet amyloid polypeptide</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
</tr>
<tr>
<td>IKK</td>
<td>IκBα kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IRE-1</td>
<td>Inositol requiring enzyme 1</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IκBα</td>
<td>Inhibitor of κBα</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun NH₂-terminal kinase</td>
</tr>
<tr>
<td>KATP</td>
<td>ATP sensitive potassium channel</td>
</tr>
<tr>
<td>KRBH</td>
<td>Krebs Ringer buffer with HEPES</td>
</tr>
<tr>
<td>L-TAU</td>
<td>Low dose taurine</td>
</tr>
<tr>
<td>LC-CoA</td>
<td>Long-chain coenzyme A</td>
</tr>
<tr>
<td>M-TAU</td>
<td>Middle dose taurine</td>
</tr>
<tr>
<td>M/I</td>
<td>Sensitivity Index</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MKP</td>
<td>Mitogen-activated protein kinase phosphatase</td>
</tr>
<tr>
<td>MitoSOX</td>
<td>Mitochondrial superoxide dye</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------------------------------------------</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate hydrogen</td>
</tr>
<tr>
<td>PBA</td>
<td>4-phenybutyrate</td>
</tr>
<tr>
<td>PDX-1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PERK</td>
<td>dsRNA-activated protein kinase-like ER kinase</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidyserine</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>Px</td>
<td>Pancreatectomized</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology2</td>
</tr>
<tr>
<td>SI</td>
<td>Sensitivity index</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SP</td>
<td>SP600125</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Sterol regulatory element-binding protein 1</td>
</tr>
<tr>
<td>TAU</td>
<td>Taurine</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with tween 20</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TPO</td>
<td>Tempol</td>
</tr>
<tr>
<td>UCP-GlcNAc</td>
<td>Uridine diphosphate N-acetylglucosamine</td>
</tr>
<tr>
<td>UCP2</td>
<td>Uncoupling protein 2</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>XBP-1</td>
<td>X-box binding protein 1</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker Diabetic Fatty</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1. Blood glucose levels during the 48h infusion period</td>
<td>71</td>
</tr>
<tr>
<td>Table 3.2. β-Cell Morphology at the end of 48h infusion period</td>
<td>72</td>
</tr>
<tr>
<td>Table 4.1. Blood glucose levels during the 48h infusion period</td>
<td>98</td>
</tr>
<tr>
<td>Table 5.1. Blood glucose levels during the 48h infusion period in rats</td>
<td>129</td>
</tr>
<tr>
<td>Table 5.2. Blood glucose levels during the 96h infusion period in mice</td>
<td>130</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1. Stimulation of insulin release by glucose</td>
<td>8</td>
</tr>
<tr>
<td>Figure 1.2. Accumulation of fatty acid esterification products under glucolipotoxic conditions</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.3. Possible sources of ROS production induced by chronic hyperglycemia</td>
<td>23</td>
</tr>
<tr>
<td>Figure 1.4. Mechanisms of glucose-induced β-cell dysfunction that involve oxidative stress</td>
<td>35</td>
</tr>
<tr>
<td>Figure 3.1. Effects of taurine, N-acetylcysteine and tempol on glucose infusion rate (Ginf)(A), insulin (B), C-peptide (C), sensitivity index (D), and disposition index (E), at the end of 48h glucose infusion period</td>
<td>73</td>
</tr>
<tr>
<td>Figure 3.2. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on insulin secretion in freshly isolated islets. Effects of hyperglycemia and tempol on islet insulin content (D)</td>
<td>74</td>
</tr>
<tr>
<td>Figure 3.3. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on islet ROS as detected by dihydro-dichlofluorescein diacetate</td>
<td>75</td>
</tr>
</tbody>
</table>
Figure 3.4. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on total islet superoxide levels as detected by hydroethidine………………………………………76

Figure 3.5. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on islet mitochondrial superoxide levels as detected by MitoSOX……………………………………77

Figure 3.6. Effects of tempol on plasma glucose levels (A), glucose infusion rate (Ginf) (B), plasma insulin levels (C), plasma C-peptide levels (D), sensitivity index (E), and disposition index (F) during the two-step hyperglycemic clamp with or without glucose infusion………...78

Figure 3.7. Effects of taurine and tempol on insulin secretion in freshly isolated islets following 96h hyperglycemia………………………………………………………………………………79

Figure 4.1. 48h Hyperglycemia Induces Activation of UPR in an Oxidative Stress Dependent Manner…………………………………………………………………………..99

Figure 4.2. Effects of Hyperglycemia and Tempol on Markers Downstream of UPR Activation…………………………………………………………………………………………100

Figure 4.3. Effects of Tempol and 4-Phenylbutyrate on GRP78 and GRP94 Levels in Pancreatic Tissue Sections at the End of 48h Glucose Infusion…………………………………………………………101

Figure 4.4. Effects of 48h Hyperglycemia on Polyubiquinated Protein Levels in Pancreatic Tissue Sections………………………………………………………………………………102
Figure 4.5. Effects of Hyperglycemia and 4-Phenylbutyrate on insulin secretion in freshly isolated islets (A) and on islet insulin content (B) following 48h infusion. .......................... 103

Figure 4.6. 4-Phenylbutyrate Decreases Activation of the IRE-1 Pathway .......................... 104

Figure 4.7. Effects of Hyperglycemia and 4-Phenylbutyrate on Markers Downstream of UPR Activation ........................................................................................................ 105

Figure 4.8. Effects of Hyperglycemia and 4-Phenylbutyrate on Total and Mitochondrial Superoxide Levels in Freshly Isolated Islets .......................... 106

Figure 4.9. 4-Phenylbutyrate Prevents Glucose-Induced β-Cell Dysfunction in vivo Assessed Using the 2-Step Hyperglycemia Clamp ................................................................. 107

Figure 4.10. Effects of hyperglycemia and 4-Phenylbutyrate on insulin secretion in freshly isolated islets (A) and on islet insulin content (B) following 96h infusion .................. 108

Figure 5.1. Effects of hyperglycemia and SP on phosphorylated c-jun levels .......................... 131

Figure 5.2. Effects of hyperglycemia and SP600125 on insulin secretion in freshly isolated islets (A), and on islet insulin content (B) ........................................................................................................ 132

Figure 5.3. Effects of SP on plasma glucose levels (A), glucose infusion rate (Ginf) (B), plasma insulin levels (C), and plasma C-peptide levels during the two-step hyperglycemic clamp with or without glucose infusion ........................................................................................................ 133
Figure 5.4. Effects of SP on insulin clearance index (A), sensitivity index (B), and disposition index (C) during the two-step hyperglycemic clamp with or without glucose infusion. 134

Figure 5.5. Effects of hyperglycemia and SP600125 on Total and Mitochondrial Superoxide Levels in Freshly Isolated Islets. 135

Figure 5.6. Effects of hyperglycemia and the superoxide dismutase mimetic TPO on phosphorylated JNK protein levels. 136

Figure 5.7. Effects of hyperglycemia and PBA on phosphorylated c-jun level. 137

Figure 5.8. Effects of hyperglycemia on insulin secretion in wildtype or JNK-1 null mice (A). Panel (B) shows the effect of hyperglycemia on islet insulin content. 138

Figure 5.9. Plasma glucose (A), glucose infusion rate (Ginf) (B), plasma insulin levels (C), and plasma C-peptide levels during the one-step hyperglycemic clamp in mice. 139

Figure 5.10. Insulin clearance index (A), sensitivity index (B), and disposition index (C) during the one-step hyperglycemic clamp in WT or JNK-1 null mice. 140

Figure 6.1. Possible mechanisms involved in the impairing effect of high glucose on β-cell function. 143
Manuscripts Arising from Completion of this Thesis


Permission to reproduce portions of the above manuscript has been obtained from the copyright owners.


Permission to reproduce portions of the above book chapter has been obtained from the copyright owners.


Introduction

1.1. Diabetes Mellitus

Diabetes Mellitus is a disease characterized by hyperglycemia, and is due to inadequate insulin secretion, absolutely or in response to insulin resistance. There are many types of diabetes. The majority of individuals with diabetes have type 2 diabetes (1), which is due to defective insulin secretion in relation to a decrease in insulin sensitivity.

1.1.1. Type 1 Diabetes Mellitus

Type 1 diabetes accounts for ~10% of all cases of diabetes mellitus (2). It is characterized by autoimmune destruction of pancreatic β-cells, which leads to severe insulin deficiency, and eventually hyperglycemia. Type 1 diabetes usually presents during childhood or adolescence, although it may also develop much later in life (2). A genetic link has been established in type 1 diabetes, although it is known to be insufficient to trigger the development of the disease. This theory is supported by the findings that show a concordance rate of 55% for type 1 diabetes in monozygotic twins (3). It has been reported that multiple environmental factors, such as exposure to viruses (such as rubella virus) and toxins, and dietary factors, also play a role in promoting the development of type 1 diabetes (4). The current treatment for type 1 diabetes is insulin therapy. Beta-cell replacement, either by pancreas or islet transplantation, has also been explored as a treatment for type 1 diabetes.
1.1.2. Type 2 Diabetes Mellitus

Over the last century, type 2 diabetes has reached epidemic proportions worldwide, and it has been estimated that the incidence of diabetes would rise to 300 million by 2025 (5). Type 2 diabetes is a multifactoral disease, and its underlying cause and pathogenesis are still not well understood. Studies conducted in monozygotic twins have demonstrated a high concordance rate in type 2 diabetes (3). Furthermore, the wide spectrum of diabetes prevalence observed in different ethnic groups, from the very low levels of 1% observed in some populations to very high levels such as those observed in the Pima Indians suggest that genetic background contributes to susceptibility in this disease (5). To date, 20 loci have been associated with type 2 diabetes (6), with the majority of these associated with decreased insulin secretion (7), due to decreased β-cell function and mass.

In conjunction with genetic predisposition, environmental factors such as physical inactivity, excess nutrient intake, and obesity, can also contribute to the development and progression of this disease (8). Currently, it is thought that type 2 diabetes occurs in genetically predisposed individuals exposed to a series of environmental factors that precipitate the onset of disease.

1.2. Hyperglycemia and Type 2 Diabetes

Chronic hyperglycemia, which is a hallmark of type 2 diabetes, is associated with a number of complications, including vascular and neurological complications (9-11). Decreasing average blood glucose levels has been shown to have profound effects on preventing complications in both type 1 (10) and type 2 diabetes (12). Chronic hyperglycemia can also induce insulin resistance and decrease β-cell function and mass, an effect known as glucotoxicity. This will be reviewed in more detail in this thesis. Although the work of this
thesis is focused on hyperglycemia in the context of type 2 diabetes, the reader should note that hyperglycemia *per se* could also contribute to β-cell dysfunction and loss in type 1 diabetes and islet transplantation (13).

### 1.2.1. Hyperglycemia and Insulin Resistance

Insulin is a hormone that is secreted by the pancreatic β-cells, and is the primary regulator of blood glucose levels. Insulin exerts its cellular effects by binding to the insulin receptor. The insulin receptor belongs to a subfamily of receptor tyrosine kinases that include the insulin-like growth factor (IGF)-I receptor and the insulin receptor-related receptor (14). These receptors consist of two α subunits, which bind insulin, and two β subunits, which are catalytically active. The α and β subunits of the receptor are linked by disulphide bonds to form an α₂β₂ heterotetrameric complex. Binding of insulin to the α subunits of the receptor leads to activation of the intracellular tyrosine kinase domains of the β subunits (15). Once activated, the insulin receptor phosphorylates the tyrosine moiety on a number of intracellular substrates including the insulin-receptor substrate (IRS) proteins (IRS-1/2/3/4), the Shc adaptor protein, Gab 1 p60 dök and Cbl. Upon tyrosine phosphorylation, these proteins interact with signaling molecules containing the SH2 domain, leading to activation of downstream signaling pathways, such as the PI3-kinase and MAP kinase pathways. These signaling pathways act to affect a number of cellular processes such as vesicle trafficking, protein synthesis, gene expression, and enzyme regulation, to regulate glucose, protein and fat metabolism (See (16) for review).

Under normal physiological conditions, insulin stimulates glucose uptake by muscle and fat, and suppresses hepatic glucose production. Furthermore, insulin promotes storage of nutrients in fat, muscle and liver by stimulating lipogenesis, protein and glycogen storage, and inhibition of lipolysis, protein degradation and glycogenolysis. However, when insulin resistance occurs, which characterizes type 2 diabetes, the metabolic effects of insulin require a
higher concentration of insulin. If insulin resistance is combined with inadequate β-cell function (i.e. the amount of insulin secreted is not able to compensate for insulin resistance), hyperglycemia ensues.

The effect of hyperglycemia per se to induce insulin resistance in vivo was first demonstrated by showing that normalization of glucose levels in the partially pancreatectomized rat model using phlorizin (a drug that inhibits tubular absorption of glucose, i.e. normalizes glucose without affecting insulin secretion), improves insulin sensitivity (17). Several mechanisms have been proposed including a role of protein kinase C (18-20), hexosamine (21-23), and oxidative stress (24; 25).

The Protein Kinase C (PKC) family is a group of enzymes that plays a key role in many cellular function (26). There are three subfamilies: 1) The conventional or classical PKC (α, βI, βII and γ), which require diacylglycerol (DAG), calcium and phosphatidylserine (PS) for activation; 2) novel PKC (δ, ε, μ, η, θ), which require DAG and PS, but not calcium for activation; and 3) atypical PKC (ζ, λ), which require only PS for activation. Intracellular hyperglycemia increases DAG primarily by increasing do novo DAG synthesis from the glycolytic intermediate dihydroxyacetone phosphate (27). The latter undergoes reduction to glycerol-3-phosphate and stepwise acylation (28) to generate DAG. PKC can phosphorylate serine residues on the insulin receptor and IRS, thereby inhibiting phosphorylation of tyrosine residues. This can lead to disruption of the insulin signaling cascade and subsequently to insulin resistance (29).

During hyperglycemia, excess intracellular glucose is shunted into the hexosamine pathway. The glycolytic intermediate fructose 6-phosphate is converted to N-acetyl glucosamine-6-phosphate by the enzyme glutamine:fructose-6-phospho amidotransferase (GFAT). Subsequently, N-acetyl glucosamine-6-phosphate is metabolized to UDP-N-
acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc is a substrate used by the enzyme N-acetylglucosamine transferase to catalyze the transfer of GlcNAc to specific serine/threonine residues of proteins via O-linkage. How increased flux through the hexosamine pathway induces insulin resistance is unclear. Many transcription factors, and nuclear/cytoplasmic proteins are dynamically modified by O-linked glycosylation, and may show reciprocal modification to phosphorylation (30). In rats, infusion of glucosamine (activator of the hexosamine pathway) led to increased O-linked glycosylation of IRS-1 and IRS-2 in skeletal muscle (23). This was associated with a reduction in tyrosine phosphorylation of IRS-1 and -2, and insulin resistance to glucose transport. However, it is of note that other studies found impaired PI3-kinase without decreased tyrosine phosphorylation of IRS-1 in glucosamine infused rats (31), and decreased PKB activation without changes in PI3-kinase activation and IRS-1 and -2 tyrosine phosphorylation in 3T3-L1 adipocytes (32). These findings suggest that activation of the hexosamine pathway may induce insulin resistance by O-linked glycosylation of proteins involved in insulin signalling; however the level at which hexosamines affect insulin signaling remains to be clarified. In addition to O-linked glycosylation, hexosamine synthesis has been reported to inhibit insulin action by stimulation of PKC (33; 34) and possibly by the induction of oxidative stress (35).

Oxidative stress, which is present in individuals with type 2 diabetes (see section 1.5.3), has also been implicated in hyperglycemia-induced insulin resistance. Reactive oxygen species can impair insulin action in multiple tissues (36). Antioxidants such as vitamin C (37), vitamin E (38), or lipoic acid (39) improve insulin-mediated glucose utilization partially. Furthermore, our lab has shown that i.v. treatment with the antioxidants taurine or N-acetylcysteine prevents insulin resistance induced by 6h hyperglycemia (40). The exact mechanism by which oxidative stress impairs insulin action is unclear, but may be linked to activation of downstream signaling
pathways such as inflammatory kinases IKK-β and JNK, PKC (41) (which can be activated by oxidative stress (42) in addition to DAG as described above), and other MAPK enzymes other than JNK.

1.3. Hyperglycemia and β-Cell Dysfunction

1.3.1. Acute Effects of Glucose on Insulin Secretion

Under normal physiological conditions, insulin is released by pancreatic β-cells in response to a variety of secretagogues. Glucose is a major regulator of insulin secretion (See Figure 1.1.). Glucose enters the β-cells through the GLUT2 transporters, which are present on the plasma membrane. Once inside the β-cells, glucose is phosphorylated by the rate limiting enzyme glucokinase to become glucose-6-phosphate. Glucose-6-phosphate then enters glycolysis and the Krebs (Tricarboxylic acid (TCA)) cycle to generate ATP. An increase in ATP to ADP ratio stimulates the closure of ATP sensitive potassium (KATP) channels. This triggers the opening of the voltage-dependent calcium channels and increased intracellular cytoplasmic calcium concentration, which subsequently causes exocytosis of insulin containing granules. Glucose stimulates insulin exocytosis by inducing fusion of a readily-releasable pool of insulin granules localized to the plasma membrane, and through the mobilization and trafficking of insulin granules from intracellular storage pools to the membrane. (43)

In addition to KATP dependent pathway, glucose can stimulate insulin secretion through a KATP independent pathway. This ATP independent pathway does not require a change in intracellular calcium and is likely accounted for by direct effects on exocytosis of the increase in ATP/ADP ratio and/or putative “coupling factors” derived from glucose or increased by glucose. One of these coupling factors is long-chain acyl CoA (LC-CoA) (44). Glucose is metabolized through glycolysis to generate pyruvate, which is then converted to citrate in the
mitochondria. Citrate can be oxidized by the Krebs cycle in the mitochondria, or it can be exported to the cytoplasm. In the cytoplasm, citrate can be converted to malonyl-CoA via the sequential action of the enzymes ATP-citrate lyase and acetyl-CoA carboxylase (ACC). Malonyl-CoA is a potent allosteric inhibitor of the mitochondrial membrane enzyme, carnitine palmitoyltransferase-1 (CPT-1), which controls transport of LC-CoA into the mitochondria for oxidation. Thus, malonyl-CoA acts to switch β-cell metabolism from fatty acid oxidation to glucose oxidation. This switch leads to an increase in cytosolic LC-CoA, which can directly stimulate insulin secretion by various mechanisms, including protein acylation (45), and stimulatory effect on protein kinase C (PKC; the majority of PKC isoforms has stimulatory effect on insulin secretion) (46).

In addition to having a direct effect on insulin secretion, glucose can also increase insulin content (which is an important determinant of insulin secretion) by increasing transcription of the proinsulin gene, translation of proinsulin, and conversion of proinsulin to insulin (43).
Figure 1.1. **Stimulation of insulin release by glucose.** Glucose enters the β-cell and is metabolized to produce ATP. The increased ATP/ADP ratio closes the KATP channels, which causes membrane depolarization and activation of the VD (Voltage-Dependent) Ca$^{++}$ channels. The increase in intracellular Ca$^{++}$ concentration [$\text{Ca}^{++}$]$_i$ triggers exocytosis. Exocytosis is also stimulated by glucose metabolism independent of KATP channels and, in part, also independent of ATP.
1.3.2. Insulin Secretory Abnormalities in Individuals with Type 2 Diabetes

In the normal physiological state, a rapid rise in blood glucose levels elicits a biphasic insulin secretory response. The first phase of insulin secretion occurs for 5-10 minutes, followed by the second phase. In patients with type 2 diabetes, this first phase of insulin secretion to glucose is no longer present. However, insulin response to secretagogues other than glucose is relatively unimpaired (47). Multiple insulin secretory defects are present, including absence of pulsatility, excess in prohormone secretion, and progressive decrease in insulin secretory capacity with time. These are due to a reduction in both β-cell function and mass. β-cell mass is reduced because of β-cell death induced by the toxic effects of glucose and fat, by amyloid and other endogenous toxins. (See (48) for review). Amyloid fibers, which are composed mainly of islet amyloid polypeptide (IAPP; a protein co-secreted with insulin), have been reported in up to 90% of subjects with type 2 diabetes compared to 10-13% in subjects with no diabetes (49). The mechanism of formation of these precipitates is still not entirely clear. It not only results from prolonged β-cell overstimulation by hyperglycemia, but is also promoted by inflammation and glucolipotoxicity. Recently, it has been suggested that toxic form of amyloidogenic proteins is not the amyloid fibers, but rather the small IAPP oligomers (50-52), which can induce β-cell apoptosis. The mechanism of how toxic IAPP oligomers induce β-cell death is unclear, but may be due to induction of endoplasmic reticulum stress (53; 54), (although this is still controversial (55)), and oxidative stress (56).

1.3.3. Chronic Effect of High Glucose on β-Cell Function and Mass

As discussed above, acute glucose elevation is a major stimulator of insulin secretion. However, chronic high glucose exposure can impair β-cell function. The idea that chronic high glucose can act as a toxin to cause β-cell dysfunction is not a new one. As early as the 1940’s, it
was demonstrated that chronic glucose injections could cause diabetes in the partially pancreatectomized cat (57). This finding was reproduced in the chronically glucose infused pancreatectomized dogs (58). Since then numerous studies have demonstrated that prolonged glucose elevation can impair β-cell function and mass. This has been demonstrated in vitro (59-64), ex vivo in the perfused pancreas (65; 66) or in freshly isolated islets of glucose infused rats (67; 68). This adverse effect of chronic glucose elevation on β-cell function and mass is known as β-cell glucotoxicity. It is important to note that the toxic effect of prolonged glucose elevation also occurs in humans. For example, Boden et al. demonstrated in humans, that 68 h of glucose elevation can impair β-cell function and induce insulin resistance (69).

1.3.4. Level of Impairment of β-Cell Function by Chronic Glucose Exposure

Chronic glucose exposure can act on multiple levels to impair β-cell function. These include impairment of 1) insulin gene transcription, 2) insulin biosynthesis, 3) ATP production, 4) late stages of insulin secretion, and 5) β-cell mass. In the following sections, the mechanisms of how chronic glucose elevation impairs the β-cell at each of these levels will be reviewed.

1.3.4.1 Insulin Gene Transcription

One of the most well studied mechanism of β-cell glucotoxicity is the impairing effect of chronic high glucose on insulin gene promoter activity. The transcriptional activity of the insulin promoter is mainly regulated by three enhancer elements: A3, E1 and C1. Mutation of these sites leads to markedly impaired insulin gene transcription (70). A3, E1 and C1 are bound by the transcription factors PDX-1, BETA2/NeuroD, and RIPE3b1/MafA respectively. Early studies demonstrating that chronic glucose can affect insulin gene transcription were performed in β-cell lines. Prolonged culture of HIT-T15 cells (a β-cell line) in high glucose led to a significant decrease in insulin gene transcription (71), and this was associated with decreased
activity of the insulin promoter, and decreased binding of PDX-1 and MafA to their respective elements (72-74). In another β-cell line, βTC-6 cells, culturing in high glucose led to decreased insulin gene transcription, but this was associated with only a decrease in MafA binding activity (64). *In vivo* evidence that high glucose impairs insulin gene transcription can be observed in animal models of type 2 diabetes. In the 90% partial pancreatectomized (Px) rat model, which are hyperglycemic, insulin mRNA, insulin content, glucose stimulated insulin secretion (GSIS), and PDX-1 expression are markedly reduced 4 weeks post Px (74). In the Zucker Diabetes Fatty Rat (ZDF rat), a genetic model of type 2 diabetes, onset of diabetes is associated with decreased insulin gene transcription and insulin content (75). Harmon *et al.* demonstrated that treatment with troglitazone in ZDF rat to prevent hyperglycemia improved PDX-1 expression, insulin gene transcription and insulin content (76). The mechanisms involved are unclear, but may be linked to oxidative stress, and subsequent activation of the JNK pathway. This will be discussed in more detail in section 1.5.6.1.

Studies have shown these same transcription factors that are important for insulin gene transcription, are also important in the regulation of other genes. For example, PDX-1 has been shown to regulate GLUT2 and glucokinase (77). Both GLUT2 and glucokinase play an important role in the β-cell’s glucose sensing mechanism, and thus alterations in the expression of these genes could predictably alter GSIS. Indeed, a marked reduction in GLUT2 protein levels has been observed in animal models of type 2 diabetes, such as the Px (74) and ZDF rat (78).

In addition to an effect of glucose on PDX-1 and MafA (as discussed above), chronic glucose is associated with increased expression of the insulin gene transcriptional repressor CCAAT/enhancer-binding protein beta (CEBPβ) (79; 80), and of the proto-oncogene c-myc (81). Increase in both transcription factors can further impair insulin gene transcription. C-myc suppresses insulin gene expression by inhibiting NeuroD-mediated transcriptional activation
(82), and its increase is postulated to reflect a loss of differentiation of β-cells exposed to elevated glucose, which could explain, in part, defective insulin secretion following chronic glucose elevation.

1.3.4.2. Insulin Biosynthesis

Insulin biosynthesis and insulin content are reduced following chronic exposure to high glucose. Although this may be secondary to a decrease in insulin gene transcription (as discussed in 1.3.4.1), there is evidence that chronic glucose elevation can specifically decrease insulin biosynthesis independent of a decrease in insulin gene transcription. One mechanism proposed for this is the role of endoplasmic reticulum (ER) stress. The ER is involved in folding, processing and exporting secretory and membrane proteins to the Golgi apparatus. When the demand for proteins exceeds the folding capacity of the ER, ER stress ensues leading to the activation of unfolded protein response, a mechanism that inhibits global protein synthesis, but increases ER chaperones and degradation of misfolded proteins to counteract ER stress. The same mechanism, when chronically activated, also initiates apoptosis of cells unable to cope with ER stress (83). That ER stress plays a role in diabetes at the β-cell level is suggested by the early destruction of β-cells in Walcott-Ralliston syndrome, which is due to a mutation in PERK, a major player in the unfolded protein response (84). It has been reported that chronic exposure to high glucose elevates ER stress markers in vitro (85-88), suggesting that glucose-induced ER stress can decrease global protein translation and hence insulin biosynthesis. It has been proposed that ER stress in β-cell glucotoxicity is due to increased folding load due to oversecretion of insulin, and/or oxidative stress (see section 1.5.6.4).

Another proposed mechanism to be involved in decreased insulin biosynthesis is the inhibition of the insulin/IGF signaling cascade in the β-cells. It has been reported that insulin release stimulated by glucose can enhance its own biosynthesis and exogenous insulin mimics
the stimulatory effect of glucose on insulin biosynthesis (89-91). A study by Andreozzi et al. showed that both chronic glucose elevation and activation of the hexosamine pathway increase serine phosphorylation of IRS-1 and impair activation of PI3-kinase/AKT/Mammalian Target of Rapamycin (mTOR) via JNK and ERK1/2 activation in a β-cell line (92). Insulin activates mTOR through the PI3-kinase/AKT pathway, which subsequently leads to phosphorylation of 4EBP-1 (eukaryote initiation factor 4E (eIF4E) binding protein). Phosphorylation of 4EBP-1 allows eIF4E to interact with eIF4G, which is critical to initiate translation (93). Therefore, insulin biosynthesis may be decreased by chronic glucose elevation due to impairment in the insulin signaling cascade.

Some argue that the effect of chronic high glucose on β-cell function is not toxic, but rather a reflection of a depletion in insulin stores due to excessive insulin secretion. It is believed that β-cell function can be restored following “β-cell rest”. The term “β-cell exhaustion” has been used to describe this phenomenon. Grill et al. demonstrated that co-infusion of diazoxide, an inhibitor of KATP channels, and hence insulin secretion (β-cell rest), with glucose for 48h in normal rats, prevented glucose induced β-cell dysfunction (94). However, β-cell exhaustion as a cause for glucotoxicity has been challenged by Moran et al. (95). In this study, HIT-T15 cells were cultured chronically with high glucose and somatostatin. Somatostatin effectively inhibited insulin secretion. However, β-cells exposed to high glucose and somatostatin still had impaired insulin gene transcription, content and GSIS, suggesting that β-cell exhaustion is not the primary cause for β-cell glucotoxicity.

1.3.4.3. ATP Production

It is well established that ATP production is essential for GSIS, thus any decrease in ATP production can impair insulin release. ATP production can be decreased by a reduction in
glucose oxidation. However, this is unlikely the main site of impairment as chronic high glucose exposure is only associated with mild reduction in glucose oxidation (60; 61).

Another more plausible mechanism for the decrease in ATP production is the upregulation or activation of uncoupling protein 2 by chronic high glucose exposure. Uncoupling proteins are located in the inner mitochondrial membrane, and can uncouple the electrochemical gradient produced by the electron transport chain from ATP synthesis, and thereby decrease ATP production. ß-cells express uncoupling protein 2 (UCP2), the only member of the UCP family located in the pancreatic islet. It is of note that although upregulation of UCP2 is a potential candidate for ß-cell glucotoxicity, the effect of UCP2 on insulin secretion is still controversial. Over-expression of UCP2 in islets has been shown to decrease insulin secretion (96). One study demonstrated that knockout of UCP2 in mice enhances insulin secretion and decreases plasma glucose (97). This is however recently challenged by another study which showed decreased glucose stimulated insulin secretion in UCP2 knockout mice (98). The discrepancy in the findings have been attributed to the difference in genetic background of the mice used (98).

Recently, a role of UCP2 in ß-cell glucotoxicity has been proposed. However, studies to date investigating UCP2 expression in ß-cell glucotoxicity have yielded conflicting results. In vitro exposure of isolated islets or INS1 cells to high glucose for at least 48h increased (61; 99), decreased (100) or had no effect (101) on UCP2 mRNA and/or protein expression. In vivo, partial pancreatectomized rats (74) and glucose infused rats (102) had increased UCP2 mRNA expression, whereas the hyperglycemic ZDF rats has been reported to have low expression of islet UCP2 mRNA (103). Interestingly, one study by Krauss et al. showed that UCP2 mRNA was unaltered in mouse islets exposed to chronic high glucose. However, UCP2 activity was increased (62), due to activation of UCP2 by superoxide (see section 1.5.5.1.). Therefore, although UCP2 appears to play a role in glucotoxicity, this may not result from changes in UCP2 expression.
1.3.4.4. Late Stages of Insulin Secretion

The majority of studies demonstrate that the impairing effect of chronic high glucose on insulin secretion is specific for glucose but not other secretagogues, suggesting interference with glucose metabolism, or any other downstream mechanisms activated by glucose. The late stages of insulin secretion, i.e. exocytosis, is a common event in insulin secretion by all secretagogues. This suggests that chronic high glucose is unlikely to impair insulin secretion by affecting the exocytosis of insulin granules. Recently, however, it has been suggested that chronic high glucose elevation can affect calcium handling and the late stages of insulin secretion (104-106). In INS-1 cells, a β-cell line, prolonged exposure to high glucose resulted in higher basal calcium levels, but a defect in insulin secretion when stimulated directly with calcium (104). Furthermore, these cells were found to express lower levels of proteins required for calcium-induced exocytosis of insulin, such as SNARE proteins, VAMP-2 and syntaxin 1. VAMP-2 was also reduced in human islets cultured in high glucose for 72 h. In another study in isolated islets, it was found that chronic high glucose reduce insulin secretion by interfering with the exit of insulin via the fusion pore, possibly due to induction of granuphilin by high glucose (105). Granuphilin is a protein important for docking of insulin granules to the fusion machinery. Overexpression of granuphilin has been reported to inhibit insulin secretion (107; 108).

1.3.4.5. β-Cell Mass

It is now well accepted that β-cell mass is constantly changing in response to changes in metabolic demands. In the presence of insulin resistance, for example, β-cell mass increases to compensate for decreased insulin action. β-cell mass is determined by the balance between β-cell regeneration (via replication and neogenesis) and β-cell death (apoptosis, necrosis and autophagy (109)) (110). It has been reported that the frequency of β-cell replication is very low in subjects with or without diabetes, with no difference between the two. However, frequency
of β-cell apoptosis is reported to be significantly higher in pancreatic tissue obtained from patients with type 2 diabetes compared to normal subjects (49). This imbalance between proliferation and apoptosis in type 2 diabetes leads to a reduction in β-cell mass.

Chronic high glucose exposure has been shown to increase β-cell apoptosis in vitro and in vivo. In cultured human islets, exposure to high glucose for 4 days induced apoptosis (111). In the desert gerbil, Psammomys obesus, a shift from low to high calorie intake increased plasma glucose levels, and this was associated with increased β-cell apoptosis (112). This toxic effect of high glucose was reproduced in primary cultured islets from the Psammonmys obesus exposed to elevated glucose concentrations (113). Many of the mechanisms involved in high glucose-induced β-cell apoptosis are similar to those involved in β-cell dysfunction. These include oxidative stress, ER stress, and inflammation, which will be discussed in later sections.

1.4. β-Cell Glucotoxicity, Lipotoxicity, and Glucolipotoxicity

Numerous studies have now demonstrated that, in addition to chronic elevation of glucose, chronic elevation of FFA levels can act to decrease both β-cell function and mass. The term β-cell lipotoxicity have been coined to describe the adverse effect of excessive FFA on the β-cells. The combined toxic effect of glucose and FFA on β-cell function has been termed glucolipotoxicity. The underlying concept is that once type 2 diabetes ensues, the increase in glucose and FFA levels, which are characteristics of diabetes, exerts additional toxic effects on the already dysfunctional β-cells, thus further aggravating the diabetic state. This leads to a vicious positive feedback cycle, deteriorating β-cell function and mass. In the following section, the effect of chronic FFA elevation on β-cell function will be briefly discussed.
1.4.1. Obesity and β-Cell Lipotoxicity

Obesity, which is clinically defined as a body mass index (BMI) of greater than or equal to 30 kg/m\(^2\), is a serious health concern in both industrialized and developing countries around the world. It has been estimated that currently over 1 billion people are overweight (114), and over 300 million (115) are obese worldwide. There is a well-known relationship between obesity and diabetes mellitus. More than 85% of individuals diagnosed with type 2 diabetes are obese (116). However, obesity is neither necessary nor sufficient for the development of type 2 diabetes, as diabetes develops in lean individuals and a high prevalence of obesity is associated with a low prevalence of type 2 diabetes in certain populations (117). Many obese individuals also do not develop diabetes, although obesity plays a role in the pathogenesis of type 2 diabetes, at least in genetically predisposed individuals.

Obesity is associated with insulin resistance. Obese individuals have elevated FFA levels, due to their more expanded (118), and lipolytically active (119) adipose tissue mass. The obesity-associated insulin resistance further increases FFA levels, due to the lack of the antilipolytic action of insulin. The mechanisms proposed for fat-induced insulin resistance are similar to those involved with glucotoxicity (see section 1.2.1), as glucose promotes fat esterification rather than oxidation. These include a role of DAG, PKC, ceramide and oxidative stress. Insulin resistance in obesity can also be due to various adipocyte-derived hormones (i.e. leptin, adiponectin and resistin), and cytokines (TNF-α and IL-6) released from the expanded adipose tissue. (See (120) for review)

Acutely, like glucose, FFA stimulates insulin secretion. In contrast, chronic elevation of FFA (>24 h) impairs GSIS. This has been demonstrated in vitro in islets (61; 121-125) or β-cell lines (126-129) and studies in situ in the perfused pancreas. In vivo, studies on the effect of prolonged FFA elevation on the β-cell function has been more controversial. Absolute GSIS
evaluated in vivo (uncorrected for insulin resistance) was found to be increased (130-132), unchanged (133-135), or decreased (136; 137). However, in most of these in vivo studies, GSIS was not corrected for insulin resistance and failure to do so likely explains the controversial results. It is now generally accepted that “β-cell lipotoxicity” occurs in vivo, when GSIS is corrected for insulin resistance (as chronic FFA elevation also induces insulin resistance). The mechanisms of FFA-induced β-cell dysfunction are not completely clear, but activation of certain isoforms of PKC (-δ (138; 139) and -ε (140)), inflammation, and oxidative stress have been implicated (see (141) for review).

1.4.2. β-Cell Glucolipotoxicity

Elevations of both glucose and FFA concentrations have been reported to act synergistically to impair β-cell function and mass. This is known as glucolipotoxicity. The exact mechanisms of glucolipotoxicity are unclear. One proposed mechanism is the increased generation of intermediate metabolites via elevated FFA esterification. It has been proposed that under circumstances when both glucose and FFA are elevated, cytosolic citrate accumulates (due to increased Krebs cycle activity), which leads to increased generation of malonyl-CoA (citrate is the precursor of malonyl-CoA). Malonyl-CoA inhibits carnitine-palmitoyl-tranferase-1 (CPT-1), which is the enzyme responsible for transport of FFA into the mitochondria. Sustained inhibition of CPT-1 leads to accumulation of long chain fatty-CoA in the cytosol, which is proposed to exert deleterious effects, either directly or via generation of lipid-derived signals such as ceramides, or diacylglycerol (DAG), on β-cell function (See Figure 1.2). DAG has been shown to activate protein kinase C (PKC), in which the isoforms δ (138; 139) and ε (140) have been implicated in β-cell lipotoxicity (the mechanisms involved are unclear, but may include changes in lipid metabolism, insulin content and insulin gene transcription by PKC ε.
activation (140), and mitochondrial dysfunction and β-cell apoptosis by PKC δ). This hypothesis was first proposed by Prentki and Corkey (142).

In addition to switching from FFA oxidation to esterification, prolonged high glucose can activate expression of genes involved in lipogenesis (143). One transcription factor that has been shown to regulate lipogenic gene expression is sterol regulatory element binding protein 1c (SREBP-1c) (144). SREBP-1c expression is elevated in islets of animal models of type 2 diabetes (145), and β-cell specific SREBP-1c transgenic mice have elevated lipogenesis and thus exaggerated lipotoxicity. Studies show that chronic glucose elevation can activate SREBP-1 (146), and dominant negative suppression of SREBP-1c activity protects against β-cell glucolipotoxicity (85).
Figure 1.2. Accumulation of fatty acid esterification products. Under circumstances when both glucose and FFA are elevated, cytosolic citrate accumulates (due to increased Krebs cycle activity), which leads to increased generation of malonyl-CoA (citrate is the precursor of malonyl-CoA) by acetyl-CoA carboxylase (ACC). Malonyl-CoA inhibits carnitine-palmitoyltranferase-1 (CPT-1), which is the enzyme responsible for transport of FFA into the mitochondria. Sustained inhibition of CPT-1 leads to accumulation of long chain fatty-CoA (LCFA-CoA) in the cytosol, which is proposed to exert deleterious effects, either directly or via generation of lipid-derived signals such as ceramides, or diacylglycerol (DAG), on β-cell function.
1.5. Reactive Oxygen Species and Oxidative Stress

1.5.1. Reactive Oxygen Species Derived from Chronic Glucose Exposure

As discussed in the above sections, various mechanisms have been proposed for the deleterious effect of glucose elevation on β-cell function and mass. These include effects of glucose on β-cell enzymes and transcription factors. Recently, it has been found that many of these mechanisms activated by glucose are linked to oxidative stress. Thus, it has been proposed that oxidative stress is the central mechanism of β-cell dysfunction associated with chronic glucose elevation.

Long-term exposure to high glucose can lead to the generation of reactive oxygen species. Endogenous reactive oxygen species (ROS), in low concentrations are important for many physiological processes (147) such as gene transcription and leukocyte function. Recent studies also demonstrate that physiological levels of ROS are important for insulin signaling (148), and normal β-cell function (149). However, when concentrations of ROS reach excessive levels for prolonged periods of time, oxidative stress ensues. β-cells are particularly vulnerable to oxidative stress, as they have intrinsically very low levels of antioxidant enzymes (150; 151). Oxidative stress can directly cause functional damage to proteins, enzymes, DNA and lipids, or can indirectly activate various stress sensitive pathways to alter cellular function (41).

1.5.2. Sites of Reactive Oxygen Species Generation

During normoglycemic conditions, glucose metabolites primarily undergo oxidative phosphorylation in the mitochondria. Oxidative phosphorylation is accompanied by ROS production in minute amounts, which is usually buffered by native antioxidant defense mechanisms such as superoxide dismutase, catalase and glutathione. However, in states of energy excess, such as chronic hyperglycemia, the high electrochemical potential generated by
the proton gradient leads to partial inhibition of Complex III of the electron transport chain. This subsequently results in the generation of superoxide radicals (152). (See Figure 1.3, for possible sites of ROS production by glucose).

In addition to oxidative phosphorylation, there is an overflow of glucose metabolites into alternative pathways during chronic hyperglycemia, which can lead to ROS generation. These include glycosylation, the hexosamine pathway, PKC activation (via DAG production), and glucose autooxidation (41).

Hyperglycemia can also activate certain enzymes to generate ROS. Glucose has been reported to activate NADPH oxidase via a PKC dependent mechanism (153), which can generate cytosolic superoxide. Chronic hyperglycemia can also increase ER oxidoreductin 1 (ERO1) activity due to increased folding of insulin in the endoplasmic reticulum (see section 1.5.6.4.).
Figure 1.3. Possible sources of ROS production induced by chronic hyperglycemia.

This figure is not intended to be all-inclusive.
1.5.3. Oxidative Stress, Type 2 diabetes and β-Cell dysfunction

Studies suggest that individuals with type 2 diabetes are subjected to chronic oxidative stress (154). Markers of oxidative stress damage are elevated in patients with type 2 diabetes (155-157). Low glutathione levels, the primary intracellular antioxidant (158) are also found in erythrocytes of patients with diabetes, a defect ameliorated by improved metabolic control (159). Oxidative stress has been suggested to play a role in β-cell dysfunction. As stated above, pancreatic β-cells have intrinsically low levels of expression and activity of antioxidant enzymes, such as superoxide dismutases (SOD-1 and 2), catalase, and glutathione peroxidases (150; 151) compared to other tissues such as the liver, thus β-cells are particularly vulnerable to oxidative stress. Chronic high glucose (75) can increase ROS generation in β-cells, and oxidative stress can result in marked impairment in GSIS (160-163). Furthermore, many proposed mechanisms of β-cell glucotoxicity are linked to oxidative stress. PKC and hexosamine pathways can induce (35; 164; 165) and are activated by oxidative stress (166-168) and the JNK and IKKβ/NFκB can be activated by oxidative stress (162; 169). Taken together, these findings suggest that high glucose may increase oxidative stress to impair β-cell function.

In animal models of type 2 diabetes, antioxidant treatment has been demonstrated to improve β-cell function. In the Zucker diabetic fatty rat, treatment with either the antioxidants N-acetylcysteine or aminoguanidine increased PDX-1 binding, insulin mRNA levels and insulin content (76). In the db/db mice, N-acetylcysteine was effective in preserving insulin content, insulin mRNA, and PDX-1 protein levels (170). Moreover, treatment with the antioxidant vitamin E had beneficial effects on glycemic control in GK rats, which was accompanied by improvement in insulin secretion and lower levels of HBA1c (171). In humans, daily supplementation with 1.5 grams of antioxidant taurine for 8 weeks had no effect on insulin secretion in patients at high risk for type 2 diabetes (172); however, intravenous infusion of the
antioxidant glutathione in patients with type 2 diabetes improved insulin secretion and glucose tolerance (173). More recently, it has been reported that islets isolated from pancreata of cadaveric organ donors with type 2 diabetes have elevated oxidative stress marker levels as well as low levels of glucose-induced insulin secretion *in vitro*. Exposure to antioxidants significantly improved β-cell function (174).

### 1.5.4. Evidence for a Role of Oxidative Stress in β-Cell Glucotoxicity and Lipotoxicity

Solid *in vitro* data have linked oxidative stress to β-cell glucotoxicity. Prolonged exposure to high glucose has been shown to increase peroxide levels in HIT-T15 cells, rodent and human islets *in vitro* (175). In HIT-T15 cells, a β-cell line, prolong culture in high glucose concentration impaired β-cell function and induced apoptosis, an effect that was prevented by either the antioxidant N-acetylcysteine or aminoguanidine (75). Treatment with either of these antioxidants in HIT-T15 cells preserved insulin promoter activity, PDX-1 binding, and levels of proinsulin mRNA (75). In cultured islets, adenovirally overexpressing the antioxidant enzyme glutathione peroxidase (which catabolizes both hydrogen peroxide and lipid peroxides to hydrogen and water) protected against ribose induced β-cell toxicity (175). Ribose is a more reducing sugar than glucose, and can cause glucotoxicity in a shorter period of time. In another study in cultured islets, overexpression of the catalytic subunit of glutamylcysteine ligase, which regulates the use of cysteine as the rate-limiting substrate to form glutathione (an abundant endogenous antioxidant), increased glutathione levels and protected the islets from oxidative stress (176). Although the majority of studies indicate that oxidative stress is an important mechanism of β-cell glucotoxicity, there are a few studies that argue against this. A study by Martens *et al.* found that oxidative stress was suppressed rather than induced following exposure of primary β-cells to high glucose (177). A more recent study by Khaldi *et al.* has also shown...
that the antioxidants N-acetylcysteine and the superoxide dismutase mimetic/catalase-like manganese(III)tetrakis (4-benzoic acid)porphyrin were ineffective in preventing β-cell dysfunction induced by exposure of isolated rat islets to high glucose for 1 week (24).

*In vivo* evidence that chronic high glucose impairs β-cell function is mainly derived from studies in animal models of type 2 diabetes. As described above, previous studies have shown that the antioxidant N-acetylcysteine was able to prevent diabetes in the Zucker Diabetic Fatty rat (ZDF) (75) and db/db mice (170), an effect due to amelioration of GSIS. This suggests that oxidative stress may also play a role in glucotoxicity *in vivo*.

In summary, there is accumulating evidence that oxidative stress is involved in high glucose-induced β-cell dysfunction. However, the mechanisms of oxidative stress-induced β-cell dysfunction are still unclear. In the next two sections, possible sites of impairment of β-cell function and downstream signaling pathways initiated by oxidative stress will be reviewed.

1.5.5. Cellular Sites of Oxidative Stress-Induced Impairment of β-Cell Function by Glucotoxicity

1.5.5.1. Glucose Oxidation and Uncoupling Protein 2

*In vitro* studies show that ROS can decrease glucose oxidation due to inhibition of mitochondrial (160) and glycolytic enzymes (163), and induce uncoupling by activating (62; 178) and/or upregulating (179; 180) uncoupling protein 2 (UCP2) in β-cells. This suggests that the effect of glucose (62) on UCP2 may be mediated, at least in part, via the generation of reactive oxygen species.
1.5.5.2. Insulin Gene Transcription and Insulin Biosynthesis

Other possible sites of action of oxidative stress are at the level of insulin gene transcription and (pro)insulin biosynthesis. As discussed above in Sections 1.3.4.1 and 1.3.4.2., glucotoxicity is associated with impaired insulin gene transcription and insulin biosynthesis. Studies in vitro show that the effect of high glucose to impair insulin gene transcription is due to induction of oxidative stress, which decreases binding of the transcription factors PDX-1 (72) and MafA (64; 181) to the insulin promoter.

Prolonged exposure of human islets to high glucose can decrease (pro)insulin biosynthesis (60); however it is unclear whether it is secondary to decreased insulin gene transcription and/or whether it is due to an independent effect of oxidative stress. Chronic glucose elevation, and activation of the hexosamine pathway, which induces oxidative stress (35), have been shown to impair (pro)insulin biosynthesis (92) via inhibition of the insulin signaling cascade in the β-cell. Decreased IRS-1 tyrosine phosphorylation and impaired activation of the downstream PI3-kinase/AKT/mTOR pathway was associated with in vitro exposure of RIN6 cells to high glucose (92). Activation of mTOR phosphorylates 4EBP-1, which allows eIF4E to interact with eIF4G (93). This step is critical to initiate translation. Thus, inhibition of mTOR activation via impairment of the insulin signaling cascade by oxidative stress may play a role in impairing (pro)insulin biosynthesis by glucose.
1.5.6. Downstream signaling mechanisms of oxidative stress–induced impairment of β-cell function by glucotoxicity

1.5.6.1. C-jun N-terminal Kinase

The C-jun N-terminal Kinase (JNK) pathway is a sub-group of the mitogen-activated protein (MAP) kinases family of signaling proteins. JNK is activated primarily by cytokines (such as TNF-α and IL-1β), and environmental stresses (182). The JNK family is encoded by three genes. The jnk1 and jnk2 genes are expressed ubiquitously, whereas jnk3 expression is restricted to the brain, heart and testes. Together, these genes are alternatively spliced to create 10 JNK isoforms (183).

Regulation of the JNK signaling pathway is very complex, and involves upstream regulators, downstream phosphatases, and scaffold proteins. Upstream regulation involves the MAP kinase kinase kinase (MAP3K), which is activated by specific stimuli (such as cytokines). Activation of MAP3K leads to phosphorylation and activation of MAP kinase kinase (MAP2K) MKK4 and MKK7, which in turn phosphorylate and activate JNK (183). JNK activation can be negatively regulated by MAPK phosphatase (MKP). Studies show that mice that have members of the MKP family knocked-out exhibit increased JNK activation (184; 185). MKP inhibition alone is also sufficient for JNK activation (186). Oxidative stress has been reported to increase JNK activation by inhibiting MAPK phosphatase (187). In addition to kinases and phosphatases, scaffold proteins, such as the JNK interacting protein (JIP) family of proteins, have also been established to be important in the regulation of JNK. Formation of JIP scaffold complexes have been reported to include both activating and inhibiting components of the JNK signaling pathway (188).
One of the major targets of JNK activation is phosphorylation of c-jun (Ser-63 and Ser-73), which together with c-fos forms the early response activator protein-1 (AP-1) transcription factor. Phosphorylation of c-jun increases its transcription activity (189). JNK can also phosphorylate other signaling molecules in the cells, which can mediate other cellular actions independent of AP-1 activation. Of particular interest in relation to type 2 diabetes is the finding that JNK can phosphorylate insulin receptor substrate-1 (IRS-1) at serine-307 residue (190), which can inhibit insulin signaling. Indeed, JNK has been reported to be abnormally elevated in various tissues such muscle, liver and adipose tissue in the diabetic state (191), and inhibition of JNK improves glucose tolerance in animal models of type 2 diabetes (192-194).

JNK activation has also been reported to be involved in oxidative stress-induced β-cell dysfunction. Oxidative stress can have direct effects on β-cell transcription factors (64; 181; 195), such as FOXO1. Oxidative stress increases FOXO1 nuclear retention (196; 197), which results in nuclear exclusion of PDX-1 (198) and consequent inhibition of insulin gene transcription. At least part of the effect of oxidative stress on FOXO1 is currently thought to be indirect and mediated by JNK (199; 200). As stated above, oxidative stress is reported to increase JNK activation by inhibiting MAPK phosphatase. Suppression of JNK has been shown to prevent ROS-induced impairment in insulin gene expression (162), and improves β-cell function in db/db mice (194). Also, islets treated with dominant negative JNK adenovirus were shown to be more effective than control islets in ameliorating hyperglycemia after transplantation (162). How JNK activation leads to impaired β-cell function is unclear. Activation of JNK by high glucose induces serine phosphorylation of insulin receptor substrates in the β-cell (92), which can impair insulin/IGF signaling, and result in the inhibition of AKT. Inhibition of AKT can lead to increased Fox-O1 nuclear localization, decreased PDX-1 nuclear localization, and thus impaired insulin gene transcription (199). JNK can also activate FOXO1
by phosphorylation independent of AKT activation (201; 202). Furthermore, inhibition of IGF/insulin signaling cascade by JNK can lead to inhibition of the mTOR pathway, which can decrease (pro)insulin biosynthesis (92).

1.5.6.2 Nuclear Factor kappa B (NFκB)

Oxidative stress is a known activator of IKKβ, which, by phosphorylating the inhibitor IkBα, activates NFκB. The role of NFκB on β-cell function is more controversial than that of JNK, because there are no reports that NFκB inhibits FOXO1 and insulin gene transcription and NFκB may actually be beneficial for GSIS (203; 204). However, inhibition of NFκB did prevent the β-cell dysfunction induced by cytokines (205). It should be noted that models of IKKβ inhibition that, in addition to inhibition of NFκB, also involves inhibition of IRS serine phosphorylation, show improvement of β-cell function (206; 207). Similar controversy relates as to whether NFκB is activated by glucose. It has been reported that glucose activates NFκB by inducing oxidative stress in rat islets (208) and that the IKKβ inhibitor salicylate protects human islets against glucotoxicity (207) and partially restores β-cell function in type 2 diabetes (209). However, in other studies in rat islets neither glucose nor oxidative stress activated NFκB (210). Thus, although there is some evidence to suggest that high glucose-induced oxidative stress activates the IKKβ-NFκB pathway to impair β-cell function, this needs to be further investigated.

1.5.6.3. β-Cell Insulin Resistance

One target of both JNK and IKKβ/NFκB is the serine/threonine phosphorylation of IRS, which impairs IGF-I/insulin signaling. Insulin and IGF-I receptors are expressed in β-cells and affect β-cell function and mass (211). Short-term effects of insulin on GSIS can be either inhibitory or stimulatory (212-215), whereas effects on insulin gene transcription and
(pro)insulin biosynthesis are stimulatory (89; 91; 216) and may serve to prevent depletion of insulin in the actively secreting β-cell. Although there is still debate about the importance of insulin for β-cell function, the majority of studies show that insulin signaling, in particular the PI3-kinase-AKT cascade, however activated (i.e., by insulin released upon glucose stimulation, directly by glucose or by other hormones (217)), plays an important and mainly stimulatory role in β-cell function and growth (91; 93; 218-220). This raises the possibility that known inducers of insulin resistance at peripheral sites, such as oxidative stress, may induce insulin resistance also at the β-cell level. However, there are only a few reports on the effect of prolonged exposure to glucose and FFA on β-cell insulin signaling and the role of oxidative stress in these effects have not been investigated. Regarding glucose, studies in RIN cells show serine 307 phosphorylation of IRS (92) by prolonged glucose exposure and reduced AKT/mTOR/4eIFBP phosphorylation in late passage glucose-cultured cells (221), effects associated with reduced (pro)insulin biosynthesis.

1.5.6.4. Endoplasmic Reticulum (ER) stress

The ER is involved in folding, processing and exporting secretory and membrane proteins to the Golgi Apparatus. When the demand for proteins exceeds the folding capacity of the ER or when the folding capacity of the ER is disrupted, ER stress ensues subsequently leading to the activation of the unfolded protein response, a mechanism that inhibits transiently global protein synthesis, increases ER chaperones levels and ER associated degradation (ERAD) components to counteract ER stress. UPR activates three distinct signal transduction pathways, which are mediated by PERK, IRE-1 and ATF-6. Under non-pathological conditions, all three components are associated with the ER chaperone GRP78 (Bip), which inhibits their activation. Once unfolded proteins accumulate in the ER, GRP78 preferentially associates with the unfolded proteins instead of PERK, IRE-1 and ATF6, resulting in activation of their
downstream mechanisms. The PERK pathway is activated by the oligomerization and autophosphorylation of PERK, which subsequently phosphorylates eIF2α leading to attenuation of general protein translation, but paradoxically increases the translation of the transcription factor ATF4. This transcription factor increases the expression of many genes that encode for protein chaperones, ERAD components, and antioxidant enzymes. Activation of IRE-1 leads to the splicing of XBP-1 mRNA and subsequently translation of the transcription factor XBP-1. Release of GRP78 from ATF6 results in the translocation of ATF6 to the Golgi apparatus where it is subsequently cleaved and activated. Both XBP-1 and cleaved ATF6 act to induce expression of genes encoding protein chaperones and ERAD machinery (See (222). for review).

Many different stimuli have been shown to activate UPR in β-cells, including an increase in misfolded proinsulin or islet amyloid polypeptide (IAPP), and an increase in extracellular glucose, FFA and/or cytokines levels (223). Recent studies have also demonstrated a close interrelationship between oxidative stress and ER stress (224). Oxidative stress can affect protein folding by direct protein modification, chaperone inactivation and/or depleting cellular glutathione levels (222), and can deplete ER calcium levels by inhibiting the sarcoplasmic reticulum Ca^{2+} ATPase (225; 226), leading to ER stress. ER stress can in turn generate more oxidative stress through increased ERO1p oxidase activity (an enzyme that transfers electrons generated by disulphide bond formation in the ER to molecular oxygen), and glutathione consumption due to reduction of mispaired disulphide bonds (227). These findings suggest that oxidative stress and ER stress can form a vicious cycle to impair β-cell function.

1.5.7. Oxidative Stress and β-Cell Lipotoxicity

Evidence for a causal role of oxidative stress in the β-cell dysfunction induced by lipotoxicity had been negative in a study in cultured rat islets (123), whereas a study by our group in MIN6 cells indicated restoration of insulin content but not insulin secretion by the
antioxidant N-acetylcysteine (NAC) (129). Furthermore, our laboratory has demonstrated that 48h oleate infusion in rat impairs β-cell function, and that co-infusion of the antioxidants NAC, taurine or tempol reverse oleate-induced impairment in β-cell function (228). More recently, we have shown that oral treatment with the antioxidant taurine in humans prevented lipotoxicity induced by i.v. infusion of Intralipid+heparin (fat infusate) (229). It is interesting to note that many of the mechanisms suggested to be involved in FFA-induced β-cell dysfunction via oxidative stress are similar to those suggested for glucotoxicity, i.e. involvement of ER stress, and inflammation (See (141) for review). Thus, oxidative stress may be the central mechanism by which nutrients overload impairs β-cell function.
1.6. Summary

Glucotoxicity has been proposed to play a pathogenic role in type 2 diabetes. The underlying concept is that once type 2 diabetes ensues, the increase in glucose levels, which are characteristic of diabetes, exerts additional toxic effects on the already dysfunctional β-cells, thus further aggravating the diabetic state. This leads to a vicious positive feedback cycle, deteriorating the β-cells.

Oxidative stress is increased in patients with type 2 diabetes. Prolonged high glucose exposure can increase ROS generation, and lead to oxidative stress. Oxidative stress can act directly on various stages of the insulin production/release pathway, or can activate downstream signaling pathways, such as JNK and ER stress, to decrease β-cell function and mass (See Figure 1.4.). A role of oxidative stress has also been implicated in β-cell lipotoxicity and glucolipotoxicity. Thus, oxidative stress may be a central mechanism by which prolonged elevation of glucose and/or FFA impairs β-cell function in type 2 diabetes.
Mechanisms of high glucose-induced β-cell dysfunction that involve oxidative stress. This figure is not intended to be all-inclusive.
1.7. Rationale and Significance of the Studies

Chronic hyperglycemia is a key characteristic of type 2 diabetes, and can decrease β-cell function and mass. The resulting deterioration of β-cell function can therefore further aggravate the diabetic state, leading to a vicious cycle. The exact mechanism by which chronic glucose elevation impairs β-cell function is unclear. A number of \textit{in vitro} studies have demonstrated a causal role of oxidative stress in high glucose-induced β-cell dysfunction. \textit{In vivo}, the role of oxidative is less well understood. Previous studies have shown that antioxidants can prevent diabetes in animal models of type 2 diabetes, an effect due to amelioration of GSIS. However, these animal models are not only characterized by hyperglycemia, but also by other metabolic (i.e. hyperlipidemia) and hormonal changes associated with diabetes. Thus it is unclear whether oxidative stress plays a causal role in high glucose-induced β-cell dysfunction \textit{per se}.

Oxidative stress can activate downstream signaling pathways to impair β-cell function. Previous \textit{in vitro} studies have implicated ER stress and JNK activation in high glucose-induced β-cell dysfunction. However, whether ER stress and JNK activation are causal in high glucose-induced β-cell dysfunction \textit{in vivo} is unclear. Furthermore, it is unclear whether high glucose-induced oxidative stress increases ER stress and/or JNK activation to impair β-cell function \textit{in vivo}.

Therefore, the three studies in this thesis were performed to address 1) whether oxidative stress, ER stress, and JNK activation are causally involved in high glucose-induced β-cell dysfunction \textit{in vivo}, and 2) whether ER stress and JNK are downstream mechanisms of oxidative stress in high glucose-induced β-cell dysfunction \textit{in vivo}. These studies are significant as they 1) provide new insights into the physiology and pathophysiology of insulin secretion and into the mechanisms of β-cell dysfunction caused by glucotoxicity, and 2) lead to identification
of novel targets susceptible to nutritional and/or pharmacological intervention to prevent and/or treat β-cell dysfunction in type 2 diabetes.
1.8. General Hypothesis

Oxidative stress plays a causal role in high glucose-induced β-cell dysfunction in vivo via pathways that involve ER stress and the JNK pathway.

1.9. Specific Aims

The thesis consists of three studies that examined the potentially causal role of each factor (oxidative stress, endoplasmic reticulum stress, and JNK activation). These studies allow us to draw preliminary conclusions regarding how these factors are linked in the pathway of β-cell glucotoxicity, and suggest potential targets in the prevention of high glucose-induced β-cell dysfunction.

**Aim 1.** To investigate the causal role of oxidative stress in high glucose-induced β-cell dysfunction in vivo

**Aim 2.** To investigate the causal role of endoplasmic reticulum stress, and its link to oxidative stress in high glucose-induced β-cell dysfunction in vivo

**Aim 3.** To examine the causal role of JNK, and its link to oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo
General Methods

2.1. Procedures

2.1.1. Experimental Animal Model and Surgical Procedures

2.1.1.1. Animals

For all rat studies, 10-12 weeks old female Wistar rats (Charles River, Quebec, Canada) weighing 250-300g were used. The rats were housed in the University of Toronto’s Department of Comparative Medicine. They were exposed to a 12 h light/dark cycle. The rats were fed a Teklad Global diet containing 21% protein with 64% carbohydrate and 14% fat, Harland Teklad Global Diets, Madison, WI.

2.1.1.2. Surgery

After a week of adaptation to the facility, rats were anaesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg.ml\(^{-1}\), 1 ul.g\(^{-1}\) of body weight; Study 1) or with isofluorane (Studies 2 and 3), and indwelling catheters were inserted into the right internal jugular vein for infusions and the left carotid artery for sampling. Polyethylene catheters (PE-50; Cay Adams, Boston, MA), each extended with a segment of silastic tubing (length of 3 cm, internal diameter of 0.02 inches; Dow Corning, Midland, MI), were used. The venous catheter
was extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch. Both catheters were tunneled subcutaneously and exteriorized. The catheters were filled with a mixture of 60% polyvinylpyrrolidone and heparin (1000U.ml\(^{-1}\)) to maintain patency and were closed at the end with a metal pin. The rats were allowed a minimum 3-4 days period of post-surgery recovery before experiments, after which they were connected to the infusion apparatus. The infusion lines ran inside a tether that was fitted to the subcutaneous implant. Each rat was placed in a circular cage, and the infusion lines were protected by a tether and run through a swivel, which was suspended on top of the cage to give complete freedom of movement to the rat. Infusions were started through the jugular vein, whereas a slow infusion of heparinized saline was used to keep the carotid artery patent for sampling. All procedures were in accordance with the Canadian Council of Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto.

### 2.1.2. Infusion Period

To induce β-cell dysfunction, 37.5% glucose was infused i.v. into rats to elevate plasma glucose to 20-22mmol/l (~50µl/min) for 48h. Saline infusion (5µl/min) was used as a control. Glucose or saline was co-infused with or without antioxidants/inhibitors (see the Methods section of the individual studies for further details). Samples for glucose, insulin, and C-peptide were measured regularly after the onset of infusion. During the infusion period, rats had free access to food and water.

### 2.1.3. Islet Isolation

Pancreatic islets were isolated in overnight fasted rats, after the 48h infusion period. Rats were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg.ml\(^{-1}\), 1µl.g\(^{-1}\) of body weight). The visceral contents were exposed and rats were exanguinated through an
incision in abdominal aorta. The common bile duct was quickly isolated and a collagenase solution (Type XI, Sigma in RPMI-1640 containing 10mmol/l HEPES and 1% Penicillin) was infused into the pancreas (~15ml). The pancreas was then removed and incubated in a water bath at 37°C for 20 minutes. Following the incubation, the pancreas was subjected to vigorous shaking. The mixture was centrifuged, and the pellet was then resuspended in RPMI-1640 containing 10mmol/l HEPES, 1% Penicillin, and 7% fetal bovine serum. This mixture was passed through a 300µm filter, and centrifuged again. Islets were then isolated with other tissues by a Histopaque-1077 density gradient (230). During isolation, glucose was kept at 2.8mmol/l.

2.1.4. Evaluation of β-Cell Function Ex vivo

The islet secretion studies were performed to investigate β-cell function in an open loop system, i.e. where insulin secretion cannot be acutely influenced by the prevailing insulin resistance or by changes in insulin clearance. These studies are performed to complement the studies in vivo where the contribution of insulin resistance to insulin secretion can only be “eliminated” by calculation (i.e. DI method; see section 2.3.3.), and where the contribution of changes in insulin clearance to β-cell function is still a factor (See section 2.3.1.).

To assess β-cell function, freshly isolated islets were pre-incubated for 1h at 37°C in Krebs Ringer buffer containing 10mmol/l HEPES (KRBH) and 2.8mmol/l glucose, and incubated in triplicate for 2h at 37°C in KRBH at the following glucose concentrations: 2.8mmol/l, to evaluate non-glucose stimulated insulin secretion; 6.5mmol/l, which is basal glucose in rats; 13mmol/l, the upper physiological glucose level in rats; and 22mmol/l, which is a maximum stimulatory concentration. Insulin was measured in the supernatant with Linco’s RIA kit. The islets used for secretion study was then subjected to acid ethanol extraction, as described in (231), for insulin content measurement. Islet pellets were dried of all liquid using a
speed vacuum. One hundred microliter of acid ethanol (0.18mmol/l HCl in 70% ethanol) was added to the islet pellet and allowed to incubate at 4°C overnight. The lysate was then neutralized with 10µl of 1mol/l Na₂CO₃. Insulin in the lysate was measured using a Linco’s RIA kit.

2.1.5. Islet ROS, total and mitochondrial superoxide

ROS were measured with dihydro-dichlorofluorescein diacetate (H₂DCF-DA), which detects most ROS including hydrogen peroxide, peroxyl radical, and peroxynitrite anion (232). H₂DCF-DA does not detect superoxide directly; however superoxide can be detected indirectly via its ROS metabolites (233). Total superoxide was measured by hydroethidine (234) and mitochondrial superoxide was measured by MitoSOX (235).

2.1.5.1. Dihydro-dichlorofluorescein diacetate

Following 1h pre-incubation at 37°C in KRBH buffer containing 2.8mmol/l glucose, islets were incubated with 10µmol/l H₂DCF-DA (Molecular Probes, Invitrogen, Burlington, ON, Canada) in KRBH containing 2.8mmol/l glucose for 30 minutes at 37°C. After washing with KRBH, islet fluorescence was measured at 480nm excitation and 510nm emission using an Olympus fluorescent BX51W1 microscope. Approximately 10 islets were measured per each n number (rat).

2.1.5.2. Hydroethidine and MitoSOX

Islets isolated from glucose treated rats were maintained at 20mmol/l glucose throughout the islet isolation procedure. Following 1h pre-incubation at 37°C in KRBH buffer containing either 2.8mmol/l or 20mmol/l glucose (for glucose infused groups), islets were incubated with hydroethidine (3µmol/l) or MitoSOX (5µmol/l) (Molecular Probes) in KRBH buffer containing
2.8mmol/l glucose for 15 minutes at 37°C. After washing with KRBH, islet fluorescence was measured at 550nm excitation and 660nm emission using an Olympus fluorescent BX51W1 microscope. Approximately 10 islets were measured per n number (rat).

2.1.6. Western Blotting

Freshly isolated islets were washed in PBS, frozen in liquid nitrogen, and stored at -80°C. Frozen islets were lysed in ice-cold lysis buffer (1% Triton X-100, 20mmol/l HEPES, pH 7.4, 100mmol/l KCl, 2mmol/l EDTA, 1mmol/l PMSF, 10μg/ml leupeptin, and 10μg/ml aprotinin). The cells were lysed on ice for 30 minutes and centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was then transferred to a new tube, and the protein concentration was determined by the detergent-compatible modified Lowry microassay (BioRad), using serum albumin as the standard. Equal volumes of all samples were mixed with equal volumes of 2x sample-loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125mol/l Tris-HCl, pH 6.8), and boiled for 5 minutes. The mixture was then vortexed and subjected to SDS-PAGE. Following electrophoretic separation, protein was transferred to nitrocellulose (Study 2) or polyvinylidene fluoride (PVDF; Study 3) 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. Following this blocking step, membranes were washed in TBST and then incubated overnight with primary antibody dissolved in blocking solution (See Methods for each study for the concentrations of antibodies used). The membrane was washed 3 times with TBST, and then incubated with secondary antibodies (See Methods for each study for the concentrations of antibodies used). The membranes were washed 3 times with TBST, and developed using enhanced chemiluminescence. The bands obtained from immunoblotting were quantified by densitometry.
2.1.7. **Two-step hyperglycemic clamp**

The two-step hyperglycemic clamp was performed in conscious rats fasted overnight. In glucose-infused groups, at the end of 48h, glucose infusion was decreased to \(~5\mu l/min\) for \(~75\) minutes, which was required to achieve basal glucose without hypoglycemia. Saline/treatment infusion was continued during this period, and throughout the hyperglycemic clamp. At \(-20\) minutes, the continuous arterial infusion of heparinized saline was stopped in all rats, since the same total amount of heparinized saline was used to dilute the erythrocytes that were re-infused into the rats after plasma separation from blood samples.

Basal insulin and C-peptide were measured at \(-20\) and \(0\) minutes. At time = \(0\) minutes, an infusion of \(37.5\%\) glucose was started. Plasma glucose was maintained at \(13\) mmol/l by adjusting the rate of glucose infusion according to frequent (5-10 minutes) glycemic determinations obtained on a Beckman 2 glucose analyzer. At 120 minutes, the glucose infusion was again raised to \(22\) mmol/l until the end of the experiment (time = 240 minutes). Samples for insulin and C-peptide were taken at regular intervals. Both C-peptide and insulin were measured because they are co-secreted but cleared by different mechanisms (kidney and liver respectively). Therefore, a change in both indicates a change in secretion rather than effects on insulin clearance. The sample volume was minimized to avoid anemia. A total of \(2.5\) ml of blood was withdrawn from the rats. After removal of plasma from centrifuged whole blood samples, erythrocytes were suspended in heparinized saline (4U/ml) and re-infused into the rats. Rat insulin and C-peptide levels were measured by the Linco’s RIA kit.

In each experiment, an index of insulin sensitivity and the disposition index (a measure of \(\beta\)-cell function *in vivo*; see sections 2.1.4.) were calculated from the hyperglycemic clamp, as described in section 2.3. of this chapter. At the end of the experiment, the rats were deeply anaesthetized and the pancreas was excised for histochemical analysis. The pancreas was fixed.
immediately in 10% formalin overnight, and then transferred to 70% ethanol. Samples for were processed and embedded in paraffin within 5 days of collection.

**2.1.8. Evaluation of GSIS and β-cell Function In vivo**

C-peptide levels were taken as an index of absolute insulin secretion, as calculations of insulin secretion by C-peptide deconvolution, which is commonly performed in humans (135; 236; 237) cannot be performed in the rat. This is because the parameters of C-peptide kinetics cannot be derived in the rat, as rat C-peptide (species specific) is not available in amounts sufficient for in vivo injections. However, studies in humans have shown that the kinetics of C-peptide are not influenced by glucose or fat (237-239). Insulin secretion in vivo has to be evaluated in the context of insulin sensitivity, since the normal β-cell compensates for insulin resistance. In normal subjects, the relationship between insulin sensitivity and insulin secretion is hyperbolic, i.e., the product of insulin sensitivity and insulin secretion is a constant defined as Disposition Index (DI) and considered as a measure of β-cell function. DI was calculated by multiplying insulin sensitivity during the clamp (See Section 2.3.2. for formula), by plasma C-peptide levels during the last 40 minutes of the clamp (See Section 2.3.3. for formula). Our lab has previously shown that the relationship between C-peptide and M/I index is indeed hyperbolic in control rats (240). Therefore, we calculated DI, and this was used for in vivo evaluation of β-cell function.

**2.2. Laboratory Methods**

**2.2.1. Plasma Glucose**

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

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

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

### 2.2.2. Plasma Insulin Assay

Radioimmunoassay (RIA) kit specific for rat insulin from Linco Research Inc. (St. Charles, MO, USA) were used to determine plasma and islet insulin concentrations. This kit has 100% reactivity to rat insulin I and II. Cross-reactivity to rat proinsulin has not been tested. Insulin in the sample competes with a fixed amount of \(^{125}\text{I}\)-labelled insulin for the binding sites on the specific antibodies. A standard curve was generated using insulin standards at 0, 3, 10, 30, 100, 240 µU/ml in duplicate. \(^{125}\text{I}\)-labelled and rat insulin antibody were mixed with plasma sample. The tubes were then vortexed and incubated overnight at 4°C. Precipitating reagent was added to all tubes followed by vortexing and incubating for 30 minutes at 4°C. The tubes were then spun at 3000g for 30 minutes. The supernatant was aspirated and the radioactivity in the pellet was counted for 4 minutes in a gamma counter (Beckman Instruments, Fullerton Ca, USA). The counts (B) for each of the standards and unknowns were expressed as a percentage of the mean counts of the “0 = standard” (Bo):
% activity bound = B (Standard or Sample)/Bo x 100%

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

2.2.3. Plasma C-peptide assay

Rat C-peptide RIA kit was used to determine plasma C-peptide levels. The kit uses an antibody specific for rat C-peptide (Linco Research, Inc, St. Charles, MO, USA), with no cross-reactivity to rat insulin I and II. Cross-reactivity to rat proinsulin has not been tested. The principle is the same as insulin RIA as described above. The procedures are the same as insulin RIA with the exception of one extra day. In the first day, only rat C-peptide antibody was added followed by an overnight incubation at 4°C. In the second day, ¹²⁵I-rat C-peptide was added followed by vortexing and overnight incubation at 4°C. In the last day, precipitating reagent was added followed by vortexing and incubation for 20 minutes at 4°C. Then, the tubes were centrifuged at 3000rpm for 30 minutes. The supernatant was then aspirated and the radioactivity in the pellet was counted for 1 minute in a gamma counter. The % activity bound was calculated in the same manner as insulin RIA. The % activity bound for each standard was plotted against the known concentration to obtain standard curve. The unknown concentrations of the samples were determined by interpolation of the standard curve. The coefficient of inter-assay variation determined on reference plasma was less than 10%.
2.3. Calculations

2.3.1. Insulin Clearance

The C-peptide to insulin ratio was used as an index of insulin clearance. The C-peptide level was divided by the insulin level at each time point in the last 40 minutes of each step of the two-step hyperglycemic clamp, and the average ratio was calculated.

2.3.2. Insulin Sensitivity Index (M/I)

The insulin sensitivity during hyperglycemic clamp is calculated as the M/I index. The M/I index is calculated at each step of the two-step hyperglycemic clamp according to the following formula

\[ \text{M/I} = \frac{\text{Ginf}}{\text{Insulin}} \]

where Ginf is the rate of glucose infusion and Insulin is the plasma insulin concentration at individual time points during the last 40 minutes of each step of the hyperglycemic clamp. The average of the M/I during the last 40 minutes was used as the M/I for the specific rat. M/I is reported in units of deciliter per kilogram per minute per microunit per milliliter. There are limitations to using this method to assess insulin sensitivity at elevated insulin levels, as it has been reported that the relationship between circulating insulin levels and insulin action is not linear at insulin concentrations higher than 180\(\mu\)U/ml (241).

2.3.3. Disposition Index

The disposition index (DI), which was used as an index of insulin secretion corrected for the ambient degree of insulin resistance, was calculated at each step of the two-step hyperglycemic clamp according to the following formula,
\[ DI = \frac{M}{I} \times C\text{-peptide} \]

where \( M/I \) is calculated as described above during the last 40 minutes, and C-peptide is the C-peptide concentration at individual time points during the last 40 minutes of each step of the hyperglycemic clamp. The rationale for using this index was provided in the section 2.1.4.

2.4. Statistical Analysis

Data are presented as means +/- SE. One way non parametric analysis of variance (ANOVA) for repeated measurements followed by Tukey’s t test was used to compare differences between treatments. Calculations were performed using SAS (Cary, NC).
Study 1

Evidence for a Role of Superoxide Generation in Glucose Induced β-cell Dysfunction In Vivo*

* The results of this study are incorporated in a manuscript published in Diabetes 56(11): 2722-31, 2007
3.1. Abstract

**Objective** Prolonged elevation of glucose can adversely affect β-cell function. *In vitro* studies have linked high glucose-induced β-cell dysfunction to oxidative stress, however whether oxidative stress plays a role *in vivo* is unclear. The objective of this study was to investigate the role of oxidative stress in an *in vivo* model of high glucose-induced β-cell dysfunction.

**Research Design and Methods** Wistar rats were infused i.v. with glucose for 48h to achieve 20mmol/l hyperglycemia with/out co-infusion of one of the following antioxidants: Taurine (TAU), an aldehyde scavenger, N-acetylcysteine (NAC), a precursor of glutathione, or tempol (TPO), a superoxide dismutase mimetic. This was followed by islet isolation or hyperglycemic clamp.

**Results** Forty-eight hours glucose infusion decreased glucose stimulated insulin secretion (GSIS), and elevated ROS, total superoxide and mitochondrial superoxide in freshly isolated islets. TPO prevented the increase in total and mitochondrial superoxide, and the β-cell dysfunction induced by high glucose. However, TAU and NAC despite completely normalizing H$_2$DCF-DA-measured ROS, did not prevent the increase in superoxide and the decrease in β-cell function induced by high glucose. TPO but not TAU also prevented β-cell dysfunction induced by less extreme hyperglycemia (15mmol/l) for a longer period of time (96h). To further investigate whether TPO is effective *in vivo*, a hyperglycemic clamp was performed. Similar to the findings in isolated islets, prolonged glucose elevation (20mmol/l for 48h) decreased β-cell function as assessed by the disposition index (insulin secretion adjusted for insulin sensitivity) and co-infusion of TPO with glucose completely restored β-cell function.

**Conclusion** Our findings implicate superoxide generation in β-cell dysfunction induced by prolonged hyperglycemia.
3.2. Introduction

Type 2 diabetes is characterized by insulin resistance and defective insulin secretion. The progressive failure of pancreatic β-cells to secrete sufficient insulin to compensate for insulin resistance leads to hyperglycemia, which can in turn exert deleterious effects on β-cells (27).

Previous *in vitro* studies have shown that oxidative stress mediates the impairment in β-cell function induced by chronic exposure of cultured islets or β-cell lines to high glucose (35; 175; 176; 181; 242). However, the association between high glucose-induced β-cell dysfunction and oxidative stress has not been shown in all studies (177; 243).

*In vivo* evidence demonstrating a link between oxidative stress and high glucose-induced β-cell dysfunction is derived from studies performed in animal models of type 2 diabetes. It has been demonstrated that the antioxidant N-acetylcysteine can prevent diabetes in the ZDF rat and *db/db* mouse (75; 170), an effect due to amelioration of defective glucose stimulated insulin secretion (GSIS). To date, however, no studies have been performed to investigate the role of oxidative stress in a selective model of high glucose-induced β-cell dysfunction (i.e., in the absence of other metabolite/hormone changes related to diabetes).

To do this, we examined the effects of 48h *in vivo* exposure to elevated glucose with or without antioxidants on β-cell function in rats. Three different antioxidants, taurine (2-aminoethanesulfonic acid; TAU), N-acetylcysteine (NAC), and tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl; TPO) were tested. Taurine is an effective aldehyde scavenger (toxic aldehydes are byproducts of lipid peroxidation) due to its ability to form a Schiff base with the amino group at its β position (244). N-acetylcysteine is a scavenger of free radicals and aldehydes, and can provide cysteine for the synthesis of glutathione, an integral part of the
intracellular antioxidant defense system. Tempol is a membrane permeable and metal independent superoxide dismutase mimetic that has been shown to prevent endothelial dysfunction in streptozotocin-induced diabetic rats (245). Similar to TAU and NAC, TPO has been used *in vivo* without adverse effects (246).

Following 48h infusion of glucose/saline with/out antioxidants, β-cell function was assessed in isolated islets, and ROS and total and mitochondrial superoxide levels were measured using the fluorescent dye dihydro-dichlorofluorescein diacetate (H$_2$DCF-DA), hydroethidine and MitoSOX respectively. The effect of TPO was also evaluated *in vivo* using a hyperglycemic clamp.
3.3. Materials and Methods

3.3.1. Animals and Surgery

Female Wistar rats (250-300 g, Charles River, Canada) were cannulated, as described in the General Methods section 2.1.1.2., and in (136).

3.3.2. Ex vivo Studies

3.3.2.1. 48h Infusions

3.3.2.1.1. Groups and Treatments

Rats were randomized and infused for 48h with either: 1) Saline (SAL), 2) A variable infusion of 37.5% glucose to achieve and maintain plasma glucose at 20-22mmol/l (HG), 3) Glucose+low dose taurine (HG+L-TAU), 4) Glucose+middle dose taurine (HG+M-TAU), 5) Glucose+high dose taurine (HG+H-TAU), 6) Glucose+N-acetylcysteine (HG+NAC), 7) Glucose+tempol (HG+TPO), or 8) TAU (middle dose), NAC, or TPO without glucose infusion. TAU was given at three doses: 2.14μmol/kg.min, which reversed insulin resistance induced by high glucose (247); 2.76μmol/kg.min, which prevented β-cell dysfunction induced by 48h fat infusion (247), and 5.52μmol/kg.min. NAC was given at 2.76μmol/kg.min (equimolar to middle dose taurine), which also prevented fat-induced β-cell dysfunction (228). Tempol was given at 2.41μmol/kg.min, which protected against ROS toxicity in experimental pancreatitis (248). All antioxidants (Sigma) were dissolved in saline at pH=7.4. After 48h infusion and ~12h fasting, we performed either ex vivo studies in isolated islets, or in vivo studies, by using the two-step hyperglycemic clamp.
3.3.2.1.2. Pancreatic Islet Isolation and *Ex vivo* Evaluation of GSIS

This was performed as described in *General Methods* sections 2.1.3. and 2.1.4.

3.3.2.1.3. Islet ROS, total and mitochondrial superoxide

This was performed as described in *General Methods* section 2.1.5.

3.3.2.1.4. β-Cell Apoptosis

Apoptosis was measured in dispersed β-cells using FITC-conjugated Annexin-5 (Sigma) and propidium iodide (Sigma). Annexin-5 detects membrane phosphotidylserine flip-flop once viability is compromised (249). Propidium iodide stains dead and necrotic cells. Cells stained with annexin-5 only were identified as apoptotic. To obtain single islet cells, ~100 freshly isolated islets were picked into Ependorf tubes. One milliliter of PBS-Ca/Mg + 1 mmol/l EGTA was added to the tubes, and incubated in room temperature for 9 minutes. The supernatant was aspirated out, and 0.6ml of dispase II (0.6-2.4 units/ml; Roche) was added. The tubes were incubated for 3 minutes at 37°C. The pellet was pipetted up and down until no clumps were seen. Five hundred microliters of RPMI-1640 containing 2.8mmol/l glucose, 10mmol/l HEPES, 1% Penicillin and 7% fetal bovine serum was added, and tubes were spun down at 4000 rpm for 2 minutes. The supernatant was removed and the pellet was resuspended with 150µl of RPMI-1640 containing 2.8mmol/l glucose, 10 mmol/l Hepes, 1% Penicillin and 7% fetal bovine serum. Twenty microliters of the mixture was loaded onto coverslips, and incubated at 37°C for 2 hours. Cells were then washed with KRBH buffer, and incubated with annexin-5 (0.15µg/ml) and propidium iodide (5umol/l) in KRBH for 20 minutes at 25°C. After washing, fluorescence was measured at 480/545nm excitation and 510/610nm emission for annexin and propidium iodide respectively.
3.3.2.2. 96h Infusions

A set of rats were also infused for 96h with either: 1) saline, 2) a variable infusion of 37.5% glucose to achieve and maintain plasma glucose at 15mmol/l, 3) glucose + TAU, 5) glucose + TPO. The same doses of TAU (middle dose) and TPO were used as in the 48h infusion study. Following 96h infusion, islets were isolated and *ex vivo* evaluation of GSIS was performed as described in General Methods sections 2.1.3. and 2.1.4.

3.3.3. *In vivo* Studies

3.3.3.1. Groups and Treatments

Rats were randomized and infused for 48h with either: 1) saline (SAL), 2) a variable infusion of 37.5% glucose to achieve and maintain plasma glucose at 20-22mmol/l (HG), 3) glucose+tempol (HG+TPO), 4) TPO alone, or 5) mannitol (MAN) to control for plasma osmolarity as in (250), infused at a rate comparable to glucose infusion (~45 µl/min). After 48h infusion and overnight fasting, all groups were subjected to a two-step hyperglycemic clamp to evaluate GSIS *in vivo*.

3.3.3.2. Two-step hyperglycemic clamp

At the end of 48h, glucose infusion in HG and HG+TPO groups was decreased to ~5 µl/min for ~75 minutes, which was required to achieve basal glucose without hypoglycemia. Basal insulin and C-peptide were measured at -20 and 0 minutes. At time=0, an infusion of 37.5% glucose was started. Plasma glucose was maintained at 13mmol/l by adjusting the rate of glucose infusion according to frequent (5-10 minutes) glycemic determinations. At 120 minutes, the glucose infusion was again raised to 22mmol/l until the end of the experiment (time = 240 minutes), as described in the General Methods section 2.1.8.
3.3.4. Islet Morphology

At the end of the hyperglycemic clamp, rats were anesthetized with a ketamine:xylazine:acepromazine cocktail (87:1.7:0.4mg/ml) and pancreas was removed, fixed overnight in 10% formalin and stored in 70% ethanol. Samples were processed and embedded in paraffin within 5 days of collection.

Three sections (separated by 100µm) were immunostained for insulin using an antibody from Biomeda (Foster City, CA) coupled with peroxidase detection. Relative β-cell area was determined from the ratio between areas occupied by insulin-positive cells and area occupied by total pancreatic tissue. Beta-cell mass was determined by multiplying this ratio by pancreas weight. Individual β-cell area (index of size) was calculated by dividing insulin-positive areas by the number of nuclei within each area.

Adjacent sections (three per rat) to those used for insulin immunostaining were used to detect islet apoptotic nuclei with TUNEL staining (251).

3.3.5. Plasma Assays

Plasma glucose, insulin and C-peptide levels were measured as described in the General Methods section 2.2..

3.3.6. Calculations

Insulin clearance, the M/I index of insulin sensitivity, and disposition index were calculated during the last 40 minutes of each step of the hyperglycemic clamp, as described in the General Methods sections 2.3..
3.3.7. Statistics

Data Refer to General Methods section 2.4..
3.4. Results

3.4.1. 48h infusions

Baseline fed plasma glucose and insulin were not different between groups before the onset of the 48h infusions. In rats infused for 48h with glucose alone (HG) or in combination with antioxidants, glucose levels were elevated to ~20-22mmol/l by ~6h and were maintained at that level for the remainder of the 48h period (Table 3.1).

**Figure 3.1.A** shows that at 48h, co-infusion of taurine (TAU)(all doses) or NAC tended to increase, and TPO significantly increased the glucose infusion rate (Ginf) compared to HG (p<0.001). HG+TPO also increased Ginf vs. HG+L-TAU and HG+H-TAU (p<0.05). Plasma insulin tended to decrease compared to HG with all antioxidants (**Figure 3.1.B**). HG+TAU (all doses) and HG+NAC treated groups had significantly lower C-peptide levels than HG at 48h (**Figure 3.1.C**). Although HG+TPO tended to increase C-peptide to insulin ratio (index of insulin clearance) compared with HG alone, this was not significantly different. Co-infusion of all antioxidants with glucose improved insulin sensitivity (**Figure 3.1.D**). HG+TPO increased the disposition index (DI) compared to HG alone. However, TAU (all doses) and NAC did not have any effect on DI (**Figure 3.1.E**). Non-glucose infused groups are not shown in the graph, as in these groups Ginf=0, and thus SI and DI could not be calculated.

3.4.1.1. Glucose Stimulated Insulin Secretion in Freshly Isolated Islets

Insulin secretion at 2.8mmol/l glucose did not differ between groups. Forty-eight hours of glucose infusion increased basal insulin secretion at 6.5mmol/l (p<0.001 HG vs. SAL), but impaired GSIS at 13mmol/l and 22mmol/l glucose (p<0.001). All three doses of TAU did not prevent the increase in basal insulin secretion induced by glucose or the impairment in GSIS at 13 and 22mmol/l glucose. In fact, high dose TAU tended to accentuate the impairment in GSIS.
at 22mmol/l glucose (Figure 3.2.A). Co-infusion of NAC, similar to TAU, did not prevent the effect of high glucose either on basal or glucose stimulated insulin secretory response (Figure 3.2.B). Co-infusion of TPO did not affect insulin secretion at 6.5mmol/l glucose, but completely restored GSIS at both 13mmol/l and 22mmol/l glucose (Figure 3.2.C).

Forty-eight hours hyperglycemia drastically reduced islet insulin content. Addition of TPO doubled the insulin content (p<0.05 HG+TPO vs. HG), but this was still profoundly reduced compared to SAL (Figure 3.2.D).

### 3.4.1.2. Reactive Oxygen Species in Islets

Forty-eight hours hyperglycemia increased intracellular ROS in islets compared to SAL (p<0.01). Co-infusion of TAU (middle dose) or NAC abolished the increase in ROS induced by high glucose (Figures 3.3.A and 3.3.B). Co-infusion of TPO did not have a significant effect (Figure 3.3.C).

### 3.4.1.3. Total and Mitochondrial Superoxide Levels in Islets

Forty-eight hours glucose infusion elevated total islet superoxide (p<0.05 vs. SAL). TAU did not normalize superoxide (Figure 3.4.A). NAC tended to decrease superoxide, but this was not significant (Figure 3.4.B). In contrast, TPO prevented the increase in superoxide induced by prolonged glucose infusion (Figure 3.4.C).

Forty-eight hours glucose infusion elevated islet mitochondrial superoxide, and co-infusion of TPO completely prevented this increase (Figure 5).

### 3.4.1.4. β-Cell Apoptosis

The percentage of apoptotic cells was measured in dispersed β-cells from isolated islets using the fluorescent dyes annexin-5 and propidium iodide. β-cells stained for annexin-5 only
were identified as apoptotic. There was no detectable difference in β-cell apoptosis between groups (% apoptotic cells: SAL=1.86±0.08%; HG=1.99±0.17%; HG+TPO=1.88±0.20%; TPO=1.91±0.18%; n=3-4 per group). No difference in propidium iodide staining were observed between groups.

### 3.4.1.5. Islet Morphology

Islet morphological studies were performed in the isolated pancreas after 48h infusion and hyperglycemic clamps. β-cell mass and individual area (i.e., size) were increased ~1.5-2 fold compared to SAL. Beta-cell mass and size were similar in HG and HG+TPO (Table 3.2.). The number of β-cells per section tended to increase in HG and HG+TPO but these differences were not significant (SAL=3119±313; HG=3431±551; HG+TPO=3312±279; TPO=3083±337). Apoptotic cells were very rare without detectable differences between groups.

### 3.4.1.6. Two-Step Hyperglycemic Clamp

To further investigate whether TPO is effective in preventing high glucose-induced β-cell dysfunction in vivo, a two-step hyperglycemic clamp was performed after 48h infusions. A subset of rats were also infused with mannitol (MAN) as a control for osmolarity of the glucose infusate, and the results were not different from SAL.

Basal plasma glucose prior to the clamp was slightly lower in HG and HG+TPO (not visible because of graph scale). Plasma glucose was elevated to 13mmol/l until 120 minutes, then to 22mmol/l until 240 minutes with no difference between groups (Figure 3.6.A).

In HG rats, a lower Ginf was necessary to clamp glucose at 22mmol/l, indicating that the circulating insulin was inadequate to compensate for insulin resistance. Co-infusion of TPO completely restored Ginf to the SAL levels (Figure 3.6.B).
Basal insulin and C-peptide levels (-20 minutes to 0 minutes) were higher in HG and HG+TPO rats than SAL (p<0.001). In response to increasing glucose levels, plasma insulin and C-peptide rose as expected. Absolute insulin and C-peptide levels were higher in HG compared to SAL (p<0.001) at 13mmol/l, and tended to be higher at 22mmol/l. However, the response of insulin and C-peptide to the rise in glucose was blunted in HG compared to SAL, suggesting β-cell dysfunction. TPO tended to decrease absolute insulin and C-peptide levels, and increased the response of insulin and C-peptide to the rise in glucose (Figures 3.6.C and 3.6.D).

The C-peptide/Insulin ratio (index of insulin clearance) was lower in HG than SAL at 13mmol/l glucose (p<0.05 vs. SAL; SAL = 4.43±0.26 vs. HG = 3.08±0.25). This was partially prevented by the co-infusion of TPO (HG+TPO = 3.65±0.26; N.S. vs. SAL or HG). Similar intergroup differences in C-peptide to insulin ratio failed to reach significance at basal and 22mmol/l glucose.

Insulin sensitivity index (M/I index) was decreased in HG compared to SAL at both 13mmol/l and 22mmol/l glucose. Decreased insulin sensitivity was partially prevented by the co-infusion of TPO at 22mmol/l glucose (Figure 3.6.E).

Although insulin and C-peptide levels were elevated in HG, the disposition index (DI), which represents the ability of the β-cell to compensate for insulin resistance, was lower in HG compared to SAL. TPO was able to completely restore DI during both steps of the clamp (Figure 3.6.F).

### 3.4.2. 96h Infusions

To investigate the effect of antioxidants in a more chronic, less extreme model of high glucose-induced β-cell dysfunction, GSIS in isolated islets was assessed following 96h hyperglycemia (plasma glucose = ~15mmol/l) with/out TPO or TAU. Ninety-six hours
hyperglycemia increased insulin secretion at 6.5mmol/l glucose, but impaired GSIS at 22mmol/l glucose. Insulin secretion at 13mmol/l glucose was unaffected. Similar to our findings following 48h glucose infusion, both antioxidants did not prevent the increase in basal insulin secretion induced by hyperglycemia, and only TPO was able to prevent the impairment in GSIS at 22mmol/l glucose (Figure 3.7.).
3.5. Discussion

In this study, the role of oxidative stress in high glucose-induced β-cell dysfunction was investigated using ex vivo and in vivo models. Chronic hyperglycemia has been well documented to induce β-cell dysfunction in vitro (60; 63; 71; 252) and ex vivo (65; 66; 76). Consistent with previous results, we found that β-cell function was impaired ex vivo in isolated islets and also in vivo, as assessed by the hyperglycemic clamp following 48h of glucose infusion.

During the hyperglycemic clamp, absolute insulin and C-peptide levels were elevated in rats that received 48h glucose infusion. However, insulin resistance was also induced in these animals. Insulin secretion in vivo has to be evaluated in the context of insulin sensitivity, since the normal β-cell compensates for insulin resistance by increasing insulin secretion, independent of plasma glucose. In subjects with normal glucose tolerance, insulin secretion and sensitivity are linked through a hyperbolic relationship (253; 254), i.e. the product of insulin sensitivity and insulin secretion is a constant. This constant is defined as the disposition index (DI), and is a measure of β-cell function (including β-cell ability to compensate for insulin resistance). DI was decreased in glucose-infused rats, demonstrating that β-cell dysfunction was induced by 48h hyperglycemia. Aside from a decreased DI, there was also a marked defect in the response of insulin and C-peptide to a rise in plasma glucose, which further demonstrates β-cell dysfunction in glucose-infused rats.

Increased insulin secretion was observed at basal glucose (~6.5mmol/l) both in isolated islets and in vivo before the onset of the hyperglycemic clamp. The mechanisms whereby chronic glucose exposure increases basal insulin secretory response are unclear. It has been proposed that this increase may be due to induction of glycolytic genes by glucose. In INS-1 cells, prolonged glucose elevation has been shown to increase basal insulin secretion, and this
was associated with increased protein expression of phosphofructokinase 1, glyceraldehyde 3-phosphate dehydrogenase, and pyruvate kinase (255). It has also been suggested that an increase in low-$K_m$ glucose-phosphorylating activity due to increased expression of hexokinase may account for the increase in basal insulin secretion (256; 257). Other proposed mechanisms for the increase in basal insulin secretion include an increase in islet stores of glycogen and triglycerides (which breakdown during basal glucose levels to stimulate insulin secretion), increase in constitutive insulin secretion, and increase in $\beta$-cell mass. However, based on the findings that an increase in insulin secretion was not observed in islets at 2.8mmol/l, it is unlikely that these latter mechanism are involved. From our findings, we postulate that this increase in insulin secretion at low glucose is likely not due to oxidative stress, since all three antioxidants did not prevent this effect.

Beta-cell mass was increased by short-term glucose infusion as previously reported (258-260), mainly because of $\beta$-cell hypertrophy (also as reported previously at 48h (258; 259)). There was no detectable change in islet cell apoptosis as detected by TUNEL and annexin-V staining. Although these findings suggest that the 48h glucose infusion model is likely a model of $\beta$-cell dysfunction rather than loss, it should be noted that detection of a real change in apoptosis is very difficult $in vivo$ due to removal of apoptotic cells by macrophages (261).

The mechanism whereby prolonged exposure to high glucose impairs $\beta$-cell function is not completely understood, and is probably due to oxidative stress-dependent and independent pathways. The studies that have linked oxidative stress to high glucose-induced $\beta$-cell dysfunction have mainly been performed $in vitro$ (35; 175; 176; 181; 242), or in animal models of type 2 diabetes (76; 177), which in addition to hyperglycemia have many other metabolic alterations that can affect $\beta$-cell function. Thus, it is important to study the effect of chronic hyperglycemia $per se$ on $\beta$-cell function $in vivo$. 

65
Unexpectedly, the antioxidants TAU and NAC, which we have previously shown to prevent lipid-induced β-cell dysfunction *in vivo* and in isolated islets (same doses as in this study), were not able to prevent high glucose-induced β-cell dysfunction in isolated islets (228) (The effects of TAU and NAC were not assessed by the hyperglycemic clamp since TAU and NAC did not show any effect on the DI at 48h). One possible explanation may be that a higher dose of TAU and NAC is required to prevent high glucose-induced than lipid induced β-cell dysfunction. However, this is unlikely, since 1) co-infusion of TAU or NAC completely normalized H$_2$DCF-DA-measured ROS, and 2) doubling the dose of TAU (compared to the dose infused with lipids) was still ineffective. Another more plausible explanation may be that different types of ROS are important for β-cell dysfunction induced by glucose and lipids. TAU is effective in scavenging toxic aldehydes. NAC contains an –SH group, thus is capable of reducing oxidized proteins. NAC is also a precursor of intracellular glutathione. Thus, it is possible that toxic aldehyde generation and glutathione depletion play a lesser role in glucose-than lipid-induced β-cell dysfunction.

In contrast to TAU and NAC, the antioxidant TPO was completely effective in preventing β-cell dysfunction induced by prolonged hyperglycemia in isolated islets and *in vivo*. Interestingly TPO did not decrease total ROS (as detected by H2DCF-DA) induced by prolonged glucose infusion. This was not surprising since TPO is a superoxide dismutase mimetic, i.e. it converts superoxide to hydrogen peroxide. Instead, TPO did reduce total superoxide in islets of glucose treated rats, unlike TAU or NAC. Therefore, our findings show that the effect of antioxidants to prevent high glucose-induced β-cell dysfunction parallels their ability to normalize superoxide, but not H2DCF-DA-detected ROS, suggesting that superoxide generation is important in our *in vivo* model of high glucose-induced β-cell dysfunction. Our findings are in accordance with an *in vitro* study by Krauss *et al.* (62), which showed that
overexpression of Mn superoxide dismutase, but not glutathione peroxidase 1 (which decreases H$_2$O$_2$) prevented high glucose-induced β-cell dysfunction.

In the β-cell, superoxide can be generated in various ways. One possible source of superoxide is the mitochondrial electron transport chain. During hyperglycemia, superoxide production is increased due to greater electron flow in the electron transport chain. Studies have shown that mitochondrial production of ROS can impair insulin secretion (62; 163). Our findings show that high glucose-induced β-cell dysfunction in vivo is indeed associated with increased mitochondrial superoxide levels, and this increase was completely prevented by the co-infusion of TPO.

Increased mitochondrial superoxide can affect β-cell function in several ways. Superoxide can decrease mitochondrial glucose oxidation. Superoxide can also activate uncoupling protein 2, resulting in decreased ATP produced by glucose oxidation, and thus decreased GSIS (62). The fact that insulin content was only mildly improved by TPO suggest that after 48h, TPO is mainly acting on the insulin secretory process, presumably by increasing glucose oxidation (160) or decreasing uncoupling (62) via decreased mitochondrial superoxide.

In addition to the mitochondria, the cytosol can also generate superoxide via the activation of NADPH oxidase. Beta-cells have been shown to express NADPH oxidase (262), and components of this enzyme are elevated in islets of animal models of type 2 diabetes (263). Furthermore, glucose can activate NADPH oxidase via protein kinase C (153). When NADPH was inhibited in islets of $db/db$ mice, insulin content was partially restored (263), suggesting that cytosolic superoxide may also be important in β-cell dysfunction. Here, we showed that the mitochondria are an important site of superoxide production in a model of high glucose-induced β-cell dysfunction. However, further studies need to be performed to assess the role of cytosolic superoxide production. This will be discussed further in the General Discussion section.
It is recognized that our glucose infusion model is short-term and extreme in nature, and that 48h glucose infusion does not induce β-cell dysfunction in humans (69). However, there is evidence that high glucose-induced β-cell dysfunction does occur in humans following exposure to hyperglycemia (13mmol/l) for longer than 68h (69). Thus, we wished to determine the effect of antioxidants in a less extreme, more chronic model of high glucose-induced β-cell dysfunction, which can be reproduced in humans. We showed that similar to 48h infusion, TPO but not TAU prevented β-cell dysfunction induced by 96h of hyperglycemia (plasma glucose=~15mmol/l). Further prolongation of in vivo infusion is not feasible and more chronic glucose exposure requires in vitro studies (264) or studies in hyperglycemic but not hyperlipidemic diabetic models such as Goto-Kakizaki (GK) rats. Future studies in these models may reveal that not only TPO but also other antioxidants are beneficial, perhaps due to their effect on insulin gene transcription (75; 181).

Previously, we have shown that TAU and NAC (247) (similar dose to this study) were effective in preventing the decrease in insulin sensitivity induced by 6h of hyperglycemia. In this study, we showed that following 48h hyperglycemia, TAU and NAC increased insulin sensitivity in glucose-infused rats. Despite TAU and NAC improving insulin sensitivity, both antioxidants did not increase DI. Oxidative stress has been shown to play a role in both hyperglycemia-induced insulin resistance and hyperglycemia-induced β-cell dysfunction, thus it is unclear why TAU and NAC were effective in the former but not the latter. It is possible that in high glucose-induced β-cell dysfunction, the main site of dysfunction is the mitochondria (i.e. due to induction of UCP2 and/or inhibition of mitochondrial oxidation by superoxide). This is supported by the findings that TPO only very partially prevented the decrease in insulin content, but completely prevented β-cell dysfunction induced by glucose (as discussed above). In contrast, in high glucose-induced insulin resistant, it is possible that the main site of dysfunction
is due to an impairment of insulin signaling pathway by oxidative stress. Since insulin signaling molecules, such as IRS-1, are localized to the cytosol, it is possible that cytosolic ROS generation (possibly due to cytosolic production of reactive aldehydes and to cytosolic glutathione depletion, which is decreased by TAU and NAC respectively), leads to activation of downstream signaling pathways, such as JNK to impair insulin signaling. Our lab has reported that with increased FFA elevation, oxidative stress can activate the stress sensitive pathways JNK and IKKβ pathways to induce hepatic insulin resistance (unpublished findings). To investigate these possibilities, one approach would be to determine the differences in sites and types of ROS production (i.e. by using fluorescent dyes such as MitoSOX) in β-cells and insulin sensitive tissues in the presence of high glucose. Another approach would be to use transgenic mice. For example, to determine the relative role of mitochondrial superoxide production in high glucose-induced β-cell dysfunction and insulin resistance, mitochondrial superoxide dismutase (MnSOD) overexpressing mice can be infused with glucose, and insulin sensitivity and β-cell function can be measured respectively using the hyperinsulinemic-euglycemic and hyperglycemic clamp. To investigate the role of hydrogen peroxide production, the glutathione peroxidase 1 overexpressing mice can be utilized. Glutathione peroxidase is an important antioxidant enzyme for detoxifying hydrogen peroxide (265).

**In vivo**, metabolic compensation for insulin resistance involves both increased GSIS and decreased insulin clearance. The C-peptide to insulin ratio (index of insulin clearance) was lower at some points after prolonged hyperglycemia consistent with a reduction in insulin clearance. Addition of TPO tended to reverse the decrease in C-peptide to insulin ratio, although not significantly, presumably because this ratio is not a good indicator of insulin clearance. Therefore, it cannot be excluded that a TPO-induced increase in insulin clearance stimulates β-cell compensation for insulin resistance, thus contributing to improved β-cell
function in vivo. Nevertheless, data from isolated islets clearly show a direct β-cell effect of TPO.

In conclusion, our study demonstrates that β-cell dysfunction induced by 48-96h glucose elevation in vivo is due to superoxide generation. Furthermore, this study suggests that superoxide dismutase mimetics are of potential interest to preserve β-cell function in type 2 diabetes.
Table 3.1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>Glucose (mM)</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL n=10</td>
<td>0</td>
<td>5.62 ± 0.22</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.81 ± 0.70</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>5.84 ± 0.21</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.63 ± 0.20</td>
<td>-</td>
</tr>
<tr>
<td>HG n=11</td>
<td>0</td>
<td>5.88 ± 0.25</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>21.57 ± 1.16</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>21.72 ± 0.72</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>20.48 ± 0.81</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>HG+L-TAU n=8</td>
<td>0</td>
<td>5.56 ± 0.34</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>21.9 ± 0.84</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>20.1 ± 0.37</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>21.78 ± 1.66</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>HG+M-TAU n=5</td>
<td>0</td>
<td>5.08 ± 0.33</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>20.82 ± 0.99</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>19.62 ± 0.87</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>19.28 ± 0.31</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>HG+TAU n=6</td>
<td>0</td>
<td>5.1 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>20.41 ± 1.08</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>19.62 ± 1.00</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>20.18 ± 0.59</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>HG+NAC n=7</td>
<td>0</td>
<td>5.31 ± 0.14</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>20.18 ± 1.00</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>19.68 ± 1.00</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>18.47 ± 0.88</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>HG+TPO n=10</td>
<td>0</td>
<td>5.27 ± 0.085</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>19.74 ± 0.63</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>20.74 ± 0.63</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>19.41 ± 0.65</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>TAU n=5</td>
<td>0</td>
<td>5.25 ± 0.26</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.4 ± 0.18</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>5.33 ± 0.08</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.4 ± 0.18</td>
<td>NS</td>
</tr>
<tr>
<td>NAC n=5</td>
<td>0</td>
<td>5.58 ± 0.75</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>6.15 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>5.45 ± 0.45</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.9 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>TPO n=6</td>
<td>0</td>
<td>5.38 ± 0.22</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.49 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>5.2 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.29 ± 0.073</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3.1. Blood glucose level during the 48h Infusion Period. Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose+low dose taurine (HG+L-TAU, 2.12 µmol/kg.min); 4) glucose + middle dose taurine (HG+M-TAU, 2.76 µmol/kg.min); 5) glucose + high dose taurine (HG+H-TAU, 5.52 µmol/kg.min); 6) glucose + N-acetylcysteine (HG+NAC, 2.76 µmol/kg.min); 7) glucose+TPO (HG+TPO, 2.41 µmol/kg.min); 8) middle dose taurine (TAU, 2.76 µmol/kg.min); 9) N-acetylcysteine (NAC, 2.76 µmol/kg.min); 10) tempol (TPO, 2.41 µmol/kg.min). Blood glucose levels were elevated to ~20mmol/l glucose in all glucose-infused groups. No significant differences were detected between all glucose-infused groups. Data are means±SE.
Table 3.2. β-Cell Morphology at the end of 48h Infusion Period. Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + TPO (HG+TPO, 2.41 µmol/kg.min); 4) tempol (TPO, 2.41 µmol/kg.min). Data are means ± SE. *p<0.05 vs. SAL, †p<0.01 vs. SAL, ‡p<0.001 vs. SAL

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pancreas Weight (g)</th>
<th>% β-cell area</th>
<th>β-cell mass (mg/pancreas)</th>
<th>Individual β-Cell Area (µm²/β-cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL (n=6)</td>
<td>1.30 ± 0.16</td>
<td>0.66 ± 0.044</td>
<td>8.71 ± 1.29</td>
<td>213.52 ± 10.69</td>
</tr>
<tr>
<td>HG (n=5)</td>
<td>1.56 ± 0.17</td>
<td>1.01 ± 0.096†</td>
<td>16.19 ± 3.06‡</td>
<td>345.78 ± 5.28*</td>
</tr>
<tr>
<td>HG+TPO (n=7)</td>
<td>1.42 ± 0.10</td>
<td>1.02 ± 0.068†</td>
<td>14.48 ± 1.49‡</td>
<td>342.19 ± 10.51*</td>
</tr>
<tr>
<td>TPO (n=4)</td>
<td>1.12 ± 0.11</td>
<td>0.71 ± 0.029</td>
<td>7.88 ± 0.64</td>
<td>239.30 ± 9.78</td>
</tr>
</tbody>
</table>
Figure 3.1. Effects of taurine, N-acetylcysteine and tempol on glucose infusion rate (Ginf) (A), insulin (B), C-peptide (C), sensitivity index (D), and disposition index (E), at the end of 48h glucose infusion period. Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + low dose taurine (HG+L-TAU, 2.12 µmol/kg.min); 4) glucose + middle dose taurine (HG+M-TAU, 2.76 µmol/kg.min); 5) glucose + high dose taurine (HG+H-TAU, 5.52 µmol/kg.min); 6) glucose + N-acetylcysteine (HG+NAC, 2.76 µmol/kg.min); 7) glucose + TPO (HG+TPO, 2.41 µmol/kg.min. The non-glucose infused groups are not shown in this graph, as in these groups Ginf=0, glucose, insulin, and C-peptide are basal, and SI and DI cannot be calculated. At 48h, co-infusion of TAU (all doses) or NAC tended to increase, and TPO significantly increased Ginf compared to HG (A). Plasma insulin levels tended to decrease compared to HG with all antioxidants (B). C-peptide was decreased by TAU (all doses) and NAC, but not by TPO (C) vs. HG alone. All three antioxidants improved insulin sensitivity (D), but only TPO increased the disposition index (E) vs. HG alone. Data are means ± SE. # p<0.05 vs. HG, ¶ p<0.01 vs. HG, * p<0.001 vs. HG
Figure 3.2. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on insulin secretion in freshly isolated islets. Effects of hyperglycemia and tempol on islet insulin content (D). Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + low dose taurine (HG+L-TAU, 2.12 µmol/kg.min); 4) glucose + middle dose taurine (HG+M-TAU, 2.76 µmol/kg.min); 5) glucose + high dose taurine (HG+H-TAU, 5.52 µmol/kg.min); 6) glucose + N-acetylcysteine (HG+NAC, 2.76 µmol/kg.min); 7) glucose + TPO (HG+TPO, 2.41 µmol/kg.min); 8) middle dose taurine (TAU, 2.76 µmol/kg.min); 9) N-acetylcysteine (NAC, 2.76 µmol/kg.min); 10) tempol (TPO, 2.41 µmol/kg.min). (A-C) 48h glucose infusion increased insulin secretion at 6.5mmol/l glucose, but impaired GSIS at 13mmol/l and 22mmol/l glucose. TPO but not TAU or NAC completely restored GSIS at both 13mmol/l and 22mmol/l glucose. All antioxidants did not affect the increase in insulin secretion at 6.5mmol/l glucose. (D) Insulin content was depleted following 48h glucose infusion. Addition of TPO doubled the insulin content, but this was still profoundly reduced compared to SAL. Data are means ± SE. *p<0.001 vs. SAL, a p<0.001 vs. SAL, b p<0.05 HG+TPO vs. HG.
Figure 3.3. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on islet ROS as detected by dihydro-dichlorofluorescein diacetate (H$_2$DCF-DA). Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + taurine (HG+TAU, 2.76 µmol/kg.min); 4) glucose + N-acetylcysteine (HG+NAC, 2.76 µmol/kg.min); 5) glucose + TPO (HG+TPO, 2.41 µmol/kg.min); 6) taurine (TAU, 2.76 µmol/kg.min); 7) N-acetylcysteine (NAC, 2.76 µmol/kg.min); 8) tempol (TPO, 2.41 µmol/kg.min). Data are expressed as % of SAL ± SE (a SAL control rat was studied on each experiment day). Approximately 10 islets were measured per n number (rat). Panel D shows representative fluorescent images of the islets. Forty-eight hours glucose infusion increased H$_2$DCF-DA-detected ROS. Coinfusion of TAU or NAC abolished the increase in ROS induced by high glucose (A and B). TPO did not have a significant effect (C). *p<0.05 vs. SAL, †p<0.01 vs. SAL.
Figure 3.4. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on total islet superoxide levels as detected by hydroethidine. Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + taurine (HG+TAU, 2.76 µmol/kg.min); 5) glucose + N-acetylcysteine (HG+NAC, 2.76 µmol/kg.min); 6) glucose + TPO (HG+TPO, 2.41 µmol/kg.min); 7) taurine (TAU, 2.76 µmol/kg.min); 8) N-acetylcysteine (NAC, 2.76 µmol/kg.min); 9) tempol (TPO, 2.41 µmol/kg.min). Forty-eight hours glucose infusion increased total islet superoxide level. Co-infusion of TAU and NAC did not normalize superoxide (A and B). TPO abolished the increase induced by 48h glucose infusion (C). Data are expressed as % of SAL ± SE (a SAL control rat was studied on each experiment day). Approximately 10 islets were measured per n number (rat). Panel D shows representative fluorescent images of the islets. # p<0.05 vs. SAL.
Figure 3.5. Effects of hyperglycemia and taurine (A), N-acetylcysteine (B) and tempol (C) on islet mitochondrial superoxide levels as detected by MitoSOX. Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + TPO (HG+TPO, 2.41µmol/kg.min); 4) tempol (TPO, 2.41 µmol/kg.min). Forty-eight hours glucose infusion increased mitochondrial superoxide levels, and co-infusion of TPO completely abolished this increase. Data are expressed as % of SAL ± SE (a SAL control rat was studied on each experiment day). Approximately 10 islets were measured per n number (rat). Panel D shows representative fluorescent images of the islets. *p<0.001 vs. SAL
Figure 3.6. Effects of tempol on plasma glucose levels (A), glucose infusion rate (Ginf) (B), plasma insulin levels (C), plasma C-peptide levels (D), sensitivity index (E), and disposition index (F) during the two-step hyperglycemic clamp with or without glucose infusion. Rats were infused for 48h with either: 1) saline (SAL), 2) a variable infusion of 37.5% glucose to achieve and maintain plasma glucose at 20-22mmol/l (HG), 3) glucose + tempol (HG+TPO; 2.41µmol/kg.min), 4) TPO alone (2.41µmol/kg.min), or 5) mannitol (MAN) to control for plasma osmolarity, infused at a rate comparable to glucose infusion (~45µl/min). Data are means ± SE. a p<0.05 HG, HG+TPO vs. SAL at basal glucose, b p<0.001 HG+TPO vs. HG at 13mmol/l, c p<0.05 HG+TPO vs. SAL at 13mmol/l glucose, d p<0.001 HG vs. SAL at 22mmol/l, e p<0.001 HG, HG+TPO vs. SAL at basal and 13mmol/l glucose, f p<0.01 vs. SAL, * p<0.001 vs. SAL.
**Figure 3.7.** Effects of taurine and tempol on insulin secretion in freshly isolated islets following 96h hyperglycemia. Rats were treated for 96h with 1) saline (SAL); 2) glucose, to maintain glycemia at ~15mmol/l (HG15); 3) glucose + middle dose taurine (HG15+M-TAU, 2.76 µmol/kg.min); 4) glucose + TPO (HG15+TPO, 2.41 µmol/kg.min). Ninety-six hours hyperglycemia (plasma glucose=15mmol/l) increased insulin secretion at 6.5mmol/l glucose, but impaired insulin secretion at 22mmol/l glucose. No impairment was observed at 13mmol/l glucose. Co-infusion of TPO but not TAU completely prevented β-cell dysfunction induced by 96h hyperglycemia at 22mmol/l glucose. Data are means ± SE.  
# p<0.001 vs. SAL, ▲ p<0.01 vs. SAL,
Study 2

High Glucose-Induced β-Cell Dysfunction *In Vivo*: Link Between Oxidative Stress and Endoplasmic Reticulum Stress

* The results of this study are incorporated in a manuscript in revision in Diabetes
4.1. Abstract

Objective Recent studies have implicated oxidative stress and endoplasmic reticulum stress in high glucose-induced β-cell dysfunction. However, the link between the two is unknown. The objective of this study was to determine the link between ER stress and oxidative stress in high glucose-induced β-cell dysfunction in vivo.

Research Design and Methods Wistar rats were infused i.v. with glucose for 48h to achieve 20mmol/l hyperglycemia with or without co-infusion of either the superoxide dismutase mimetic tempol (TPO), or the chemical chaperone 4-phenylbutyrate (PBA). This was followed by assessment of β-cell function, and measurement of ER stress markers and superoxide in islets.

Results Forty-eight hours of glucose infusion impaired β-cell function in vivo, and this was accompanied by increased islet superoxide levels, and enhanced unfolded protein response (UPR) pathway activation (increased XBP-1 mRNA splicing and phospho-eIF2α levels). Downstream markers of UPR were also elevated by glucose. Co-infusion of TPO, which decreased islet superoxide, reduced ER stress markers and prevented high glucose-induced β-cell dysfunction in vivo. Co-infusion of the chemical chaperone PBA with glucose prevented the increase in spliced XBP-1, but not phospho-eIF2α. PBA decreased islet superoxide levels by glucose, and prevented high glucose-induced β-cell dysfunction in vivo. PBA also prevents β-cell dysfunction induced by less extreme hyperglycemia (15mmol/l) for a longer period of time (96h).

Conclusion These findings suggest: 1) ER stress plays a causal role in high glucose-induced β-cell dysfunction in vivo, and 2) there is a link between oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo.
4.2. Introduction

One proposed mechanism of high glucose-induced β-cell dysfunction is endoplasmic reticulum stress. The endoplasmic reticulum (ER) is involved in folding, processing and exporting secretory and membrane proteins to the Golgi apparatus. When the demand for proteins exceeds the folding capacity of the ER, ER stress ensues leading to the activation of the unfolded protein response (UPR), a mechanism that transiently inhibits global protein synthesis and increases ER chaperones and degradation of misfolded proteins to counteract ER stress (266). Prolonged in vitro exposure of β-cell lines or islets to glucose has been demonstrated to increase ER stress markers (85-88), and over-expression of the ER chaperone GRP78 has been shown to partially prevent high glucose-induced β-cell dysfunction in vitro in INS-1 cells (267). However, it is currently unknown whether ER stress plays a causal role in high glucose-induced β-cell dysfunction in vivo.

Oxidative stress plays a causal role in high glucose-induced β-cell dysfunction both in vitro and in vivo (27; 268). Both ER stress (269) and oxidative stress (174) are increased in islets of subjects with diabetes. Recent studies demonstrate a close interrelationship between oxidative stress and ER stress in β-cells (270; 271). Oxidative stress can induce ER stress, and vice versa (266). However, whether there is a link between oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo is not known.

In Study 1, we have demonstrated that 48h glucose infusion increases superoxide levels, and that co-infusion the superoxide dismutase mimetic Tempol, prevents high glucose-induced β-cell dysfunction in vivo. The objective of this study was to investigate whether 1) ER stress plays a causal role in high glucose-induced β-cell dysfunction in vivo, and 2) there is a link between ER stress and oxidative stress in high glucose-induced β-cell dysfunction. To examine
the causal role of ER stress, the effect of prolonged hyperglycemia with or without the chemical chaperone 4-phenylbutyrate on β-cell function was assessed in ex vivo isolated islets and in vivo using the hyperglycemic clamp. To examine the link between oxidative stress and ER stress, markers of UPR activation and ROS were measured in isolated islets following 48h in vivo exposure to high glucose with or without the antioxidant tempol or the chemical chaperone 4-phenylbutyrate (PBA).

4.3. Material and Methods

4.3.1. Animals and Surgery

Female Wistar rats (10-12 weeks old, 250-300g, Charles River, Canada) were cannulated, as described in the General Methods section 2.1.1.2..

4.3.2. 48h Infusion Period

4.3.2.1. 48h Infusion of Tempol

Rats were infused for 48h with either: 1) Saline (SAL), 2) A variable infusion of 37.5% glucose maintain plasma glucose at 20-22 mmol/l (HG), 3) Glucose+Tempol (HG+TPO, 2.41μmol/kg.min), or 4) Tempol (TPO), as in Study 1. In Study 1, we have shown that TPO prevents high glucose-induced increase in total and mitochondrial superoxide in isolated islets, and prevents high glucose-induced β-cell dysfunction both ex vivo in isolated islets and in vivo (assessed by the 2-step hyperglycemic clamp). In this study, we studied the effect of TPO on ER stress markers. Following 48h infusion and overnight fasting, we isolated islets to perform
insulin secretion studies and detection of ER stress markers. We also performed immunohistochemistry.

4.3.2.2. 48h Infusion of PBA

Rats were infused for 48h with either: 1) SAL, 2) HG as above, 3) Glucose+4-Phenylbutyrate acid (HG+PBA), or 4) PBA. PBA acts as a chemical chaperone to improve protein folding (272). PBA was given at a dose of 2.08umol/kg.min, adapted from a dose that was shown to alleviate ER stress, and normalized glycemia in ob/ob mice (273). PBA (Sigma) was dissolved in saline, pH=7.4. Following 48h infusion and overnight fasting, we carried out one of the following protocols 1) insulin secretory studies, superoxide (total and mitochondrial) measurements, and ER stress marker detection in isolated islets, 2) immunohistochemistry, or 3) 2-step hyperglycemic clamp.

4.3.2.3. Pancreatic Islet Isolation and Ex vivo Evaluation of glucose stimulated Insulin Secretion (GSIS)

This was performed as described in General Methods sections 2.1.3. and 2.1.4..

4.3.2.4. RNA Isolation and Real Time Polymerase Chain Reaction

Total RNA was extracted from isolated islets using Trizol Reagent (Invitrogen). RNA was transcribed to single stranded cDNA, which was used for real-time PCR analysis using the TaqMan Gene Expression System (Applied Biosystems) as in (274). Primers for rat ATF-4 (Rn00824644_gl), GRP78 (Rn01435771_g1), CHOP (Rn00492098_g1), and rat IL-1β (Rn00676333_gl) were obtained from Applied Biosystems. (Performed by L. Zhang from Dr. Allen Volchuk’s lab).
4.3.2.5. Measurement of XBP-1 mRNA Splicing

Total RNA was extracted as described above. Spliced XBP-1 mRNA was detected as in (274). (Performed by L. Zhang from Dr. Allen Volchuk’s lab).

4.3.2.6. Western Blot Analysis

Western blots were performed as in (274). The following primary antibodies were used: phospho-eukaryotic initiation factor-2α (eIF2α) (Cell Signaling, #9721, 1:500), anti-KDEL (StressGen, SPA-827, 1:1,000) and γ-tubulin (Sigma, T6557, 1:1,000). (Performed by L. Zhang from Dr. Allen Volchuk’s lab).

4.3.2.7. Total and Mitochondrial Superoxide

This was performed as described in General Methods section 2.1.5.

4.3.2.8. Immunohistochemistry

At the end of the hyperglycemic clamp (both TPO and PBA studies), rats were anesthetized with a ketamine:xylazine:acepromazine cocktail (87:1.7:0.4mg/ml) and pancreas was removed, fixed overnight in 10% formalin and stored in 70% ethanol. Samples were processed and embedded in paraffin within 5 days of collection. Tissue sections were deparaffinized and rehydrated with a series of xylene and ethanol washes. Slides were then permeabilized with 0.1% Triton X-100. For antigen retrieval, slides were transferred into boiled sodium citrate buffer and microwaved for 30 minutes. Slides were incubated in blocking solution (10% goat serum with 0.2% saponin in PBS) for 3h at room temperature. For KDEL staining, primary antibodies (mouse monoclonal anti-KDEL (Stressgen #SPA-827), and guinea pig anti-insulin antibody (Dako) at dilutions of 1:200) were added in blocking solution overnight.
at 4°C. Secondary antibodies (Oregon Green goat anti-mouse IgG and Cy5-conjugated donkey anti-guinea pig IgG) at dilutions of 1:200 in blocking solution were added for 2h at room temperature. For ubiquitin staining, primary antibodies (rabbit polyclonal anti-ubiquitin (Dako), 1:100, and guinea pig anti-insulin antibody (Dako) at dilutions of 1:200) were added in blocking solution overnight at 4°C. Secondary antibodies (Oregon Green goat anti-rabbit IgG and Cy5-conjugated donkey anti-guinea pig IgG) at dilutions of 1:200 in blocking solution were added for 2h at room temperature. Images were acquired using a Zeiss LSM510 META laser scanning confocal microscope. (Performed by L. Zhang from Dr. Allen Volchuk’s lab).

4.3.2.9. Two-Step Hyperglycemic Clamp

At the end of 48h, glucose infusion in HG and HG+PBA groups was decreased to ~5µl/min for ~75 minutes, which was required to achieve basal glucose without hypoglycemia. Basal insulin and C-peptide were measured at -20 and 0 minutes. At time=0 minutes, an infusion of 37.5% glucose was started. Plasma glucose was maintained at 13mmol/l by adjusting the rate of glucose infusion according to frequent (5-10 minutes) glycemic determinations. At 120 minutes, the glucose infusion was again raised to 22mmol/l until the end of the experiment (time = 240 minutes). The clamp details are described in the General Methods section 2.1.8..

4.3.3. Ninety-six Hours Infusions

A set of rats were also infused for 96h (4 days) with either: 1) saline (SAL4), 2) a variable infusion of 37.5% glucose to achieve and maintain plasma glucose at 15 mmol/l (HG4), 3) glucose + PBA (HG4+PBA), 4) PBA without glucose (PBA4). The same dose of PBA was used as in the 48h infusion study. Following 96h infusion, islets were isolated and *ex vivo*
evaluation of GSIS and insulin content were performed as described in General Methods sections 2.1.3. and 2.1.4..

4.3.4. Plasma Assays

Plasma glucose, insulin and C-peptide levels were measured as described in the General Methods section 2.2..

4.3.5. Calculations

The C-peptide/insulin ratio index of insulin clearance, the M/I index of insulin sensitivity, and disposition index were calculated during the last 40 minutes of each step of the hyperglycemic clamp, as described in the General Methods section 2.3..

4.3.6. Statistics

Refer to General Methods section 2.4.
4.4. Results

4.4.1. 48h Infusions

4.4.1.1. Hyperglycemia, Oxidative Stress and Activation of the Unfolded Protein Response

Baseline fed plasma glucose and insulin were not different between groups before the onset of the 48h infusions. In rats infused for 48h with glucose alone (HG) or in combination with TPO (HG+TPO), glucose levels were elevated to ~20-22mmol/l by ~6h and were maintained at that level for the remainder of the 48h period, as in Study 1.

ER stress activates three sensor proteins, PERK, IRE-1 and ATF-6, which mediate the UPR. To examine whether prolonged hyperglycemia induces ER stress in pancreatic β-cells, we first monitored activation of two of these pathways, PERK and IRE1 (ATF-6 was monitored indirectly via detection of chaperone mRNA levels).

Upon activation by ER stress, PERK phosphorylates eIF2α, which attenuates general protein translation. As shown in Figures 4.1.A and B, hyperglycemia increased the phosphorylation of eIF2α (p<0.01 vs. SAL), and co-infusion of TPO with glucose completely reversed this effect. TPO alone did not have any significant effect on eIF2α phosphorylation.

Activation of IRE-1 results in splicing of the XBP-1 mRNA, which leads to enhanced XBP-1 translation. Hyperglycemia increased XBP-1 mRNA splicing (p<0.001 vs. SAL), and co-infusion of TPO with glucose partially reversed this effect (p<0.001 HG+TPO vs. HG). TPO alone appeared to slightly increase XBP-1 splicing (Figures 4.1.C and D).

Activation of the UPR pathways leads to increased expression of a number of genes including the pro-apoptotic factor CHOP and ER chaperones such as GRP78 and GRP94 (275).
Forty-eight hours glucose infusion increased CHOP (p<0.001 vs. SAL) and GRP78 mRNA levels (p<0.01 vs. SAL). Co-infusion of TPO prevented the high glucose-induced up-regulation of CHOP mRNA, but not GRP78 mRNA (p=NS HG vs. HG+TPO). TPO alone did not have any significant effect on GRP78 or CHOP mRNA levels (Figure 4.2.A).

Forty-eight hours glucose infusion also increased the protein levels of GRP78 (p<0.001 vs. SAL) and GRP94 (p<0.001 vs. SAL) (Figure 4.2.B). Co-infusion of TPO did not decrease, and tended to further increase these chaperone levels. TPO alone had no significant effect on GRP78 and GRP94 protein levels (Figure 4.2.B). Consistent with the western analysis, immunostaining pancreas sections with α-KDEL antibody, which recognizes GRP78 and GRP94, showed a more intense staining in HG compared to control islets (Figures 4.3.A and B). Co-infusion of TPO with HG had no effect on α-KDEL staining indicating TPO does not reduce HG-induced chaperone levels (Figure 4.3.C).

The accumulation of polyubiquitinated proteins is a characteristic of ER stress, and is increased by high glucose in vitro (276). Consistent with this finding, we show that 48h glucose infusion increased polyubiquitinated protein levels compared to SAL (Figure 4.4.).

4.4.1.2. Effect of Chemical Chaperone 4-Phenylbutyrate on High glucose-induced β-Cell Dysfunction ex vivo

Baseline fed plasma glucose and insulin levels were similar between the experimental groups before the onset of the 48h infusion. In rats infused for 48h with glucose alone or in combination with PBA, glucose levels were elevated to ~20mmol/l by ~6h and were maintained at that level for the remainder of the 48h period (Table 4.1.).

Following 48h infusion, islets were isolated and insulin secretion was measured. Insulin secretion at 2.8mmol/l glucose did not differ between groups. Forty-eight hours glucose
infusion increased insulin secretion at 6.5mmol/l, but impaired GSIS at 13mmol/l and 22mmol/l glucose. Co-infusion of PBA with glucose slightly increased insulin secretion at 6.5mmol/l glucose, and completely restored GSIS at both 13mmol/l and 22mmol/l glucose (Figure 4.5.A).

Forty-eight hours hyperglycemia reduced islet insulin content. Co-infusion of PBA did not improve islet insulin content (Figure 4.5.B).

4.4.1.3. 4-Phenylbutyrate and Activation of the Unfolded Protein Response

To investigate the effect of PBA on activation of UPR, the PERK and IRE pathways were monitored. As shown in Figures 4.6.A and B, co-infusion of PBA with HG did not have any effect on eIF2α phosphorylation. However, PBA co-infusion did significantly reduce spliced XBP-1 levels (p<0.001 HG+PBA vs. HG) (Figures 4.6.C and D), suggesting that PBA decreases activation of IRE-1. Interestingly, PBA alone without glucose infusion also increased eIF2α phosphorylation and spliced XBP-1.

Co-infusion of PBA partially reduced CHOP and GRP78 mRNA induced by 48h hyperglycemia (Figure 4.7.A). Similar to TPO, co-infusion of PBA tended to increase GRP78 protein, and significantly increase GRP94 compared to HG (Figure 4.7.B). PBA alone without glucose slightly increased CHOP and GRP78 mRNA, and GRP94 and 78 protein levels. Consistent with our western data, immunostaining for α-KDEL shows that both HG and HG+PBA have similar levels of GRP78 and 94 (Figure 4.3.D).

4.4.1.4. Total and Mitochondrial Superoxide Levels in Islets

Forty-eight hours glucose infusion elevated total, and mitochondrial islet superoxide levels, as previously reported (268). Co-infusion of PBA with glucose significantly lowered both total (p<0.001 HG+PBA vs. HG) and mitochondrial (p<0.05 HG+PBA vs. HG) islet
superoxide. PBA alone without glucose infusion had a tendency to reduce total superoxide, but had no effect on mitochondrial superoxide levels (Figure 4.8).

### 4.4.1.5. Two-Step Hyperglycemic Clamp

To further investigate whether PBA is effective in preventing high glucose-induced β-cell dysfunction *in vivo*, a two-step hyperglycemic clamp was performed after 48h infusions. Basal plasma glucose prior to the clamp was lower in HG and HG+PBA. Plasma glucose was elevated to 13mmol/l until 120min, then to 22mmol/l until 240min with no difference between groups (Figure 4.9.A).

In HG rats, a lower glucose infusion rate (Ginf) was necessary to clamp glucose at 22mmol/l, indicating that the circulating insulin was inadequate to compensate for insulin resistance. Co-infusion of PBA completely restored Ginf (Figure 4.9.B).

Basal insulin and C-peptide levels (-20min to 0min) were higher in HG and HG+PBA rats than SAL (p<0.001). In response to increasing glucose levels, plasma insulin and C-peptide rose as expected. HG elevated plasma insulin levels at both 13mmol/l (p<0.001) and 22mmol/l (p<0.05) glucose, and C-peptide at 13mmol/l glucose (p<0.001). Co-infusion of PBA tended to lower plasma insulin, but had a tendency to increase C-peptide levels at 22mmol/l (Figures 4.9.C and D).

The C-peptide/Insulin ratio (index of insulin clearance) was lower in HG than SAL at 13mmol/l glucose (p<0.05 vs. SAL; SAL = 3.66±0.26 vs. HG = 2.11±0.09) and 22mmol/l glucose (p<0.05 vs. SAL; SAL = 3.08±0.20 vs. HG = 2.10±0.09). This was partially prevented by the co-infusion of PBA (HG+PBA = 2.56±0.17 at 13mmol/l glucose; 2.51±0.21; N.S. vs. SAL or HG at both glucose levels).
Sensitivity index (M/I index) was decreased in HG compared to SAL at both 13mmol/l and 22mmol/l glucose. Co-infusion of PBA tended to prevent this decrease in insulin sensitivity (Figure 4.9.E).

Although insulin and C-peptide levels were elevated in HG, the disposition index (DI), which represents the ability of the β-cell to compensate for insulin resistance, was lower in HG compared to SAL. PBA was able to completely restore DI during both steps of the clamp (Figure 4.9.F).

4.4.1.6. Interleukin-1β mRNA expression

To explore the possibility that increased IL-1β production by the β-cells in response to prolonged hyperglycemia is inducing oxidative stress and/or ER stress to impair β-cell function, we measured IL-1β mRNA levels in isolated islets. No difference in IL-1β mRNA expression was detected between groups (IL-1β mRNA expressed relative to actin: SAL=1.26±0.014; HG=1.27±0.020; HG+PBA=1.25±0.014).

4.4.2. 96h Infusions

Ninety-six hours of hyperglycemia increased insulin secretion at 2.8mmol/l and 6.5mmol/l glucose, but impaired GSIS at 22mmol/l glucose. There was also a tendency of prolonged hyperglycemia to decrease GSIS at 13mmol/l glucose. PBA did not prevent the increase in insulin secretion at 2.8 and 6.5mmol/l induced by hyperglycemia, but was able to prevent the impairment in GSIS at 22mmol/l glucose (Figure 4.10.A). Ninety-six hours of hyperglycemia reduced islet insulin content. The addition of PBA increased insulin content (~2 fold) (Figure 4.10.B).
4.5. Discussion

Oxidative stress is a well established mechanism by which chronic high glucose induces β-cell dysfunction (27). Recent studies have demonstrated a close interrelationship between oxidative stress and ER stress (224). Oxidative stress can affect protein folding by direct protein modification, chaperone inactivation and/or depleting cellular glutathione levels (222), and can deplete ER calcium levels (225; 226), leading to ER stress. ER stress can in turn generate more oxidative stress through increased ERO1p oxidase activity (an enzyme that transfers electrons generated by disulphide bond formation in the ER to molecular oxygen), and glutathione consumption due to reduction of mispaired disulphide bonds (227). In Study 1, we have shown that TPO, a superoxide dismutase mimetic, decreases superoxide levels and prevents high glucose-induced β-cell dysfunction ex vivo and in vivo (268). In this study, we found that TPO decreases markers of UPR activation induced by glucose, such as phospho-eIF2α, spliced XBP-1, GRP78 and CHOP mRNA. Interestingly, TPO did not decrease GRP78 and GRP94 protein levels, which may reflect the longer half-life of proteins versus mRNA. This can be investigated by measuring time dependent changes in GRP78 and GRP94 mRNA and protein levels in islets exposed to HG with or without TPO. It is also possible that co-infusion of TPO results in a milder degree of ER stress than glucose infusion alone, which allows the β-cell to increase expression of chaperones at the post-transcriptional levels (277). However, we cannot exclude that TPO by itself induces more ER stress and/or affects the UPR, as indicated by the tendency of ER chaperone proteins to be increased by the co-infusion of TPO, and by the small increase in spliced XBP-1 mRNA induced by TPO alone.

The observation that TPO prevents high glucose-induced increase in ER stress markers supports the notion that ER stress lies downstream of oxidative stress. However, there is also evidence that ER stress can induce oxidative stress (270; 271). In the present study, the
chemical chaperone 4-phenylbutyrate PBA reduced not only total but also mitochondrial superoxide levels in isolated islets. It has been reported that accumulation of unfolded protein in the ER can cause Ca\(^{2+}\) leakage from the ER, and uptake of Ca\(^{2+}\) into the mitochondria (278; 279). This can lead to disruption of the electron transport chain and superoxide generation (280).

Co-infusion of PBA with glucose decreased XBP-1 splicing, and CHOP and GRP78 mRNA. Unexpectedly, PBA did not prevent high glucose-induced increase in phospho-eIF2\(\alpha\). Why this occurs is unclear. It is possible that PBA decreases ER stress only partially and not enough to decrease PERK activation. To investigate this possibility, the effect of PBA on the branches of UPR can be studied in a milder model of high glucose-induced \(\beta\)-cell dysfunction (i.e. the 96h glucose infusion model). It is possible that in a milder model, PBA can also decrease phospho-eIF2\(\alpha\) levels. PBA alone increased phospho-eIF2\(\alpha\) levels and also induced a small increase in markers of UPR activation, suggesting that it may cause some ER stress. Interestingly, increased phospho-eIF2\(\alpha\) did not parallel high glucose-induced \(\beta\)-cell dysfunction, which suggests that this is likely not caused by eIF2\(\alpha\)-mediated translational attenuation. Other factors, such as induction of oxidative stress and/or activation of other branches of the UPR, may be involved. The finding that co-infusion of PBA restores GSIS, but not insulin content after 48h hyperglycemia suggests that PBA is mainly acting on other processes than insulin biosynthesis, i.e. ATP production and/or exocytosis to improve \(\beta\)-cell function. In our study, we show that co-infusion of PBA reduces total and mitochondrial superoxide levels. Mitochondrial superoxide can decrease glucose oxidation (160) and increase activation of uncoupling protein 2 (62) (which dissipates the proton gradient in the electron transport chain), and thereby reduce ATP production by glucose oxidation. This can lead to decreased GSIS.
Prolonged glucose exposure impairs β-cell function in part by decreasing insulin gene transcription (71). Both oxidative stress (162) and ER stress (281) can activate JNK, and activation of this pathway has been implicated in high glucose-induced impairment in insulin gene transcription (162). We have also found that inhibition of JNK prevents β-cell dysfunction induced by 48h glucose infusion without completely restoring insulin content (see Study 3), thus, we cannot exclude that ER stress is impairing β-cell function by decreasing insulin gene transcription. It is possible that because PBA is directly affecting translation (as indicated by increased phospho-eIF2α), changes in insulin content are difficult to detect and/or improved insulin biosynthesis does not result in improved insulin content because of concomitant improvement of insulin secretion.

To determine whether PBA is effective in vivo, we performed 2-step hyperglycemic clamps. Co-infusion of PBA prevents high glucose-induced β-cell dysfunction in vivo, as assessed by the disposition index (an in vivo measure of β-cell function, including the β-cell ability to compensate for insulin resistance). PBA tended to improve insulin sensitivity. Insulin sensitivity during a hyperglycemic clamp mainly reflects peripheral insulin sensitivity because glucose production is totally suppressed by glucose and high insulin. It has been previously reported that PBA reduces obesity-induced ER stress and consequently improves insulin sensitivity in the liver (273). We have shown that PBA partially prevents high glucose-induced decrease in insulin clearance (as indicated by C-peptide to insulin ratio). Insulin clearance is mainly a liver function, and is generally proportional to hepatic insulin sensitivity. Thus, PBA may be preventing high glucose-induced hepatic insulin resistance. Since PBA can increase insulin clearance, it cannot be excluded that in vivo this stimulates β-cell compensation for insulin resistance, thus contributing to improved β-cell function. Nevertheless, data from isolated islets clearly show an effect of PBA on insulin secretion.
As in Study 1, following 48h glucose infusion, increased insulin secretion was observed at basal glucose (~6.5mmol/l) not only *in vivo* (i.e., in response to insulin resistance), but also in isolated islets. Possible mechanisms for this increase are described in Study 1 Discussion section. This effect is likely not due to oxidative stress or ER stress, since both TPO and PBA did not prevent this effect. After 96h, there was an increase in insulin secretion also at 2.8mmol/l glucose, which was not affected by TPO (See Study 1), or PBA.

In Study 1, we have reported no detectable changes in islet cell apoptosis following 48h glucose infusion. Although 48h hyperglycemia increased CHOP mRNA, the effect was modest and both TPO and PBA prevented this increase. We have attempted to measure CHOP protein by western blotting and immunohistochemistry, but the results were variable due to the well known specificity problem of the CHOP antibodies (282). In the absence of demonstrated CHOP protein elevation or apoptosis, one may argue that glucose may not induce ER stress, but perhaps nonspecific upregulation of the UPR. However, we have recently been able to show increased levels of polyubiquinated proteins, which are characteristic of misfolded proteins, in our model. One limitation of our model is that it is short-term and extreme in nature, and that 48h glucose infusion does not induce β-cell dysfunction in humans (69). However, there is evidence that high glucose-induced β-cell dysfunction occurs in humans following exposure to hyperglycemia (13mmol/l) for 68h (69). In Study 1, we have reported that TPO is able to prevent 96h high glucose-induced β-cell dysfunction. In this study, we investigated whether PBA is effective in this model. Similar to 48h infusion, co-infusion of PBA with glucose prevents high glucose-induced β-cell dysfunction following 96h. However, unlike the 48h model, PBA partially prevents high glucose-induced decrease in insulin content. The underlying mechanism for this is unclear. One possibility is that PBA may be acting on different processes.
of insulin secretion in the two models (i.e., mainly on ATP production in the 48h model versus insulin gene transcription/translation in the 96h model).

In conclusion, our study suggests that ER stress plays a causal role in high glucose-induced β-cell dysfunction in vivo, and that there is a reciprocal link between oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo. Furthermore, this study suggests that chemical chaperones are of potential interest to preserve β-cell function in type 2 diabetes.
Table 4.1. Blood glucose level during the 48h Infusion Period. Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + 4-phenylbutyrate (HG+PBA, 2.08 µmol/kg.min); or 4) 4-phenylbutyrate (PBA, 2.08 µmol/kg.min). Blood glucose levels were elevated to ~20mmol/l glucose in all glucose-infused groups. No significant differences were detected between HG and HG+PBA groups. Data are means ± SE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>Glucose (mM)</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL n=14</td>
<td>0</td>
<td>5.34 ± 0.14</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.28 ± 0.14</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>5.44 ± 0.16</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.14 ± 0.06</td>
<td>-</td>
</tr>
<tr>
<td>HG n=16</td>
<td>0</td>
<td>5.58 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>20.58 ± 1.05</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>20.59 ± 0.42</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>19.925 ± 0.87</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>HG+PBA n=12</td>
<td>0</td>
<td>5.56 ± 0.34</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>19.4 ± 0.84</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>20.1 ± 0.60</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>19.13 ± 0.91</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>PBA n=11</td>
<td>0</td>
<td>5.56 ± 0.13</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>5.38 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>5.4 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>4.01 ± 0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>
**Figure 4.1.** Forty-eight Hours Hyperglycemia Induces Activation of UPR in an Oxidative Stress Dependent Manner. Rats were treated for 48h with saline (SAL); glucose, to maintain glycemia at ~20-22mmol/l (HG); glucose+Tempol (HG+TPO, 2.41 µmol/kg.min); Tempol (TPO, 2.41 µmol/kg.min) as in Study 1. (A) shows representative immunoblots of phospho-eIF2α (an indicator of PERK activation). Each lane represents islet lysates from one animal. As a positive control, cultured INS1 cells were treated or not with thapsigargin (Tg) for 6h. (B) Western blots for phospho-eIF2α were quantified and results are expressed as % of SAL normalized by tubulin. * p<0.01 vs. SAL. (C) XBP-1 cDNA (Unspliced (uXBP-1, 480 bp) and spliced form of XBP-1 (sXBP-1, 454 bp) was amplified by RT-PCR using primers that flank the intron excised by IRE1 exonuclease activity. Each lane represents islet lysates from one animal. As a positive control, cultured INS1 cells were treated with thapsigargin (Tg) for 1h. (D) shows the mean of the ratio of sXBP-1 to uXBP-1 (as a % of SAL). * p<0.001 vs. SAL;  b p<0.05 HG+TPO vs. HG. (B and D) Data are means±SE. n number indicates the number of rats studied.
**Figure 4.2.** Effects of hyperglycemia and Tempol on Markers Downstream of UPR Activation. (A) Total RNA was isolated from islets isolated from rats treated for 48h with saline (SAL); glucose, to maintain glycemia at ~20-22mmol/l (HG); glucose+Tempol (HG+TPO, 2.41 µmol/kg.min); Tempol (TPO, 2.41 µmol/kg.min). Real-time PCR was used to analyze the relative mRNA levels of CHOP and GRP78. Results are expressed as target gene relative to β-actin. Data are means ± SE. ¶p<0.01 vs. SAL; *p<0.001 vs. SAL. (B) shows representative immunoblots with the α-KDEL antibody that recognizes GRP78 and GRP94. Each lane represents islet lysates from one animal. Western blots were quantified and results are expressed as % of SAL normalized by tubulin. n number indicates the number of rats studied. Data are means ± SE. ¶p<0.01 vs. SAL; *p<0.001 vs. SAL.
Figure 4.3. Effects of Tempol and 4-Phenylbutyrate on GRP78 and GRP94 Levels in Pancreatic Tissue Sections at the End of 48h Glucose Infusion. Pancreatic tissue sections obtained from rats treated for 48h with (A) saline (SAL), (B) glucose, to maintain glycemia at ~20-22mmol/l (HG), (C) glucose+tempol (HG+TPO, 2.41 µmol/kg.min), (D) glucose+4-phenylbutyrate (HG+PBA, 2.08 µmol/kg.min), (E) tempol (TPO, 2.41 µmol/kg.min), and (F) 4-phenylbutyrate (PBA, 2.08 µmol/kg.min). Sections were stained with KDEL (green) and insulin (red) antibodies.
Figure 4.4. Effects of 48h Hyperglycemia on Polyubiquinated Protein Levels in Pancreatic Tissue Sections. Pancreatic tissue sections obtained from rats treated for 48h with saline (SAL), or glucose, to maintain glycemia at ~20-22mmol/l (HG). Sections were stained for ubiquitin (green) and insulin (red). Polyubiquinated proteins were not detected in SAL treated islets, but were present in HG treated islets.
Figure 4.5. Effects of hyperglycemia and 4-phenylbutyrate on insulin secretion in freshly isolated islets (A) and on islet insulin content (B) following 48h infusion. Rats were treated for 48h with saline (SAL); glucose, to maintain glycemia at ~20-22mmol/l (HG); glucose+4-phenylbutyrate (HG+PBA, 2.08 µmol/kg.min); 4-phenylbutyrate (PBA, 2.08 µmol/kg.min). (A) shows that 48h glucose infusion impaired GSIS at 13mmol/l and 22mmol/l glucose, and co-infusion of PBA completely prevented this. (B) shows that insulin content was depleted following 48h glucose infusion. Addition of PBA did not prevent this decrease. Data are means ± SE. *p<0.01 vs. SAL; **p<0.001 vs. SAL.
Figure 4.6. 4-phenylbutyrate Decreases Activation of the IRE-1 Pathway. Rats were treated for 48h with saline (SAL); glucose, to maintain glycemia at ~20-22mmol/l (HG); glucose+4-phenylbutyrate (HG+PBA, 2.08 µmol/kg.min); 4-phenylbutyrate (PBA, 2.08 µmol/kg.min). (A) shows representative immunoblots of phospho-eIF2α (an indicator of PERK activation). Each lane represents islet lysates from one animal. As a positive control, cultured INS1 cells were treated or not with thapsigargin (Tg) for 6h. (B) Western blots were quantified and results are expressed as % of SAL normalized by tubulin. ¶p<0.01 vs. SAL; *p<0.001 vs. SAL. (C) XBP-1 cDNA (Unspliced (uXBP-1, 480 bp) and spliced form of XBP-1 (sXBP-1, 454 bp) was amplified by RT-PCR using primers that flank the intron excised by IRE1 exonuclease activity. Each lane represents islet lysates from one animal. As a positive control, cultured INS1 cells were treated with thapsigargin (Tg) for (1h) and two independent experiments are shown. (D) shows the mean of the ratio of sXBP-1 to uXBP-1 (as a % of SAL). *p<0.001 vs. SAL; ¤p<0.05 HG+PBA vs. HG. (B and D) Data are means ± SE. n number indicates the number of rats studied.
Figure 4.7. Effects of hyperglycemia and 4-Phenylbutyrate on Markers Downstream of UPR Activation. Rats were treated for 48h with saline (SAL); glucose, to maintain glycemia at ~20-22mmol/l (HG); glucose+4-phenylbutyrate (HG+PBA, 2.08 µmol/kg.min); 4-phenylbutyrate (PBA, 2.08 µmol/kg.min). (A) Total RNA was isolated from islets of rats treated for 48h. Real-time PCR was used to analyze the relative mRNA levels of ATF4, CHOP and GRP78. Results are expressed as target gene relative to β-actin. Data are means ± SE. #p<0.05 vs. SAL; ¶p<0.01 vs. SAL; *p<0.001 vs. SAL. (B) shows representative immunoblots with the α-KDEL antibody that recognizes GRP78 and GRP94. Each lane represents islet lysates from one animal. Western blots were quantified and results are expressed as % of SAL normalized by tubulin. n number indicates the number of rats studied. Data are means ± SE. #p<0.05 vs. SAL; ¶p<0.01 vs. SAL; *p<0.001 vs. SAL.
**Figure 4.8.** Effects of hyperglycemia and 4-Phenylbutyrate on Total and Mitochondrial Superoxide Levels in Freshly Isolated Islets. Rats were treated for 48h with saline (SAL); glucose, to maintain glycemia at ~20-22mmol/l (HG); glucose+4-phenylbutyrate (HG+PBA, 2.08 µmol/kg.min); 4-phenylbutyrate (PBA, 2.08 µmol/kg.min). Following 48h, islets were isolated and incubated with hydroethidine (3µmol/l) (A) or MitoSOX (5µmol/l) (B). Approximately 20 islets were measured per n number (rat). Forty-eight hours glucose infusion increased total and mitochondrial superoxide levels. Co-infusion of PBA with glucose completely prevented this effect. Data are expressed as % of SAL ± SE (a SAL control rat was studied on each experiment day). *p<0.05 vs. SAL; †p<0.01 vs. SAL

![Graph A: Total Superoxide](image.png)

![Graph B: Mitochondrial Superoxide](image.png)
Figure 4.9. 4-Phenylbutyrate Prevents Glucose-Induced β-Cell Dysfunction in vivo Assessed Using the 2-Step Hyperglycemic Clamp. Rats were treated for 48h with saline (SAL); glucose, to maintain glycemia at ~20-22mmol/l (HG); glucose+4-phenylbutyrate (HG+PBA, 2.08 µmol/kg.min); 4-phenylbutyrate (PBA, 2.08 µmol/kg.min). At time=0, an infusion of 37.5% glucose was started. Plasma glucose was maintained at 13mmol/l by adjusting the rate of glucose infusion according to frequent (5-10min) glycemic determinations. At 120min, the glucose infusion was again raised to 22mmol/l until the end of the experiment (time=240min). Samples for insulin and C-peptide were taken at regular intervals. (A) Plasma glucose levels. (B) Glucose infusion rate (Ginf), (C) Plasma insulin, (D) Plasma C-peptide, (E) Sensitivity Index, and (F) Disposition Index during the 2-step hyperglycemic clamp. Data are means ± SE. n represents the number of rats studied. ^ Units of sensitivity index (M/I Index) = µmol.kg⁻¹.min⁻¹ glucose infusion per pmol/l insulin. ¡p<0.01 HG vs. SAL, p<0.001 HG+PBA vs. SAL at basal; ¡p<0.05 HG+PBA vs. SAL at 13mmol/l; ¡p<0.05 HG vs. SAL at 22mmol/l; ¡p<0.001 HG, HG+PBA vs. SAL at basal; *p<0.001 HG, HG+PBA vs. SAL at 13mmol/l; f p<0.05 HG vs. SAL at 13mmol/l. #p<0.05 vs. SAL; "p<0.01 vs. SAL; *p<0.001 vs. SAL
Figure 4.10. Effects of hyperglycemia and 4-phenylbutyrate on insulin secretion in freshly isolated islets (A) and on islet insulin content (B) following 96h infusion. Rats were treated for 96h with 1) saline (SAL4); 2) glucose, to maintain glycemia at ~15mmol/l (HG4); 3) glucose+4-phenylbutyrate (HG4+PBA, 2.08 µmol/kg.min); 4) 4-phenylbutyrate (PBA4, 2.08 µmol/kg.min). (A) Ninety-six hours glucose infusion increased insulin secretion at 6.5mmol/l glucose, but impaired GSIS at 22mmol/l glucose. Co-infusion of PBA completely prevented glucose-induced decrease in GSIS at 22mmol/l glucose. (B) Addition of PBA with glucose approximately doubled the insulin content, but this was still profoundly reduced compared to SAL. Data are means ± SE. # p<0.05 vs. SAL; ¶ p<0.01 vs. SAL; * p<0.001 vs. SAL; a p<0.05 HG+PBA vs. HG.
Study 3

High glucose-induced β-Cell Dysfunction *In Vivo*: Evidence for a Causal Role of C-jun N-terminal Kinase Pathway*

* The results of this study are incorporated in a manuscript submitted to Diabetes. Current status: Invited new submission
5.1. Abstract

Objective

Oxidative stress can activate the c-jun N-terminal kinase (JNK) pathway, however whether JNK activation is causal in high glucose-induced β-cell dysfunction is unclear. Therefore, the objective of this study was to investigate 1) the causal role of JNK activation in high glucose-induced β-cell dysfunction in vivo, and 2) whether JNK activation is a downstream mechanism of oxidative stress in high glucose-induced β-cell dysfunction.

Research Design and Methods

High glucose-induced β-cell dysfunction was investigated in the presence or absence of JNK inhibition. JNK inhibition was achieved using either 1) the JNK specific inhibitor SP600125 (SP) or 2) JNK-1 null mice. 1) Normal Wistar rats were infused i.v. for 48h with saline or glucose (hyperglycemia ~20mmol/l) with or without SP. 2) JNK-1 null mice and their littermate wildtype controls were infused i.v. for 96h with saline or glucose (hyperglycemia ~13mmol/l). Following the glucose infusion periods in rats and mice respectively, β-cell function was assessed in isolated islets or in vivo using a hyperglycemic clamp. To investigate whether JNK activation is a downstream mechanism of oxidative stress, superoxide levels (total and mitochondrial) were measured in isolated rat islets following 48h infusion with glucose/saline with or without SP. Phosphorylated JNK (marker of JNK activation) was also measured using western blot in islets exposed to glucose/saline with or without the superoxide dismutase mimetic Tempol.

Results
Forty-eight hours or ninety-six hours hyperglycemia in rats and mice respectively, impaired β-cell function in isolated islets and *in vivo* (as indicated by the disposition index calculated from the hyperglycemic clamp). Inhibition of JNK using either SP or JNK-1 null mice prevented glucose induced β-cell dysfunction in isolated islets and *in vivo*.

Forty-eight hours of hyperglycemia increased total and mitochondrial superoxide levels in isolated rat islets. This increase was not prevented by SP co-infusion. Phosphorylated JNK levels in isolated rat islets were also increased by glucose infusion. Co-infusion of TPO with glucose completely prevented this increase.

**Conclusion**

These data suggest that 1) JNK pathway is causally involved in high glucose-induced β-cell dysfunction *in vivo*, and 2) JNK activation is a downstream mechanism of oxidative stress in high glucose-induced β-cell dysfunction *in vivo*. 
5.2. Introduction

The activity of c-jun N-terminal kinase (JNK), which is known to be activated by various stress signals (182; 283), is abnormally elevated in the diabetic state (191). Increased JNK activation has been shown to mediate obesity-induced insulin resistance by interfering with the insulin signaling cascade (190; 192; 194; 284). Inhibition of JNK ameliorates glucose tolerance in animal models of type 2 diabetes by improving insulin sensitivity (192-194), and insulin biosynthesis (194). Furthermore, genetic evidence shows that increased JNK activity caused by a loss of function mutation of islet-brain-1, the human and rat homolog of mouse JNK-interacting protein-1, is associated with late onset type 2 diabetes in humans (285). These findings suggest a central role of JNK in the pathophysiology of type 2 diabetes.

Numerous studies, including ours (268), have shown that oxidative stress, which is present in the diabetic state (154), plays a role in high glucose-induced β-cell dysfunction both in vitro (35; 175; 181; 242) and in vivo (75; 170; 268). Oxidative stress can activate the JNK pathway in various tissues, including pancreatic islets (162). Oxidative stress-induced activation of JNK has been demonstrated to impair insulin gene transcription and secretion by inducing the nucleocytoplasmic translocation of the transcription factor pancreatic and duodenal homeobox factor-1 (PDX-1), and thereby reducing PDX-1 DNA-binding activity (199; 286). PDX-1 regulates multiple genes (287), including insulin, GLUT2 and glucokinase, and a reduction in expression or binding of PDX-1 leads to suppressed insulin gene transcription (27).

It has been reported that inhibition of JNK can prevent high glucose-induced β-cell dysfunction and apoptosis in human islets and in a β-cell line (288). In vivo, islets over-expressing dominant negative JNK have preserved insulin gene expression when transplanted into the streptozotocin-induced diabetic nude mice (162). However, whether these islets were protected from hyperglycemia-induced impairment in GSIS per se is not known.
The objective of this study is to investigate the causal role of JNK activation in a selective in vivo model of high glucose-induced β-cell dysfunction (i.e., in the absence of other metabolite/hormone changes such as hyperlipidemia and relative insulin deficiency hyperinsulinemia which are present in diabetes), and determine whether JNK activation is a downstream mechanism of oxidative stress. The effect of prolonged hyperglycemia with or without JNK inhibition on β-cell function was evaluated both in isolated islets and in vivo using the hyperglycemic clamp. JNK inhibition was achieved either by using 1) the JNK specific inhibitor SP600125, or 2) JNK-1 null mice. SP600125 is a reversible ATP-competitive inhibitor that is selective for JNK (289). Three isoforms of JNK exists: JNK-1, JNK-2 and JNK-3 (182) (See Introduction 1.5.6.1.) SP600125 inhibits all 3 isoforms of JNK (289). Since JNK-1 is the main isoform associated with type 2 diabetes (192), JNK-1 null mice were used in this study.

5.3. Materials and Methods

5.3.1. Animals and Surgery

Rats: Female Wistar rats (Charles River, Canada), weighing 250-300g, were cannulated as described in General Methods section 2.1.1. Mice: The right jugular vein of 10-12 weeks old male JNK-1 null mice or their littermate wildtype controls (Jackson Laboratory 004319) was cannulated using a two-part catheter consisting of PE-10 and silastic tubings for infusion. The free catheter end was exteriorized at the back of the neck through a subcutaneous tunnel and filled with heparinized saline (40 units/ml). Mice were allowed 4-5 days post-surgery recovery before infusions. All procedures were approved by the Animal Care Committee of the University of Toronto.
5.3.2. Study in Rats

5.3.2.1. Infusion Period

Rats were randomized and infused for 48h with either: 1) saline (SAL), 2) a variable infusion of 37.5% glucose to achieve and maintain plasma glucose at 20-22mmol/l (HG), as in our previous studies (268), 3) glucose + SP600125 (Sigma) (HG+SP), 4) SP600125 alone without glucose infusion (SP). Plasma glucose was maintained at ~20mmol/l by adjusting the rate of glucose infusion according to frequent (every 2-3 h) glycemic determinations. SP was dissolved in 20% (2-hydroxypropyl)-β-cyclodextrin (Sigma H107), pH=7.4, and given at a dose of 0.05µmol/kg.min. This is a similar dose shown to inhibit JNK in rats in a model of adjuvant-induced arthritis (290).

To investigate the link between JNK and oxidative stress and ER stress, rats were also infused for 48h with 5) glucose + Tempol (HG+TPO, 2.41µmol/kg.min), 6) Tempol alone (TPO) as in Study 1, 7) glucose + 4-Phenylbutyrate (HG+PBA, 2.08µmol/kg.min), 8) 4-Phenylbutyrate alone (PBA) as in Study 2.

5.3.2.2. Pancreatic Islet Isolation and Ex vivo Evaluation of glucose stimulated Insulin Secretion (GSIS).

This was performed as described in General Methods sections 2.1.3. and 2.1.4..

5.3.2.3. Total and mitochondrial superoxide

This was performed as described in General Methods section 2.1.5..
5.3.2.4. Western Blot

Western blots were performed as described in General Methods section 2.1.6., on islet lysates of animals infused for 48h as described in 5.3.2.1. and 3.3.2.2.. The following primary antibodies were used: phospho-c-jun (Ser73) (Cell Signaling, #9164, 1:500), total c-jun (Cell Signaling, #9165, 1:500), phospho-JNK (Thr183/Tyr185) (Santa Cruz, #sc-12882, 1:100), total JNK (Santa Cruz, sc-571, 1:1000), and actin (Sigma, #A2066, 1:500).

5.3.2.5. Two-step hyperglycemic clamp

At the end of 48h, glucose infusion in HG and HG+SP groups was decreased to ~5µl/min for ~75 minutes, which was required to achieve basal glucose without hypoglycemia. Basal insulin and C-peptide were measured at -20 and 0 minutes. At time = 0, an infusion of 37.5% glucose was started. Plasma glucose was maintained at 13mmol/l by adjusting the rate of glucose infusion according to frequent (5-10 minutes) glycemic determinations. At 120 minutes, the glucose infusion was again raised to 22mmol/l until the end of the experiment (time = 240 minutes). The clamp details are described in the General Methods section in 2.1.7..

5.3.2.6. Calculations

The C-peptide to insulin ratio index of insulin clearance, the M/I index of insulin sensitivity, and disposition index for the rat were calculated during the last 40 minutes of each step of the hyperglycemic clamp, as described in the General Methods sections 2.3..
5.3.3. Studies in Mice

5.3.3.1. Infusion Period

JNK1-null (Jackson Lab, Bar Harbor, Maine) or wildtype (WT) littermates were infused for 96h with either 1) saline (WT SAL; KO SAL) or 2) a variable infusion of 50% glucose to elevate plasma glucose (hyperglycemia ~13mmol/l) (WT HG; KO HG).

5.3.3.2. Pancreatic Islet Isolation

Pancreatic islets were isolated in 5 h fasted mice, following 96h infusion, as previously described (291). The pancreatic duct was perfused with 3 ml of collagenase V (0.8 mg/ml) in RPMI-1640 containing 2.8mmol/l glucose, 10 mmol/l Hepes, 1% Penicillin. The pancreas was then excised and digested for 20 minutes at 37 °C. Islets were hand-picked from acinar tissue debris, and transferred into Krebs Ringer buffer containing 10mmol/l HEPES (KRBH) and 2.8 mmol/l glucose.

5.3.3.3. Ex vivo Evaluation of GSIS

The freshly isolated islets were pre-incubated for 1h at 37°C in Krebs Ringer buffer containing 10mmol/l HEPES (KRBH) and 2.8mmol/l glucose, and incubated in duplicate for 2 h at 37°C in KRBH at the following glucose concentrations: 6.5mmol/l and 22mmol/l. Insulin was measured in the supernatant with Linco’s RIA kit. The islet pellets were subjected to acid-ethanol extraction for measurement of insulin content (268).

5.3.3.4. One-step hyperglycemic clamp

The hyperglycemic clamp was performed in 5h fasted mice. At the end of 96h, glucose infusion was stopped for 90 minutes in glucose infused- WT and JNK-1 null mice to bring plasma glucose back to basal levels. Basal insulin and C-peptide were measured at 0min. At
time = 0, an infusion of 37.5% glucose was started. Plasma glucose was maintained at 22mmol/l by adjusting the rate of glucose infusion according to frequent (every 10 minutes) glycemic determinations from tail blood. Samples for insulin and C-peptide were taken at the last 20 minutes of the 120 minutes hyperglycemic clamp. Blood glucose was measured using the Hemocue analyzer II (HemoCue, Lake Forest, CA).

5.3.3.5. Calculations

M/I index and disposition index were calculated as described in General Methods Section 2.3. during the last 20 minutes of the hyperglycemic clamp.

5.3.4. Plasma Assays

Insulin and C-peptide levels were measured as described in the General Methods section 2.2.

5.3.5. Statistics

Data Refer to General Methods section 2.4.
5.4. Results

5.4.1. Studies in Rats

5.4.1.1. Forty-eight Hours Infusion Period

Baseline fed plasma glucose and insulin were not different between groups before the onset of the 48h infusions. In rats infused for 48h with glucose alone (HG) or in combination with SP, glucose levels were elevated to ~20-22mmol/l by ~6h and were maintained at that level for the remainder of the 48h period (Table 5.1).

5.4.1.2. Effect of SP600125 on JNK Activation

To determine whether SP is inhibiting JNK in islets in vivo, western blot was performed in islet lysate for phospho-c-jun at serine 73 (site phosphorylated by JNK), and total c-jun. JNK activation has been shown to increase both serine phosphorylation and total c-jun protein levels (292). Forty-eight hours of glucose infusion increased phosphorylation of c-jun (calculated as ratio of phosphorylated c-jun to total c-jun) and total c-jun. This was prevented by the co-infusion of SP. SP alone tended to slightly decrease phospho-cjun, although this effect was not significant. (Figure 5.1)

5.4.1.3. Glucose Stimulated Insulin Secretion in Freshly Isolated Islets

Insulin secretion at 2.8mmol/l glucose did not differ between groups. Forty-eight hours glucose infusion increased basal insulin secretion at 6.5mmol/l, but impaired GSIS at 13mmol/l and 22mmol/l glucose (p<0.001 vs. SAL). Co-infusion of SP did not affect insulin secretion at 6.5mmol/l glucose, but completely restored GSIS at both 13mmol/l and 22mmol/l glucose (Figure 5.2A). SP alone without glucose did not have any significant effect.
Forty-eight hours of hyperglycemia drastically reduced islet insulin content. The addition of SP significantly increased insulin content (~1.5 fold), but this was still profoundly reduced compared with SAL (Figure 5.2.B).

5.4.1.4. Two-Step Hyperglycemic Clamp

To further investigate whether SP is effective in preventing high glucose-induced β-cell dysfunction in vivo, a two-step hyperglycemic clamp was performed after 48h infusions. Plasma glucose was elevated to 13mmol/l until 120min, then to 22mmol/l until 240min with no difference between groups (Figure 5.3.A). In HG rats, a lower glucose infusion rate (Ginf) was necessary to clamp glucose at 22mmol/l, indicating that the circulating insulin was inadequate to compensate for hyperglycemia-induced insulin resistance (p<0.05 vs. SAL). Unexpectedly, co-infusion of SP with glucose further reduced Ginf at 22mmol/l glucose (p<0.001 vs. SAL) (Figure 5.3.B).

Basal insulin and C-peptide levels (-20min to 0min) were higher in HG and HG+SP rats than SAL (p<0.001). In response to increasing glucose levels, plasma insulin and C-peptide rose as expected. HG elevated plasma insulin levels at both 13mmol/l (p<0.001) and 22mmol/l (p<0.05) glucose, and C-peptide at 13mmol/l glucose (p<0.001). Co-infusion of SP tended to lower plasma insulin, but significantly elevated plasma C-peptide levels at 22mmol/l (p<0.05 HG+SP vs. SAL and HG) (Figures 5.3.C and D).

The C-peptide to insulin ratio, an index of insulin clearance, was lower in HG than SAL at both 13mmol/l and 22mmol/l glucose (p<0.05 vs. SAL). Co-infusion of SP significantly elevated insulin clearance (p<0.01 vs. HG at 13mmol/l glucose; p<0.001 vs. HG at 22mmol/l glucose). Insulin clearance by HG+SP tended to be higher than SAL, although this was not
significant. SP alone without glucose infusion also tended to increase insulin clearance (Figure 5.4.A).

Insulin sensitivity index (M/I index) was decreased in HG compared to SAL at both 13mmol/l and 22mmol/l glucose. Insulin sensitivity was not changed by the co-infusion of SP at both 13mmol/l and 22mmol/l glucose (Figure 5.4.B). Although insulin and C-peptide levels were elevated in HG, the disposition index (DI), which represents the ability of the β-cell to compensate for insulin resistance, was lower in HG compared to SAL. SP was able to completely restore DI during both steps of the clamp (Figure 5.4.C). SP without glucose did not have any significant effect on DI.

### 5.4.1.5. Total and Mitochondrial Superoxide Levels

Forty-eight hours glucose infusion elevated total (p<0.05 vs. SAL) and mitochondrial (p<0.01 vs. SAL) superoxide levels. SP did not prevent the increase in total and mitochondrial superoxide induced by prolonged glucose infusion. SP alone without glucose did not have any significant effects on islet superoxide compared to SAL, although it did have a tendency to elevate mitochondrial superoxide (Figures 5.5.). Mitochondrial superoxide levels in HG+SP tended to be higher than SP, but did not reach statistical significance (p=0.07) (Figures 5.5.B). Thus, it cannot be excluded that the increase in mitochondrial superoxide in HG+SP is due to an effect of SP alone.

### 5.4.1.6. Effect of Tempol on JNK Activation

To determine whether JNK activation is a downstream mechanism of oxidative stress, phospho-JNK levels were measured in islets isolated from animals treated with the superoxide dismutase mimetic TPO. TPO prevented the increase in JNK phosphorylation (calculated as the
ratio of phosphorylated JNK to actin) induced by high glucose. TPO alone tended to decrease JNK phosphorylation, but this was not significant compared to SAL (Figure 5.6).

5.4.1.7. Effect of the Chemical Chaperone PBA on JNK Activation

To determine whether JNK activation is downstream of endoplasmic reticulum stress, phospho- and total c-jun levels were measured in islets isolated from animals treated with the chemical chaperone 4-phenylbutyrate (PBA). PBA prevented the increase in phosphorylation of c-jun (calculated as ratio of phosphorylated c-jun to total c-jun) induced by high glucose. PBA alone did not have any significant effect (Figure 5.7).

5.4.2. Studies in Mice

5.4.2.1. Ninety-six Hours Infusions

In both wildtype (WT) and JNK-1 null (KO) mice receiving glucose infusion, hyperglycemia was elevated to ~13mmol/l throughout the 96h infusion. No significant differences in blood glucose levels were found between these groups. (Table 5.2)

5.4.2.2. Glucose Stimulated Insulin Secretion in Freshly Isolated Islets

Insulin secretion at 6.5mmol/l glucose did not differ between groups. Ninety-six hours glucose infusion significantly impaired GSIS at 22mmol/l glucose. In contrast, JNK-1 null mice infused with glucose had similar GSIS to WT SAL or KO SAL mice. GSIS between WT SAL and KO SAL was not significantly different (Figure 5.8.A).

Ninety-six hours hyperglycemia decreased islet insulin content (by ~50%) in WT mice. In contrast, no decrease in islet insulin content was observed in KO HG mice (Figure 5.8.B).
5.4.2.3. One-Step Hyperglycemic Clamp

To further investigate whether JNK-1 null mice are protected from high glucose-induced β-cell dysfunction in vivo, a one-step hyperglycemic clamp was performed after the 96h infusion period. Plasma glucose was elevated and maintained at 22 mmol/l for 120 minutes (Figure 5.9.A). In WT HG mice, a lower glucose infusion rate (Ginf) was needed to clamp glucose at 22 mmol/l indicating that the circulating insulin was inadequate to compensate for insulin resistance (p<0.01). In contrast, Ginf in KO HG mice was similar to WT SAL and KO SAL mice (Figure 5.9.B).

Basal insulin and C-peptide levels tended to be slightly higher in glucose infused compared to saline infused mice, although this was not significant. Clamp insulin and C-peptide levels in WT HG mice was similar to saline infused mice. In contrast KO HG had significantly higher insulin and C-peptides levels (Figures 5.9.C and D).

The C-peptide to insulin ratio (index of insulin clearance) was not significantly different between groups (Figure 5.10.A). WT HG mice had a lower sensitivity index (M/I index) compared to saline infused mice (Figure 5.10.B). KO HG tended to have a higher M/I index than WT HG, although this was not significant. The disposition index (DI) was significantly reduced only in WT HG mice (p<0.01 vs. all) (Figure 5.10.C).
5.5. Discussion

The aim of this study was to investigate whether activation of JNK is causally linked to high glucose-induced β-cell dysfunction *in vivo*, and determine whether JNK activation is a downstream mechanism of oxidative stress. Using either 1) the JNK specific inhibitor SP600125, or 2) the JNK-1 null mice, we have demonstrated that inhibition of JNK protects against high glucose-induced β-cell dysfunction, assessed both *ex vivo* in isolated islets and *in vivo* using the hyperglycemic clamp. We have also demonstrated that the SP does not prevent high glucose-induced superoxide generation, and that the superoxide dismutase mimetic TPO prevents high glucose-induced JNK activation. These findings suggest that JNK pathway is causally linked to high glucose-induced β-cell dysfunction *in vivo*, and that JNK activation is a downstream mechanism of oxidative stress.

As previously shown in Studies 1 and 2, 48h glucose infusion impaired GSIS both *ex vivo* and *in vivo* in rats. Similar to our rat findings, we report for the first time that 96h of mild hyperglycemia at ~13mmol/l in mice impairs β-cell function *ex vivo* in isolated islets and *in vivo*. We have attempted to establish a model in mice that is more comparable to that in rats (i.e. 48h of hyperglycemia at ~20mmol/l), however, we find that mice do not tolerate this level of hyperglycemia well. Thus, we established a milder and more feasible model in mice. It is interesting to note that humans also do not tolerate glucose infusion well (293). It is unclear whether a threshold exist at which a stimulatory effect of glucose becomes detrimental to β-cell function. This should be further investigated in the mouse model. However, from our findings, it is clear that 13mmol/l glucose is enough to cause β-cell dysfunction. For future studies, we believe that the mouse, rather than the rat model may be the preferred model used to study high glucose-induced β-cell dysfunction, as it more closely mimics glucotoxicity in humans (more chronic and milder levels of hyperglycemia).
The mechanism whereby prolonged exposure to high glucose impairs β-cell function is not completely understood. Glucose activates JNK, and JNK inhibition prevents high glucose-induced β-cell dysfunction \textit{in vitro} (288). Furthermore, it has been shown that over-expression of dominant negative JNK in islets preserves insulin gene transcription after transplantation into the streptozotocin-induced diabetic nude mice (162), suggesting that JNK activation may be involved in β-cell glucotoxicity. However, in the diabetic milieu, in addition to hyperglycemia, many other metabolic alterations are present, which can potentially affect β-cell function. Thus, in this present study, we investigated the role of JNK in a more selective \textit{in vivo} model of glucose induced β-cell dysfunction.

Using a co-infusion of the JNK specific inhibitor SP600125, we show that β-cell dysfunction in islets of glucose infused rats can be prevented. Calculation of DI during the hyperglycemic clamp demonstrates that SP prevents high glucose-induced β-cell dysfunction also \textit{in vivo}. However, despite this, co-infusion of SP decreased glucose infusion rate (Ginf), which is an indicator of the whole body’s ability to dispose of glucose. A change in Ginf can be due to changes in β-cell function, insulin sensitivity, and/or insulin clearance. It is likely that the decrease in Ginf in HG+SP is due to an increase in insulin clearance, since whole-body insulin sensitivity (assessed by the M/I index) was unchanged. Insulin clearance is mainly a liver function, and is generally inversely proportional to insulin resistance. Thus, it is possible that SP is improving high glucose-induced hepatic insulin resistance in the liver. In contrast, SI during the hyperglycemic clamp is mostly a measure of peripheral insulin sensitivity, and this was not affected by the JNK inhibitor. Since SP increases insulin clearance, it cannot be excluded that a SP-induced increase in insulin clearance stimulates β-cell compensation for insulin resistance, thus contributing to improved β-cell function \textit{in vivo}. Nevertheless, data from isolated islets clearly show a direct β-cell effect of SP.
As we have shown in Study 1, no detectable change in islet viability or apoptosis is observed following 48h hyperglycemia. Thus, the effect of SP is most likely due to an improvement of β-cell function rather than mass. We have not measured β-cell mass or apoptosis in our mouse model. However, one study reported no changes in β-cell mass or death following 96h glucose infusion in mice (294), although this was in a slightly milder model of hyperglycemia.

In rats, increased insulin secretion was observed at basal glucose (6.5mmol/l) both in isolated islets and in vivo before the onset of the hyperglycemic clamp. SP does not prevent the effect of previous hyperglycemia to increase insulin secretion at 6.5mmol/l glucose, suggesting that this effect is independent of JNK activation. As previously discussed in Study 1, this effect may be due to induction of glycolytic genes by glucose (255). This increase in basal insulin secretion was not observed in mice, likely due to milder hyperglycemia in the mouse model.

Similar to our findings with SP, 96h hyperglycemia in mice decreases GSIS only in wildtype but not JNK-1 null mice in isolated islets and in vivo, indicating that decreased JNK activation prevents high glucose-induced β-cell dysfunction. JNK-1 null mice have been reported to be more insulin sensitive than WT littermate controls (192). However, in our study, we did not observe any differences in insulin sensitivity between JNK-1 null and WT mice, as assessed by the sensitivity index from the hyperglycemic clamp. This may be is linked to the age in which the mice were tested (10 weeks in our study compared to 16 weeks in other studies which showed differences in insulin sensitivity), and the fact that we did not perform a hyperinsulinemic-euglycemic clamp.

Unlike SP, Ginf was not decreased by prolonged hyperglycemia in the JNK-1 null mice (compared with a reduction in Ginf in HG+SP group) during the hyperglycemic clamp. Calculation of ratio between C-peptide and insulin indicates no significant changes in insulin
clearance due to glucose or chronic JNK-1 lack. The difference between our findings in mice and rats are likely explained by 1) differences in the degree and duration of hyperglycemia between the rat and mouse model, 2) acute (SP) versus chronic (JNK-1 null) inhibition of JNK, and preferential liver versus islet effects of SP. In addition, we cannot exclude that the different findings may be related to 3) differences in the isoforms inhibited by SP (isoforms 1-3), and the JNK-1 null mice and 4) species specificity (rat versus mouse). In humans, prolonged glucose infusion is associated with a significant reduction in insulin clearance (69). In this respect, the rat model, which has a more pronounced effect of high glucose on insulin clearance than mice, may be a more relevant model to study high glucose-induced decrease in insulin clearance.

It is unclear how JNK is activated by prolonged glucose elevation. In Study 1, we demonstrated that prolonged hyperglycemia increases total and mitochondrial superoxide levels in islets, and impairs β-cell function. Co-infusion of the superoxide dismutase mimetic TPO completely prevents high glucose-induced β-cell dysfunction. In the present study, we demonstrate that co-infusion of SP with high glucose does not prevent the increase in total and mitochondrial superoxide levels. It is important to note that SP alone tended to increase mitochondrial superoxide levels, suggesting that the inhibitor alone may have some cytotoxic effect. Thus, it cannot be excluded that the increase in mitochondrial superoxide levels observed in HG+SP islets are due an effect of the inhibitor alone. Nevertheless, we show that co-infusion of TPO does completely prevent high glucose-induced increase in JNK activation (measured by phosphorylated JNK levels). These findings suggest that JNK activation lies downstream of oxidative stress to impair β-cell function. Our findings are consistent with other in vitro studies, which show that oxidative stress activates JNK (162; 199; 286).

In addition to oxidative stress, prolonged glucose elevation has been reported to increase endoplasmic reticulum stress (85-87) and cytokine production (111), i.e. IL-1β, in β-cells, and
both have been shown to activate JNK (295; 296). In this study, we show that co-infusion of the chemical chaperone, which prevented high glucose-induced β-cell function (as shown in Study 2), decreased JNK activation, as indicated by the decreased ratio of phospho- to total c-jun. These findings suggest that JNK activation is a downstream mechanism of ER stress. As described in Study 2, we did not find an increase in IL-1β mRNA expression following 48h hyperglycemia. This suggests that high glucose-induced cytokine production is not likely an inducer of JNK activation.

JNK activation can impair β-cell function through a number of mechanisms. In insulin sensitive tissue, it is known that activated JNK phosphorylates serine residues on insulin receptor substrate, leading to decreased tyrosine phosphorylation and subsequent impaired insulin signaling (190). In the β-cells, JNK activation has also been reported to impair the insulin signaling cascade. Kawamori et al. demonstrates that activation of JNK decreases the activity of Akt in the pancreatic β-cell line HIT-T15 (199). This was followed by decreased phosphorylation of FOXO1, and subsequent translocation of FOXO1 to the nucleus. FOXO1 has been reported to inhibit PDX-1 gene transcription. Furthermore, FOXO1 exhibits counterlocalization to PDX-1 in β-cells (198). Thus, increased FOXO1 translocation to the nucleus by JNK activation can decrease binding of PDX-1 to the insulin gene promoter, and subsequently lead to decreased insulin gene transcription. In addition to impairing insulin gene transcription, activation of JNK can impair insulin biosynthesis. Andreozzi et al.(92) show that both prolonged glucose elevation and increased activation of hexosamine pathway activate JNK, increase serine phosphorylation of IRS-1 (serine 307), and impair activation of PI3 kinase/Akt/Mammalian Target of Rapamycin (mTOR) in a β-cell line. Activation of mTOR is important for translation initiation. Thus inhibition of this pathway can lead to impaired insulin biosynthesis.
JNK can also affect β-cell function via its main target, c-jun. C-jun is a basic Zip transcription factor phosphorylated by JNK. It has been reported that c-jun can suppress insulin gene transcription via inhibition of E1-mediated transcription (297; 298). In our study, we have reported that prolonged glucose elevation increases both phosphorylation and total c-jun levels, and that co-infusion of SP prevents these effects. Thus, it is possible that JNK-induced activation of c-jun is involved, at least in part, in the suppression of insulin biosynthesis by glucose. In agreement with these studies that JNK activation impairs insulin gene transcription and/or biosynthesis, we show that prolonged hyperglycemia decreases islet insulin content. Inhibition of JNK using either SP or JNK-1 null mice during prolonged hyperglycemia improves islet insulin content, which may contribute to prevention of high glucose-induced β-cell dysfunction. In the rat model, JNK inhibition results in partial restoration of insulin content, perhaps due to greater hyperglycemia, but to complete recovery of insulin secretion. This likely indicates that insulin biosynthesis is rate limiting for insulin secretion in this model, although it cannot be excluded that JNK affects other processes involved in insulin secretion.

In conclusion, our study demonstrates that inhibition of JNK, using either 1) the JNK specific inhibitor SP600125, or 2) the JNK-1 null mice protects against high glucose-induced β-cell dysfunction \textit{ex vivo} and \textit{in vivo}. Our study also suggests that JNK activation is a downstream mechanism of oxidative stress. These findings suggest that JNK inhibitors are of potential interest in the preservation of β-cell function in type 2 diabetes.
Table 5.1. Blood glucose level during the 48h Infusion Period in Rats. Rats were treated for 48h with 1) Saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + SP600125 (HG+SP, 0.05 µmol/kg.min); or 4) SP600125 (SP, 0.05 µmol/kg.min). Blood glucose levels were elevated to ~20-22mmol/l glucose in all glucose-infused groups. No significant differences were detected between all glucose-infused groups. Data are means ± SE.
Table 5.2. Blood glucose level during the 96h Infusion Period in Mice. Wildtype or JNK-1 null mice were treated for 96h with either 1) saline (WT SAL, KO SAL) or 2) glucose, to maintain glycemia at \( \sim 13 \text{mmol/l} \) (WT HG, KO HG). Glucose infusion significantly elevated blood glucose levels in both wildtype and JNK-1 null mice compared to saline infusion. No significant differences in blood glucose levels were detected between WT HG and KO HG. Data are means ± SE.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>Glucose (mM)</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT SAL</td>
<td>0</td>
<td>9.82 ± 0.42</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>9.47 ± 0.30</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>8.56 ± 0.45</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>9.23 ± 0.40</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>7.74 ± 0.28</td>
<td>-</td>
</tr>
<tr>
<td>WT HG</td>
<td>0</td>
<td>8.81 ± 0.32</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>11.74 ± 0.29</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>12.23 ± 0.75</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>13.42 ± 0.54</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>14.08 ± 0.78</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>KO HG</td>
<td>0</td>
<td>8.82 ± 0.53</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>13.43 ± 0.85</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>12.37 ± 0.51</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>12.25 ± 0.37</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>12.90 ± 0.54</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>KO SAL</td>
<td>0</td>
<td>9.18 ± 0.36</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>8.88 ± 0.32</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>9.17 ± 0.32</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>8.94 ± 0.43</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>7.49 ± 0.27</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 5.1. Effects of hyperglycemia and SP on phosphorylated c-jun levels. (A) shows representative immunoblots of phosphorylated c-jun (p-c-jun), total c-jun and actin. Each lane represents islet lysates from one animal. (B) Western blots were quantified and results are expressed as the ratio of phosphorylated c-jun to total c-jun as a percentage of SAL. Phosphorylated c-jun normalized to total c-jun levels was increased by glucose infusion, and decreased by the co-infusion of SP. \(^a\) p<0.001; \(^b\) p<0.05
Figure 5.2. Effects of hyperglycemia and SP600125 on insulin secretion in freshly isolated islets (A), and on islet insulin content (B). Rats were treated for 48h with 1) saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + SP600125 (HG+SP, 0.5 µmol/kg.min) or 4) SP600125 without glucose (SP, 0.5µmol/kg.min). (A) Forty-eight hours glucose infusion increased insulin secretion at 6.5mmol/l glucose, but impaired glucose stimulated insulin secretion (GSIS) at 13mmol/l and 22mmol/l glucose. SP completely restored GSIS at both 13mmol/l and 22mmol/l glucose. (B) Insulin content was depleted following 48h glucose infusion. Addition of SP increased islet insulin content by ~1.5 fold, but this was still profoundly reduced compared to SAL. Data are means ± SE. *p<0.001 vs. all; *p<0.001 vs. SAL and SP; *p<0.05 HG+SP vs. HG.
Figure 5.3. Effects of SP on plasma glucose levels (A), glucose infusion rate (Ginf) (B), plasma insulin levels (C), and plasma C-peptide levels during the two-step hyperglycemic clamp with or without glucose infusion. Glucose levels were superimposable in all groups during both steps of the clamp (A). HG group had a significantly lower Ginf than SAL at 22mmol/l. Co-infusion of SP with glucose tended to further decrease Ginf at 22mmol/l glucose (B). Insulin levels were significantly higher in HG compared to SAL both at basal and during the clamp. Co-infusion of SP tended to reduce clamp insulin levels at both 13 and 22mmol/l glucose (C). C-peptide levels at basal and 13mmol/l glucose were significantly increased by glucose infusion. Co-infusion of SP tended to increase C-peptide levels at basal and 13mmol/l glucose, and significantly at 22mmol/l glucose compared to HG (D). Data are means ± SE. a p<0.05 HG vs. SAL at 22mmol/l; b p<0.001 HG+SP vs. SAL and SP at 22mmol/l glucose; c p<0.001 HG, HG+SP vs. SAL and SP; d p<0.001 HG, HG+SP vs. SAL and SP; e p<0.01 HG vs. SAL and SP; f p<0.001 HG, HG+SP vs. SAL; g p<0.05 HG+SP vs. SAL.
Figure 5.4. Effects of SP on insulin clearance index (A), sensitivity index (B), and disposition index (C) during the two-step hyperglycemic clamp with or without glucose infusion. (A) Insulin clearance index was decreased by glucose infusion. Co-infusion of SP significantly increased insulin clearance index both at 13 and 22mmol/l glucose. (B) Insulin sensitivity, as determined by the sensitivity index was reduced by glucose, and not prevented by co-infusion of SP. $^A$Units of sensitivity index (M/I Index) = µmol.kg$^{-1}$.min$^{-1}$ glucose infusion per pmol/l insulin. (C) Disposition index was significantly decreased by glucose infusion and prevented by SP co-infusion. Data are means ± SE. $^a$ p<0.05; $^b$ p<0.01; $^c$ p<0.001; $^d$ p<0.001 vs. SAL and SP; $^e$ p<0.05 vs. SAL; $^f$ p<0.01 vs. SAL and SP.
Figure 5.5. Effects of hyperglycemia and SP600125 on Total and Mitochondrial Superoxide Levels in Freshly Isolated Islets. Rats were treated for 48h with 1) saline (SAL); 2) glucose, to maintain glycemia at ~20-22mmol/l (HG); 3) glucose + SP600125 (HG+SP, 0.5 µmol/kg.min) or 4) SP600125 without glucose (SP, 0.5 µmol/kg.min). Following 48h, islets were isolated and incubated with hydroethidine (3µmol/l) (A) or MitoSOX (5µmol/l) (B). Approximately 20 islets were measured per n number (rat). Forty-eight hours glucose infusion increased total and mitochondrial superoxide levels. Co-infusion of SP with glucose did not have any significant effect on both total and mitochondrial superoxide levels. Data are expressed as % of SAL ± SE (a SAL control rat was studied on each experiment day). *p<0.05 vs. SAL; †p<0.01 vs. SAL
Figure 5.6. Effects of hyperglycemia and the superoxide dismutase mimetic TPO on phosphorylated JNK protein levels. Western blots were performed on islet lysates for phosphorylated JNK following 48h infusion with glucose (HG) or saline (SAL) with or without the superoxide dismutase mimetic tempol (TPO). Western blots were quantified and results are expressed as the ratio of phosphorylated JNK to actin expressed as a percentage of SAL. Phosphorylated JNK was increased by glucose infusion as shown above, and decreased by the co-infusion of TPO. \( a \ p<0.05; \ b \ p<0.01 \)
**Figure 5.7. Effects of hyperglycemia and PBA on phosphorylated c-jun levels.** (A) shows representative immunoblots of phosphorylated c-jun (p-c-jun), total c-jun and actin. Each lane represents islet lysates from one animal. (B) Western blots were quantified and results are expressed as the ratio of phosphorylated c-jun to total c-jun as a percentage of SAL. Phosphorylated c-jun normalized to total c-jun levels was increased by glucose infusion, and decreased by the co-infusion of SP. \(^a\) p<0.001; \(^b\) p<0.01
Figure 5.8. Effects of hyperglycemia on insulin secretion in wildtype or JNK-1 null mice (A). Panel (B) shows the effect of hyperglycemia on islet insulin content. Wildtype (WT) or JNK-1 null mice were treated for 96h with either saline (SAL) or glucose, to maintain glycemia at ~13mmol/l (HG). (A) Ninety-six hours glucose infusion significantly impaired GSIS at 22mmol/l glucose in WT mice. In contrast, JNK-1 null mice infused with glucose had similar GSIS to WT SAL or KO SAL mice. (B) Insulin content was depleted following 96h glucose infusion only in WT HG mice. Data are means ± SE. *p<0.05 vs. all.
Figure 5.9. Plasma glucose (A), glucose infusion rate (Ginf) (B), plasma insulin levels (C), and plasma C-peptide levels during the one-step hyperglycemic clamp in mice. Glucose levels were superimposable in all group during the clamp (A). In WT HG mice, a lower glucose infusion rate (Ginf) was needed to clamp plasma glucose at 22mmol/l indicating that the circulating insulin was inadequate to compensate for insulin resistance (B). In contrast, Ginf in KO HG mice was similar to control WT SAL and KO SAL mice. Basal insulin (C) and C-peptide (D) levels tended to be slightly higher in glucose infused compared to saline infused mice, although this was not significant. Clamp insulin and C-peptide levels in WT HG mice was similar to saline infused mice. In contrast KO HG had higher insulin and C-peptides levels. Data are means ± SE. \(^a\) p<0.01 WT HG vs. WT SAL, \(^b\) p<0.05 WT HG vs. KO SAL; \(^c\) p<0.01 WT HG vs. all; \(^d\) p<0.05 KO HG vs. all; \(^d\) p<0.05 KO HG vs. WT SAL.
Figure 5.10. Insulin clearance index (A), sensitivity index (B), and disposition index (C) during the one-step hyperglycemic clamp in WT or JNK-1 null mice. (A) The C-peptide to insulin ratio (index of insulin clearance) was not significantly different between groups. (B) WT HG mice had a lower sensitivity index compared to WT SAL. KO HG tended to have a higher sensitivity index than WT HG, although this was not significant. Units of sensitivity index = μmol.kg⁻¹.min⁻¹ glucose infusion per pmol/l insulin. (C) The disposition index (DI) was significantly reduced only in WT HG mice. Data are means ± SE.  a p<0.001; b p<0.01; c p<0.01 vs. all.
General Discussion

Oxidative Stress as a Causal Mediator of β-Cell Glucotoxicity

The majority of *in vitro* studies have shown that oxidative stress plays an important role in β-cell glucotoxicity (27). *In vivo* evidence for a role of oxidative stress has been limited, and has mainly shown that antioxidants can prevent diabetes in animal models of type 2 diabetes (ZDF rat and *db/db* mouse) by ameliorating defective glucose stimulated insulin secretion (75; 170). Although these animal models are characterized by chronic hyperglycemia, they also have other hormonal (i.e. leptin receptor deficiency) and metabolic (i.e. hyperlipidemia) alterations that can potentially affect β-cell function. Thus the first objective of my thesis was to investigate the role of oxidative stress in a selective *in vivo* model of β-cell glucotoxicity (i.e. in the absence of metabolic and hormonal alterations).

In my first study, I found that 48h glucose infusion impaired β-cell function *ex vivo* and *in vivo*, and this was associated with higher levels of total and mitochondrial superoxide in the islets. Co-infusion of the superoxide dismutase mimetic Tempol, prevented the increase in total and mitochondrial superoxide, and prevented β-cell dysfunction induced by high glucose. These findings suggest that superoxide generation plays an important role in high glucose-induced β-cell dysfunction *in vivo*. These results lead us to build a hypothetical scheme (*Figure 6.1.*), which suggests possible mechanism by which prolonged glucose infusion impairs β-cell
function. These mechanisms are partly validated by the results from our Study 2 and 3. The scheme will be further discussed later in the chapter.
Figure 6.1. Possible mechanisms involved in the impairing effect of high glucose on β-cell function. This scheme was partly validated by the results from our studies with tempol, 4-phenylbutyrate and SP600125, and is discussed in chapter 6 of this thesis.
Superoxide generation can impair β-cell function at multiple sites. In the mitochondria, oxidative stress can decrease glucose oxidation via inhibition of mitochondrial enzymes. Superoxide can also upregulate and/or activate uncoupling protein 2, resulting in decreased ATP produced by glucose oxidation, and thus decreased GSIS (62) in vitro. Recently, a role of UCP2 in β-cell glucotoxicity has been proposed. However, studies to date investigating UCP2 expression in β-cell glucotoxicity have yielded conflicting results. Interestingly, one study shows that 72h exposure of mouse islets increases mitochondrial superoxide, which activates UCP2 without altering UCP2 mRNA levels (62). Currently, it is unknown whether mitochondrial superoxide increases UCP2 activity in a selective in vivo model of β-cell glucotoxicity. The fact that insulin content was only mildly improved by TPO suggest that after 48h, TPO is mainly acting on the insulin secretory process, presumably by increasing glucose oxidation (160) or decreasing uncoupling (62) via decreased mitochondrial superoxide. These findings prompt further study of β-cell glucotoxicity in the whole body or β-cell specific UCP2 null mice, as well as measurements of glucose oxidation (using 14CO2 methodology) and ATP production.

Another level of impairment that can be involved in β-cell dysfunction caused by high glucose-induced oxidative stress is decreased insulin biosynthesis and insulin gene transcription. Studies in vitro show that the effect of high glucose to impair insulin gene transcription is due to induction of oxidative stress, which decreases binding of the transcription factors PDX-1 (72) and MafA (64; 181) to the insulin promoter. This can be due to either a direct effects of oxidative stress on β-cell transcription factors (181; 195), and/or due to activation of various signaling pathways by oxidative stress. One pathway that can be activated by oxidative stress is the JNK pathway. Recent reports demonstrate that JNK activation induces the
nucleocytoplasmic translocation of the pancreatic transcription factor PDX-1 via a FOXO1 dependent mechanism, and thereby reduce PDX-1 binding activity to the insulin promoter (286). It is of note that although the prevalent notion is that FOXO1 decreases insulin gene transcription, one study found that FOXO1 upregulates transcription factors important for β-cell function (299). The JNK pathway will be discussed in more detail later in this chapter.

Oxidative stress is also a known activator of IKKβ, which by phosphorylating the inhibitor IκBα, activates NFκB. It has been reported that glucose activates NFκB by inducing oxidative stress in rat islets (208) and that the IKKβ inhibitor salicylate protects human islets against glucotoxicity (207) and partially restores β-cell function in type 2 diabetes (209). However, a role of NFκB in β-cell glucotoxicity has not been consistently shown in all studies (210). There are no reports that IKKβ can directly affect transcription factors such as PDX-1 or FOXO1. However, IKKβ has been shown to impair the insulin signalling cascade, and impairment of the insulin signaling cascade can increase FOXO1 nuclear translocation and decrease PDX-1 binding to the insulin promoter (199). Recently, our laboratory has shown that salicylate, an inhibitor of IKK-β, prevents fat-induced β-cell dysfunction. Although it would be very interesting to investigate the effects of salicylate in a glucotoxic model, this is likely not feasible due to its inhibitory effect on COX, which can lead to renal vasoconstriction. In the presence of hyperglycemia, this can lead to fluid overload. Alternatively, glucotoxicity can be studied in the β-cell specific IKK-β null mice, which our lab is in the process of breeding.

Other possible mechanism downstream of oxidative stress is p38 MAPK pathway. Oxidative stress has been shown to activate this pathway in islets; however, inhibition of p38 MAPK did not protect against oxidative stress-induced impairment of insulin gene expression and secretion (162). Thus, further studies of the p38 MAPK pathway are currently unwarranted.
More recently, the effects of oxidative stress on β-cell dysfunction have been attributed to NAD depletion via activation of poly(ADP-ribose) polymerase (PARP), a DNA-repair enzyme that is activated by DNA strand breaks. NAD depletion can lead to decrease activity of Sirt1, an NAD dependent deacetylase that is expressed in β-cells (300). Sirt1 can directly act as a transcription factor, and repress UCP2 expression (300), thereby regulating insulin secretion. Sirt1 has also been reported to deacetylate and modulate the function of the transcription factor FOXO1 (301; 302). These findings suggest that increased oxidative stress-induced DNA damage by prolonged hyperglycemia may lead to decreased Sirt1 activity, and thereby induce β-cell dysfunction. Indeed, mice overexpressing Sirt1 in β-cells has been reported to have more efficient glucose handling due to enhanced glucose stimulated insulin secretion (303). Thus, it would be very interesting to investigate the effect of Sirt1 overexpression on β-cell glucotoxicity. This could be achieved by using the β-cell specific Sirt1 overexpressing mice, or by using activators of Sirt1. In endothelial cells, it has been reported that mitochondrial superoxide can activate PARP and inhibit the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (304). Thus, in addition to depleting NAD, PARP may also impair β-cell function by inhibiting glycolytic enzymes, and hence glucose oxidation. Thus, future studies focused on investigating glucotoxicity in the PARP-null mice may help address the role of PARP activation in impairing β-cell function. These mice have normal fetal and postnatal development, but have inherent genomic instability and are high sensitive to DNA damage induced by γ-radiation and alkylating agents (305).

In Study 1, I found that only tempol, but not taurine nor N-acetylcysteine, prevented high glucose-induced β-cell dysfunction. We postulate from these findings that superoxide generation rather than toxic aldehyde generation and glutathione depletion, which are prevented by taurine and N-acetylcysteine, plays a greater role in high glucose-induced β-cell dysfunction.
Our findings are in accordance with an *in vitro* study by Krauss et al. (62), which showed that overexpression of Mn superoxide dismutase (decreases mitochondrial superoxide), but not glutathione peroxidase 1 (which decreases H$_2$O$_2$) prevents β-cell dysfunction induced by 72h of hyperglycemia. However, based on other *in vitro* studies (35; 175; 176; 181; 242), and studies in animal models of type 2 diabetes (75; 170), which show that increasing antioxidant capacity that does not specifically reduce superoxide, prevents β-cell dysfunction. This raises the possibility that in more prolonged models of glucotoxicity, other types of ROS may play a role. In study 1, I have attempted to investigate this possibility by utilizing a more prolonged (96h) model of glucotoxicity. However, similar to 48h infusion, TPO but not taurine, prevented high glucose-induced β-cell function. Because prolonging glucose infusion for longer is not feasible, studies using *in vitro* glucotoxicity models or animal models that are hyperglycemic, but not hyperlipidemic (i.e. the streptozotocin rat, or the GK rat) may be required. Using more prolonged models of glucotoxicity would also allow one to investigate of the effect of oxidative stress on β-cell apoptosis. No changes in apoptosis were observed in our short-term infusion model of glucotoxicity. High glucose-induced oxidative stress has been shown to induce β-cell apoptosis (249; 306); thus investigating of the effects of TPO and other antioxidants on β-cell mass in glucotoxic models is warranted.

Superoxide can be generated in various ways in cells. One of these is by the electron transport chain in the mitochondria. The observations that mitochondrial superoxide was increased by 48h high glucose and decreased by TPO suggest that increased mitochondrial superoxide plays a role in high glucose-induced β-cell dysfunction. However, whether mitochondrial superoxide is causal in high glucose-induced β-cell dysfunction is unknown. Future glucotoxicity studies focused on overexpressing of superoxide dismutase in the
mitochondria, such as using the Mn superoxide dismutase (mitochondrial isoform) overexpressing mouse, would help address the role of mitochondrial superoxide.

The cytosol can also generate superoxide via the activation of NADPH oxidase. Glucose can activate NADPH oxidase via protein kinase C (153), and activation of PKC-β2 has been implicated in high glucose-induced β-cell dysfunction in vitro (307). When NADPH was inhibited in islets of db/db mice, insulin content was partially restored (263), suggesting that cytosolic superoxide may also be important in β-cell dysfunction. I have performed a pilot study to determine the role of NADPH oxidase in β-cell glucotoxicity. My preliminary findings show that co-infusion of apocynin, an inhibitor of NADPH oxidase (which our lab has shown to reduce superoxide and prevent β-cell lipotoxicity), does not prevent β-cell dysfunction induced by 48h hyperglycemia. These findings suggest that NADPH oxidase is not a significant source of superoxide generation, at least following 48h hyperglycemia. However, these findings do warrant future experiments to assess the role of NADPH oxidase in more chronic models of glucotoxicity. Future glucotoxicity studies in Cu/Zn superoxide dismutase (cytosolic isoform) overexpressing mice would also shed light into the role of cytosolic superoxide generation in β-cell glucotoxicity.

If indeed superoxide is produced mainly in the mitochondria, one important question is how mitochondrial superoxide production increases activation of kinases, such as JNK, and IKK-β (as discussed above), which are predominantly in the cytosol, to impair β-cell function. One possibility is that mitochondrial superoxide induces ER stress (this will be discussed in more detail latter in the chapter). Activation of the unfolded protein response in response to ER stress has been shown to activate JNK (281) and IKKβ (308) pathways. Alternatively, mitochondrial superoxide may combine with nitric oxide, which is present in the mitochondria (309), to form peroxynitrite. Peroxynitrite is a very strong oxidant and has been associated with
cytokine-induced β-cell damage (310). It shows remarkable stability compared to superoxide as it is not a free radical (311), thus allowing a greater opportunity for peroxynitrite to diffuse from the mitochondrial into the cytosol or other cellular compartments (such as the nucleus to cause DNA damage) to activate kinases. It should be noted however, that there are reports that N-acetylcysteine can protect against the adverse effects of peroxynitrite (312). Since N-acetylcysteine does not prevent high glucose-induced β-cell dysfunction in our 48h model, a role for peroxynitrite is unlikely. However, peroxynitrite generation may be an important source of cellular damage in more long-term models of glucotoxicity and warrant future investigation.

Although TPO was able to completely prevent high glucose-induced β-cell dysfunction in our model, it is still possible that not all pathways of β-cell glucotoxicity diverge or converge onto oxidative stress. In the rat model, presumably treatments are given at high enough dose to inhibit pathways below basal so that other pathways that may be activated may not result in any effect, due to overcompensation by the inhibited pathway. Thus, the observation that all treatments are completely effective, at least in the short-term, is not unusual. Other pathways of β-cell glucotoxicity independent of oxidative stress have been reported, which include a role for endoplasmic reticulum stress, protein kinase C, hexosamine pathway, and inflammatory cytokines, which will be discussed below. These pathway that may be involved β-cell glucotoxicity, which are independent of oxidative stress is represented by an arrow that points directly (or via ER stress) from prolonged hyperglycemia to β-cell dysfunction in Figure 6.1.

Endoplasmic reticulum stress can be induced by oxidative stress as described later in the section. However, increased protein folding load due to over-secretion of insulin in response to high glucose can also induce ER stress independent of oxidative stress.

Chronic glucose elevation has been shown to activate PKC-β2, and impair insulin gene expression by inducing c-myc in rat islets (307). Oxidative stress has been shown to activate
PKC in islet; however, inhibition of this pathway did not protect against oxidative stress-induced impairment of insulin gene expression and secretion (162). It is well known that glucose can increase de novo synthesis of DAG (313) and activate PKC (314). Thus, this may be a pathway by which glucose can impair β-cell function independent of oxidative stress. How certain isoforms of PKC impair β-cell function is unclear. Mice overexpressing kinase-negative protein kinase C-δ are protected against high fat diet-induced β-cell dysfunction by inhibiting mitochondrial dysfunction and FOXO1 activation, and protecting against cell death (139). Mice null for PKC-ε have also been shown to be protected from fat-induced β-cell dysfunction (140), by amplifying pathways of GSIS via increasing lipolysis (315). Whether PKC-δ and –ε isoforms are involved in high glucose-induced β-cell dysfunction is unknown. PKC-β2 has been implicated in high glucose-induced decrease in insulin gene transcription in vitro. No studies have investigated its effect on β-cell glucotoxicity in vivo, although the PKC-β knockout mice are available (20). Thus future studies examining glucotoxicity in these transgenic mice would shed insight into the role of the different isoforms of PKC in high glucose-induced β-cell dysfunction.

Activation of the hexosamine pathway has been shown to be important in high glucose-induced β-cell dysfunction. While one study show that hexosamine synthesis impairs β-cell function by generating oxidative stress (35), others have shown that β-cell dysfunction by hexosamine can be due to increased O-linked glycosylation of proteins. Glucosamine treatment was shown to increase O-linked glycosylation of IRS-1 and IRS-2 in the RIN rat β-cells, leading to impaired activation of the PI3-kinase/Akt signaling pathway, and decreased β-cell survival (316). Glucosamine treatment also increased O-linked glycosylation of AKT, which increased β-cell death in the βTC-6 cell line (317). Another study showed that PDX-1 can be modified by
O-linked glycosylation, and this is correlated with decreased DNA binding activity and insulin secretion in MIN6 cells (318).

Sustained exposure or high concentration of IL-1β can impair insulin secretion and promote apoptosis (319). High glucose concentrations have been to stimulate IL-1β and IL-1β dependent proinflammatory factors in cultured human and rodent islets (320; 321). Furthermore, high glucose concentrations have been reported to induce apoptosis and expression of death receptor Fas in an IL-1 dependent manner (111; 112; 322). Stimulation of the IL-1β receptor can lead to NFκB activation, which although still controversial, can impair β-cell function (see Introduction section 1.5.6.2). There have been reports that JNK can also be activated by IL-1β (323). Interestingly recent studies show that high glucose-induced 1L-1β production actually require reactive oxygen species (324). Thus, the impairment of β-cell function by IL-1β may not be completely independent of oxidative stress. In our study, we have measured IL-1β mRNA expression in islets following 48h glucose infusion (see Study 2), but no increase in IL-1β was observed. This suggests that the involvement of the IL-1β pathway in our model of high glucose-induced β-cell dysfunction is unlikely. However, it cannot be excluded that the increase in IL-1β may occur post-transcriptionally, and that other proinflammatory cytokines are involved.

In addition to hyperglycemia, patients with type 2 diabetes have elevated FFA levels because of relative insulin deficiency as well as obesity (expanded fat mass). Elevated FFA can induce insulin resistance and decrease β-cell function and mass (lipotoxicity). Oxidative stress has been reported to play an important role in β-cell lipotoxicity (27). Like glucose, FFA can induce ROS in β-cells and can impair β-cell function at all levels (141), including an effect on UCP2, glucose oxidation, insulin biosynthesis, and insulin gene transcription. Although both glucose and fat-induced β-cell dysfunction likely involves oxidative stress, findings from our
laboratory suggest that the type of ROS, and site of ROS generation, involved are different. Whereas TAU and NAC did not prevent high glucose-induced β-cell dysfunction, they completely prevented fat-induced β-cell dysfunction. Furthermore, using fluorescent imaging, we have preliminary data to show that cytosolic but not mitochondrial superoxide is elevated by fat infusion. This is different from our findings with glucose, that show that both total and mitochondrial superoxide is elevated. Consistent with these findings, preliminary studies from our lab show that apocynin, an inhibitor of NADPH oxidase (a possible site of cytosolic superoxide generation), prevents fat- but not high glucose-induced β-cell dysfunction. It is possible that with glucose elevation, superoxide generation is involved, and this likely involves the mitochondria. In contrast, fat-induced β-cell dysfunction mainly involves cytosolic superoxide and other ROS that may be derived from cytosolic superoxide and are decreased by NAC and TAU. Because both glucose and FFA levels are elevated in type 2 diabetes, it is of importance to investigate the role of oxidative stress in the presence of both glucose and FFA elevation (β-cell glucolipotoxicity). Recently, a model of glucose and FFA elevation has been described (325), thus it would be interesting to investigate the effects of TPO and other antioxidants in this model.

In summary, the results of Study 1 suggest that superoxide generation plays a causal role in high glucose-induced β-cell dysfunction. However, how high glucose-induced superoxide generation leads to β-cell dysfunction remains to be elucidated. Further work to address this issue is warranted.

The Link Between Endoplasmic Reticulum Stress and Oxidative Stress in β-Cell Glucotoxicity
Endoplasmic reticulum stress has also been shown to play a role in diabetes. Obesity-induced insulin resistance has been linked to ER stress, and treatment with chemical chaperones, which improves protein folding, prevents this adverse effect. ER stress has also been linked to β-cell dysfunction. Recent genome studies show a link between WFS1 single nucleotide polymorphisms and increased risk for type 2 diabetes (326; 327) (WFS1 is a gene highly expressed in the β-cells, and maintains ER homeostasis by modulating the unfolded protein response (328)). Islets from subjects with type 2 diabetes have higher levels of ER stress markers (269), and glucose (85-87) can increase ER stress markers. Recently, over-expression of the ER chaperone GRP78 partially prevented high glucose-induced β-cell dysfunction in vitro in INS-1 cells (267). These studies point to ER stress as a key causal mediator of β-cell glucotoxicity and reveal chemical chaperones as a potential target for treatment. However, it is unknown whether ER stress plays a causal role in high glucose-induced β-cell dysfunction in vivo.

Recent studies demonstrate a close interrelationship between oxidative stress and ER stress in β-cells (270; 271). Since we have implicated oxidative stress in our model of high glucose-induced β-cell dysfunction (Study 1), this prompted us to investigate the link between oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo. No study to date has investigated this link in the context of β-cell glucotoxicity.

In study 2, we found that 48h hyperglycemia increases activation of the unfolded protein response, an indirect measure of ER stress. Co-infusion of the superoxide dismutase mimetic tempol prevented activation of the UPR, suggesting that high glucose-induced oxidative stress induces ER stress to impair β-cell function. We further found that the chemical chaperone 4-phenylbutyrate, which improves protein folding, completely prevented high glucose-induced β-cell dysfunction. Interestingly, treatment with 4-PBA also completely prevented high glucose-
induced increase in total and mitochondria superoxide. These results suggest that ER stress plays a causal role in high glucose-induced β-cell dysfunction in vivo, and that there is a reciprocal link between oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo, which is depicted in Figure 6.1. This study also suggests that chemical chaperones are of potential interest to preserve β-cell function in type 2 diabetes. Furthermore, our findings suggest that a combined strategy to enhance β-cell protection against both ER stress and oxidative stress may be required for prevention and/or treatment of β-cell dysfunction in type 2 diabetes.

Although we have shown an interrelationship between superoxide generation and ER stress, it is unknown which occurs first. The mitochondria and endoplasmic reticulum interact both physically and functionally, and are in close proximity to each other forming endomembrane networks (329). Thus, it is possible that superoxide in the mitochondria leaks to the ER due to its close proximity to induce ER stress. It has been shown that oxidative stress can induce ER stress by affecting protein folding by direct protein modification, chaperone inactivation and/or depleting cellular glutathione levels (222), and can deplete ER calcium levels by inhibiting SERCA (225; 226). ER stress can in turn generate more oxidative stress through increased ERO1p oxidase activity (an enzyme that transfers electrons generated by disulphide bond formation in the ER to molecular oxygen), and glutathione consumption due to reduction of mispaired disulphide bonds (227). ER stress can also cause calcium leakage from the ER, which can be taken up by the mitochondria matrix via the mitochondria Ca\(^{2+}\) uniporter (329), leading to depolarization of the electron transport chain and superoxide generation (266). Although it would be very interesting to investigate these mechanisms, the experiments involved are very technically difficult or not feasible to perform. For example, there is currently no available inhibitor of ERO1p to assess this enzyme as a site of superoxide generation. The
measurement of ER calcium levels although possible, is very technically difficult (330). One experiment that may be feasible is to measure mitochondrial calcium levels following chaperone treatment. However, it should be noted that in one study in MKR mice, a model characterized by hyperglycemia but no hyperlipidemia, mitochondrial calcium uptake in β-cells was decreased rather than increased (331). This may be, however, due to mitochondrial dysfunction in these mice.

In addition to oxidative stress, over-secretion of insulin due to glucose itself and to compensation for insulin resistance, can overwhelm the folding capacity of the ER to induce ER stress. This is supported by our findings that high glucose treatment abolishes islet insulin content, suggesting that β-cell exhaustion may play a role in our model of glucotoxicity. A study by Grill et al. demonstrated that co-infusion of diazoxide, an inhibitor of KATP channels, and hence insulin secretion (β-cell rest), with glucose for 48h in normal rats, prevented glucose induced β-cell dysfunction (94). Future studies investigating whether the effect of diazoxide is mediated through a reduction in ER stress would help address the role of insulin over-secretion, and ER stress in high glucose-induced β-cell dysfunction.

ER stress can impair β-cell function by a number of mechanisms. ER stress can activate the unfolded protein response, and specifically the PERK branch, to decrease insulin global translation. However, we do not think this is likely in our model as PBA prevented β-cell dysfunction without reversing high glucose-induced increase in eIF-2α. Another possibility is that activation of the unfolded protein response by ER stress leads to activation of JNK and NFκB. Both of these pathways have been implicated in impairing insulin gene transcription and/or insulin biosynthesis (See Introduction section 1.5.6.). It is of note these pathways can be activated by the unfolded protein response directly, or by the induction of oxidative stress by ER stress. Indeed we demonstrate that the chemical chaperone reduces activation of JNK
induced by glucose, as induced by the decrease in p-cjun to total c-jun (See study 3). In addition to impairing insulin biosynthesis, ER stress can also act to decrease ATP production and/or insulin exocytosis to impair β-cell function. It is possible that ER stress, by inducing mitochondrial superoxide production, increases UCP2 activity and impairs glucose oxidation, leading to β-cell dysfunction. Following 48h infusion, we found that co-infusion of PBA restores GSIS, but not insulin content after 48h hyperglycemia. Interestingly, following 96h infusion, PBA was able to partially prevent the decrease in insulin content. The underlying mechanism for this is unclear. It is possible that in the short-term, high glucose-induced ER stress mainly impairs mitochondrial function to decrease β-cell function (i.e. effects of UCP2). However, in more prolonged conditions, high glucose-induced ER stress plays a greater role in impairing insulin biosynthesis. These findings warrant future studies to investigate these possibilities.

Prolonged glucose elevation has been reported to increase cytokine production, such as IL-1β in β-cells (111). IL-1β can induce ER stress in β-cells in vitro via the induction of ER Ca²⁺ leak through JNK activation (332). This suggests the possibility that increased IL-1β signaling induces ER stress to impair β-cell function in this model. In our study, we did not find any changes in IL-1β mRNA expression following 48h hyperglycemia. However we cannot exclude changes in IL-1β expression at the protein level. One possible way to determine the causal role of IL-1β would be infuse the IL-1β receptor antagonist with glucose. Also, other cytokines such as IL-6 are elevated in islets isolated from animal models of type 2 diabetes (320; 333). Thus, we cannot exclude that other cytokines than IL-1β are involved in our model. In contrast to my findings with glucose, I have recently demonstrated that 48h Intralipid-heparin (IH) infusion increases IL-1β mRNA in islets of pre-diabetic diabetes-prone Biobreeding rats (an animal model of type 1 diabetes), which paralleled a decrease in GSIS. This increase in IL-
1β mRNA expression and decrease in GSIS by IH infusion was not observed in the control diabetes-resistant Biobreeding rats or Wistar Furth rats. Furthermore, I have showed that the IL-1 receptor antagonist completely prevents the decrease in GSIS in the diabetes-prone Biobreeding rats ex vivo (Tang et al. unpublished findings). These findings suggest that an increase susceptibility to islet inflammation may be important for the induction of IL-1β by fat. Thus, it would be interesting to investigate also the effects of glucose infusion on β-cell function in the diabetes-prone Biobreeding rat, and determine whether IL-1β is induced in this model. It is possible that an initial susceptibility to islet inflammation, which has been suggested to occur in type 2 diabetes (334), is necessary for the induction of IL-1β by high glucose. It would also be interesting to investigate the effect of the IL-1 receptor antagonist in both diabetes-prone Biobreeding rat and normal Wistar rats infused with glucose. In the latter, as stated above, although no increase in IL-1β mRNA was observed in islets exposed to high glucose, there may be still changes in IL-1β protein levels.

In more chronic conditions, ER stress may lead to β-cell death. Several components of ER stress signaling have been shown to contribute to β-cell loss, including IRE-1 JNK signaling (281), CHOP (335) and glycogen synthase kinase (GSK3β) (336). We do not observe apoptosis with our models of high glucose-induced β-cell dysfunction, although a slight increase in CHOP mRNA expression was observed. Future studies should focus on investigating ER stress in more chronic models of high glucose-induced β-cell dysfunction such as the pancreatectomized or Goto-Kakizaki (GK) rats, or in an in vitro model of glucotoxicity, which would allow investigation of β-cell apoptosis in the context of ER stress.

In summary, the results of Study 2 demonstrate that ER stress is causally involved in high glucose-induced β-cell dysfunction, and that there is a reciprocal link between oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo.
The Role of C-jun N-terminal Kinase in β-Cell Glucotoxicity

C-jun N-terminal kinase (JNK) activity is abnormally elevated in various tissues such as the muscle, liver and adipose tissue, in the diabetic state (191). Inhibition of JNK ameliorates glucose tolerance in animal models of type 2 diabetes by improving insulin sensitivity (192-194), and insulin biosynthesis (194). Furthermore, genetic evidence shows that increased JNK activity caused by a loss of function mutation of islet-brain-1, the human and rat homolog of mouse JNK-interacting protein-1, is associated with late onset type 2 diabetes in humans (285). These findings suggest a central role of JNK in the pathophysiology of type 2 diabetes.

JNK inhibition can prevent high glucose-induced β-cell dysfunction and apoptosis in human islets and in a β-cell line (288). In vivo, islets over-expressing dominant negative JNK have preserved insulin gene expression when transplanted into the streptozotocin-induced diabetic nude mice (162). However, whether these islets were protected from hyperglycemia per se is not known. Therefore, the objective of Study 3 was to investigate whether JNK activation plays a role in high glucose-induced β-cell dysfunction in vivo.

In Study 3, we show that suppression of JNK using either 1) the pharmacological inhibitor SP600125, or 2) JNK-1 null mice, prevents high glucose-induced β-cell dysfunction ex vivo and in vivo, and this was associated with an improvement in islet insulin content. Thus, these findings suggest a causal role of JNK in high glucose-induced β-cell dysfunction.

Oxidative stress has been shown to activate JNK and impair insulin gene transcription and insulin biosynthesis. In Study 1, we have implicated superoxide generation in β-cell glucotoxicity. To investigate whether JNK activation is a possible mechanism linking oxidative stress to high glucose-induced β-cell dysfunction, we measured total and mitochondrial
superoxide levels in islets from rats treated with glucose with or without the JNK inhibitor SP600125. We show that JNK inhibitor does not reduce superoxide levels (total and mitochondrial), suggesting that oxidative stress is upstream of JNK activation. However, it should be noted that these findings could also suggest that oxidative stress and JNK may act independently to impair β-cell function. To further investigate the link between oxidative stress and JNK, we determined whether the superoxide dismutase mimetic Tempol decreases JNK activation. We show that Tempol completely prevents glucose-induced increase in phosphorylated JNK levels (marker of JNK activation). Our findings suggest that JNK activation is a downstream mechanism of oxidative stress in high glucose-induced β-cell dysfunction.

It has also been reported that endoplasmic reticulum stress can activate JNK via the IRE-1 branch of the unfolded protein response. We found in this study that co-infusion of the chemical chaperone PBA, which prevents high glucose-induced β-cell dysfunction as we have shown in Study 2, prevents activation of JNK. These findings provide evidence that high glucose-induced ER stress activates JNK to impair β-cell function.

Based on our Study 1 and 2, we postulate that glucose-induces both oxidative stress and ER stress. Both these stress induces one another, forming a vicious cycle that activates JNK, and possibly other pathways, to impair β-cell function (See Figure 6.1). It should be noted that IL-1β has also been implicated in β-cell glucotoxicity, and can activate JNK independent of oxidative stress and ER stress (337) (in Figure 6.1, this is depicted as an arrow directly from hyperglycemia to JNK activation). However, as described in previous sections, we did not find an increase in IL-1β mRNA levels in islet exposed to glucose.

JNK can impair β-cell function through a number of mechanisms. One mechanism is via its main target, c-jun. Insulin gene transcription is regulated by its enhancer. C-jun has been
shown to suppress insulin gene transcription by inhibiting transcriptional activation by the insulin enhancer (298; 338). JNK activation can also impair insulin biosynthesis and/or transcription by direct effects on FOXO1 (200; 201), and impairment of the insulin signaling cascade. Like insulin sensitive tissues, β-cells express the insulin receptor and the insulin signaling molecules. The insulin signaling cascade has been found important not only for β-cell growth, but also secretion, including insulin gene transcription (89; 91; 216). Accordingly, it has been reported that β-cell specific insulin receptor knockout mice have a reduction in GSIS (91). It cannot be excluded, however, that this effect is due to deletion of insulin receptor in the hypothalamus. Kawamori et al. demonstrates that activation of JNK decreases the activity of AKT in the pancreatic β-cell line HIT-T15 (199). This was followed by translocation of FOXO1 to the nucleus, decreased binding of PDX-1 to the insulin gene promoter, and subsequently decreased insulin gene transcription. It has also been reported by Andreozzi et al. (92) that both prolonged glucose elevation and increased activation of hexosamine pathway activate JNK, increase serine phosphorylation of IRS-1, and impair activation of PI3-kinase/AKT/Mammalian Target of Rapamycin (mTOR) in a β-cell line. Activation of mTOR is important for translation initiation. Thus inhibition of this pathway can lead to impaired insulin biosynthesis. Whether these pathways are involved in our model of high glucose-induced β-cell function is unclear and needs to be further investigated.

In addition to glucose, FFA can activate JNK to impair β-cell function. Palmitate, a saturated FFA, has been reported to inhibit insulin gene transcription by activating JNK and impairing serine phosphorylation of IRS-1 (339). Oleic acid (the most predominant FFA in the body) can also increase JNK activation (340; 341) in vitro, but to a much lesser extent than palmitate. Thus, in conditions of glucolipotoxicity, it is possible that JNK plays an even greater
role in impairing β-cell function. This points to the importance of investigating the role of JNK in a model of β-cell gluolipotoxicity.

In summary, the results of Study 3 suggest that JNK plays a causal role in high glucose-induced β-cell dysfunction. However, how high glucose-induced JNK activation leads to β-cell dysfunction remains to be elucidated. Future work to address this issue is warranted.
Summary and Conclusions

Summary of Each Study in the thesis

*In vitro* studies have linked high glucose-induced β-cell dysfunction to oxidative stress, however whether oxidative stress plays a role *in vivo* is unclear. In Study 1, we show that prolonged glucose infusion impaired β-cell function both *in vivo* and *ex vivo*. This was associated with an increase in both total and mitochondrial superoxide levels. Co-infusion of the superoxide dismutase mimetic TPO with glucose, prevented the increase in total and mitochondrial superoxide levels, and prevented β-cell dysfunction *ex vivo* and *in vivo*.

In Study 2, we investigated whether ER stress is a downstream mechanism of oxidative stress, and whether ER stress plays a causal role in high glucose-induced β-cell function *in vivo*. We found that prolonged glucose infusion increased ER stress markers, and co-infusion of the superoxide dismutase mimetic TPO prevented this increase. We also show that the co-infusion of chemical chaperone 4-phenylbuoytrate with glucose partially prevented the increase in ER stress markers, and completely prevented high glucose-induced β-cell dysfunction *ex vivo* and *in vivo*.
The role of JNK activation in high glucose-induced β-cell dysfunction is unclear. In Study 3, we showed that inhibition of JNK pathway, using either the pharmacological inhibitor SP600125, or JNK-1 null mice, prevent high glucose-induced β-cell dysfunction ex vivo and in vivo. Furthermore, TPO and PBA prevented high glucose-induced JNK activation. These findings suggest that JNK plays a causal role in high glucose-induced β-cell dysfunction, and JNK activation lies downstream of oxidative stress and ER stress in high glucose-induced β-cell dysfunction in vivo.

General Summary

By using an in vivo glucose infusion model, the studies in this thesis have demonstrated that 1) decrease in superoxide, reduction of ER stress, and suppression of the JNK pathway prevent high glucose-induced β-cell dysfunction, and 2) high glucose-induced oxidative stress and ER stress are linked, and both induce JNK activation in vivo.

General Conclusion

The results from this thesis suggest that oxidative stress, ER stress and JNK activation are causally involved in high glucose-induced β-cell dysfunction, and 2) High glucose-induced oxidative stress and ER stress are linked, and both impair β-cell dysfunction via JNK activation in vivo. Based on these findings, we reject our original hypothesis that high glucose-induced oxidative stress impairs β-cell dysfunction in vivo via pathways that involve ER stress and JNK. The results from this thesis suggest that oxidative stress, ER stress and JNK are potential targets through nutrition (e.g. dietary antioxidants) and pharmacological agents (i.e. antioxidants,
chaperones, JNK inhibitors) to prevent or treat high glucose-induced β-cell dysfunction. Furthermore, the results from this thesis suggest that treatments that target hyperglycemia (i.e. inhibitors of renal reabsorption of glucose), may be of therapeutic interest to improve β-cell function in type 2 diabetes. These inhibitors are currently in clinical trials for the treatment of type 2 diabetes (342).
Limitations

The short-term glucose infusion used in our studies is a convenient and useful model to raise glucose levels in vivo, and to investigate the mechanisms of glucose per se on β-cell function. However, this model is associated with certain limitations. One of the greatest limitations is that the mechanisms implicated in this model cannot be validated in humans. Unlike rats, humans do not tolerate hyperglycemia (due to glucose infusion) well (293). There has only been one study which showed impaired β-cell function following 68 h of mild (~13mmol/l) hyperglycemia in humans (69). Alternatively, glucotoxicity can be studied in individuals with type 2 diabetes. However, as discussed previously, type 2 diabetes is associated with other metabolic changes (such as hyperlipidemia) than glucose, which can affect β-cell function. Thus, it would be very difficult to tease out the effect of prolonged glucose elevation per se on β-cell function.

Another limitation to this model is its short-term and extreme nature. Thus, it can be argued that our model does not represent glucotoxicity in humans and is not physiological. However, high glucose-induced β-cell dysfunction does occur in humans following exposure to hyperglycemia (13mmol/l) for 68h (69). We have investigated the effect of TPO (Study 1) and PBA (Study 2) in a less extreme, more chronic model of high glucose-induced β-cell dysfunction (96h at 15mmol/l glucose) and the findings were very comparable to the 48h study. It would be important to investigate β-cell glucotoxicity in even more prolonged models.
However, further prolongation of *in vivo* infusion is not feasible. Thus, more chronic glucose exposure may require *in vitro* studies (264) or studies in hyperglycemic but not hyperlipidemic diabetic models, such as Goko-Kakizaki (GK) rats or the transgenic MKR mice. The GK rat is a non-obese Wistar sub-strain that develops type 2 diabetes (343). The MKR mouse expresses a dominant-negative IGF-1 receptor mutation specifically in skeletal muscle, and develops hyperglycemia and type 2 diabetes in the absence obesity (344). It is of note however that it is not possible to induce β-cell glucotoxicity by oral feeding of glucose (due to an increase in insulin secretion and decrease in glucose feeding). Partial pancreatectomy can lead to hyperglycemia (345), however, islets of these animals are directly affected by the treatment and these animals are subject to many metabolic and hormonal changes that may interfere with the effect of hyperglycemia. Nevertheless, studies have used this rat model, together with phlorizin (a drug that inhibits renal tubular reabsorption of glucose) to investigate the effects of glucotoxicity (346).

Chronic glucose elevation has been reported to induce β-cell death, and has been proposed to act as a secondary force to decrease β-cell mass following the onset of diabetes. In our model of β-cell dysfunction induced by 48h glucose infusion, we observe no changes in β-cell apoptosis. Even following 96h glucose infusion, it has been reported that β-cell apoptosis does not change significantly. Thus, our model is one of a model of β-cell dysfunction rather than loss. Many of the mechanisms that are studied in this thesis (oxidative stress, ER stress and JNK activation) are not only involved in β-cell dysfunction, but also β-cell apoptosis. Thus, future studies should investigate these pathways in more chronic models of β-cell glucotoxicity that are associated with decreased β-cell mass. These can include using an *in vitro* model of glucotoxicity (β-cell lines, mouse or human islets), or *in vivo* models such as GK rats, or MKR mice as described above.
In addition to sustained hyperglycemia, it has been reported that patients with type 2 diabetes are associated with greater fluctuations of glucose levels than normal patients (347; 348). Recently, it has been shown that intermittent high glucose induced greater impairment of insulin secretion in human islets compared to those exposed to sustained high glucose (349). Interestingly, oxidative stress and endoplasmic reticulum stress were found to be more significantly increased with intermittent glucose exposure compared to sustained glucose exposure in rat islets and INS-1 cells (88). Thus, future studies of glucotoxicity utilizing a model that alternates glucose levels from high to low may be useful in mimicking glucotoxicity in patients with type 2 diabetes.

In obese glucose intolerant patients, postprandial hyperglycemia occurs on top of the already high FFA levels. In subjects with type 2 diabetes, both glucose and FFA levels are chronically elevated. Therefore, the combined effects of glucotoxicity and lipotoxicity are of great clinical importance. Glucotoxicity may add to lipotoxicity, or may have a permissive or synergistic effect on lipotoxicity as it favors fat esterification, generating lipotoxic signals (350). The synergistic interaction between glucotoxicity and lipotoxicity is associated with triglyceride accumulation (44; 350) and decreased insulin gene expression (350; 351). This effect is known as glucolipotoxicity. Future studies should address the effect and mechanisms of β-cell glucolipotoxicity.

We have demonstrated that prolonged hyperglycemia impairs insulin sensitivity, as indicated by the sensitivity index calculated from the hyperglycemic clamp. Thus, it cannot be excluded that the effect of TPO, PBA and the JNK inhibitor to prevent high glucose-induced β-cell dysfunction is secondary to an improvement in insulin sensitivity. Also, sensitivity index calculated from the hyperglycemia is known to be less accurate than that obtained from the hyperinsulinemic-euglycemic clamp. Ideally, a hyperinsulinemic-euglycemic and
hyperglycemic clamp should be performed on the same rat to calculate the disposition index. However, our preliminary findings show that performing both clamps in the same animal increases invasiveness and stress. Nevertheless, we have performed ex vivo studies in isolated islets, and have shown that the DI corresponds to β-cell function ex vivo. Although we have performed studies ex vivo; it can still be argued that previous exposure to insulin resistance during the 48h may have “memory effect” on the β-cell. Thus, to completely eliminate the indirect effect of insulin resistance on insulin secretion, in vivo studies with β-cell specific transgenic/knockout mice, or in vitro studies would have to be performed. However, it is known that a more prolonged exposure to glucose is necessary to induce glucotoxicity in rat islets. It has been reported that 3 days exposure of rat islets at 20mmol/l glucose does not impair insulin secretion (35). Furthermore, exposure of isolated rat islets at 30 mmol/l for the same duration does not induce ROS (175). Therefore, it will not be possible to completely mimic the in vivo glucotoxic conditions (48h exposure at 20mmol/l glucose) in vitro.
Future Directions

1. The findings from Study 1 suggest that increased superoxide generation is important in high glucose-induced β-cell dysfunction. However, the site of superoxide is unknown. Multiple sites including the mitochondria electron transport chain, and NADPH oxidase in the cytosol, have been reported to produce superoxide levels in the β-cell. In this regard, future work should examine whether increasing the superoxide scavenging capacity in the individual cellular compartments can prevent high glucose-induced β-cell dysfunction. This can be achieved by studying glucotoxicity in mice overexpressing the MnSOD (mitochondrial isoform), or Cu/ZnSOD (cytosol isoform). Moreover, it is unclear how oxidative stress impairs β-cell function. The study presented in this thesis has linked oxidative stress to endoplasmic reticulum stress and JNK activation. However, many other pathways remain unexplored. In particular, it would be interesting to examine the role of UCP2 activation in high glucose-induced β-cell dysfunction (perhaps by using the β-cell specific UCP2 null mice), as high glucose-induced mitochondrial superoxide has been shown to increase UCP2 activation in vitro.

2. In Study 2, we have established a link between oxidative stress and ER stress, and have shown that ER stress plays a causal role in high glucose-induced β-cell dysfunction in vivo. However, it is unclear how ER stress impairs β-cell function. We have shown that co-
infusion of the chaperone PBA with glucose decreases activation of JNK, suggesting that ER stress may activate JNK to impair β-cell function. Future studies should also examine ER stress in islets exposed to glucose with or without JNK inhibition to provide further evidence for a link between ER stress and JNK activation. The inflammatory pathway NFκB can also be activated by ER stress. Thus, future studies should examine whether NFκB is a downstream mechanism of ER stress in β-cell glucotoxicity. Because PBA may have some nonspecific effects on insulin sensitivity (based on the sensitivity index calculated from the hyperglycemic clamp), we are in the process of generating a β-cell specific GRP78 overexpressing mouse (GRP78 is an resident ER chaperone), which will be used to investigate glucotoxicity.

3. In Study 3, our findings suggest that JNK activation is important in high glucose-induced β-cell dysfunction in vivo. However, it is unknown how JNK impairs β-cell function. JNK has been reported to impair insulin gene transcription and insulin biosynthesis. Thus, future studies should examine the effect of glucose with or without JNK inhibition on insulin gene transcription (PCR on insulin gene transcription, PDX-1 and FOX-O1 immunostaining) and insulin biosynthesis (by leucine incorporation into proinsulin). JNK is known to impair the insulin signaling pathway in insulin sensitive tissue. Because the β-cell also expresses the insulin receptor, it would be very interesting to investigate whether JNK impairs β-cell function by inducing β-cell insulin resistance. In future studies, we plan to first examine activation of insulin signaling cascade in the β-cells (i.e. serine phosphorylation of IRS-1, activation of AKT) with or without JNK inhibition in glucotoxicity. If the insulin signaling cascade is inhibited by JNK activation, we plan to then investigate whether β-cell insulin resistance plays a causal role in high glucose-induced β-cell dysfunction, by utilizing the peroxovanadium compound, BPV, a tyrosine phosphatase inhibitor with no antioxidant
activity, in our model of glucotoxicity. Our lab has obtained preliminary data that BPV can prevent fat-induced β-cell dysfunction, possibly by improving insulin signaling in the β-cells. Because BPV has other nonspecific effects than inhibiting tyrosine phosphatase, we plan to also investigate β-cell glucotoxicity in the β-cell specific PTEN null mice, which have an upregulated insulin signaling cascade, if BPV does prove to be effective. These experiments would provide insights into the link between JNK activation and β-cell insulin resistance in glucotoxicity.


57. Lukens FDaF: Experimental Diabetes Produced by the Administration of Glucose. *Endocrinology* 42:244-262, 1948


73. Sharma A, Olson LK, Robertson RP, Stein R: The reduction of insulin gene transcription in HIT-T15 beta cells chronically exposed to high glucose concentration is associated with the loss of RIPE3b1 and STF-1 transcription factor expression. *Mol Endocrinol* 9:1127-1134, 1995


201. Matsumoto M, Accili D: All roads lead to FoxO. Cell Metab 1:215-216, 2005


pancreatic transcription factor PDX-1 through activation of c-Jun NH(2)-terminal kinase.

*Diabetes.* 52:2896-2904, 2003


310. Di Matteo MA, Loweth AC, Thomas S, Mabley JG, Morgan NG, Thorpe JR, Green IC: Superoxide, nitric oxide, peroxynitrite and cytokine combinations all cause functional impairment and morphological changes in rat islets of Langerhans and insulin secreting cell lines, but dictate cell death by different mechanisms. *Apoptosis* 2:164-177, 1997


