The Roles of Free Fatty Acids and Inflammation in β-Cell Dysfunction

by

Aleksandar Ivović

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

Department of Physiology
University of Toronto

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Abstract

β-cell lipotoxicity refers to the adverse effects of chronically elevated plasma free fatty acids on β-cell function and mass and plays an important role in type 2 diabetes. The objective of my thesis was to investigate the role of inflammation in fat-induced β-cell dysfunction in vivo. I used in vivo models of 48h intravenous fat infusion in mice followed by hyperglycemic clamps or islet secretion studies ex vivo, and in vitro models of 48h exposure to oleate and palmitate in islets. The first study showed the proinflammatory kinase IKKβ is causally involved in fat-induced β-cell dysfunction during hyperglycemic clamps in vivo, ex vivo in isolated islets of fat-infused rodents and in vitro in oleate-exposed islets. This is the first study to show directly that β-cell IKKβ plays a causal role in fat-induced β-cell dysfunction in vivo. Inflammation induces insulin resistance in the classical insulin target tissues muscle, fat and liver and the purpose of the second study was to determine whether β-cell insulin resistance plays a causal role in fat-induced β cell dysfunction. We used models of pharmacologic and genetic upregulation of insulin signaling, which were protected from fat-induced β-cell dysfunction in vivo and ex vivo. These data are the first to show that β-cell insulin resistance plays a causal role in β-cell dysfunction induced selectively by fat. The intracellular NOD1 receptor of innate immunity also affects metabolism, however its role in β-cell function is unknown and was the focus of the third study. Mouse and human islets expressed
NOD1 mRNA, the NOD1 activator FK565 decreased β-cell function and NOD1-KO islets were protected from palmitate-induced β-cell dysfunction \textit{in vitro}. \textit{In vivo}, FK565 decreased β-cell function and NOD1-KO mice were protected from palmitate-induced β-cell dysfunction. These data demonstrate that NOD1 plays a causal role in saturated-fat induced β-cell dysfunction \textit{in vivo}. 
Dedicated to Zagorka Jovanović
Acknowledgments

The amount of time and energy needed to complete a PhD project is enormous and far exceeds any other endeavor I have undertaken. I would not have been able to accomplish this without the tremendous support I have received from friends, family, colleagues and mentors.

First and foremost, I would like to thank my supervisor, Dr. Adria Giacca for everything she has done for me, starting with giving me an opportunity to work in her laboratory as an undergraduate project student. She recognized and supported my passion for diabetes research as a person living with type 1 diabetes and gave me the chance to work on a number of projects in the laboratory that only grew my interest in the field. I am very grateful for her guidance, patience and wisdom and without her I would not have been able to pursue my dream of working in a leading diabetes research laboratory and completing a PhD.

Thank you to the members of my supervisory committee, Dr. Dana Philpott, Dr. Gary Lewis, and Dr. Minna Woo for your time, patience, and advice. Thank you also for pushing and encouraging me throughout my years of research and helping me to contribute everything I could to, and receive the most from, this experience. Your help and support are greatly appreciated. Thank you to the members of my Final Supervisory Committee, Dr. Michael Wheeler and Dr. Jonathan Rocheleau for your time and your advice regarding the next steps for my thesis. Thank you also to the members of my Final Oral Examination Committee, Dr. Jonathan Schertzer, Dr. Carolyn Cummins, Dr. Cristina Nostro, and Dr. Tianru Jin. Your time and input is greatly appreciated.

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I could write another manuscript if I were to say thank you to everyone who contributed to my success, but it is important to me to say thank you to a number of my friends and colleagues outside the laboratory for their unwavering support and encouragement.

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a University of Toronto chapter of Diabetes Canada, for guiding me in advocating for changes in diabetes-related healthcare policy and for trusting me to lead the mission of Diabetes Canada in the Greater Toronto Area as the Regional Chair. These were all instrumental in motivating me to continue in diabetes research over the years.

The experience of graduate school has many ups and downs and struggles that are shared by graduate students from across the University. I was fortunate enough to find community and a support system through CUPE 3902 and in particular I would like to thank my friends and colleagues Abouzar Nasirzadeh, Dr. Erin Black, Dr. Meaghan Marian, Sean Hayes, Wayne Dealy, Jeremy Hurdis, Dan Brielmaier, Pamela Arancibia, Dr. Kiran Banerjee, Dr. Abraham Singer, Golbarg Rekabtalaei, Dr. Lama Mourad, Lincoln Rathnam, and Craig Smith and for striving to make the graduate student experience better for everyone at the University of Toronto and for supporting and encouraging me in my pursuit of a PhD. I would also like to thank the staff representatives, Rebecca Strung and Tiffany Balducci, for their help in difficult situations, especially when our laboratory and others in the Medical Sciences Building had asbestos leaks and the University administration was unwilling or unable to help. Regarding the graduate student experience, I would also like to thank Dr. Allan Kaplan for his willingness to work with myself and other graduate students to hear our concerns and begin to address them in real ways, including instituting paid medical leaves, increasing the stipend and beginning to develop professional development programs to help graduate students address the ever increasing need to look for careers outside of academia.

I am very grateful to my close friends and my significant other Emily Clare and her family for their support, advice and positivity. Finally, I am extremely grateful to my mother and father for their advice, their love and their constant support, especially my mother. Since I was diagnosed with diabetes, she went above and beyond to keep me healthy and to make sure I had all the support I needed. She also strongly encouraged me to pursue a PhD related to diabetes and she is a major reason that I chose to do so. She passed away from cancer just before I began the degree. This thesis is dedicated to her.
# Table of Contents

Dedication ................................................................................................................................. iv

Acknowledgments ..................................................................................................................... v

List of Abbreviations .................................................................................................................. xiii

List of Tables ............................................................................................................................... xviii

List of Figures .............................................................................................................................. xix

Manuscripts arising from completion of this thesis ............................................................... xxii

Collaboration manuscripts of the author of this thesis developed in the same laboratory .... xxiii

1 Introduction ............................................................................................................................... 1

1.1 Type 2 diabetes mellitus ...................................................................................................... 3

1.2 Obesity, insulin resistance and inflammation .................................................................... 4

1.2.1 Insulin signaling ............................................................................................................... 4

1.2.2 Obesity and inflammation .............................................................................................. 6

1.2.2.1 Adipose tissue ............................................................................................................. 6

1.2.2.2 Skeletal muscle .......................................................................................................... 9

1.2.2.3 Liver ........................................................................................................................... 11

1.2.2.4 Brain .......................................................................................................................... 12

1.2.2.5 Intestinal microbiome ............................................................................................... 13

1.3 Inflammation and metabolic stress .................................................................................... 14

1.4 The pathogenesis of type 2 diabetes ................................................................................... 15

1.5 Free fatty acids and β-cell function ................................................................................... 16

1.5.1 Mechanisms of insulin secretion .................................................................................... 16

1.5.2 Acute effects of free fatty acids on β-cell insulin secretion ............................................ 22

1.5.2.1 Free fatty acid metabolism in the β-cell .................................................................... 22

1.5.2.2 Free fatty acids and protein acylation ....................................................................... 23
1.5.2.3 Free fatty acid esterification and signaling in glucose-stimulated insulin secretion ........................................... 23
1.5.2.4 FFA-activated G-protein coupled receptors and insulin secretion .......... 24
1.5.2.5 Other nutrient secretagogues .................................................. 25
1.5.3 Chronic effects of free fatty acids on β-cell insulin secretion ......................... 26
1.5.4 Free fatty acids and β-cell apoptosis ........................................... 32
1.6 Chronic elevation of free fatty acids, initiation of inflammation, and mechanisms of β-cell dysfunction ................................................................. 32
1.6.1 Reactive oxygen species and oxidative stress ....................................... 33
1.6.2 Endoplasmic reticulum stress .......................................................... 36
1.6.3 Inflammation .................................................................................. 37
  1.6.3.1 Cytokines .................................................................................. 38
  1.6.3.2 Immune cells .............................................................................. 39
  1.6.3.3 Free fatty acids and inflammatory signaling .................................... 39
  1.6.3.4 Inflammation and β-cell insulin resistance .................................... 45
1.7 Inflammation and type 2 diabetes .................................................................. 46
1.8 Summary ............................................................................................... 47
1.9 Rationale and significance of the studies ...................................................... 48
1.10 General hypothesis .................................................................................. 49
1.11 Specific aims .......................................................................................... 50
1.12 Studies ..................................................................................................... 51
2 General Methods .......................................................................................... 53
2.1 Procedures ................................................................................................. 53
  2.1.1 Experimental animal model and surgical procedures ............................ 53
    2.1.1.1 Animals ..................................................................................... 53
    2.1.1.2 Surgery .................................................................................... 54
2.1.2 Preparation of infused solutions and the infusion period ........................................... 55
2.1.3 Islet isolation .................................................................................................................. 56
2.1.4 Evaluation of β-cell function *ex vivo* ........................................................................ 56
2.1.5 Evaluation of β-cell function *in vitro* ......................................................................... 57
2.1.6 Hyperglycemic clamp .................................................................................................. 57

2.2 Laboratory methods ......................................................................................................... 59
  2.2.1 Plasma glucose ............................................................................................................ 59
  2.2.2 Plasma free fatty acids assay ....................................................................................... 60
  2.2.3 Plasma insulin assay (RIA) ........................................................................................ 60
  2.2.4 Plasma insulin assay (ELISA) ..................................................................................... 61
  2.2.5 Plasma C-peptide assay (RIA) ..................................................................................... 62
  2.2.6 Plasma C-peptide assay (ELISA) ................................................................................. 63

2.3 Calculations .................................................................................................................... 64
  2.3.1 Insulin sensitivity index (M/I) .................................................................................... 64
  2.3.2 Evaluation of GSIS and β-cell function *in vivo* ......................................................... 64
    2.3.2.1 Disposition index (DI) .......................................................................................... 65

2.4 Statistical analysis ............................................................................................................ 65

3 Study 1 – IKKβ inhibition prevents fat-induced beta-cell dysfunction *in vitro and in vivo* ................................................................................................................................. 66
  3.1 Abstract .......................................................................................................................... 67
  3.2 Introduction ..................................................................................................................... 68
  3.3 Methods .......................................................................................................................... 70
    3.3.1 Animals ...................................................................................................................... 70
    3.3.2 Surgeries and intravenous infusions .......................................................................... 70
    3.3.3 Hyperglycaemic clamps ........................................................................................... 71
    3.3.4 Hyperinsulinaemic-euglycaemic clamps .................................................................. 71
3.3.5  Ex vivo studies in islets ........................................................................................................71
3.3.6  Phosphorylated IκBα assay .................................................................................................72
3.3.7  NFκB p65 activity assay .......................................................................................................72
3.3.8  ROS measurements ..............................................................................................................72
3.3.9  Real time RT-PCR .................................................................................................................73
3.3.10 Studies in cultured islets ......................................................................................................73
3.3.11 Western blots .........................................................................................................................73
3.3.12 Plasma assays .........................................................................................................................74
3.3.13 Statistics ...............................................................................................................................74

3.4 Results ........................................................................................................................................75
3.4.1 Hyperglycaemic clamps in rats ...............................................................................................75
3.4.2 Hyperinsulinaemic-euglycaemic clamps ...............................................................................78
3.4.3 Ex vivo studies in rat islets ......................................................................................................79
3.4.4 Hyperglycaemic clamps in mice .............................................................................................81
3.4.5 Studies in ‘beta-cell specific’ IKKβ-deficient (IKKβΔbeta-cell) mice ..................................83
3.4.6 In vitro studies in islets ...........................................................................................................86

3.5 Discussion ..................................................................................................................................89

4 Study 2 – β-cell insulin resistance plays a role in fat-induced β-cell dysfunction in vitro and in vivo .........................................................................................................................93
4.1 Abstract .....................................................................................................................................94
4.2 Introduction ...............................................................................................................................95
4.3 Methods .....................................................................................................................................97
4.3.1 Animals ..................................................................................................................................97
4.3.2 Cannulation surgeries and intravenous infusions .................................................................97
4.3.3 Pancreatic islet isolation .......................................................................................................98
4.3.4 Hyperglycemic clamps, insulin sensitivity index, disposition index and insulin clearance index..........................98

4.3.5 Ex vivo studies in islets .................................................................................................................98

4.3.6 In vitro studies in islets ..................................................................................................................99

4.3.7 Western blots ...............................................................................................................................99

4.3.8 Plasma assays ...............................................................................................................................100

4.3.9 Statistics .......................................................................................................................................100

4.4 Results ............................................................................................................................................101

4.4.1 Studies in rats ...............................................................................................................................101

4.4.1.1 In vivo hyperglycemic clamps .................................................................................................101

4.4.1.2 Ex vivo studies in islets ..........................................................................................................107

4.4.1.3 In vitro studies in islets ........................................................................................................108

4.4.1.4 Measurement of Akt phosphorylation ....................................................................................109

4.4.2 Studies in mice .............................................................................................................................109

4.4.2.1 Mouse weights and PTEN protein expression .........................................................................109

4.4.2.2 In vivo hyperglycemic clamps ...............................................................................................111

4.4.2.3 Ex vivo studies in islets ..........................................................................................................113

4.5 Discussion .......................................................................................................................................114

5 Study 3 – NOD1 plays a causal role in palmitate induced β-cell dysfunction in vitro and in vivo .................................................................118

5.1 Abstract .........................................................................................................................................119

5.2 Introduction ....................................................................................................................................120

5.3 Methods .........................................................................................................................................121

5.3.1 Animals .......................................................................................................................................121

5.3.2 Human islets ..............................................................................................................................121

5.3.3 Microarray and qPCR analyses .................................................................................................121
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACOD</td>
<td>Acyl-CoA oxidase</td>
</tr>
<tr>
<td>ACS</td>
<td>Acyl-CoA synthetase</td>
</tr>
<tr>
<td>AMPK</td>
<td>5' adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activating protein1</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated specklike protein</td>
</tr>
<tr>
<td>ATF-6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>B420</td>
<td>Animalis subsp lactis 420</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl2-associated death promoter</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl2-associated X protein</td>
</tr>
<tr>
<td>βIRKO</td>
<td>Beta-cell specific insulin receptor knockout</td>
</tr>
<tr>
<td>BMS</td>
<td>BMS-345541</td>
</tr>
<tr>
<td>BPV</td>
<td>Bisperoxovandate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CBR</td>
<td>Cannabinoid receptor</td>
</tr>
<tr>
<td>CCR2</td>
<td>C-C chemokine receptor 2</td>
</tr>
<tr>
<td>CMPF</td>
<td>3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid</td>
</tr>
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<td>CON</td>
<td>Control</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CPE</td>
<td>Carboxypeptidase E</td>
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<tr>
<td>CPT-1</td>
<td>Carnitine palmitoyltransferase1</td>
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<tr>
<td>Cre</td>
<td>Cre recombinase</td>
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<tr>
<td>CXCL1</td>
<td>C-X-C motif chemokine ligand 1</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage-associated molecular patterns</td>
</tr>
<tr>
<td>DI</td>
<td>Disposition index</td>
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<tr>
<td>DKO</td>
<td>Double knockout</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
</tbody>
</table>
FADH2  Flavin adenine dinucleotide
FAM  Fluorescein amidite
FFA  Free fatty acids
FOXO1  Forkhead box protein O1
GINF  Glucose infusion rate
GIP  Glucose-dependent insulinotropic polypeptide
GIPR  GIP receptor
GK  Goto-Kakizaki
GLP-1  Glucagon-like peptide-1
GLP1R  GLP1 receptor
GPCRs  G-protein coupled receptors
GPX4  Glutathione peroxidase 4
GRP78  Glucose-regulated protein 78
GRX  Glutaredoxin
GSH  Glutathione reductase
GSIS  Glucose stimulated insulin secretion
H2DCF-DA  Dihydro-dichlorofluorescein-diacetate
HbA1c  Hemoglobin A1c
HRP  Horseradish peroxidase
iE-DAP  D-glutamyl-meso-diaminopimelic acid
IGF  Insulin-like growth factor
IGF-1R  Insulin-like growth factor-1 receptor
IH  Intralipid and heparin
IkBα  Inhibitor of (nuclear factor) kappa B alpha
IKKβ  Inhibitor of (nuclear factor) kappa B kinase beta
IKKβ_{beta-cell}  Beta-cell specific IKK beta deficient
IL-1  Interleukin-1
IL-1R1  IL-1 receptor type 1
IL-1Ra  IL-1 receptor antagonist
IMTG  Intramyocellular triacylglycerol
iNOS  Inducible nitric oxide synthase
IP3  Inositol 1,4,5 trisphosphate
IRAk  IL-1 receptor-associated kinase
IRE-1  Inositol-requiring enzyme 1
IRF3  Interferon regulatory factor 3
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IkBα</td>
<td>Inhitor (of nuclear factor) kappa B alpha</td>
</tr>
<tr>
<td>JNK</td>
<td>C-Jun amino-terminal kinase</td>
</tr>
<tr>
<td>KATP</td>
<td>ATP sensitive potassium channel</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>Potassium Voltage-Gated Channel Subfamily J Member 11</td>
</tr>
<tr>
<td>Kv</td>
<td>Voltage-gated potassium channels</td>
</tr>
<tr>
<td>LC-CoA</td>
<td>Long chain-CoA</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LRR</td>
<td>Leucine-rich repeats</td>
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<tr>
<td>M/I</td>
<td>Sensitivity index</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDA</td>
<td>Malonaldehyde</td>
</tr>
<tr>
<td>MEHA</td>
<td>3-methyl-N-ethyl-N-(B-hydroxyethyl)-aniline</td>
</tr>
<tr>
<td>MIP</td>
<td>Mouse insulin promoter</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein 88</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NAIP, C2TA, HET-E, TP1</td>
<td>Neuronal apoptosis inhibitor protein, MHC class 2 transcription activator, incompatibility locus protein from Podospora anserine, telomerase-associated protein</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NOD Leucine-rich Repeat and Pyrin domain containing 3</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>Nox</td>
<td>Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase</td>
</tr>
<tr>
<td>nPKC</td>
<td>Novel PKCs</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OLE</td>
<td>Oleate</td>
</tr>
<tr>
<td>OLO</td>
<td>Olive oil</td>
</tr>
</tbody>
</table>
PAMP  Pathogen-associated molecular pattern
PBA  Phenylbutyrate
PDH  Pyruvate dehydrogenase
PDK  Phosphoinositol-dependent kinase
PDX-1  Pancreatic and duodenal homeobox-1
PE  Polyethylene
PERK  PKR-like eukaryotic initiation factor 2α kinase
PFK  Phosphofructokinase
PGE2  Prostaglandin E2
PI  Phosphatidyl inositol
PI3K  Phosphoinositide 3 kinase
PIP2  Phosphoinositol 4,5 bisphosphate
PIP3  Phosphoinositol 3,4,5 triphosphate
PKA  Protein kinase A
PKB  Protein kinase B
PKC  Protein kinase C
PLC  Phospholipase C
PMSF  Phenylmethane sulfonyl fluoride
PNS  Parasympathetic nervous system
PPAR  Peroxisome proliferator-activated receptor
PRR  Pattern recognition receptors
PTEN  Phosphatase and tensin homolog
PVDF  Polyvinylidene difluoride
RIA  Radioimmunoassay
RIPA  Radioimmunoprecipitation assay
RIPK1  Receptor-interacting serine/threonine-protein kinase 1
ROS  Reactive oxygen species
S6K  S6 kinase
SAL  Saline
SENP1  Sentrin/sumo-specific protease 1
SERCA  Sarcoendoplasmic reticulum pump calcium ATPase
SFA  Saturated fatty acids
SLY  Salicylate
SNS  Sympathetic nervous system
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element binding protein 1c</td>
</tr>
<tr>
<td>SUMO</td>
<td>Small ubiquitin-like modifier</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β-activated protein kinase 1</td>
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<td>TBK1</td>
<td>TRAF family member-associated NF-κB activator-binding kinase 1</td>
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<td>TCA</td>
<td>Tricarboxylic acid</td>
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<td>TINSAL-T2D</td>
<td>Targeting Inflammation Using Salsalate for T2D</td>
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<td>Toll-IL-1R</td>
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<td>TIRAP</td>
<td>Toll-IL-1 receptor domain-containing adapter protein</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
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<td>TMB</td>
<td>3,3’,5,5’-Tetramethylneizidine</td>
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<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumor necrosis factor receptor-associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adapter molecule</td>
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<td>TRIF</td>
<td>TIR domain-containing adapter inducing interferon-β</td>
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<td>TSC1</td>
<td>Tuberous sclerosis complex 1</td>
</tr>
<tr>
<td>TXNIP</td>
<td>Thioredoxin-interacting protein</td>
</tr>
<tr>
<td>Tyr-PO32</td>
<td>Tyrosine phosphorylation</td>
</tr>
<tr>
<td>UCP2</td>
<td>Uncoupling protein 2</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VD</td>
<td>Voltage dependent</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
</tr>
</tbody>
</table>
List of Tables

Table 3.1 Plasma FFA, triacylglycerol, glucose and insulin levels after 48h infusions ............ 75

Table 3.2 mRNA levels of inflammatory markers in freshly isolated islets of rats infused with Saline (SAL), Oleate (OLE), Oleate + Salicylate (OLE+SLY) or Salicylate alone (SLY) ........ 80

Table 4.1 Plasma FFA levels during the 48h infusion period of rats infused with oleate or olive oil and with or without BPV. .......................................................... 102
List of Figures

Figure 1.1 Signal transduction by the insulin receptor. ................................................................. 6

Figure 1.2 Stimulation of insulin secretion by glucose................................................................. 17

Figure 1.3 The disposition index (DI) curve in states of normal and impaired β-cell function. .. 27

Figure 1.4 Mechanisms of FFA-induced inflammation and sites of impairment of β-cell function.
.................................................................................................................................................. 52

Figure 3.1 Plasma glucose, glucose infusion rate (GINF), insulin, and C-peptide during two-step
hyperglycaemic clamps following 48h oleate or olive oil infusion in rats................................. 76

Figure 3.2 Sensitivity Index (M/I) and Disposition Index (DI) during two-step hyperglycaemic
clamps with/without 48h oleate or olive oil infusion in rats......................................................... 77

Figure 3.3 Effects of oleate and olive oil on FFA, insulin, glucose infusion rate (GINF) and
insulin sensitivity during hyperinsulinaemic-euglycaemic clamps following 48h oleate or olive
oil infusion in rats. .......................................................................................................................... 78

Figure 3.4 Insulin secretory response to glucose, phosphorylated IkappaBalpha, and active
nuclear NFkB of freshly isolated islets of fat-infused rats. ......................................................... 79

Figure 3.5 Effects of salicylate on ROS levels of freshly isolated islets of fat-infused rats........ 81

Figure 3.6 Plasma glucose, GINF, plasma insulin, C-peptide, sensitivity index and disposition
index during hyperglycaemic clamps in mice infused with oleate with or without BMS.............. 82

Figure 3.7 IKKβ protein expression in islets and hypothalamus of, plasma FFA levels in, and
insulin secretory response to glucose in islets of IKKβ-knockout mice...................................... 83

Figure 3.8 FFA levels, plasma glucose, glucose infusion rate (GINF), plasma insulin, plasma C-
peptide, sensitivity index (M/I), insulin clearance index, and disposition index (DI) during
hyperglycemic clamps in IKKβ knockout mice.............................................................................. 85
Figure 3.9 Insulin secretion, IκBα and phosphorylated AMPKα expression in islets exposed for 48h to oleate with or without salicylate and insulin secretion in islets exposed to oleate with or without BMS .......................................................... 86

Figure 3.10 Serine phosphorylated IRS-1 and PGE2 levels in cultured islets exposed for 48h to oleate (OLE) or vehicle control (CON) with/without salicylate (SLY) and insulin secretion in islets exposed to oleate with or without a COX-2 inhibitor ................................................. 87

Figure 4.1 Plasma FFA, plasma glucose, glucose infusion rate (GINF), plasma insulin and plasma C-peptide during two-step hyperglycemic clamps with/without 48h oleate or olive oil infusion and with/without co-infusion of BPV in rats ................................................................. 104

Figure 4.2 Sensitivity index (M/I), insulin clearance index and disposition index (DI) during two-step hyperglycemic clamps with/without 48h oleate or olive oil infusion and with/without co-infusion of BPV in rats ........................................................................................................ 106

Figure 4.3 Insulin secretory response to glucose of freshly isolated islets of 12 week old normal female Wistar rats treated for 48h with oleate or olive oil with or without BPV ............................................. 107

Figure 4.4 Insulin secretory response to glucose and phosphorylated Akt in cultured islets exposed for 48h to oleate or vehicle with or without BPV ............................................................... 108

Figure 4.5 PTEN protein expression in islets and hypothalamus of PTEN knockout mice and plasma FFA levels, plasma glucose, and glucose infusion rate (GINF) in PTEN knockout mice during hyperglycemic clamps ........................................................................... 110

Figure 4.6 Plasma insulin, plasma C-peptide, sensitivity index (M/I), insulin clearance index, and disposition index (DI) in PTEN knockout mice during hyperglycemic clamps and insulin secretion in islets of heterozygous PTEN-knockout mice .............................................................................. 112

Figure 5.1 NOD1 mRNA expression in mouse and humans islets ......................................................... 124

Figure 5.2 β-cell function in vitro and in vivo in the presence or absence of a NOD1 activator.125
Figure 5.3 Plasma glucose, glucose infusion rate/Δglucose, plasma insulin, plasma C-peptide, sensitivity index/Δglucose, and insulin clearance index during a hyperglycemic clamp following injection of a NOD1 activator or vehicle control.......................................................... 126

Figure 5.4 Insulin secretion in NOD1KO and wild-type islets in the presence or absence of palmitate or oleate................................................................. 127

Figure 5.5 Plasma FFA, plasma glucose, glucose infusion rate (GINF), plasma insulin, plasma C-peptide, sensitivity index (M/I), insulin clearance index and disposition index (DI) during hyperglycemic clamps in NOD1KO/WT mice infused with/without ethylpalmitate............. 129
Manuscripts arising from completion of this thesis


2) **Ivovic A**, Oprescu AI, Eversley JA, Wheeler MB, Woo M, Giacca A. β-cell insulin resistance plays a causal role in fat-induced β-cell dysfunction *in vitro* and *in vivo*. *(Endocrinology, in revision; en.2017-00501).*

3) **Ivovic A**, Desai T, Koulaian K, Ye K, Mori Y, Maisonneuve C, Prentice KJ, Wheeler MB, Philpott DJ, Giacca A. NOD1 plays a causal role in palmitate-induced β-cell dysfunction *in vitro* and *in vivo*. *(Invited to resubmit to Diabetes; DB17-0534, Brief Report).*
Collaboration manuscripts of the author of this thesis developed in the same laboratory


Introduction

Diabetes mellitus is one of the greatest health concerns of the 21st century. As of 2015, there were an estimated 415 million people with diabetes around the world and this number is projected to grow by more than 50% to reach 642 million by 2040. Global expenditures related to diabetes are already more than USD670 billion and make up 12% of total global healthcare expenditures. The greatest rise in prevalence is in low- and middle-income countries, which has been attributed to the increase in urbanization and the adoption of a Western diet and lifestyle (1).

Diabetes mellitus refers to a group of metabolic disorders, characterized primarily by high glucose levels caused by a defect in or absence of the insulin-producing β-cells which are the predominant cell type in the islets of Langerhans in the pancreas. β-cells are responsible for maintaining glucose homeostasis. This applies even in the context of excess nutrients or insulin resistance, which is an inefficiency of insulin in performing its primary role of facilitating glucose uptake and inhibiting glucose production (2). Therefore, the decrease in β-cell mass and/or the impairment of β-cell function, leading to the body’s inability to respond adequately to increases in blood glucose, is the factor that leads to hyperglycemia and defines diabetes mellitus of all types.

The most common types of diabetes mellitus are type 1 and type 2. Type 1 diabetes accounts for approximately 10% of all cases of diabetes. It ensues following an autoimmune attack against β-cells (3). The exact cause is unclear, but involves both a genetic predisposition (4) and an environmental trigger (5) and most frequently develops in children and adolescents. Typical T1D
has a sudden onset and is defined by the destruction of all or most β-cells (3). Therefore, insulin injection therapy is required from the beginning of the disease.

In contrast, type 2 diabetes (T2D), which makes up approximately 90% of cases, has a more gradual progression. It is now known to involve a genetic predisposition (6), however environmental and lifestyle factors play a critical role in its development. Diets high in fat and sugar sweetened beverages, as well as a sedentary lifestyle and age greatly increase the risk of T2D, which is characterized by both insulin resistance and β-cell dysfunction (7). Insulin resistance generally occurs first and can be compensated for by an increase in insulin secretion from β-cells, however in predisposed individuals β-cells are eventually unable to compensate adequately (8). β-cells are also directly damaged by circulating free fatty acids (FFA), which are elevated in T2D and obesity, the strongest risk factor for T2D (7). The resulting impairment in β-cell function leads to hyperglycemia. As the deterioration of β-cells progresses, treatment progresses from lifestyle changes, to oral medications that improve insulin sensitivity or stimulate insulin secretion, and finally to daily insulin injections.

Gestational diabetes mellitus is the next most common type of diabetes and occurs in 3% - 14% of pregnancies (9). From 25% to 50% of women with gestational diabetes develop T2D following pregnancy and the risk of T2D in the offspring is also increased (10). It is not clear why some cases lead to T2D while others do not, however recent studies have identified an increase in the molecule 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) as a possible mechanism through which gestational diabetes leads to T2D (11). The mechanistic pathway involved and the relevance to T2D remain to be investigated.

Regardless of the type, chronic hyperglycemia is what leads to the increased risk of morbidity and premature death. Chronic hyperglycemia leads to a number of complications, largely classified as either macrovascular or microvascular (12). Macrovascular complications include cardiovascular disease (including stroke, cerebrovascular disease, peripheral vessel disease), caused by glucose-mediated damage to major blood vessels and contributed to by other factors associated with T2D such as hyperlipidemia and hypertension. Microvascular complications refers to the deterioration of smaller peripheral vessels leading to retinopathy and nephropathy and contributing to neuropathy, and are directly linked to hyperglycemia. Neuropathy is mainly caused by metabolic
changes that are also due to hyperglycemia. In addition to these long-term complications, short-term complications also occur. This includes ketoacidosis and coma due to insulin insufficiency as well as severe hypoglycemia (13).

Fortunately, with adequate control of the disease, complications can be prevented and indeed the rates of complications have decreased significantly over the last several decades (14,15). Nonetheless, the global rates of diabetes continue to rise at a significant cost to patients and to healthcare systems worldwide. This is primarily due to the increasing prevalence of T2D, which is largely attributed to Western-style high-fat, high-sugar diets and is strongly correlated with obesity (1).

1.1 Type 2 diabetes mellitus

According to the International Diabetes Federation, approximately 9% of adults globally have T2D, with the greatest prevalence in developed countries, while the greatest increase in incidence is in the developing world (1). As mentioned above, T2D is characterized by both a defect in insulin signaling and an impairment in β-cell function, with hyperglycemia resulting ultimately from the latter. Although the exact mechanisms underlying these processes are not known, it is clear that both genetic and environmental factors contribute to the development of T2D.

It has been shown that concordance between monozygotic twins is up to 90% (16,17), which highlights the importance of genetic predisposition. The predisposition to T2D is now known to involve several genes (6), however it is still largely unknown what the relative contribution is of different genes or how they interact. Additionally, certain human populations are known to be at a higher risk of T2D (18). Most recently, epigenetic modifications of DNA have been identified to play a role in T2D which accounts for part of the role of environmental factors, especially high-fat diet (19). Notably, it has been shown in rats that high-fat feeding leads to epigenetic modifications that are inherited by offspring, which as a result, have impaired insulin secretion and glucose tolerance (20).

Obesity is the strongest predictor of T2D and 80% of people with T2D have obesity. The rate of increase in obesity is in line with that of T2D (1). This is largely due to the effect of the chronically
elevated FFA levels seen in obesity to impair insulin signaling and β-cell function. This can occur for three reasons: 1) an increase in fat mass, providing increased substrate for lipolysis (21,22); 2) an increase in visceral adipose tissue, which has a high rate of lipolysis (23,24); and 3) an increase in lipolysis due to insulin resistance (21,25) as insulin promotes fat storage (26,27). Importantly, the increase in FFA results in elevated tissue fat, which directly impairs cell functions. Although T2D had previously been classified as adult-onset diabetes, there is a growing number of children being diagnosed with the disease, and, similar to the increase seen in developing countries, this has been attributed to obesity (28,29). Early studies suggested that chronic hyperglycemia was responsible for β-cell dysfunction, which led to the concept of glucotoxicity (30) and it was thought that lipotoxicity, the damaging effect of chronically elevated FFA levels, could lead to diabetes only in the context of glucotoxicity (31). However, there is now substantial evidence to support a role for lipotoxicity in the initiation and progression of T2D (32–34). Furthermore, glucotoxicity requires the presence of hyperglycemia, which can only occur once β-cells have already begun to deteriorate, thus lipotoxicity is likely critical in the initiation of T2D, while both glucotoxicity and lipotoxicity contribute to its progression. In particular, abdominal obesity, which is characterized by more metabolically active adipose tissue, is now known to contribute significantly to insulin resistance and β-cell dysfunction (35). Furthermore, the insulin resistance that ensues impairs the anti-lipolytic effect of insulin, which leads to a further increase in circulating FFA, thereby accentuating the effect of FFA on β-cell dysfunction and insulin resistance (24).

### 1.2 Obesity, insulin resistance and inflammation

#### 1.2.1 Insulin signaling

The insulin signaling pathway consists of a number of well-characterized steps (Figure 1.1). Insulin is a peptide hormone that initiates signaling by binding to its heterotetrameric insulin receptor which consists of two extracellular α subunits and two transmembrane β subunits (36). The β subunits contain intracellular tyrosine kinase domains and once activated by insulin, phosphorylate each other, allowing for binding of docking proteins, such as insulin receptor substrates (IRS)-1/2/3/4/5/6 (37–40). The most well-defined of these are IRS-1 and IRS-2, which both play important roles in the metabolic signaling pathway of insulin (41,42). IRS is phosphorylated by the insulin receptor on specific tyrosine residues which allows for docking and
phosphorylation of the lipid kinase phosphoinositide-3 kinase (PI3K) (43). Activated PI3K then phosphorylates phosphoinositol-4,5-phosphate (PIP₂) to form phosphoinositol-3,4,5-phosphate (PIP₃), thereby recruiting phosphoinositol-dependent kinase (PDK)-1 and protein kinase B (PKB)/Akt to the cell membrane allowing for phosphorylation of Akt by PDK-1 and PDK-2, leading to its activation (44).

Akt signaling leads to a number of downstream effects, including phosphorylation and activation of AS160 which initiates translocation of the glucose transporter GLUT4 to the cell membrane in insulin target tissues (muscle, adipose) (45). Akt also phosphorylates and inhibits forkhead box protein O1 (FOXO1), thereby preventing its translocation to the nucleus and subsequent transcription of gluconeogenic genes (46). Akt also phosphorylates tuberous sclerosis complex 1 (TSC1), thereby inhibiting it by dissociating the TCS1/TCS2 complex, allowing for activation of mammalian target of rapamycin (mTOR), which stimulates protein translation through activation of S6 kinase (S6K) and eukaryotic initiation factor 4E (eIF4E) (47,48).

Insulin also signals through another pathway that stimulates growth and proliferation. Following insulin binding to its receptors, adaptor proteins such as son-of-sevenless (SOS) and Grb2 can bind either the insulin receptors, IRS proteins, or IRS like proteins such as Shc and activate downstream signaling molecules to initiate cell growth and proliferation (49). This pathway is not significantly affected by insulin resistance since it does not depend exclusively on IRS tyrosine phosphorylation, the inhibition of which is a major cause of insulin resistance (50).
Figure 1.1 Signal transduction by the insulin receptor. Signal transduction by the insulin receptor. Insulin binding to its receptor results in tyrosine phosphorylation (-Tyr-PO32) of the insulin receptor and IRS (Insulin Receptor Substrates) 1-6. Phosphatidylinositol (PI) 3 Kinase (PI3K) and Grb2 bind to phosphorylated IRS and activate the PI3K and mitogen-activated protein kinase (MAPK) pathways, respectively. Akt is downstream of PI3K and forkhead box protein O1 (FOXO1) and mammalian target of rapamycin (mTOR) are downstream of Akt. When activated, the inhibitor of kappa B kinase β (IKKβ) and c-Jun amino-terminal kinase (JNK) serine phosphorylate IRS and thereby prevent its tyrosine phosphorylation and subsequent downstream signaling. When PTEN is activated, it dephosphorylates PIP3, thereby preventing Akt activation. Not shown in this figure are tyrosine phosphorylation of Shc and PI3K- and MAPK-independent pathways such as the CAP-Cbl pathway, which also contributes to the metabolic actions of insulin.

1.2.2 Obesity and inflammation

1.2.2.1 Adipose tissue

Obesity is defined by a body mass index greater than 30 and is caused by greater intake than output of energy (51). The excess energy is stored in adipose tissue, which expands over time, increases circulating FFA levels and leads to fat deposition in other tissues. Despite this circulating FFA increase being modest (typically a 50% increase), its chronicity brings about toxic effects. This is referred to as lipotoxicity and has been largely attributed to the increase in circulating FFA and
intracellular lipid accumulation that have damaging effects on metabolic tissues causing insulin resistance (7).

Early in the study of fat-induced insulin resistance, it was shown by Randle and colleagues that an increase in circulating FFA increases fatty acid metabolism and in turn impairs glucose metabolism, leading to impaired glucose uptake and glycogen storage (52). Since then, many studies have shown that FFA and their metabolites also impair the insulin signaling pathway in the insulin target tissues. This has been linked in part to activation of inflammatory pathways (as explained below, in part is due to toxic lipid metabolites, i.e. DAG activation of PKC and formation of ceramide) and it is now recognized that obesity and T2D are characterized by a state of chronic low-grade inflammation.

The initial finding that clearly demonstrated the connection between obesity and inflammation was an elevation of the proinflammatory cytokine, tumour necrosis factor α (TNF-α), in the adipose tissue of obese mice (53). It is not clear what initiates inflammation in obesity, however there are a number of possibilities. Metabolic stress (oxidative stress, endoplasmic reticulum [ER] stress) resulting from excess fatty acids is known to initiate inflammatory signaling (54). It is also possible that inflammatory sensors detect fatty acids or products that are released into the circulation in response to high-fat feeding (55). A high fat diet can increase intestinal permeability and thereby facilitate translocation of the bacterial cell wall component lipopolysaccharide (LPS) from the intestinal lumen into the circulation, leading to activation of the proinflammatory toll-like receptor 4 (TLR4) (56). In adipose tissue, excessive expansion resulting from the increased storage of fat in adipocytes can result in decreased vascularization and subsequent hypoxia and cell death. Excess cell expansion may also result in cell damage. Cell damage can alter adipose tissue cytokine secretion and the release of cell debris may initiate inflammatory signaling and further production of cytokines (57). In line with this, TNF-α is secreted by adipose tissue and its level in the circulation correlates with obesity (58). Furthermore, it provides a mechanism linking the obesity-induced inflammation to insulin resistance. Upon binding of its receptor, TNF-α induces activation of the proinflammatory kinases c-Jun amino-terminal kinase (JNK) (59) and the inhibitor of kappa B kinase β (IKKβ) (60).
JNK is a member of the mitogen-activated protein kinase (MAPK) family of stress kinases that respond to various stressors, including cytokines and metabolic stress. Once activated, JNK phosphorylates c-Jun, which forms part of the activating protein-1 (AP-1) that translocates to the nucleus in order to initiate transcription of genes involved in cell differentiation, proliferation, and apoptosis (61). IKKβ is part of a heterotrimeric protein complex, which also consists of the α subunit, and the γ subunit that is responsible for the inhibition of the complex. Upon activation, IKKβ phosphorylates the inhibitor of kappa B α (IκBα), leading to its degradation. Under basal conditions, IκBα is bound to the nuclear factor kappa B (NF-κB) and thereby sequesters it in the cytoplasm. The release of IκBα allows for nuclear translocation of NF-κB subunits, which leads to the transcription of a number of inflammatory proteins, including cytokines as well as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX) enzymes, which are involved in the proinflammatory response (60).

Both JNK and IKKβ are known to serine phosphorylate IRS-1, which prevents its tyrosine phosphorylation, and therefore activation, by the insulin receptor (50). In vitro incubation of adipocytes with TNF-α impairs insulin signaling and glucose uptake through JNK-mediated serine phosphorylation of IRS-1 (62). Indeed, TNFα is a key factor in insulin resistance in rodents, however its importance in humans is still controversial (63–65).

Over the past several years, there has been accumulating evidence implicating an important role of the interleukin-1 (IL-1) family of cytokines in fat-induced inflammation and insulin resistance. IL-1β was among the first identified and has potent proinflammatory properties that are highly regulated at the levels of expression, processing and secretion as well as through antagonism by the IL-1 receptor antagonist (IL-1Ra). Production of IL-1β is regulated by the multiprotein inflammasome NLRP3 (nucleotide-binding oligomerization domain [NOD]-like leucine-rich repeat and pyrin domain containing) (66). It consists of the adaptor protein apoptosis-associated specklike protein (ASC), containing card, caspase-1 which cleaves pro-IL-1 to mature IL-1, and NLRP3. The NLRP3 inflammasome has been shown to play a major role in metabolic inflammation and insulin resistance and is known to be activated in response to reactive oxygen species (ROS). Although the upstream signaling molecules in the IL-1β signaling pathway differ from those of the TNF-α pathway, it too results in the activation of both IKKβ and JNK1, and thereby serine phosphorylation of IRS-1 (67,68).
Another proinflammatory cytokine that was identified early as an important inflammatory mediator of fat-induced insulin resistance is IL-6. IL-6 signals through a pathway involving the suppressor of cytokine signaling (SOCS) 3. SOCS proteins are negative regulators of cytokine signaling that bind and inhibit Janus-associated kinases downstream of cytokine receptors (49). However, they also bind the insulin receptor and inhibit its activation (69). This prevents the binding of IRS-1, which SOCS proteins are also known to bind and degrade (70). Nevertheless, IL-6 is also known to have beneficial effects, as explained below.

Macrophage infiltration is known to accompany obesity-induced inflammation. Adipose tissue secretes chemokines, such as monocyte chemoattractant protein-1 (MCP-1) which attracts immune cells by binding to their C-C chemokine receptor 2 (CCR2) receptors (71). MCP-1 production in adipocytes increases with increasing fat intake and MCP-1 is highly expressed in adipose tissue of obese animals and humans (72). Deletion of MCP-1 (73) or CCR2 (74) improves high-fat diet induced insulin resistance. Interestingly, deletion of IKKβ specifically in myeloid-lineage protects mice from high-fat diet induced insulin resistance (75).

In contrast to proinflammatory cytokines, expression of the anti-inflammatory adipokine adiponectin is decreased in adipose tissue and circulation of obese animals and patients with obesity (76). The mechanism of action of adiponectin appears to involve activation of AMP-activated protein kinase (AMPK) and subsequent inhibition of acetyl-CoA carboxylase (ACC) as well as activation of peroxisome proliferator-activated receptor α (PPARα) (77), which promotes fatty acids uptake and oxidation (78). Notably, adiponectin levels are increased by agonists of PPAR-γ, another PPAR that regulates fatty acid storage and glucose metabolism (78). These PPAR-γ agonists are used in T2D treatment (79).

1.2.2.2 Skeletal muscle

The effect of obesity and ectopic fat deposition on skeletal muscle inflammation and insulin resistance is of particular importance as skeletal muscle accounts for approximately 80% of postprandial glucose uptake (80) and is the main site of FFA utilization (81). FFA in skeletal
muscle are stored as intramyocellular triacylglycerol (IMTG) and are an important source of energy (81), but they can be detrimental in excess. Upregulation of FFA oxidation via the Randle cycle increases intracellular acetyl-CoA which inhibits pyruvate dehydrogenase resulting in decreased glucose oxidation and in conditions of energy excess increases citrate which inhibits phosphofructokinase, resulting in decreased glucose utilization.

Furthermore, studies in both animals and humans have shown that increased IMTG in skeletal muscle strongly correlates with insulin resistance (82,83). However, the increase in IMTG itself does not appear to impair muscle insulin signaling, but rather is indicative of increased FFA uptake and esterification which also leads to the production of lipid metabolites such as diacylglycerol (DAG) and ceramide. These lipid metabolites have been shown to be associated with insulin resistance in humans infused with lipid (84,85) and cause insulin resistance in high fat-fed mice (86). DAG leads to the activation of conventional and novel protein kinase C (PKC) isoforms (87) and ceramide leads to activation of JNK (88) and atypical PKC (89) and each of these impairs insulin signaling through serine phosphorylation of IRS-1. Ceramide also leads to activation of an Akt phosphatase. Interestingly, in trained athletes, a high IMTG content is not associated with insulin resistance and this is likely due to frequent turnover of the IMTG pool and more efficient coupling of lipolysis to FFA oxidation (90), thereby preventing the accumulation of toxic lipid intermediates. This suggests that a decreased level of β-oxidation in obesity is a critical factor in the accumulation of lipid metabolites and in turn insulin resistance. However, other studies have shown that β-oxidation is not decreased with exposure of muscle to elevated FFA levels, but rather that it is not coupled to the TCA cycle due to a rate of β-oxidation that outpaces the TCA cycle and depletes TCA intermediates such as citrate (91). This excess and incomplete oxidation leads to an increase in ROS as well as the formation of acylcarnitines, both of which may contribute to insulin resistance. Acylcarnitines may contribute to insulin resistance through modification of mitochondrial proteins or an increase in NFκB activation (92), however a direct link between acylcarnitines and insulin resistance has not yet been established. These two pathways (i.e. accumulation of lipid intermediates vs. incomplete FFA oxidation) may both play a role in insulin resistance to some extent or may each be predominant in different contexts and this remains to be determined.
Additionally, skeletal muscle insulin signaling is impaired by inflammatory mediators released in other tissues, namely adipose tissue (93) and liver (94). Inflammatory cytokines, such as TNFα and IL-6 have been shown to induce insulin resistance in muscle cells in vitro (95,96) and with infusion in humans in vivo (97) resulting in decreased glucose uptake and glycogen synthesis. However, some studies have shown that IL-6 does not induce insulin resistance, and when it is produced by muscle during exercise, leads to activation of AMPK which promotes glucose uptake and fatty acid oxidation in muscle (98). A number of myokines such as IL-15 (99), decorin (100), irisin (101), and myonectin (102) have also been discovered recently and may play important and mostly beneficial roles in metabolism. They are involved in muscle growth (100) and in fat metabolism (102). Notably, myonectin, which is primarily secreted by skeletal muscle in response to muscular contraction during exercise, promotes fatty acid uptake in adipocytes and hepatocytes and is decreased with obesity (102).

1.2.2.3 Liver

The liver is another important site of ectopic fat deposition and inflammation in obesity. The liver resident macrophages, Kupffer cells, make up 5% of the total cell population (94) and although their numbers do not increase during obesity, their activation does, along with inflammatory signaling and gene expression in liver (94). Obesity is associated with non-alcoholic fatty liver disease (NAFLD) which can lead to the more advanced inflammatory stage of steatohepatitis (103). Patients with NAFLD have increased TNFα, IL-1β and IL-18 expression in the liver, which contributes to liver disease and inflammation as well as insulin resistance in NAFLD (104).

Similar mechanisms as in adipose and muscle tissue, such as IKKβ activation, are involved in fat-induced hepatic inflammation and insulin resistance. Both hepatocyte- and myeloid-specific deletion of IKKβ prevent hepatic insulin resistance (75). It has also been shown that IL-6 derived from adipose tissue impairs hepatic insulin sensitivity (105). The decrease in insulin signaling in the liver impairs insulin-mediated suppression of glucose production, which contributes to hyperglycemia. Insulin by itself increases lipogenesis (fatty acid synthesis and esterification), although it decreases VLDL secretion. However, under conditions of insulin resistance, hepatic lipogenesis is increased by IL-6 and TNFα (106,107). This is likely due to SOCS-induced increase
in sterol regulatory element binding protein 1c (SREBP-1c), a key regulator of hepatic fatty acid synthesis (108), and this increase may also be contributed to by endoplasmic reticulum (ER) stress (109).

As in muscle, lipid metabolites have also been shown to impair insulin signaling in the liver. High-fat diet induces hepatic accumulation of DAG which is associated with hepatic insulin resistance (110,111). As described above, DAG activates PKC and it has been shown that PKC levels are elevated in livers of humans with T2D (112). Importantly, deletion of PKC-δ in mice prevents high fat diet-induced hepatic steatosis and insulin resistance while PKC-δ overexpression causes hepatic insulin resistance (113). PKC-ε knockdown in rats has also been shown to prevent hepatic insulin resistance. The mechanism of action by which PKC-ε decreases hepatic insulin sensitivity involves interaction with the insulin receptor which prevents phosphorylation of IRS (114). Accumulation of ceramide also leads to hepatic insulin resistance and this is prevented with inhibition of ceramide synthesis (115).

1.2.2.4 Brain

The role of the brain, specifically the hypothalamus, in metabolic homeostasis is primarily to balance energy intake with energy expenditure (116). In response to increased levels of insulin and the hormone leptin in the circulation, signaling in the hypothalamus results in decreased food intake (117). Excess energy or saturated fat, however, has been shown to induce hypothalamic inflammation and prevent both leptin and insulin signaling, thereby preventing an appropriate attenuation of hunger and decrease in food intake (118). The effects of FFA to induce hypothalamic inflammation and hypothalamic insulin resistance have been shown to also reduce energy expenditure, and more recently, to have metabolic effects on peripheral tissues, such as decreasing muscle and hepatic insulin sensitivity as well as β-cell insulin secretion (119,120).

In high-fat fed mice, IKKβ activation specifically in the brain results in hypothalamic insulin resistance and an increase in food intake and body weight gain (120). Accordingly, inhibition of IKKβ in the brain improves hypothalamic insulin sensitivity and prevents weight gain, leading to improved glucose tolerance (120). Brain specific deletion of JNK also improves hypothalamic, as
well as adipose tissue, hepatic, and muscle insulin sensitivity and glucose tolerance in high fat-fed mice (121). Interestingly, obesity-induced hypothalamic inflammation has been shown to also impair β-cell insulin secretion (122). In addition, in the hypothalamus both insulin and nutrients (glucose, fatty acids and amino acids) signal to the liver to inhibit gluconeogenesis via the vagus nerve and this effect is impaired with high fat feeding. Importantly, inhibition of TNFα or TLR4 in the hypothalamus prevents high fat diet-induced hypothalamic leptin resistance and obesity, and restores the vagal-mediated suppression of gluconeogenesis in the liver (123).

### 1.2.2.5 Intestinal microbiome

The first evidence for the critical role of the intestinal microbiota in metabolism was the finding that the composition of bacterial communities in the intestine differed significantly between lean and obese animals (124). This was also demonstrated in humans and it was found that weight loss was able to change the composition to a lean phenotype (125). Later, it was shown that germ-free mice are protected from high fat diet-induced insulin resistance, weight gain and glucose intolerance (126), suggesting the gut microbiota are required for the impairing effects of high fat diet on insulin sensitivity and other metabolic parameters. However, changes in insulin sensitivity in these studies were associated with changes in body weight. Nonetheless, it has been shown that an increase in circulating bacterial cell wall products directly leads to insulin resistance, as described below. Additionally, there may be differences between the microbiota that lead to obesity and those that cause insulin resistance (127). Notably, infusion of intestinal microbiota from lean subjects into patients with metabolic syndrome improved insulin sensitivity without any effect on weight (128).

Research into the mechanisms through which bacteria affect metabolism reveal that high fat feeding increases the level of LPS in the circulation and this increase is termed metabolic endotoxemia (129). Treatment with antibiotics has been shown to decrease gut LPS, intestinal permeability and expression of TNFα and to lead to a decrease in body weight, insulin resistance and glucose intolerance (129,130). It has also been shown that adipose tissue from high fat-fed mice contain live bacteria, translocated from the intestine, that cause low-grade chronic inflammation (131). In β-cells, treatment with LPS increased TLR4 expression and decreased
insulin content and secretion in one study (132). In another, it was found that TLR4 deletion protects from fat-induced β-cell dysfunction (55). Finally, in one study, high fat diet-fed mice were treated with the probiotic bacterial strain *Bifidobacterium animalis* subsp. *lactis* 420 (B420) to assess its effect on bacterial translocation and metabolic parameters (131). B420 has been shown to bind to the intestinal mucosa and exclude LPS-containing gram negative bacteria (133) as well as to improve the integrity of the intestinal epithelial cell layer (134). Accordingly, treatment with B420 decreased intestinal adherence and translocation of gram-negative bacteria and protected against high fat diet-induced inflammation, insulin resistance and glucose intolerance (131).

The relative contribution of microbiota to inflammation and impairment in insulin sensitivity and glucose homeostasis is still unclear and studies have shown there are genetic differences between individuals in the response of the microbiome to high fat diets (135). There is much more to be dissected to understand the contribution of the gut microbiome, but it is clear that it plays a critical role in the initiation and progression of insulin resistance, obesity and T2D.

### 1.3 Inflammation and metabolic stress

In addition to inflammation, metabolic stress such as oxidative stress and ER stress play important roles in fat-induced insulin resistance and can both induce and be induced by inflammation. As mentioned above, an excess in FFA can lead to the production of ROS, such as through incomplete β-oxidation or PKC-mediated activation of the membrane protein NADPH oxidase (Nox), and ROS can impair normal cell function by modifying proteins, lipids and DNA (136,137). ROS are also known to activate JNK (138), IKKβ (139) and NLRP3 (140) and thereby initiate inflammatory signaling, which leads to a further increase in ROS and a perpetuating cycle of cellular damage leading to the progression of metabolic dysfunction. The ER is the primary site of protein folding and experiences functional stress in response to nutrient excess due to the increase in synthesis of proteins involved in nutrient storage and metabolism (141). ER stress is measured by activation of the unfolded protein response (UPR), which consists of three transmembrane sensor proteins: PKR-like eukaryotic initiation factor 2α kinase (PERK); inositol-requiring enzyme 1α (IRE-1α); and activating transcription factor 6 (ATF-6) (142). In basal conditions, they are bound and held inactivated by glucose-regulated protein 78 (GRP78), which dissociates from the UPR proteins in
response to misfolded proteins in the cell (143). Together, these proteins inhibit protein synthesis, increase degradation of proteins in the ER and upregulate transcription of chaperone proteins that assist in proper protein folding (142). If the UPR is unable to restore normal protein folding, apoptotic pathways are initiated (144). Furthermore, proteins of the UPR are known to activate both IKKβ and JNK, in particular JNK. IRE-1α has been shown to phosphorylate and activate both IKKβ and JNK (145,146) and PERK can decrease translation of IκBα leading to increased NF-κB activity and subsequent transcription of proinflammatory cytokines and other proinflammatory mediators such as iNOS and COX-2, which leads to further ER stress as well as oxidative stress (147,148). The cycle between oxidative stress, ER stress and inflammation continues throughout obesity and without intervention leads to metabolic dysfunction, including insulin resistance and β-cell dysfunction.

1.4 The pathogenesis of type 2 diabetes

As described above, T2D is characterized by both insulin resistance and β-cell dysfunction. The FFA-induced impairment of insulin signaling in various tissues requires β-cells to increase the level of insulin secretion in order to compensate and maintain glucose homeostasis. Insulin resistance is present relatively early in the pathogenesis of obesity-associated diabetes and in many individuals, β-cells are able to compensate for insulin resistance (149). However, in predisposed individuals, the β-cells are eventually unable to compensate, which leads to glucose intolerance and hyperglycemia (149). Furthermore, β-cells are directly damaged by FFA in a way that induces oxidative and ER stress as well as inflammation, eventually leading to β-cell dysfunction and apoptosis. Therefore, it is both the stress induced by insulin resistance and the impairing effect of FFA that decrease β-cell function. Since normoglycemia can be maintained even in the context of insulin resistance, this dysfunction in the β-cells is the earliest event required for glucose intolerance and the development of diabetes, and is compounded by the toxic effect of progressive hyperglycemia. Similar to lipotoxicity, glucotoxicity involves oxidative stress, ER stress and inflammation, and in addition, high glucose levels decrease fatty acid oxidation and promote esterification into toxic signaling molecules such as DAG and ceramide when FFA are elevated. The combination of elevated FFA and hyperglycemia leads to the progressive worsening of β-cell function in T2D (31).
1.5 Free fatty acids and β-cell function

1.5.1 Mechanisms of insulin secretion

Insulin is produced, stored and secreted by pancreatic β-cells. Secretion is regulated by a number of secretagogues and potentiating metabolic coupling factors. Glucose is the most potent secretagogue and the major regulator of insulin secretion (150). It enters the β-cell through the GLUT2 glucose transporter in rodents and primarily through the GLUT1 transporter in humans (151). Both GLUT1 and GLUT2 are abundant in β-cells and do not require insulin for activation or translocation. Once in the cell, glucose is phosphorylated by the rate-limiting enzyme glucokinase to form glucose-6-phosphate. This allows for it to be further metabolized through the glycolytic pathway, leading to the formation of pyruvate. Pyruvate can then enter the mitochondrion and be further metabolized through the tricarboxylic acid (Krebs) cycle to generate ATP. The production of ATP increases the cellular ATP:ADP ratio which acts on ATP sensitive potassium (K\text{ATP}) channels, causing them to close. This leads to the depolarization of the β-cell which activates voltage-gated calcium channels. When calcium enters the β-cell, it stimulates fusion of insulin containing vesicles with the cell membrane and the release of insulin into the circulation (Figure 1.2).
The release of insulin in response to an increase in glucose is biphasic both in vitro and in vivo (152). The first phase is a rapid release that lasts up to 10 minutes and is followed by a slowly developing second phase of release (152). In prediabetes, the first phase is reduced, while the second phase remains intact, whereas in T2D the first phase is almost completely abolished and the second phase is reduced (153). This biphasic release does not occur in vivo with meals that result in a slow increase in glucose (153), suggesting the biphasic pattern may have evolved as a mechanism to respond to rapid increases in glucose induced either by high-glycemic meals or increases in catecholamines during stress responses. Additionally, basal insulin secretion occurs between meals when blood nutrient levels are much lower than postprandi ally and this contributes up to half of the daily insulin release (154). Previous understanding of the mechanism through which insulin vesicle release is coupled to glucose metabolism is that first phase secretion results from the release of granules already docked to the plasma membrane, whereas the second phase...
involves the recruitment of granules from further inside the cytoplasm to the cell membrane (155,156). However, a recently developed model proposes that both the first and second phase of insulin secretion involve the recruitment of vesicles (157). In this model, the major distinctions between the first and second phase are that the insulin granules in the first phase are closer to the membrane (but not docked) and that the granule pools recruited in the second phase are recruited through remodeling of the actin cytoskeleton in response to glucose (158). Further studies and more sensitive techniques are needed to fully understand the mechanisms of biphasic insulin secretion.

β-cell glucose stimulated insulin secretion (GSIS) has a threshold of 2.8mM and is concentration dependent from 5 – 20 mM with the highest response rate at 5-8 mM glucose(154,159–161). Glucose metabolism in β-cells is unique in that it is driven by changes in nutrient availability in the circulation rather than the requirement for energy substrates as in the case in other tissues, such as muscle, that take up glucose in response to energy expenditure and depletion. This occurs because glucose is transported into the β-cell through the GLUTs along a glucose concentration gradient and has a high K_m (inverse measure of affinity) of 15-20 mM which is therefore not limiting (162). This results in an equalization between the extracellular and intracellular glucose concentrations. Importantly, glycolysis is controlled by glucokinase, which, as alluded to above, is a hexokinase enzyme isoform with very low glucose affinity (163). Since the use of glucose-6-phosphate by other pathways, such as glycogen synthesis or the pentose-phosphate shunt, is minimal in β-cells (164), glucose uptake and usage is determined primarily by the activity of glucokinase in the cell, in combination with the levels of glucose inside and outside the cell. Furthermore, β-cells have low levels of lactate dehydrogenase (LDH) and high activity of pyruvate dehydrogenase (PDH) and pyruvate carboxylase (165). This ensures sufficient levels of both oxidative phosphorylation through the Krebs cycle and anaplerotic metabolism (i.e. the replenishment of Krebs cycle intermediates). Synthesis of Krebs cycle intermediates, such as oxaloacetate, is needed to allow for the cycle to continue (166). These adaptations of the β-cell ensure the coupling of glucose stimulation to insulin secretion by enhancing the glycolytic flux and activity of the Krebs cycle.

Glycolysis in the β-cell is coupled to mitochondrial oxidative phosphorylation through the glycerol-3-phosphate shuttle and the malate-aspartate shuttle which provide reducing equivalents
to the mitochondria for the generation of ATP by transferring protons through the formation of flavin adenine dinucleotide (FADH$_2$) and nicotinamide adenine dinucleotide (NADH) and H+, respectively. These shuttles also sustain glycolysis by transferring NAD+ to the cytosol and are highly upregulated in β-cells (165).

Mitochondrial metabolism is essential for coupling glucose metabolism to insulin secretion. It produces both the ATP which leads to the exocytosis of insulin containing granules via K$_{ATP}$ dependent and independent mechanisms, which include the effect of ATP on granule priming and raising calcium levels independent of K$_{ATP}$ (167), and the additional metabolites involved in the amplification of GSIS. This process is largely driven by anaplerosis (164). The key driver in this process is pyruvate carboxylase which produces oxaloacetate leading to the formation of citrate by condensation of oxaloacetate with acetyl-CoA via citrate synthase and thereby synthesis of downstream intermediates of the Krebs cycle. Once the levels of Krebs cycle intermediates are elevated, they may leave the Krebs cycle and mitochondrion and play other roles in the regulation of insulin secretion. Notably, citrate may exit the mitochondrion and serve as a substrate for the synthesis of malonyl-CoA via the formation of acetyl-CoA by citrate lyase, followed by its carboxylation by acetyl-CoA carboxylase (ACC). Malonyl-CoA is an inhibitor of carnitine palmitoyltransferase-1 (CPT-1), which is an essential step for fatty acid β-oxidation as it transports long chain fatty acids into the mitochondrion. Therefore, its inhibition favours glucose over fatty acid oxidation and increases the cytosolic levels of long chain-CoA (LC-CoA), which serve to potentiate GSIS through various mechanisms, as described below (168).

In addition to serving as a substrate for malonyl-CoA synthesis, citrate can be converted to isocitrate in the cytosol by cytosolic aconitase. Isocitrate itself can also exit the mitochondrion. Regardless of the source, once in the cytosol, isocitrate is converted to α-ketoglutarate by cytosolic, NADP-dependent, isocitrate dehydrogenase (169). The production of NADPH through this process and through conversion of malate to pyruvate by malic enzyme is known to enhance GSIS. Inhibition of enzymes that lead to NADPH production decreases GSIS (170,171) as does treatment with NADP, which decreases the NADPH/NADP ratio (172). NADPH serves as a co-factor for glutathione reductase (GSH), allowing it to reduce glutathione, which can in turn reduce glutaredoxin (GRX). GRX regulates posttranslational modifications of t-SNARE proteins involved in insulin secretion and co-treatment with GRX and NADPH has been shown to increase
insulin granule exocytosis (172). One recent study showed that this may be through GRX-mediated reduction and activation of the redox sensitive sentrin/sumo-specific protease 1 (SENP1) (173). SENP1 deSUMOylates proteins of the exocytotic machinery by removing small ubiquitin-like modifier (SUMO) peptides, which have been shown to decrease insulin granule exocytosis in β-cells through SUMOylation of the exocytotic calcium sensor synaptotagmin VII, although the decrease in exocytosis likely involves SUMOylation of other proteins of the exocytotic machinery as well (174). Importantly, SENP1 deletion impairs insulin secretion and glucose homeostasis and SENP1 activation through the isocitrate-NADPH-GSH-GRX pathway increases insulin secretion and restores insulin exocytosis in β-cells of human donors with T2D (173). NADPH may also act on voltage-gated potassium channels (Kv). These channels are activated following GSIS in order to repolarize β-cells. NADPH has been shown to prevent their activation, which may contribute to a more sustained second-phase of insulin release (175).

Exposure of mouse islets to α-ketoglutarate has also been shown to stimulate insulin secretion (176), which may be a direct or indirect effect of α-ketoglutarate. It can act directly on K\textsubscript{ATP} channels, resulting in their closure (177), it can be converted to glutamate, which one group suggests has a stimulatory effect on exocytosis (178), and it can serve as a substrate for various α-ketoglutarate-dependent hydroxylases that modify proteins involved in oxidative metabolism, thereby enhancing oxidative metabolism (179). Finally, α-ketoglutarate can also re-enter the Krebs cycle where it is converted to succinyl-CoA, which can then lead to the production of GTP by serving as a substrate for succinyl-CoA synthetase, the inhibition of which has been shown to decrease GSIS (180). GTP contributes to the potentiation of GSIS by increasing intracellular calcium concentrations, although the mechanisms involved are not completely clear.

The molecular machinery that β-cells possess and the intricate internal regulation of their metabolic pathways allow β-cells to be self-sufficient, independent of external inputs, such as from the nervous system. Nonetheless, the autonomic nervous system is involved in the adaptive control of β-cells in response to food intake or stress (181). Even the presence of food in the mouth results in increased insulin secretion, mediated by the parasympathetic nervous system (PNS). The PNS continues to stimulate insulin secretion as food is absorbed and it acts primarily by releasing acetylcholine, which binds to the M\textsubscript{3} muscarinic receptors expressed in β-cells. These receptors are G-protein coupled receptors (GPCRs) coupled to the G\textsubscript{q} protein which mediates its downstream
effects through activation of phospholipase C (PLC). PLC hydrolyzes PIP$_2$ to inositol 1,4,5-trisphosphate (IP$_3$) and DAG, leading to the release of calcium from the ER and activation of PKC, respectively, which together result in the enhancement of insulin secretion (182). The sympathetic nervous system (SNS), on the other hand, responds to stress and decreases insulin secretion, primarily through the release of noradrenaline (183). β-cells express both α$_2$ and β$_2$ adrenoreceptors (184) and although β$_2$ activation increases insulin secretion by elevating cyclic adenosine monophosphate (cAMP) through the G$_s$ protein associated with the β$_2$ receptor (185), the predominant effect of adrenaline is to decrease insulin secretion through activation of the α$_2$ adrenoreceptor. The α$_2$ adrenoreceptor is a GPCR coupled to G$_i$ and therefore inhibits cAMP production, resulting in decreased insulin secretion (183). cAMP activates protein kinase A (PKA), which has been shown to phosphorylate voltage-gated calcium channels and $K_{ATP}$ channels as well as translocation and docking components of the exocytotic machinery, thereby increasing insulin secretion (186–188). cAMP also has a PKA-independent effect of increasing insulin secretion via exchange protein directly activated by cAMP (Epac) (188,189).

In addition to the nervous system, incretin hormones activate GPCRs to regulate insulin secretion in response to food intake. Incretins are characterized by their role in enhancing insulin secretion following meals. The two predominant incretins are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP1). GIP is secreted primarily by the K-cells of the proximal small intestine and GLP1 is secreted primarily by the L-cells of the ileum and colon, where it is cleaved from proglucagon by prohormone convertase 1 (190). Activation in β-cells of the GIP receptor (GIPR) or the GLP1 receptor (GLP1R), each of which is a GPCR linked to G$_s$, enhances insulin secretion by stimulating the production of cAMP (191,192). GIP deletion has been shown to impair GSIS in response to oral, but not intraperitoneal, glucose administration (193), however administration of synthetic GIP is not effective in restoring insulin secretion in people with T2D (194,195). On the other hand, GLP1, which is also required for normal GSIS following oral glucose administration (196,197), is very effective in improving glycemic control in people with T2D. Agonists of GLP1 are currently used to treat T2D and their beneficial effects on glycemic control are likely due at least in part to enhanced postprandial insulin secretion (198–202).
1.5.2 Acute effects of free fatty acids on β-cell insulin secretion

1.5.2.1 Free fatty acid metabolism in the β-cell

Glucose metabolism contributes to the production of lipid metabolites which play important signaling roles in the insulin secretion process. As described above, malonyl-CoA shifts fuel metabolism from FFA to glucose and the ability of FFA to potentiate insulin secretion depends on the availability of glucose (168). Lipid signaling molecules can only be synthesized in the presence of sufficient glucose levels. Otherwise, any FFA present in the β-cell are oxidized, following their conversion to acyl-CoA by acyl-CoA synthetase (ACS), and do not accumulate. Although FA oxidation produces ATP, the process depletes the FA that serve as sources for the signaling molecules needed to potentiate insulin secretion (mechanism discussed below). In addition, FA oxidation produces less ATP than glucose due to the uncoupling effect of FFA whereby phosphorylation is dissociated from oxidation. Therefore, the stimulatory role of FFA in insulin secretion is mainly in potentiating insulin secretion in the presence of glucose rather than directly inducing insulin secretion.

FFA may enter the β-cell through passive diffusion and do not require active transport (203), however they may also be transported through the fatty acid translocase CD36 (204). FFA may become integrated into the phospholipid bilayer or may move from cell to cell (203). Additionally, islets express lipoprotein receptors and lipoprotein lipase thus allowing for lipoproteins to serve as a source of fatty acids (205,206). FFA metabolism is determined primarily by the supply of FFA, in addition to glucose availability (168). Under basal conditions, intracellular FFA are converted to FA-CoA and oxidized. With glucose stimulation, however, their oxidation is inhibited by malonyl-CoA and they are used for the rapid synthesis of FA-CoA by long-chain acyl-CoA synthase and play a number of important signaling roles that enhance GSIS such as the activation of protein kinase C and acylation of proteins that promote insulin granule exocytosis (207,208). Fasting has been shown to decrease the ability of β-cells to secrete insulin in response to glucose and this is due to the depletion of FFA esterification products during fasting (209). Indeed, FFA are required for efficient glucose stimulation of insulin secretion. The response depends on the intracellular concentration of LC-CoA and the duration of exposure, and the potency of FFA to potentiate GSIS increases with chain length and degree of saturation (210,211).
1.5.2.2 Free fatty acids and protein acylation

LC-CoA modulates signaling effectors and is used as a substrate for protein acylation in β-cells (207). Protein acylation directs proteins to appropriate membrane sites, stabilizes protein-protein interaction and regulates enzymatic activities (212). FFA have been shown to stimulate insulin secretion independent of glucose and this was attributed in part to protein acylation (213). Indeed, LC-CoA have been shown to directly facilitate the fusion of insulin granules with the plasma membrane (214) and to modify the activity of ion channels directly or through their incorporation into the plasma membrane (215). However, LC-CoA have also been shown to bind to the Kir6.2 subunit of K_\text{ATP} channels and potently activate these channels thereby decreasing insulin secretion (216–218). The differences may be due to different concentrations of LC-CoA or duration of exposure to FFA. In the context of glucose stimulation, there is decreased LC-CoA oxidation which would be expected to increase cytosolic LC-CoA, as found in some studies (207,219), however there is also an increase in the production of glycerol-3-phosphate, which serves as a substrate for the esterification of FFA and perhaps decreased lipolysis. Indeed, it has also been reported that glucose stimulation leads to a decrease in cellular levels of LC-CoA (220). The discordant results may be due to differences in time of analysis following stimulation with glucose.

1.5.2.3 Free fatty acid esterification and signaling in glucose-stimulated insulin secretion

The esterification of FA-CoA leads to the rapid generation of monoacylglycerol (MAG) and DAG. Studies have suggested that MAG production increases GSIS. MAG is thought to enhance insulin secretion by binding to the exocytosis modulating protein Munc13-1 (221), the knockdown of which decreases GSIS (222). DAG has also been shown to bind to Munc-13-1 and stimulate vesicle release (223). Additionally, it plays an important role in GSIS through the activation of PKC. There are 11 different PKC isoforms which differ in their cellular location and requirements of calcium and lipids for activation (208). These are divided into three major classes: 1) conventional PKCs (cPKC; α, βI, βII, and γ) the activation of which is calcium and DAG dependent; 2) novel PKCs (nPKC; δ, ε, η, θ, and μ) the activation of which is DAG but not calcium dependent; and 3) atypical PKCs (aPKC; ζ and λ) which are calcium and DAG insensitive and are primarily regulated by
phosphatidylinositol-4-phosphate and other lipids. The activation of PKCs involves their translocation from the cytosol to the plasma membrane (224). In β-cells, it has been reported that the cPKCs α and βII, and to varying degrees all the novel and atypical isoforms are expressed (224–226).

PKC phosphorylates exocytotic proteins (214) as well as voltage-gated calcium channels (227) and may phosphorylate and inhibit $K_{ATP}$ channels (228), each of which promotes insulin secretion. PKC may also activate adenylate cyclase which catalyzes the conversion of ATP to cAMP and thereby activates PKA (186,229), although it is not clear whether PKC has this effect in β-cells. However, chronic elevation of FFA leads to PKC-mediated serine phosphorylation of IRS-1 and -2 in muscle (230) and similar mechanisms may exist in β-cells, leading to impairment of β-cell function (231). PKCδ activation in particular may contribute to the decrease in β-cell mass in diabetes by inhibiting Akt phosphorylation and thereby decreasing β-cell mitogenesis (231). In addition, PKC-ε, which does not appear to affect GSIS in the absence of a fat challenge, has been shown to impair insulin secretion in high-fat fed mice and in islets exposed to palmitate. The exact mechanisms are not clear, but involve a decrease in FFA esterification and lipolysis in favour of FFA oxidation, thereby preventing the potentiating effect of FFA on GSIS (232).

1.5.2.4 FFA-activated G-protein coupled receptors and insulin secretion

FFA are known to activate the G$_q$-protein coupled FFA receptor GPR40. GPR40 is highly expressed in β-cells and insulin secreting cell lines and its activation is thought to account for half the effect of FFA in potentiating GSIS (233,234). GPR40 responds to saturated fatty acids of 12-16 carbons and unsaturated fatty acids of 18-20 carbons in length in a dose-response manner (235). FFA binding to GPR40 activates PLC, leading to the release of calcium from the ER and activation of PKC, which together stimulate insulin secretion (233,234). However, the role of GPR40 in lipotoxicity induced by chronic elevation of FFA is not clear. One study found that deletion of GPR40 protected against high-fat diet induced glucose intolerance and, conversely, GPR40 overexpression impaired glucose tolerance (236). Other studies found no effect of GPR40 deletion on glucose tolerance in high fat diet and genetic models of diabetes (237,238). Furthermore, additional studies found that GPR40 deletion actually increases susceptibility to high fat diet-
induced glucose intolerance (233) and that GPR40 overexpression protects against genetically-induced diabetes (239). The cause of the discrepancies is unknown but may be due to the different genetic backgrounds of the mice used in the different studies. Importantly, the GPR40 agonist fasiglifam (TAK-875) has been found to improve glycemic control in a phase III clinical trial (240). However, this trial was terminated due to liver toxicity induced by fasiglifam and additional investigation into safer GPR40 agonists is currently underway (241).

GPR41 and GPR43 are also expressed in β-cells (242) and are activated by short chain fatty acids derived from bacterial fermentation of fibre in the colon (243). Their role in β-cell function is not yet clear, however initial studies have shown that GPR41 activation impairs GSIS (244), while in the case of GPR 43, its activation stimulates, and deletion impairs, GSIS (245). Another study showed that deletion of both GPR41 and GPR43 improves glucose tolerance in T2D (242). Further studies are needed to determine the role of each of these receptors in β-cell function and to determine their specific activators and mechanisms of action.

### 1.5.2.5 Other nutrient secretagogues

Amino acids are also able to stimulate insulin secretion, primarily through the sequential deamination of glutamine to glutamate by glutaminase and glutamate to the Krebs cycle intermediate α-ketoglutarate by glutamate dehydrogenase (246), which is allosterically activated by leucine (247). Interestingly, receptors for sugars (248) and amino acids (249) have been found to be expressed in β-cells. The sweet taste receptor Tas1R2 may be involved in fructose stimulated potentiation of GSIS (248) and the amino acid taste receptor Tas1R1/Tas1R3 may mediate the action of glutamate and arginine to stimulate insulin secretion (249). Lipid derivatives, such as lysophosphatidylcholine and oleoylethanolamide, have also been shown to play a role in β-cell function as they stimulate insulin secretion via activation of GPR119, which is expressed in β-cells and operates via cAMP-dependent pathways (250,251). Although enhanced GSIS (250) and protection against glucose intolerance (252) has been achieved through the activation of GPR119, its deletion does not appear to impair glucose tolerance. The role of GPR119 in β-cell function is still under investigation, although it is emerging as an important therapeutic target for the treatment of T2D (253). Another group of G-protein coupled receptors expressed on β-cells that are activated
by lipid derivatives, such as arachidonylethanolamide and 2-arachidonoylglycerol, are the cannabinoid receptors (CB1R and CB2R) (254). These receptors, however, have an inhibitory effect on GSIS (255,256). Lastly, metabolism of the polyunsaturated ω-6 fatty acid arachidonic acid produces prostaglandins, such as prostaglandin E2 (PGE2). β-cells express prostaglandin receptors and their activation by PGE2 is known to impair GSIS by decreasing the cellular levels of cAMP and in turn decreasing insulin gene transcription mediated by cAMP response elements in insulin gene promoters (257).

1.5.3 Chronic effects of free fatty acids on β-cell insulin secretion

In contrast to the stimulatory effect of FFA on insulin secretion in the short-term, chronic exposure (>24h) of β-cells to FFA has a number of deleterious effects that result in a decrease in insulin secretion and therefore hyperglycemia and T2D. Studies have shown that 24-48h in vitro incubation of islets with FFA may enhance basal insulin secretion (258), but reduces insulin synthesis, depletes insulin stores and reduces GSIS (258–270). Ex vivo studies also showed that although 3 hour lipid infusion enhances GSIS, GSIS is reduced after 48h of infusion (271).

A number of in vivo studies, however, have shown discordant results as some found decreases in GSIS in response to chronic FFA (272–275), while others found no change (276–283) or even increases (284–286). These studies used different fat preparations, durations of infusion, methods of assessment of glucose tolerance, and had subjects with differences in metabolic parameters and genetic predisposition to fat-induced β-cell dysfunction. These are important variables to consider, however the most important factor that likely accounts for the differences between studies is the level of insulin resistance induced by fat and whether this was taken into consideration during the assessment of β-cell function.

In vivo studies of β-cell function require the calculation of the disposition index (DI), developed by Bergman et al. (287) and Kahn et al. (288), rather than the measure of insulin in the plasma alone. Insulin resistance causes a compensatory increase in insulin secretion in healthy and even to an extent in damaged β-cells (287,288) and thus the level of insulin secreted must be considered in relation to the level required to maintain glucose homeostasis. In a metabolic state that includes
both insulin resistance and glucose intolerance, it is possible that the level of insulin in the circulation would be higher than that found in a state of normal insulin sensitivity and glucose tolerance. This should not be interpreted as enhanced β-cell function. In this case, β-cells would be attempting to compensate for insulin resistance but a state of glucose intolerance would indicate that even this elevated level of insulin is insufficient to maintain glucose homeostasis, which is a sign of β-cell dysfunction. Therefore, relative rather than absolute insulin secretion must be calculated to determine β-cell function in vivo. This is accomplished through the DI calculation as it is the product of insulin secretion and insulin sensitivity, which have a hyperbolic relationship where the constant of the hyperbolic curve represents the DI (287–289). In fully functioning β-cells, any decrease in insulin sensitivity is matched by a corresponding increase in insulin secretion along a hyperbola that is different (higher DI) from the hyperbola of impaired β-cells, although it is also possible for insulin levels to increase instead by a decrease in insulin clearance (see Chapter 8). Importantly, impaired beta cells are unable to match the level of insulin sensitivity with the appropriate level of insulin secretion and thus follow a left-shifted hyperbola (lower DI, i.e. lower insulin secretion for a corresponding decrease in insulin sensitivity, Figure 1.3).

**Figure 1.3 The disposition index (DI) curve in states of normal and impaired β-cell function.** The DI is the product constant of insulin sensitivity and insulin secretion. As insulin sensitivity decreases, insulin secretion increases such that the relationship between the two is hyperbolic and the constant of the hyperbola is the DI. Healthy β-cells have appropriate compensation for insulin resistance, which is depicted by the solid hyperbola, whereas dysfunctional β-cells have incomplete compensation for insulin resistance, depicted by the dashed hyperbola that is shifted to the left, representing a lower DI. Figure modified from Stumvoll et al. (289,290).
Indeed, in the context of the DI, or relative insulin secretion, the results of the in vivo studies are more consistent. One study showed that although 48h infusion of the lipid emulsion intralipid and heparin (IH) in rats decreased insulin secretion in isolated islets ex vivo, it increased secretion in vivo (284). Insulin resistance was also induced, but not taken into account in assessing β-cell function, therefore it is not clear whether the increase in GSIS was sufficient to compensate. A study in rats performed in our laboratory used 48h infusion of oleate or IH to determine the effect on GSIS and found that both fat infusions had an impairing effect (273). Importantly, the glucose infusion rate, which is an assessment of glucose tolerance, required to maintain a plasma glucose level of 22mM during hyperglycemic clamps was decreased, indicating impaired glucose tolerance and β-cell dysfunction. The impairing effect of oleate was greater than that of IH and this may be due to the differences in the fatty acids as IH consists mostly of polyunsaturated fatty acids whereas oleate is a monounsaturated fatty acid.

Similar results were found in humans. Boden et al. showed that 48h infusion of the fat emulsion Liposyn and heparin in healthy humans increased GSIS (286). However, the glucose infusion rate decreased in the first 24h. In the last 24h, the decrease was less than in the first 24h and did not reach statistical significance, however the glucose was maintained at 8.6mM, rather than a maximally stimulatory rate of 16.7 or greater, which may be necessary to assess differences in β-cell function. Additionally, the control group had an average BMI greater than 25 (i.e. overweight) while the lipid-infused group was of normal weight, which makes it difficult to interpret the apparent, but statistically insignificant decrease in the glucose infusion rate in the second 24h period. Magnan et al. also showed that 48h infusion increased GSIS, but it induced insulin resistance and did not indicate whether the DI changed (285). Paolisso et al. did show that 24h IH infusion in healthy subjects decreases GSIS and the degree of impairment was correlated with the level of increase in plasma FFA, which in this study was threefold after 24h (272), rather than the twofold increase in the other studies (285,286).

In order to assess the effect of fat on β-cell function directly, our laboratory collaborated with Dr. Lewis’s laboratory to determine the effect of FFA on the DI. A 90 minute infusion of IH induced insulin resistance, but was accompanied by a corresponding increase in GSIS, resulting in a DI that did not differ from control. However, following a 48h infusion, there was no change in GSIS to compensate for the fat-induced insulin resistance, and therefore the DI was decreased (276). A
similar study showed that 48h IH infusion did not affect GSIS, but decreased the DI in healthy humans and interestingly, decreased it to a lesser extent in a group of subjects from the First Nations Oji-Cree population, which is known to have a high risk of T2D. The less potent effect of IH was attributed to the lower baseline β-cell function associated with predisposition to diabetes (277). Another laboratory showed that family history of T2D actually increased the susceptibility to fat induced β-cell dysfunction as a 48h lipid infusion decreased both GSIS and DI compared to control treatment and to control subjects infused with fat (274). The difference between the susceptibility between the subjects in these studies may be due to the level of β-cell impairment at the time of the study or to differences in the nature of the genetic susceptibility. Another study showed that 24h IH infusion did not affect GSIS, but induced insulin resistance and decreased DI in both control subjects and those with a family history of impaired glucose tolerance. However, the DI in the first phase of GSIS only decreased in subjects with the history of impaired glucose tolerance (279). Interestingly, one study from our laboratory also found that diabetes-prone BioBreeding rats, which are genetically predisposed to developing T1D, have increased susceptibility to IH-induced β-cell dysfunction. This is likely due to the presence of insulitis caused by inflammation and immune cell infiltration of islets (291). Although this is model of T1D, inflammation is now known to play a critical role in the development of T2D, as discussed later in this chapter.

Additional studies have shown that 12h or 48h IH infusion does not affect GSIS but induced insulin resistance and decreased DI in overweight or obese men (280–282) and adolescents (292). Results from one study from our group suggest that obesity predisposes to fat-induced β-cell dysfunction (275). However, this study did not have a nonobese control group and the results were compared only to an earlier study in which the nonobese subjects were on average 25 years old (276) compared to 50 years old in the obese subjects in the later study. Nonetheless, our group has shown that in rats there is increased susceptibility to fat-induced β-cell dysfunction in obese Zucker rats (293).

Studies in humans have also suggested that existing T2D may increase susceptibility to fat-induced β-cell dysfunction. Although 16h IH infusion did not affect GSIS (278) and only with 48h infusion did GSIS decrease (275) in subjects with obesity and T2D, either duration of infusion induced insulin resistance and decreased DI (275,278) and to a greater extent in those with T2D (278). This
may have been due to the elevated fasting plasma glucose levels, which when combined with elevated FFA levels caused by IH infusion led to glucomlipotoxicity in this group. However, our group has also shown in a group of subjects with T2D that IH infusion, which decreased GSIS in healthy obese subjects, did not affect GSIS in those with T2D. This was likely due to the level of impairment in β-cell function already present in the subjects with T2D (275).

Notably, the level of saturated fat in lipid infusions has been shown to affect the degree of β-cell impairment. One study showed that with 24h intravenous infusion of two different lipid emulsions and heparin there was no change in GSIS for either of them while there was induction of insulin resistance, the extent of which was increased with a higher level of saturated fat (294). The DI was not assessed, but the lack of an increase in GSIS in the presence of insulin resistance implies the DI was decreased and more severely so with the lipid emulsion that had the greater proportion of saturated fat. One study from our group found that ingestion of fat emulsions high in either saturated fatty acids (SFA), monounsaturated FA (MUFA) or polyunsaturated FA (PUFA) over 24h had different effects. Although SFA ingestion did not decrease GSIS, it was the only treatment that induced insulin resistance without a corresponding increase in secretion, suggesting a decrease in DI, which would be in line with the tendency for the glucose infusion rate to decrease in this group compared to the other treatment groups (295). However, in rats, an increase in the degree of FFA saturation may not enhance the impairing effect of FFA on β-cell function. One collaborative study of our laboratory showed that a diet high in soybean oil decreased glucose-stimulated insulin secretion from the perfused pancreas whereas a diet high in lard oil did not (296). Furthermore, rats that were infused intravenously with soy oil had an impairment in DI (not reported in the paper), while rats infused with lard oil had no impairment. It is not clear whether the discrepancies are due to different saturated and unsaturated fat mixtures and in order to better understand the specific effect of fatty acid type, studies with specific fatty acids will be required. Although this may not currently be feasible in humans, infusion of different fatty acids in animals is now possible. Species-specificity may, however, affect the translational value of these studies.

Elevations of glucose and FFA together have been shown to act synergistically to impair β-cell function through an effect known as glucomlipotoxicity. One study in rats found that the combination of elevated FFA, via 48h IH infusion, and elevated glucose, via glucose infusion + dexamethasone treatment, decreased GSIS to a greater extent than elevation of either FFA or
glucose alone (271). A study with our collaborators found that in humans a 24h infusion of a combination of IH and glucose did not decrease DI to a greater extent than IH did alone (297). However, the glucose level was only elevated to 7.5 mM, which actually increased DI when glucose was infused alone and the model used in this study may not have represented true glucolipotoxicity. Another study showed that a higher elevation of glucose to a level of 12.6 mM was sufficient to impair GSIS whereas elevation to 8.8 mM glucose was not (298). Nonetheless, it is possible that there would have been a greater decrease in DI with the co-infusion in the study with our collaborators (297) if the hyperglycemic clamps had clamped glucose levels at a maximally stimulatory level of approximately 20 mM, as in our previous collaborative studies (276), rather than at 10 mM, as in this study (297). This potentially would have allowed for detection of defects in β-cell function (assessed using the DI) which may not have been detectable with submaximal stimulation (278). Further studies in humans would help to better understand glucolipotoxicity, however they are difficult to perform due to inflammation of the i.v. infusion site, swelling, nausea and electrolyte imbalance with prolonged glucose infusions.

Studies in vitro and in animals have examined mechanisms involved in β-cell glucolipotoxicity. The effect of glucose to increase the supply of Krebs cycle intermediates, such as citrate, and to promote the synthesis of LC-CoA, as described above, can lead to toxic accumulation of lipid metabolites such as DAG and ceramide when glucose and FFA are chronically elevated, as in glucolipotoxicity. This leads to impaired insulin secretion and insulin gene transcription (260,299). Glucolipotoxicity has also been shown to impair GSIS by uncoupling oxidative phosphorylation and increasing ROS (300). Chronic elevation of glucose and insulin can also increase expression of the lipogenic transcription factor SREBP-1c (301–303). Overexpression of SREBP-1 in β-cells increased lipogenesis and worsened β-cell impairment induced by fat (304). Conversely, suppression of SREBP-1c prevented β-cell glucolipotoxicity (305).

In summary, these studies support the notion that elevation of circulating FFA impairs β-cell function in vivo in rodents as well as humans and that a predisposition to impaired glucose tolerance or T2D may increase susceptibility to a fat-mediated impairment in β-cell function. Many studies using high-fat diets also provide strong evidence in support of a role of fat to impair β-cell function (131,143,261,296,306–321). However, these studies lead to changes in the gut microbiota, secretion of incretin hormones from the gut and changes in weight that confound the
interpretation of the effects of fat on β-cell function. Therefore, the fat-infusion studies described above are necessary to separate the effect of fat itself from other metabolic alterations associated with obesity and T2D and have indeed highlighted the detrimental effect of fat on β-cell function.

1.5.4 Free fatty acids and β-cell apoptosis

In addition to the effects of FFA on β-cell function, FFA have a negative effect on β-cell mass in the long term. Studies have shown that β-cell mass can continue to increase for as long as 16-20 weeks of high-fat feeding in rodents (307,308), however, similar to the increase in absolute GSIS in the presence of FFA-induced insulin resistance, the increase in β-cell mass is insufficient to compensate for the insulin resistance. Furthermore, with diabetes, there is evidence of β-cell apoptosis (307,308,310,322) and senescence (310), suggesting there is a decrease in functional β-cell mass. FFA-induced β-cell apoptosis is thought to be the mechanism through which β-cell mass ultimately is decreased in the long term in T2D (311,323). The mechanisms proposed to explain FFA-induced apoptosis in part overlap with those involved in FFA-induced β-cell dysfunction, with an important role attributed to oxidative stress, ER stress, and inflammation (as described in the following sections), which gradually impair β-cell function long before they decrease β-cell mass (290).

1.6 Chronic elevation of free fatty acids, initiation of inflammation, and mechanisms of β-cell dysfunction

It is well established that FFA can directly impair β-cell function, independent of their effect on insulin sensitivity, although many of the contributing mechanisms are similar to those involved in the fat-induced impairment of insulin signaling in peripheral tissues. The Randle cycle has been suggested to play a role in fat-induced β-cell dysfunction by stimulating β-oxidation and increasing intracellular acetyl-CoA, citrate and NADH, thereby decreasing the activation of enzymes required for glycolysis (PFK), glucose oxidation (PDH) and in turn, GSIS (52). In support of this, studies have shown that fat-induced β-cell dysfunction is associated with increased β-oxidation (262,263).
ACC mRNA has been shown to decrease in response to fat, while CPT-1 is upregulated, thereby metabolism is shifted from synthesis to oxidation (262,264). Notably, inhibiting β-oxidation through inhibition of CPT-1 prevents fat-induced β-cell dysfunction (259). However, other studies have also shown that the glucose utilization rate, levels of glucose-6-phosphate, and activity of PDH in β-cells is unaffected with exposure to fat (263,320), suggesting other mechanisms are involved in the fat-mediated decrease in insulin secretion. In line with this, the induction of oxidative stress, ER stress and inflammation are now known to occur in response to chronic exposure of β-cells to elevated levels of FFA and lead to their dysfunction. In β-cells, these processes lead to impairments at different levels of function, including insulin gene transcription, proinsulin biosynthesis and insulin secretion.

1.6.1 Reactive oxygen species and oxidative stress

Although ROS, in particular H₂O₂, at low concentrations have been shown to stimulate insulin secretion and even to be required for GSIS acutely (136), chronically elevated levels of ROS lead to oxidative stress. Oxidative stress is of interest in understanding the development of T2D because of its role in initiating β-cell damage as well as the elevated levels of oxidative stress markers found in people with T2D (324). As described above, β-cells play a central role in energy homeostasis by metabolizing macronutrients to produce ATP for insulin secretion, but this also involves the production of ROS (325,326). Long-term elevation of FFA, which increases the level of fatty-acid oxidation, has harmful effects on β-cells mediated at least in part by oxidative stress. This occurs when the level of ROS surpasses the ability of antioxidant enzymes to scavenge them (325). β-cells have relatively low levels of these enzymes (327,328) and a low capacity to store triglycerides, which makes them especially susceptible to lipid-induced oxidative stress (32,329).

It has been suggested that storage of FFA as triglycerides protects β-cells against lipotoxicity (330), but chronic FFA flux into β-cells may overwhelm this potential defense mechanism, allowing for excess ROS accumulation.

Within the β-cell, mitochondria are the greatest source of ROS, particularly superoxide anion (O₂⁻) which is generated by the electron transport chain (ETC) during cellular respiration (326). Increased flux through the ETC occurs with an excess of FFA which leads to high levels of O₂⁻.
production (136). Conversely, FFA have been shown to act as protonophores and can spontaneously move from the outer to the inner lipid layer of the inner mitochondrial membrane in a protonized form. This leads to uncoupling of oxidation from ATP production and decreased ROS production as the proton gradient and in turn transmembrane potential decreases (331). However, with prolonged exposure to FFA, it appears that the predominant effect of FFA is to increase ROS production through the ETC, through changes in mitochondrial membrane composition, and through other mechanisms, leading to impairment in β-cell function. Peroxisomes have also been shown to play an important role in FFA-induced oxidative stress as they produce hydrogen peroxide (H$_2$O$_2$) via β-oxidation of very long-chain FFA (326). In addition, recent evidence implicates a significant role for the membrane associated enzyme, NADPH oxidase (Nox), in the production of cytosolic O$_2^-$ in β-cells (325,332). High levels of FFA can overstimulate Nox through activation of PKC, which can itself be activated by ROS (333). Once generated, O$_2^-$ can be converted by the antioxidant enzyme superoxide dismutase (SOD) to H$_2$O$_2$, which is a less reactive form of ROS (334). H$_2$O$_2$ may then be converted to H$_2$O and O$_2$ by another antioxidant enzyme, catalase (334). As noted above, however, β-cells have much lower levels of antioxidant enzymes than do other tissues (327,328), which allows for rapid accumulation of O$_2^-$ when FFA levels are elevated. Furthermore, O$_2^-$ can be converted into hydroxyl radical (OH•) which is the most reactive form of ROS, capable of damaging DNA directly (334). In addition, metabolic stressors such as inflammation and ER stress, as well as ceramides are triggers of ROS production (335).

Our laboratory has demonstrated a direct role for oxidative stress in β-cell lipotoxicity in vitro in islets exposed for 48h to FFA, ex vivo in islets isolated from rats infused intravenously for 48h with olate and in vivo during hyperglycemic clamps in rats following 48h oleate infusion. An olate-induced decrease in GSIS was prevented by co-infusion of oleate with the antioxidants N-acetylcysteine (NAC) or taurine, both of which scavenge lipid peroxidation products (265). Similarly, co-infusion of NAC, taurine, or Tempol (a SOD mimetic) prevented the impairment of GSIS in isolated islets of olate-infused rats and prevented an increase in islet ROS accumulation (265). In vitro, taurine also prevented a decrease in GSIS and an increase in ROS after 48 hour exposure of MIN6 cells to olate and isolated islets to olate and palmitate (265). Oral taurine was also found to protect against fat-induced β-cell dysfunction in vivo in humans. NAC did not have a protective effect, which was likely due to low bioavailability after oral administration (280).
Although one *in vitro* study did not find a protective effect of NAC nor an effect of FFA to increase islet ROS (299), others have found that in rats, NAC protects against fat-induced β-cell dysfunction caused by either IH infusion (336) or high fat diet (313). Similarly, taurine has been shown to protect against high fat diet induced β-cell dysfunction *in vivo* (314) and in islets of high fat diet-fed mice (337).

More recent studies in our laboratory have investigated mechanisms by which FFA may lead to an increase in ROS and shown that oleate-induced β-cell dysfunction is associated with generation of cytosolic superoxide which leads to lipid peroxidation and in turn formation of the reactive aldehyde malonaldehyde (MDA). Inhibition of Nox prevents this increase in cytosolic superoxide and MDA and prevents oleate-induced β-cell dysfunction *in vivo* which suggests that Nox plays a causal role in fat-induced β-cell dysfunction (137). Similarly, overexpression of the cell membrane peroxidase glutathione peroxidase 4 (GPX4), which reduces lipid peroxides and prevents formation of MDA, protected against oleate-induced β-cell dysfunction *in vitro, ex vivo and in vivo* (266), further implicating lipid peroxidation as a causal mediator in fat-induced β-cell dysfunction. A number of studies have proposed mechanistic links between ROS and β-cell dysfunction. Elevated ROS can decrease binding of the transcription factors PDX-1 and MafA (324), which regulate insulin gene transcription (338), and thereby decrease insulin gene expression and β-cell insulin content (324, 339), although it is unclear whether this is a direct effect of ROS. As in peripheral tissues, ROS overproduction in β-cells can activate the inflammatory kinases JNK and IKKβ and impair β-cell function via effects in the β-cell insulin signaling cascade and through transcription of proinflammatory genes. Importantly, a decrease in GSIS has been shown to occur with increased uncoupling protein 2 (UCP2) activation. UCP2 is a mitochondrial inner membrane protein that is upregulated in response to oxidative stress and uncouples oxygen consumption from ATP production, thereby decreasing ATP production and insulin secretion (340). This oxidative stress-mediated increase in UCP2 has been shown to decrease GSIS in the context of lipotoxicity (315, 341). Additionally, overexpression of UCP2 in isolated β-cells has been shown to impair GSIS (342–344), while both knockdown of UCP2 in mouse islets and pharmacological inhibition of UCP2 increase GSIS (345, 346). There is also evidence that decreased UCP2 protects against the impairment of insulin secretion induced by a high fat diet (307, 315). However, UCP2 also decreases ROS production (347), which could prevent β-cell dysfunction, especially in the long term. FFA can also increase UCP2 expression independent of ROS production. FFA modulate the
activity and expression of the transcription factors PPAR and SREBP-1, which are thought to regulate UCP2 expression (348,349). Nonetheless, further studies are needed to better understand the role of UCP2 in fat-induced β-cell dysfunction and the mechanisms through which UCP2 is regulated.

1.6.2 Endoplasmic reticulum stress

In β cells the ER is responsible for synthesizing and folding insulin molecules in response to glucose stimulation and to increasing metabolic demand. Proper protein folding requires appropriate levels of ATP and calcium as well as a favourable oxidizing environment to allow for the formation of disulfide bonds (350). The dependence of the ER on these conditions makes it susceptible to various stressors, including FFA. β-cells contain the proteins of the UPR (351), which are activated under stressful conditions and often able to restore normal protein folding. The UPR is essential for normal β-cell function and deletion of components of the UPR decreases proinsulin processing, insulin secretion and glycemic control and increases the serum ratio of proinsulin:insulin and apoptosis (352). With insulin resistance, production of insulin in β-cells can increase by more than 10-fold (353) and proinsulin can account for 50% of the total amount of proteins synthesized in the ER of the β-cell, eventually exceeding the capacity of the ER (354). If the UPR is unable to reduce the accumulation of unfolded proteins, the ER induces a pro-apoptotic response (352). FFA have been shown to induce ER stress in human islets (355) and markers of ER stress are increased in islets of cadaveric donors with T2D (356,357). FFA have been shown to activate the three branches of the UPR in β-cells and overexpression of GRP78 prevents palmitate-induced ER stress and subsequent β-cell apoptosis in vitro (144). Furthermore, high fat fed mice have hyperproinsulinemia, which is indicative of ER stress and the inability of the ER to appropriately process proinsulin (358). Importantly, a heterozygous mutation in the Ins2 gene, which characterizes the Akita mouse model, leads to proinsulin misfolding and subsequent ER-stress induced apoptosis and diabetes (359).

Incubation of islets with either oleate or palmitate depletes ER calcium stores, possibly through inhibition of the sarcoendoplasmic reticulum pump calcium ATPase (SERCA) activity, which may result from changes in ER lipid composition, leading to protein misfolding (355). Palmitate has
been reported to induce ER stress by increasing the saturated fat content of the ER (360) or by increasing ceramide synthesis (267). Notably, inhibition of ceramide synthesis decreased ER stress and apoptosis induced by palmitate (361,362). Fat exposure has also been shown to decrease protein trafficking from the ER to the Golgi apparatus (268). One study showed that carboxypeptidase E (CPE), which is required for the processing of proinsulin to insulin, is decreased with exposure of islets to palmitate and its degradation has been shown to induce ER stress (363). The effect of palmitate on CPE was attributed to palmitate metabolism rather than to palmitate itself as a non-metabolizable homolog of palmitate, 2-bromopalmitate, did not decrease CPE protein levels. Further, calcium influx, which has been shown to be induced by palmitate (364), played a causal role in CPE degradation as inhibition of calcium influx via voltage-gated calcium channels prevented CPE degradation and importantly, calcium has been implicated in the stability of CPE (363). Studies have also shown that ER stress is involved in fat-induced β-cell dysfunction in humans. Sodium phenylbutyrate (PBA), which acts as a chemical chaperone and is known to reduce ER stress, partially prevented the decrease in β-cell dysfunction induced by IH infusion (365). However, studies in rodents from our laboratory (unpublished) did not show upregulation of ER stress markers with either IH or oleate in vivo and whether ER stress markers are upregulated with palmitate remains to be determined as in vivo palmitate infusion has only recently been made possible. In addition to the effect of ER stress to induce ROS, as mentioned above, oxidative stress is another pathway through which FFA induce ER stress (322,366). Finally, proinflammatory cytokines have been shown to induce ER stress in islets. IL-1β mediated iNOS transcription and the subsequent NO production led to calcium depletion in the ER through downregulation of SERCA2b and the resulting inability of the ER to maintain a calcium gradient, which is required for protein folding (366).

1.6.3 Inflammation

Studies have shown that inflammatory signaling and production of cytokines and chemokines occurs directly in islet cells, including in both β-cells (367) and immune cells (368). Greater numbers of immune cells as well as cytokine and chemokine levels has been found in islets of patients with T2D (316,369,370) and of all animal models of T2D investigated to date (316,371). IL-1β is now recognized as a master regulator of inflammation and its expression level is increased
in β-cells of animal models (372) of T2D and the level in human β-cells is greater in patients with T2D than in those of BMI-matched controls (370). Although the negative regulator of IL-1β, IL-1Ra, is highly expressed in human islets under normal conditions, it is decreased with T2D (373). Additionally, a global gene expression analysis in human islets found a group of co-expressed genes, enriched for IL-1 related genes, associated with T2D and decreased insulin secretion (374). Gene arrays of human islets and β-cell lines further revealed an increased expression of many inflammatory markers induced by FFA (375,376). In addition, the most strongly up-regulated gene in human islets in response to exposure to high glucose is thioredoxin-interacting protein (TXNIP) (377), which is also elevated in islets of animal models of T2D (378,379) and is known to activate the NLRP3 inflammasome (380). Importantly, reducing inflammation has been shown to improve β-cell function and glucose homeostasis (55,317,319).

1.6.3.1 Cytokines

Cytokines can mediate adaptive responses to damage or stress in β-cells and thereby enhance proliferation and insulin secretion (381), however a chronic increase in cytokine signaling overwhelms the β-cell and results in the transition from an adaptive response to one that is detrimental for β-cell function. Notably, IL-1β secretion from β-cells is likely initiated as an adaptive response (382,383), but eventually leads to an overproduction of cytokines and recruitment of macrophages (372,382). Chronic IL-1β mediated inflammatory signaling is known to decrease insulin mRNA, insulin processing and GSIS (384,385) and also regulates other cytokines and chemokines, leading to recruitment of immune cells to β-cells (370). As mentioned above, inflammation can be initiated by ROS or ER stress and it has been shown that the presence of these stressors sensitizes β-cells to IL-1β induced apoptosis (386). The ability of IL-1β to impair GSIS is also enhanced when combined with exposure of islets to either TNFα (387) or IL-6 (385). IL-6 is elevated in islets of high fat fed mice (316), Goto-Kakizaki (GK) rats (316,372) and db/db mice (316,319) and TNFα expression is increased in GK rats (372) and db/db mice (319), however the roles of TNFα and IL-6 on their own in β-cell function are less clear than that of IL-1β. TNFα has been shown to impair GSIS in vitro (388) and its inhibition improves glucose metabolism in patients with rheumatoid arthritis (389), however TNFα antagonism has been ineffective in improving β-cell function in T2D (65,390). Prolonged incubation of human or mouse islets with
IL-6 impairs GSIS (391). However, IL-6 also enhances glucagon-like peptide-1 (GLP-1) production and secretion during high fat feeding, which stimulates β-cell proliferation and function through the activation of β-cell GLP-1 receptors (392). Therefore, β-cell specific effects and the effect on insulin secretion of IL-6 remain to be determined.

1.6.3.2 Immune cells

Studies have shown that infiltrating macrophages greatly increase islet cytokine levels (316) and that recruitment and activation of IL-β producing macrophages plays an essential role in islet inflammation (55,393). Macrophage infiltration has also been shown to increase production of IL-6 and the chemokine IL-8 in islets, leading to further inflammation and immune cell infiltration (316). Chemokine expression and immune cell infiltration of islets of the non-obese T2D model, GK rats, are prevented with IL-1Ra and this is associated with improved glycemia and β-cell insulin processing (372,394). Importantly, palmitate-induced β-cell dysfunction has been shown to be mediated in part by macrophage infiltration and the depletion of macrophages in palmitate-infused mice has been shown to decrease IL-1β production, increase PDX-1 mRNA expression in islets and improve insulin secretion ex vivo and in vivo (55).

1.6.3.3 Free fatty acids and inflammatory signaling

Oleate and palmitate have both been shown to stimulate IL-1β secretion and its downstream proinflammatory mediators in rodent and human islets (269,316,370). FFA-induced inflammation has been shown to require IL-1 receptor activation in both mouse and human islets as FFA induced cytokine and chemokine production was inhibited by IL-1Ra or an anti-IL1β antibody (269). Palmitate has also been shown to induce the local production of IL-6 (316) and increases chemokine secretion from human (269,270) and mouse islets (269). The media from these islets led to the recruitment of macrophages through IL-8. Additionally, saturated fatty acids have been shown to induce expression of the chemokines C-X-C motif chemokine ligand 1 (CXCL1) and MCP-1 in both mouse (269) and human islets (269,270), leading to macrophage recruitment. Our
laboratory has shown that 48h infusion with a triglyceride emulsion and heparin (291) or with oleate (Chapter 3) in rats induced islet expression of proinflammatory factors.

Two main mechanistic pathways may link elevated FFA to inflammation: receptor and non-receptor mediated pathways. TLR signaling is a prominent receptor mediated inflammatory pathway and as mentioned above, TLRs are transmembrane proinflammatory PRRs which are activated by pathogen-associated molecular patterns (PAMPs) and may be activated by FFA. TLRs have three domains: 1) an ectodomain which contains leucine-rich repeats (LRR) for the recognition of PAMPs; 2) a transmembrane domain; and 3) a cytosolic Toll-IL-1R (TIR) domain for downstream signaling. To date, 13 TLRs (TLR1-13) have been identified, although only TLR1-11 have been found in humans. Once activated by their ligands, TLRs undergo dimerization and may also require co-receptors for full activation (395). TLR4, for example, requires the co-receptor CD14 for LPS-mediated activation (396). Once this complex of receptors forms, adapter molecules from two signaling pathways are recruited. The predominant pathway that is involved in signaling of almost all TLRs is initiated by the recruitment of the adapter protein MyD88 (myeloid differentiation primary response protein 88), which in turn recruits IRAK1/2/4/M (IL-1 receptor-associated kinases 1, 2, 4, and M). IRAK kinases phosphorylate and activate TRAF6 (tumor necrosis factor receptor-associated factor 6), which ubiquitinates TAK1 (transforming growth factor-β-activated protein kinase 1) and facilitates its binding to IKKβ. TAK1 then activates IKKβ, which in turn phosphorylates IκBα, leading to NF-κB nuclear translocation and transcription of proinflammatory genes. In addition, TAK1 phosphorylates two MAPK kinase family members, MKK3 and MKK6, which activate JNK and p38. MyD88 signaling by certain TLRs also requires binding of another adapter protein, TIRAP (Toll-IL-1 receptor domain-containing adapter protein) to the TLR in order to link TLR to MyD88 and initiate intracellular signaling and control inflammatory responses (397). Furthermore, there is a MyD88-independent pathway, which signals through the adaptor protein TRIF (TIR domain-containing adapter inducing interferon-β) in response to TLR3 and TLR4 activation, however with TLR4 TRAM (TRIF-related adapter molecule) is also required for TRIF recruitment (398,399). Similar to the role of TIRAP in linking TLR to MyD88 in the MyD88 pathway, TRAM in the TRIF pathway acts to link TLR to TRIF. Once recruited, TRIF activates the kinases TBK1 (TRAF family member-associated NF-κB activator-binding kinase 1) and RIPK1 (receptor-interacting serine/threonine-protein kinase 1). TBK1 phosphorylates interferon regulatory factor 3 (IRF3), allowing for its translocation to the
nucleus and transcription of the interferon type 1 cytokines. Similar to the MyD88 pathway, activation of RIPK1 leads to polyubiquitination of TAK1, leading to NF-κB nuclear translocation (395,399). TLR4 is the only TLR which uses both pathways. The MyD88 pathway initiates the early phase of NF-κB activation, while TRIF initiates the late phase and both phases are required for maximal production of proinflammatory cytokines (400–402).

Similar to TLRs, the NOD receptors NOD1 and NOD2, which are the best characterized of the NLR receptors, are PRRs that respond to PAMPs from specific bacterial products as well as damage-associated molecular patterns (DAMPs) associated with cell stress, however NODs are cytosolic rather than transmembrane receptors (403). NODs consist of three domains: 1) a central NACHT (NAIP [neuronal apoptosis inhibitor protein], C2TA [MHC class 2 transcription activator], HET-E [incompatibility locus protein from Podospora anserine] and TP1 [telomerase-associated protein]) domain that mediates oligomerization; 2) a LRR C-terminal for ligand binding; and 3) a caspase recruitment domain (CARD) for protein-protein interaction with downstream signaling proteins (403). Upon recognition of NOD ligands by the LRR domain, ATP-dependent self-oligomerization is induced via the NACHT domain and activation of RIPK2, which contains a CARD domain, is initiated through CARD-CARD interaction between the NOD receptor and RIPK2. RIPK2 then recruits a signaling complex that includes TAK1, leading to activation of IKKβ, JNK, and p38 and ultimately their downstream effectors NF-κB and AP-1 (404–406). NOD receptors are involved in innate immune responses to infection and in activation of the adaptive immune system (403). It has been suggested that they also interact with TLRs in proinflammatory signaling, however there is limited direct evidence for this and the mechanisms involved are not clear (407). There is also evidence that NOD2 has an anti-inflammatory effect that dampens the proinflammatory response of TLRs (408). Further investigation is required to better understand NOD signaling, however it is clear that NOD receptors are important activators of the innate immune system.

FFAs, in particular saturated fatty acids, may activate TLRs by forming lipid rafts in plasma membranes, facilitating dimerization and subsequent activation of TLRs (409). These lipid rafts are thought to be composed primarily of saturated fatty acids, whereas unsaturated fatty acids may in fact disrupt the lipid order and composition of the rafts and inhibit TLR signaling (410,411). Another possible mechanism through which saturated fatty acids activate TLRs is through direct
binding. Saturated fatty acids make up the endotoxic moiety of LPS Lipid A and it is possible that the FFA alone can activate TLRs, although it remains unclear whether this occurs (412,413). Alternatively, saturated fatty acids, such as palmitate, serve as substrates for ceramide synthesis, which has been shown to activate TLRs (414,415). The fatty acid translocase CD36 has also been shown to bind to and stimulate TLR2 in response to saturated fatty acids (416). Finally, activation of TLR4 by FFA has been suggested to occur indirectly via FFA binding to the hepatic secretory glycoprotein fetuin A, which in turn binds TLR4 (417), however this is still debated and it appears that this mechanism may not be relevant in humans (418). Although the exact mechanisms of TLR activation by FFA remain to be determined, there is strong evidence that FFA can stimulate production of inflammatory cytokines through the activation of TLR2 (261,269) or TLR4 (55,269).

TLR4 activation has been shown to inhibit insulin secretion in rat islets (419) and human β-cells (132). In line with this, islets of high-fat fed mice and of db/db mice have elevated TLR4, cytokine expression and macrophage infiltration, associated with decreased insulin secretion in islets and progressive hyperglycemia in mice (261,319), whereas whole-body deletion of TLR4 significantly reduces blood glucose in these mice (320,420). Islets of TLR2 (261) or TLR4 (55) knockout mice have also been shown to be partially protected from FFA-induced proinflammatory cytokine production, whereas deletion of MyD88, the downstream effector of both TLR and IL-1β signaling, is completely protective (269), suggesting an essential role of IL-1 β. Nonetheless, it is possible that the combination of TLR2 and TLR4 activation is sufficient to induce proinflammatory cytokine production in response to FFA and further studies in TLR2/4 double knockout islets would help to better understand the relative contributions of TLRs versus IL-1β.

In vivo, one study showed that infusion of palmitate for 72h in mice induced islet expression of chemokines, infiltration of macrophages, and impaired insulin secretion and that this was prevented by deletion of either TLR4 or MyD88 (55). Palmitate has been shown to increase expression of the chemokine CCL2 through activation of TLR4 in vitro and in vivo in mice leading to macrophage infiltration and subsequent reduction in insulin gene transcription and β-cell function (55). Additionally, palmitate induced IL-1β expression has been shown to be TLR-2 dependent in islets and TLR2 deletion decreased islet IL-1β expression and improved insulin secretion in HFD-fed mice (261). TLR activation has also been shown to prime the induction of IL-1β transcription and synthesis of pro-IL-1β prior to its cleavage by caspase-1 (421) and to increase expression of components of the NLRP3 inflammasome (422). Finally, the NOD1
intracellular receptor of innate immunity has been shown to play a role in fat-induced glucose intolerance in one study (131). β-cell function was not assessed directly, however glucose tolerance is a reflection of β-cell function, therefore these results suggest NOD1 activation plays a role in fat-induced inflammation and β-cell dysfunction. Further studies are required to determine the role of NOD1 in β-cell function directly.

Receptor-independent FFA-induced inflammation occurs through the induction of ER stress, ROS production, and ceramide synthesis. Notably, ROS can activate the NLRP3 inflammasome (140). Under basal conditions, the antioxidant protein thioredoxin is associated with TXNIP. In the presence of elevated FFA however, thioredoxin senses an increase in ROS and dissociates from TXNIP, which in turn binds to and activates the NLRP3 inflammasome leading to processing and secretion of IL-1β (380). In one study using a one-year long high fat diet in mice, deletion of NLRP3 or ASC led to a reduction of islet IL-1β protein expression and macrophage infiltration and an improvement in insulin secretion (317).

Common to both receptor (TLR, NOD, cytokines) and metabolic stressor (ER stress, ROS, ceramide) mediated inflammation is the activation of the inflammatory kinases IKKβ and JNK. In vitro studies in our laboratory using islets from JNK1-null mice have shown that JNK1 is involved in β-cell lipotoxicity in a manner dependent on the fatty acid degree of saturation, as palmitate-induced β-cell dysfunction was prevented in islets of JNK1-null mice, although oleate-induced β-cell dysfunction was not (unpublished). Saturated fat has been shown in several studies to activate JNK in β-cells (260,323,423,424). A possible mechanism through which palmitate may activate JNK is the synthesis of the pro-apoptotic second messenger, ceramide (260,423). Palmitate, but not oleate, has been shown to decrease insulin gene transcription (424), an effect that has been attributed to the synthesis of ceramides (260,423). Furthermore, inhibitors of ceramide synthesis prevented FFA-induced β-cell apoptosis (323).

As described above, FFA-induced ER stress can lead to JNK activation (141,146). High-fat feeding causes ER stress and IRE1α activation, which can lead to JNK activation and insulin resistance in liver and adipose tissue. Furthermore, JNK inhibition prevents ER-stress induced IRS-1 serine phosphorylation (141,146,425). Recently, it has also been shown that β-cell specific overexpression of the ER chaperone GRP78 prevents high fat diet-induced glucose intolerance
and islet ER stress (143). Notably, in the INS-1 β-cell line palmitate, but not oleate, led to IRE1α activation and apoptosis, which was partially prevented by JNK inhibition (355). Recently, it has been shown that the non-receptor tyrosine kinase, c-Src, is required for saturated fatty acid-induced JNK activation in vivo and in vitro (426). However, c-Src is not required for ER stress and activation of the UPR (426). The relative importance of ER stress and c-Src in fat-induced JNK-mediated β-cell dysfunction in vivo is not known, but needs to be determined to better understand the mechanisms of FFA-induced β-cell dysfunction.

Short term activation of NF-κB is required for β-cell survival and has been shown to enhance GSIS and PDX-1 expression (427). However, its long-term activation by FFA, which can occur through ROS-mediated activation of IKKβ (139) is detrimental for β-cells. One study using INS-1 cells found that FFA-induced β-cell apoptosis was associated with NF-κB activation, and was inhibited with the IKK-specific inhibitor I229 (428). An IKKβ inhibitor also prevented diet-induced diabetes and increased pancreatic insulin stores in Psammonys obesus gerbils (321). The gerbils were fed a high-energy diet for four weeks which led to diabetes with control treatment, but diabetes was prevented with daily oral administration of the IKKβ inhibitor.

As in peripheral tissues, activation of NF-κB in β-cells stimulates the transcription of COX-2 and iNOS, which have been shown to play important roles in FFA-induced β-cell dysfunction (311,323). COX-2 metabolizes arachidonic acid from the plasma membrane to produce PGE2 and thereby decrease insulin secretion (429). iNOS expression induces production of nitric oxide which can impair β-cell function through the generation of reactive nitrogen species (311,323,430,431).

Many studies provide evidence that implicates causal roles of ROS, ER stress and inflammation in FFA-induced β-cell dysfunction. It is clear that the initiation of any of these contributes to the activation of the others and it is also interesting to note that islet inflammation is associated with early insulin resistance (432). Studies in rodents have also shown that inflammation and macrophage infiltration of islets occur as early as eight weeks before the onset of diabetes (316), providing evidence for inflammation as an initiating event in its development. However, further investigation is required to determine the relative contribution of ROS, ER stress and inflammation and the order in which they are initiated.
1.6.3.4 Inflammation and β-cell insulin resistance

As in peripheral tissues, IKKβ and JNK1 activation in β-cells is known to lead to phosphorylation of serine/threonine residues of IRS, which impairs β-cell insulin signaling and thus β-cell function. The insulin receptor, insulin-like growth factor-1 receptor (IGF-1R) and their intracellular signaling molecules are present in β-cells and are known to be important for β-cell growth, proinsulin biosynthesis and insulin gene transcription (433–436). The presence of the insulin receptor in β-cells is required for β-cell growth as β-cell specific insulin receptor knockout (βIRKO) mice show an age-dependent decrease in β-cell mass (435). Accordingly, mice with β-cell specific deletion of the PIP3 phosphatase and negative regulator of insulin signaling, PTEN (phosphatase and tensin homolog) have increased β-cell mass due to enhanced PI3K signaling in β-cells (437). Furthermore, PTEN is upregulated in animal models of obesity-induced diabetes (437,438) and its overexpression impairs β-cell function (439). Importantly, exposure of islets to insulin upregulates insulin gene transcription and proinsulin biosynthesis (433,436), an effect that may prevent depletion of β-cell insulin content following glucose-induced insulin secretion.

The effect of β-cell insulin signaling on insulin secretion per se may be inhibitory or stimulatory according to studies in vitro (440–443). Insulin stimulation has been shown to increase intracellular calcium concentrations in β-cells through an IRS-1-dependent mechanism. IRS-1 activation by insulin leads to IRS-1 binding to and inhibition of SERCA, the purpose of which is to pump calcium from the cytosol into the ER (444). Therefore, SERCA inhibition leads to an increase in cytosolic calcium and in turn exocytosis of insulin granules (444). In contrast, insulin has been shown to inhibit insulin secretion by opening ATP-dependent potassium channels through the formation of PIP3 by PI3K activation (445,446). The contradictory results are likely due to β-cells undergoing different durations of exposure to insulin with different concentrations of insulin and glucose in vitro. In vivo, exogenous insulin administration, although it decreases concomitant endogenous insulin secretion by a neural reflex (447), has been shown to potentiate subsequent GSIS in humans (448–450).

Notably, in vitro studies have shown that incubation of INS-1 cells with FFA, such as oleate (231,451) or palmitate (452), impairs the IGF-1 induced activation of Akt (231,451,452) as well as insulin signaling and induces β-cell apoptosis (451). It has also been shown that palmitate
induced JNK activation decreased IRS-1 and IRS-2 phosphorylation and in turn impaired insulin gene transcription (453). Furthermore, adenoviral-mediated overactivation of Akt prevented the oleate-induced apoptosis (451) whereas a model of genetically mediated reduction in Akt activity increased susceptibility to high fat diet-induced β-cell dysfunction (318). This suggests impairment of the insulin signaling pathway in β-cells is involved in β-cell lipotoxicity, but further studies are required to better understand the role of β-cell insulin signaling in fat-induced β-cell dysfunction.

1.7 Inflammation and type 2 diabetes

Since the identification of inflammation as a major factor in the development and progression of T2D, a number of studies have been undertaken to determine whether inhibition of inflammatory pathways can improve glucose homeostasis. As described above, in patients with rheumatoid arthritis studies have shown that TNFα inhibition improves glucose homeostasis (389), although trials for diabetes or obesity have not (65,390). This may be due to small sample sizes in studies and the issue needs to be further assessed. In contrast, clinical studies of IL-1β inhibition have shown that it improves glucose levels and β-cell function and decreases inflammatory markers in patients with T2D (454). Similarly, the prodrug salsalate, which is a dimer of the IKKβ inhibitor salicylate and is metabolized to produce salicylate, improves glucose tolerance. Studies have shown that it is capable of improving insulin sensitivity, fasting glucose levels, and long-term glucose homeostasis, as determined by decreases in glycated hemoglobin levels (455–457). Although insulin sensitivity is consistently improved with treatment of salsalate, the effect on insulin secretion and glycemia is less consistent (281,455–457). In contrast, treatment with IL-1β inhibitors has been shown consistently to improve insulin secretion and glucose homeostasis (454,458–463). Overall, however, the effects of currently available anti-inflammatory therapy are not sufficient for the prevention and treatment of T2D, which highlights the need to gain a better understanding of the inflammatory signaling pathways involved in FFA-induced β-cell dysfunction and T2D in order to identify other therapeutic targets.
1.8 Summary

It is now well established that T2D is associated with obesity, characterized in part by an elevation in circulating FFA levels. Prolonged elevation of FFA eventually results in lipotoxicity, which is a critical mechanism through which obesity can lead to T2D. FFA are known to induce insulin resistance and impair β-cell function, which are characteristic features of T2D. Insulin resistance generally develops early in obesity and increases the demand on β-cells to produce greater amounts of insulin in order to compensate. Over time, this can lead to an impairment of β-cell function due to metabolic stress. Importantly, FFA also directly act on β-cells to induce an impairment in their function. A critical mechanism through which FFA induce insulin resistance is through the activation of inflammatory signaling pathways in insulin target cells and the activation of the immune system. FFA-induced inflammation is now known to play a causal role in the development of β-cell dysfunction as well. The exact mechanisms involved and their relative importance are not clear, but likely involve the activation by FFA of proinflammatory receptors and FFA-mediated activation of proinflammatory kinases via ROS, ER stress and ceramide, which result in β-cell dysfunction in part via inducing insulin resistance at the level of the β-cell. In Figure 1.4 the mechanisms proposed to be involved in FFA-induced inflammation and β-cell dysfunction are summarized and the mechanisms investigated in this thesis are in bold.
1.9 Rationale and significance of the studies

Inflammation is a characteristic feature of obesity and T2D and its role in the pathogenesis of T2D is well established. However, inflammatory signaling in response to FFA in β-cells specifically is not yet well defined. Although preliminary studies in our laboratory have shown that pharmacological inhibitors of one of the major regulators of inflammation, IKKβ, prevent FFA-induced β-cell dysfunction, these inhibitors have systemic and potentially, non-specific effects. Therefore, more specific models of IKKβ inhibition in β-cells are needed. Furthermore, an important mechanism through which IKKβ activation impairs function of peripheral tissues is through the induction of insulin resistance and the same mechanism has been proposed to be involved in the β-cell. Preliminary studies in our laboratory have shown that an upregulator of the insulin signaling pathway, the tyrosine phosphatase inhibitor bisperoxovandate (BPV), prevents FFA-induced β-cell dysfunction. However, treatment with BPV also upregulates all growth factor signaling pathways and thus other models of upregulation of the insulin signaling cascade in β-cells are needed. Finally, upstream of IKKβ is the NOD1 receptor, which has only recently been shown to play an important role in nutrient metabolism. NOD1/2 double KO mice are protected from high-fat diet induced inflammation and insulin resistance (464). Importantly, high-fat diet induced glucose intolerance is prevented in NOD1-null mice (131). Changes in glucose tolerance reflect changes in β-cell function which implicates NOD1 in FFA-induced β-cell dysfunction and warrants further investigation to directly determine whether NOD1 is involved.

The studies performed and described in my thesis use unique and selective in vivo models relevant to T2D to reveal molecular mechanisms involved in FFA-mediated β-cell dysfunction. These studies 1) provide new insights into the pathophysiology of β-cell dysfunction caused by lipotoxicity; 2) support the rationale for FFA lowering therapies in preventing deterioration of β-cell function in T2D; and 3) identify novel targets for nutritional and or pharmacological intervention to prevent and treat β-cell dysfunction in obesity associated T2D.
1.10 General hypothesis

FFA-induced β-cell dysfunction \textit{in vivo} is mediated through the inflammatory kinase IKKβ, and involves β-cell insulin resistance and stimulation of the intracellular NOD1 receptor of innate immunity.
1.11 Specific aims

**Aim 1.** To investigate whether IKKβ in β-cells plays a causal role in FFA-induced β-cell dysfunction *in vivo*.

**Aim 2.** To investigate whether upregulation of insulin signaling in β-cells can prevent FFA-induced β-cell dysfunction *in vivo*.

**Aim 3.** To investigate whether NOD1 plays a causal role in FFA-induced β-cell dysfunction *in vivo*.
1.12 Studies

This thesis consists of three studies designed to investigate the role of inflammation induced by FFA in β-cell dysfunction *in vivo*. This involved establishing the causal role of β-cell specific IKKβ in FFA-induced β-cell dysfunction (Study 1), investigating the role of one of the downstream mechanisms initiated by IKKβ, insulin resistance, in the β-cell (Study 2), and beginning to investigate whether an important upstream activator of IKKβ, NOD1, is involved in FFA-induced β-cell dysfunction (Study 3). These studies have provided insights into the inflammatory mechanisms involved in FFA-induced β-cell dysfunction and established novel potential targets for its prevention or treatment.
Figure 1.4 Mechanisms of FFA-induced inflammation and sites of impairment of β-cell function. The figure is not intended to be all-inclusive. Mechanisms studied in this thesis are in bold.
2. General Methods

2.1 Procedures

2.1.1 Experimental animal model and surgical procedures

2.1.1.1 Animals

All experiments in rats were performed using 11-13 week old female Wistar rats (Charles River, Quebec, Canada) weighing 250-300g. Female rats were chosen to allow for comparison of the effect of FFA on β-cell function with that of high fat feeding in female Zucker diabetic fatty (ZDF) rats from previous studies. Female ZDF rats are a model of prediabetes that develop diabetes when fed a high fat diet, whereas male ZDF rats develop diabetes even in the absence of high fat feeding (293).

Mice used for experiments were born in the Division of Comparative Medicine of the University of Toronto. For all the in vivo studies, 11-13 week old knockout mice and their littermate controls were used. Ear clips were taken from mice at 3-4 weeks of age for genotyping. Mouse DNA was extracted and amplified using the KAPA Mouse Genotyping Kit according to the manufacturer’s protocol (Catalog #KK7352, Kapa Biosystems, Wilmington, MA, USA). The genotypes of these mice are described in the Methods section of each study.

The animals were housed in the Division of Comparative Medicine of University of Toronto. They were exposed to a 12 h light/dark cycle. The animals were fed a Teklad Global 2018 diet containing
24% protein, 58% carbohydrate and 18% fat by calories (Harland Teklad Global Diets, Madison, WI).

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.

2.1.1.2 Surgery

Following a week of adaptation to the animal facility, rats were anesthetized with 2% isofluorane 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 2.7 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. A subcutaneous implant was inserted to allow for the rats to be attached to the tether that would be used to protect the infusion lines. The catheters were filled with heparinized saline (1000U.ml⁻¹) to maintain patency and were closed at the end with a metal pin. The rats were allowed a 3-4 day 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 the 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.

For infusions in mice, an indwelling catheter was inserted into the right internal jugular vein under general anesthesia with 2% isofluorane and sterile conditions. Polyethylene catheters (PE-10; Cay Adams, Boston, MA), extended with a segment of silastic tubing (length of 1.7 cm, internal diameter of 0.012 inches; Dow Corning, Midland, MI), were used. The venous catheter was extended to the level of the right atrium and tunneled subcutaneously and externalized. The catheters were filled with heparinized saline (1000U/ml heparin) to maintain patency and knotted at the end. The mice were allowed a 3-5 day 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 mice.

2.1.2 Preparation of infused solutions and the infusion period

We have established an in vivo model of β-cell lipotoxicity in rodents (137,265,266), for which we have used a 48 h intravenous infusion of oleate, a monounsaturated fatty acid, bound to bovine serum albumin (BSA). The BSA is used to prevent the detergent action of the fatty acid, and in this way, the oleate infusion can be given to animals through a central i.v. line (465). 48h i.v. infusion of oleate bound to BSA was given. To avoid the surfactant activity of direct infusion of FFA, rats were also infused for 48 h with an emulsion of 20% olive oil, which is a triglyceride mixture containing 71% oleate. Heparin was added to the olive oil emulsion as in (296) to activate lipoprotein lipase, which releases FFA from the triglycerides of olive oil. Additionally, in study 3, ethylpalmitate/vehicle was infused in mice. Palmitate is the most abundant saturated fatty acid in the circulation (466) and was only used in study 3 since it was not possible to solubilize palmitate prior to the use of a method (55) developed after the completion of studies 1 and 2. Also, ethylpalmitate was used in study 3 because only saturated and not unsaturated fatty acids have been shown to activate NOD1, which was the focus of study 3. Ethylpalmitate has been shown to be safe for infusion in mice and since rodent blood rapidly hydrolyzes fatty acid ethyl esters, this infusion elevates plasma FFA levels (55). During the 48 h infusions the animals had free access to food and water. Samples for FFAs, glucose, insulin and C-peptide were taken at 0 and 48 hours after the onset of the saline or fat infusion in both rats and mice and in rats, additional samples for these were taken at 18 and 24 hours after the onset of the infusions. The fat emulsions were freshly prepared as in our previous studies (296,467) and protected from light. Saline infusion (5μl/min) was used as a control for oleate in BSA as we have shown that BSA does not have an effect on the parameters under study in our model (273). Islet isolation or a hyperglycemic clamp was performed in overnight-fasted rats and 4h-fasted mice at the end of the 48 h infusions.
2.1.3 Islet isolation

Pancreatic islets were isolated in overnight-fasted rats and 4h-fasted mice, after the 48h infusion period. Animals were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg.ml\(^{-1}\), 1.5μl.g\(^{-1}\) and 2.5μl.g\(^{-1}\) of body weight in rats and mice, respectively). The visceral contents were exposed and the animals were exsanguinated through an incision in the abdominal aorta for sample collection and to decrease the risk of serum-mediated inhibition of collagenase (468,469) from possible bleeding during surgery. The common bile duct was quickly isolated and a collagenase solution (Type XI in rats and type V in mice, Sigma in RPMI-1640 containing 10mmol/l HEPES and 1% Penicillin) was infused into the pancreas (~15 ml for rat islets and ~3 ml for mouse islets). The pancreas was then removed and incubated in a water bath at 37\(^{\circ}\)C for 20 minutes for rat islets and 15 minutes for mouse islets. Following the incubation, the pancreas was subjected to vigorous shaking to break the pancreas. Thereafter, the mouse islets were handpicked under a dissecting microscope. For rat islets, 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 by a Histopaque-1077 density gradient (470). During isolation, glucose was kept at 2.8mmol/l (starvation media) for both rat and mouse islets (137,265,266,471–473). Following islet isolation, islets were pooled together and from the pool, sets of 40 mouse islets (2 glucose concentrations * 10 islets/per well * duplicate experiments) or 60 rat islets (4 glucose concentrations * 5 islets/well * triplicate experiments) were incubated in control or treatment conditions. Therefore, ‘n’ in in vitro experiments refers to one of these sets of 40 or 60 islets. For ex vivo experiments, ‘n’ refers to one animal as islets cannot be pooled for ex vivo experiments due to the intravenous infusion of fat that occurs in vivo.

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. To assess β-cell function, freshly isolated islets were pre-incubated for 1h at 37\(^{\circ}\)C in Krebs Ringer buffer containing 10mmol/l HEPES (KR BH) and 2.8mmol/l
glucose, and incubated in triplicate (rat islets) or duplicate (mouse islets) for 2h at 37°C in KRBH at the following glucose concentrations:

Rat islets: 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 (137,265,266,471,472,474,475).

Mouse islets: 6.5mmol/l, which is basal glucose in mice and 22mmol/l, which is a maximum stimulatory concentration (137,266,476,477).

Insulin was measured in the supernatant with Linco’s RIA kit.

2.1.5 Evaluation of β-cell function in vitro

Islets were isolated using the same protocol as in the ex vivo studies. Batches of 200–300 (rats) or 50–70 (mice) islets of similar size were collected and maintained at 37°C in a 5% CO₂ atmosphere in suspension in 20 ml RPMI medium without antioxidants, containing 10% fetal bovine serum, with or without oleate (0.4 mmol/l in 0.5% FFA-free BSA) or palmitate (0.4 mmol/l in 0.5% FFA-free BSA), and different agents according to the experimental protocols, for 48 h. At the end of the 48 h period, islet insulin secretion was determined through static incubation. The same determinations were performed as described above for ex vivo studies.

2.1.6 Hyperglycemic clamp

In rat experiments, the two-step hyperglycemic clamp was performed in conscious, overnight-fasted rats. 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 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.

Post-infusion FFA, and basal insulin and C-peptide were measured at -20 and 0 minutes. At time = 0 minutes, an infusion of 37.5% glucose was started. We did not use a glucose bolus because
we wished to avoid possible arrhythmias caused by an oleate bolus from the dead space of the infusion line. Plasma glucose was maintained at 13 mmol/l 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 further raised to 22mmol/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 in different tissues (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 2.5ml of blood was withdrawn from the rats. After removal of plasma from centrifuged whole blood samples, erythrocytes were suspended in heparinized saline (4U/ml) and re-infused into the rats.

In mouse experiments, the hyperglycemic clamp was performed in conscious, 4h-fasted mice. Control/treatment infusion was continued during the hyperglycemic clamp.

Samples for post-infusion FFA, and basal insulin and C-peptide were obtained from the tail vein at 0 minutes and an infusion of 37.5% glucose was started. As with rats, we did not use a glucose bolus in order to avoid possible arrhythmias. Plasma glucose was maintained at 22 mmol/l for a period of 2h, by adjusting the rate of glucose infusion according to frequent (10 minutes) glycemic determinations obtained from the tail vein and measured on a HemoCue 201 Glucose Analyzer. Samples for insulin and C-peptide were taken again at the end of the clamp. The sample volume was minimized to avoid anemia. A total of ~600µl of blood was drawn from the mice.

In each experiment for both mice and rats, an index of insulin sensitivity and the disposition index (a measure of β-cell function in vivo) were calculated from the hyperglycemic clamp (see Calculations).
2.2 Laboratory methods

2.2.1 Plasma glucose

Plasma rather than whole blood glucose is measured as the glucose that is transported in the blood is transported in the plasma. Importantly, measuring whole blood, as many glucose meters do, can give inaccurate readings if there are changes in hematocrit, which there are during hyperglycemic clamps due to blood loss.

In rats, plasma glucose concentrations were measured by the glucose oxidase method using a Beckman 2 Glucose Analyzer (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. 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.

In mice, plasma glucose concentrations were measured using the HemoCue Glucose 201 Analyzer and HemoCue Glucose 201 Microcuvettes from HemoCue Inc. (CA, USA). The microcuvette is filled with approximately 5 μL of undiluted whole blood. The glucose reaction is a modified glucose dehydrogenase method in which a tetrazolium salt is used to obtain a quantification of glucose in visible light. α-D-glucose is transformed to β-D-glucose using mutarotase. Glucose dehydrogenase acts as a catalyst for the oxidation of β-D-glucose, to form NADH, which in the presence of diaphorase produces a coloured formazan with MTT, a tetrazolium salt. The formazan is quantified photometrically using a two wavelength photometric method at 667 nm and 840 nm.
To ensure the stability of the final reaction, measurement of microcuvettes containing blood samples was performed no later than 40 seconds after the microcuvette was filled with a blood sample. Once the microcuvette is placed in the analyzer, the analyzer finds the steady-state of the chemical reaction after 40-240 seconds and the result appears on the display of the analyzer. The microcuvette is then discarded, the cuvette holder is left in the loading position, and a new sample may be measured.

2.2.2 Plasma free fatty acids assay

Plasma levels of FFA were analyzed using a colorimetric kit under enzymatic reaction from Wako Industrials (Neuss, Germany). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA produced is oxidized by adding acyl-CoA oxidase (ACOD), which generates H₂O₂. H₂O₂, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(B-hydroxyethl)-aniline (MEHA) with 4-aminophenazone to form the final reaction product, which is a purple colored adduct. This can be measured colorimetrically at 550 nm. The results are correct to within 1.1%. The reactions of this assay is listed below:

\[
\begin{align*}
\text{FFA + ATP + CoA} \xrightarrow{ACS} & \text{Acyl-CoA + AMP + PPi} \\
\text{Acyl-CoA + O}_2 \xrightarrow{ACOD} & \text{2,3-trans-Enoly-CoA + H}_2\text{O}_2 \\
\text{2 H}_2\text{O}_2 + 4\text{-aminoantipyrine + MEHA} \xrightarrow{POD} & \text{Final Reaction Product + 3 H}_2\text{O}
\end{align*}
\]

2.2.3 Plasma insulin assay (RIA)

Radioimmunoassay (RIA) kit specific for rat/mouse insulin from Linco Research Inc. (St. Charles, MO, USA) was used to determine plasma and islet insulin concentrations. Radioimmunoassay (RIA) is a very sensitive assay that quantifies antigen concentrations using specific antibodies. A known concentration of radioactive-labelled antigen (tracer) is incubated
with a known amount of antibody and the two specifically bind to each other. When the unknown quantity of the unlabeled antigen is added to the reaction medium, it competes with labeled tracer for the limited number of binding sites on the antibody. As the concentration of the unlabeled antigen increases, more of it binds to the antibody and displaces the tracer, thus reducing the ratio of antibody-bound tracer to free tracer. The free and bound tracers are separated and the radioactivity is counted with gamma counter. A calibration standard curve is set up with increasing concentrations of standard unlabeled antigen and the amount of antigen in the unknown sample can be calculated.

This kit has 100% reactivity to rat insulin I and II and mouse insulin. Cross-reactivity to rat/mouse proinsulin has not been tested. Insulin in the sample competes with a fixed amount of $^{125}$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. $^{125}$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” (B₀):

$$\% \text{ activity bound} = \frac{B}{B_0} \times 100$$

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 inter-assay variation determined on reference plasma is less than 10%.

2.2.4 Plasma insulin assay (ELISA)

High sensitivity mouse insulin immunoassay kit from AIS Antibody and Immunoassay Services (Li Ka Shing Faculty of Medicine, Hong Kong University, Hong Kong) was also used in study 3
(mice) to determine plasma insulin levels as it allows for detection of lower levels of insulin and avoids the need to use radioactivity. The results are comparable to those of the RIA.

This assay is an enzyme-linked immunosorbent assay (ELISA) and has 100% reactivity to mouse insulin. Cross-reactivity to mouse proinsulin has not been reported. The micro-plate used is pre-coated with a monoclonal antibody against insulin. Standards and samples are added into the well and co-incubated at room temperature for 90 minutes with a monoclonal secondary antibody conjugated to horseradish peroxidase (HRP) and shaken at 600 rpm. After a wash step, which consists of four washes to remove any unbound substances, the chromogenic substrate TMB (3,3’,5,5’-Tetramethylneizidine) is added and incubated for 15 minutes while protected from light. The assay is stopped and the optical density of the wells determined using a micro-plate reader. Colour develops in proportion to the amount of insulin bound initially. Since the increases in absorbance are directly proportional to the amount of the captured insulin, the unknown sample concentration can be interpolated from a standard curve included in each assay. All standards, controls and samples were run in duplicate.

The assay allows for detection as low as 0.2 ng/ml. Intra- and inter-assay coefficients of variation are each less than 10%.

2.2.5 Plasma C-peptide assay (RIA)

Rat/mouse 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 100% reactivity to mouse C-peptide and no cross-reactivity to rat insulin I and II. Cross-reactivity to rat/mouse 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 is less than 10%.

2.2.6 Plasma C-peptide assay (ELISA)

Mouse C-peptide ELISA from ALPCO (Salem, NH, USA) was used in study 3 (mice) to determine C-peptide levels as the RIA was no longer available when these experiments were initiated. The results are comparable to those of the RIA.

This assay has 100% reactivity to mouse C-peptide 1 and mouse C-peptide 2. It has 2% cross-reactivity to mouse and rat proinsulin 1 and no cross-reactivity with mouse and rat proinsulin 2. A 96-well microplate is coated with a monoclonal antibody specific for C-peptide. The standards, controls, and samples are added to the microplate wells with the conjugate. The microplate is then incubated for two hours at room temperature on a microplate shaker at 700-900 rpm. After the first incubation is complete, the wells are washed three times with Wash buffer and blotted dry. TMB Substrate is added, and the microplate is incubated a second time at room temperature on a microplate shaker at 700-900 rpm for 10 minutes. Once the second incubation is complete, Stop Solution is added, and the optical density (OD) is measured by a spectrophotometer at 450 nm. The microplate was analyzed within 30 minutes after the addition of the Stop Solution. The intensity of the color generated is directly proportional to the amount of C-peptide in the sample and the unknown sample concentration can be interpolated from a standard curve included in each assay. All standards, controls, and samples were run in duplicate.

The sensitivity of the assay is 7.6 pM and intra- and inter-assay coefficients of variation are each less than 10%. 


2.3 Calculations

2.3.1 Insulin sensitivity index (M/I)

The insulin sensitivity during hyperglycemic clamp is calculated as the M/I [glucose metabolism (M) divided by plasma insulin (I)] index (287,288). The M/I index is calculated according to the following formula:

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

where \( G_{\text{inf}} \) is the rate of glucose infusion and \( \text{Insulin} \) is the plasma insulin concentration at individual time points during the last 40 minutes of each step of the two-step hyperglycemic clamp in rats and the last 20 minutes of the one-step hyperglycemic clamp in mice. The average of the M/I during the last 40 minutes of each step of the two-step hyperglycemic clamp in rats and the last 20 minutes of the hyperglycemic clamp in mice was used as the M/I for the specific animal. 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 different insulin levels, as it has been reported that the relationship between circulating insulin levels and insulin action is not linear at high insulin concentrations (478).

2.3.2 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 (276,277,479) cannot be performed in rodents. This is because the parameters of C-peptide kinetics cannot be derived in them, as rodent 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 (479–481). 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 the disposition index.
(DI) and considered as a measure of β-cell function (287,288). Previous studies from our laboratory have also shown that this relationship is hyperbolic in control rats (293) and have validated the DI against β-cell function ex vivo in rodents (266,472). Therefore, we calculated DI, and this was used for in vivo evaluation of β-cell function.

2.3.2.1 Disposition index (DI)

The DI, which was used as an index of insulin secretion corrected for the ambient degree of insulin resistance, was calculated according to the following formula:

$$DI = M/I \times C\text{-peptide}$$

where M/I is the insulin sensitivity index, calculated as described above during the last 40 minutes of each step of the two-step hyperglycemic clamp in rats and the last 20 minutes of the hyperglycemic clamp in mice, and C-peptide is the C-peptide concentration at individual time points during the last 40 minutes of each step of the two-step hyperglycemic clamp in rats and the last 20 minutes of the hyperglycemic clamp in mice.

2.4 Statistical analysis

Data are presented as means ± SEM. 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 — IKKβ inhibition prevents fat-induced beta-cell dysfunction \textit{in vitro} and \textit{in vivo}

The results of this study are incorporated in a manuscript published in Diabetologia and are reproduced in this thesis with permission:


My contributions to the manuscript were performing all the β-cell specific IKKβ-knockout experiments, assisting with Western blots and \textit{in vitro} experiments in rat islets, as well as completing all the revisions to the manuscript.
3.1 Abstract

**Aims/hypothesis** We have previously shown that oxidative stress plays a causal role in beta-cell dysfunction induced by fat. Here we address whether the proinflammatory kinase IkappaBalpha kinase beta (IKKβ), which is activated by oxidative stress, is also implicated.

**Methods** Fat (oleate or olive oil) was infused intravenously in Wistar rats for 48 h with or without the IKKβ inhibitor salicylate. Thereafter, beta-cell function was evaluated *in vivo* using hyperglycaemic clamps or *ex vivo* in islets isolated from fat-treated rats. We also exposed rat islets to oleate in culture, with or without salicylate and BMS (another inhibitor of IKKβ) and evaluated beta-cell function *in vitro*. Furthermore, oleate was infused in mice treated with BMS and in beta-cell specific IKKβ-null mice.

**Results** 48h infusion of fat impaired beta-cell function *in vivo* and *ex vivo* and the dysfunction was prevented by coinfusion of salicylate or BMS. In cultured islets, 48h exposure to oleate impaired beta-cell function, an effect prevented by both inhibitors. Genetic inhibition of IKKβ also prevented fat-induced beta-cell dysfunction *ex vivo* and *in vivo*.

**Conclusions/interpretations** Our results demonstrate a causal role for IKKβ in fat-induced beta-cell dysfunction *in vitro, ex vivo* and *in vivo*. 
3.2 Introduction

Free fatty acids (FFA) have been shown to both stimulate and impair insulin secretion depending on the duration of beta-cell exposure (290). Prolonged (>24h) exposure is inhibitory and involves oxidative stress, endoplasmic reticulum (ER) stress, and inflammation (290).

Lipotoxicity, i.e. the decrease in beta-cell function and mass induced by chronically elevated FFA, plays a role in the pathogenesis of type 2 diabetes, at least in predisposed individuals (290). Previously, we demonstrated that oxidative stress mediates fat-induced beta-cell dysfunction in vivo in rats (265) and in humans (280). Oxidative stress activates IκBα kinase (IKKβ), which, by phosphorylating the inhibitor IκBα, activates the transcription factor NFκB. The effect of IKKβ/NFκB on beta-cell function is controversial. NFκB is important for cell survival and there are reports that NFκB is beneficial for glucose stimulated insulin secretion (427), unless activated by cytokines (482). However, IKKβ which, in addition to activating NFκB, induces serine phosphorylation of insulin receptor substrates (IRS) (483), decreases beta-cell function (484). It is also controversial whether fat activates IKKβ/NFκB in beta-cells: although fatty acids did not activate NFκB in INS-1 or primary rat beta-cells in one study (485), lipotoxicity was associated with NFκB activation and palmitate-induced apoptosis was inhibited by an IKKβ inhibitor in INS-1 beta-cells in another study (428). The in vivo effect of fat on beta-cell IKKβ/NFκB has not been investigated previously.

To address the role of IKKβ in fat-induced beta-cell dysfunction in vivo, rats were infused i.v. with fat to elevate plasma FFA 50% - 100% (elevation seen in obesity (486)) with or without the IKKβ inhibitor (487) salicylate for 48h. Monounsaturated fat was infused (oleate or olive oil), as in our previous study showing a role of oxidative stress in beta-cell dysfunction (265). Although oleate has been found to protect β-cells against palmitate-induced toxicity in in vitro studies (488), the prolonged effect of oleate by itself on β-cell function is mainly inhibitory (265,489). After 48h infusion, beta-cell function was evaluated in vivo using hyperglycaemic clamps, or ex vivo in isolated islets. We also used the specific IKKβ inhibitor BMS-345541 (BMS) (490) in hyperglycaemic clamps performed after 48h oleate infusion in mice. In addition, we exposed rat islets for 48h to oleate with or without salicylate or BMS in vitro. Lastly, we performed oleate infusion in ‘beta-cell specific’ IKKβ-deficient mice, followed by evaluation of beta-cell function
in vivo using hyperglycaemic clamps or ex vivo in isolated islets. In all these models, IKKβ inhibition prevented fat-induced beta-cell dysfunction.
3.3 Methods

3.3.1 Animals

All procedures were approved by the Animal Care Committee of the University of Toronto and conducted according to the Canadian Council on Animal Care Guidelines. Female Wistar rats (250-300g, Charles River, Canada) were used as in our previous studies (265,273). Female C57BL/6 mice (22-25g, Jackson Lab, Bar Harbor, Maine, USA) and male RIP2-Cre recombinase (Cre) positive or negative IKKβ floxed mice were also used (491). Female wildtype C57BL/6 mice underwent the BMS infusion studies. For studies in beta-cell specific IKKβ-null mice, IKKβF/F mice on a C57BL/6 background were crossed with RIP2-Cre mice (also on a C57BL/6 background) obtained from Jackson Lab to generate RIP2-Cre positive or RIP2-negative IKKβF/+ mice, which were interbred to generate RIP2-Cre−:IKKβF/F (floxed controls) and RIP2-Cre+:IKKβF/F (IKKβbeta−cell) mice. Cre-mediated recombination was confirmed by PCR (492).

3.3.2 Surgeries and intravenous infusions

Surgeries were performed under general anaesthesia (isoflurane, to effect). Carotid artery and jugular vein cannulation of rats has been previously described (265,273). After 3 days postsurgical recovery, the rats were randomized to the following 48h infusions: 1) NaCl (154 mmol/l) (Saline, SAL, 5 µl/min) as control; 2) Fat, either oleate (OLE, 1.3 µmol/min) prepared as in our previous studies (265,273) or 20% olive oil plus heparin (OLO, 5.5 µl/min) prepared as in (296). Olive oil is a triacylglycerol mixture containing 75% oleate, and 16% saturated fat. Heparin was added to olive oil to a final concentration of 50 U/ml to activate lipoprotein lipase, which releases FFA and glycerol from the triacylglycerol mixture of olive oil; 3) OLE or OLO plus Salicylate (0.7 µmol kg⁻¹ min⁻¹, the dose that reversed insulin resistance in (493)) and 4) Salicylate alone. We have shown that bovine serum albumin (BSA), the vehicle for oleate, has no effect on insulin secretion (266,273); heparin (494) and glycerol (495) also have no effect. A 2-step hyperglycaemic clamp or islet isolation was performed after the infusion period.
Mouse jugular vein cannulation is described in (266). In mice, oleate (0.4 μmol/min)/equivolume saline was infused for 48h (266), starting 4-5 days after surgery with or without BMS (0.12 mmol kg⁻¹ day⁻¹) (490). After 48 h infusions, mice received hyperglycaemic clamps. Sampling was through the tail vein.

3.3.3 Hyperglycaemic clamps

Insulin secretion was determined by measuring plasma insulin and C-peptide during a two-step (13 and 22 mmol/l) hyperglycaemic clamp in rats (265) and a one-step (22 mmol/l) hyperglycaemic clamp in mice (266). Insulin sensitivity (M/I) and beta-cell function (DI) were assessed during the last 40 minutes of each step of the two step hyperglycaemic clamp in rats and during the last 20 minutes of the one-step hyperglycaemic clamp in mice using the calculations described in General Methods.

3.3.4 Hyperinsulinaemic-euglycaemic clamps

Insulin sensitivity was determined using the gold standard technique, i.e. hyperinsulinaemic-euglycaemic clamps (493).

3.3.5 Ex vivo studies in islets

Islets of the in vivo infused rats and IKKβΔbeta-cell mice were isolated and insulin secretion studies were performed as in (265). ELISA for phosphorylated IκBα and active nuclear NFκB (Active Motif, Carlsbad, CA, USA), ROS measurements and RT-PCR were also performed in rat islets, as described below.
3.3.6 Phosphorylated IκBα assay

Whole islet-extract was obtained according to manufacturer’s protocol. Protein concentration was determined using the BCA protein assay (Pierce). Islet-lysates were assayed for phosphorylated IκBα by an ELISA kit previously used by our group (496). The absorbance levels were in the upper detection range of the standard curve. The intra- and inter-assay coefficients of variations are <10% and <20%, respectively (Active Motif, Carlsbad, CA, USA).

3.3.7 NFκB p65 activity assay

Nuclear proteins were extracted according to the manufacturer’s instructions. The supernatant (nuclear fraction) was kept at -80°C until analysis. The active NFκB contained in the nuclear extracts was measured by its DNA binding activity on immobilized oligonucleotides encoding a specific consensus site using a NFκB p65 transcription factor ELISA kit previously used by our group (496). We used greater than 10 times more protein than the detection limit (0.5µg nuclear extract/well) and the intra- and inter-assay coefficients of variations are <10% and <20%, respectively (Active Motif, Carlsbad, CA).

3.3.8 ROS measurements

Islets were incubated with 10 µmol/l dihydro-dichlorofluorescein-diacetate (H2DCF-DA) (D6883; Sigma) in KRBH containing 2.8 mmol/l glucose for 30 minutes (497). The medium was then replaced with fresh KRBH containing no glucose, and fluorescence was measured at 480 nm excitation and 510 nm emission with an Olympus microscope. Data were analysed using ImageMaster3.
3.3.9 Real time RT-PCR

Total rat islet RNA was prepared as in (261,291) according to manufacturer’s instructions (Qiagen, Hombrechtikon, Switzerland), and was reverse transcribed using random hexamers. Mouse primers for IL-1β, TNFα, monocyte chemoattractant protein 1 (MCP-1), IL-1 receptor antagonist (IL-1Ra), TGFβ, the macrophage marker CD68, and cyclooxygenase-2 (COX-2) from Applied Biosystems, CA, USA were used and quantitative PCR was done with fluorescein amidite (FAM)-based reference dye using commercial TaqMan gene expression assays and the 7500 Fast Real-Time PCR System according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). Changes in mRNA expression were calculated using difference of Ct values, and normalized to the housekeeping gene 18S (261,291).

3.3.10 Studies in cultured islets

Rat islets were cultured for 48h in RPMI 1640 without antioxidants containing 0.4 mmol/l oleate in 0.5% FFA-free BSA with or without 0.25 mmol/l salicylate (484) or 3 µmol/l BMS, a dose based on pilot dose-response studies. Islets were also cultured in control/oleate media with or without the COX-2 inhibitor SC-236 (10 µmol/l, based on (498)) or the COX-1 inhibitor SC-560 (100 µmol/l, based on (499)). Thereafter, glucose stimulated insulin secretion was assessed. Prostaglandin E2 (PGE2) in the medium was measured via ELISA (Enzo Life Sciences).

3.3.11 Western blots

Western blots were performed as previously described (493). Islet pellets (100~150 islets per sample) were lysed in RIPA buffer (50 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) supplemented with 1 mmol/l phenylmethane sulfonyl fluoride (PMSF), 1.5 nmol/ml aprotinin, 23 nmol/ml leupeptin, and incubated on ice for 30 min. The samples were then centrifuged at 13,000 rpm, 4°C for 10 min. The supernatant was removed and the protein concentration was measured as described above or by Bradford assay (BioRad, Canada). 30 µg of protein from each islet lysate was resolved by SDS-
PAGE, transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes and immunoblotted with antibodies against: phospho-Ser307-IRS-1 (Millipore Cat# 07-247, raised in rabbit, RRID:AB_310463, 1:500), total IKKβ (Cell Signaling Technology [CST] Cat# 2370, raised in rabbit, RRID:AB_2122154, 1:100), phospho-Thr172-AMPKα (CST Cat# 2535, raised in rabbit, RRID:AB_331250, 1:1000), total AMPKα (CST Cat# 2793, raised in mouse, RRID:AB_915794, 1:1000), total IκBα (Santa Cruz Biotechnology Cat# sc-371, raised in rabbit, RRID:AB_2235952, 1:250), α-actinin (CST Cat# 3134S, raised in rabbit, RRID:AB_2223798, 1:1000), β-actin (Abcam Cat# ab6276, RRID:AB_2223210, raised in mouse, 1:10,000) or γ-tubulin (Sigma-Aldrich Cat# T6557, RRID:AB_477584, raised in mouse, 1:1,000). Secondary anti-mouse (Cell Signaling Technology [CST] Cat# 7076, raised in horse, RRID:AB_330924, 1:10,000) and anti-rabbit (CST Cat# 7074, raised in goat, RRID:AB_2099233, 1:10,000) IgG antibodies conjugated to horse radish peroxidase and enhanced chemiluminescence system (Amersham Biosciences, Quebec, QC, Canada) were used for detection. For phospho-Thr172-AMPKα, AMPKα, IκBα and α-actinin Kodak Imager 4000pro (Carestream, USA) was used to image membranes. The bands obtained from immunoblotting were quantified by scanning densitometry (Sicon, Suffolk, UK).

3.3.12 Plasma assays

Plasma glucose in rats was measured on a Beckman Analyser II (Beckman, Fullerton, CA) and in mice was measured on a HemoCue Glucose 201 Analyser (HemoCue Inc., CA, USA). Plasma FFA were measured with an enzymatic colorimetric kit (Wako Industries, Neuss, Germany). Radioimmunoassays specific for rat/mouse insulin and C-peptide (Linco, St. Charles, MO) were used to determine their plasma concentrations. Plasma triacylglycerol levels were measured by a colorimetric kit (Boehringer Mannheim, Laval, QC, Canada).

3.3.13 Statistics

Statistics were performed as described in General Methods.
3.4 Results

3.4.1 Hyperglycaemic clamps in rats

Rats were infused i.v. with saline (SAL), oleate (OLE) or olive oil plus heparin (OLO), with or without the IKKβ inhibitor salicylate (SLY). After 48h infusion, plasma FFA were ~1.5-fold higher with OLE or OLO and triacylglycerol levels were elevated by OLO (Table 3.1). OLE, OLO or SLY did not affect plasma glucose or insulin (Table 3.1).

Table 3.1 Plasma FFA, triacylglycerol, glucose and insulin levels after 48h infusions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FFA (mmol/l)</th>
<th>Triacylglycerol (mmol/l)</th>
<th>Glucose (mmol/l)</th>
<th>Insulin (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL (n=12)</td>
<td>0.693 ± 0.026</td>
<td>0.17±0.019</td>
<td>5.8±0.2</td>
<td>79±12</td>
</tr>
<tr>
<td>OLE (n=10)</td>
<td>1.050±0.118**</td>
<td>0.21±0.014</td>
<td>5.9±0.1</td>
<td>72±13</td>
</tr>
<tr>
<td>OLO (n=7)</td>
<td>0.925±0.104*</td>
<td>0.60±0.063***</td>
<td>5.5±0.2</td>
<td>110±80</td>
</tr>
<tr>
<td>OLE + SLY (n=8)</td>
<td>1.123±0.168**</td>
<td>0.25±0.020</td>
<td>5.2±0.1</td>
<td>68±14</td>
</tr>
<tr>
<td>OLO + SLY (n=11)</td>
<td>1.027±0.095*</td>
<td>0.54±0.057***</td>
<td>5.0±0.1</td>
<td>82±15</td>
</tr>
<tr>
<td>SLY (n=9)</td>
<td>0.580±0.095</td>
<td>0.15±0.024</td>
<td>5.4±0.1</td>
<td>57±90</td>
</tr>
</tbody>
</table>

Data are mean ± SEM
Rats were treated with: saline; oleate at 1.3 μmol/min; OLO (20% olive oil infusate containing 50 U/ml heparin) at 5.5 μl/min; oleate + salicylate at 0.7 μmol kg\(^{-1}\) min\(^{-1}\); OLO + salicylate; or salicylate only
*p<0.05, **p<0.01 and ***p<0.001 compared with saline and salicylate only

Following the 48h infusions, we evaluated beta-cell function in vivo using two-step hyperglycaemic clamps (13 mmol/l and 22 mmol/l, Figure 3.1a,b). The glucose infusion rate (GINF) necessary to maintain the clamp was lower with OLE or OLO, suggesting reduced insulin secretion and/or sensitivity (Figure 3.1c,d). In OLE+SLY or OLO+SLY, GINF was similar to control (Figure 3.1c,d).
Figure 3.1 Plasma glucose, glucose infusion rate (GINF), insulin, and C-peptide during two-step hyperglycaemic clamps following 48h oleate or olive oil infusion in rats. a, c, e, g Rats were treated with Saline (SAL, n=12), Oleate at 1.3 μmol/min (OLE, n=10), Oleate+Salicylate at 0.7 μmol kg⁻¹ min⁻¹ (OLE+SLY, n=8), or Salicylate alone (SLY, n=9); b, d, f, h Rats were treated with Saline (SAL, n=12), Olive oil (20% olive oil infusate containing 50U/ml heparin) at 5.5 μl/min (OLO, n=7), Olive oil+Salicylate (OLO+SLY, n=11), or Salicylate alone (SLY, n=9). ** p<0.01 vs. all, throughout the clamp. *** p<0.001 vs. all, throughout the clamp.

Clamp plasma insulin was lower in OLE and was restored in OLE+SLY, whereas in OLO and OLO+SLY insulin was not different from control (Figure 3.1e,f). Clamp C-peptide showed the same pattern as insulin (Figure 3.1g,h), indicating unchanged absolute insulin secretion in OLO-treated groups. The insulin sensitivity index M/I=GINF/Insulin (500) was not affected by OLE.
(Figure 3.2a), as previously found in the same model (265,273) and was also unaffected by salicylate. In OLO, M/I was reduced, indicating insulin resistance (Figure 3.2b). The decrease in M/I was prevented by salicylate. The evaluation of beta-cell function in vivo should take into account the ambient insulin sensitivity, as normal beta-cells increase insulin secretion in response to insulin resistance along a hyperbola, characterised by a constant Disposition Index (DI) (288). DI is an established index of beta-cell function (288), that we have previously validated in rodents (266,291). The DI was impaired in both OLE and OLO groups (Figure 3.2c,d), indicating reduced beta-cell function, but the impairment was completely prevented by salicylate. Salicylate alone had no effect on DI.

Figure 3.2 Sensitivity Index (M/I) and Disposition Index (DI) during two-step hyperglycaemic clamps with/without 48h oleate or olive oil infusion in rats. Groups are described in Figure 1 legend. ** p<0.01 vs. all groups at the same glucose concentration.
3.4.2 Hyperinsulinaemic-euglycaemic clamps

When evaluated by hyperinsulinaemic clamp, insulin sensitivity tended to be decreased by OLE (n=4), and was significantly decreased by OLO (n=3) compared to SAL (n=4) (Figure 3.3).

**Figure 3.3** Effects of oleate and olive oil on FFA, insulin, glucose infusion rate (GINF) and insulin sensitivity during hyperinsulinaemic-euglycaemic clamps following 48h oleate or olive oil infusion in rats. Rats were treated with Saline (SAL, n=4), Oleate (OLE, n=4), or Olive Oil (OLO, n=3). During 30 min preceding the clamp (‘basal period’), measurements were taken at 10-min interval for plasma glucose, insulin and FFA. At the onset of the clamp, an infusion of human insulin at 5 mU kg\(^{-1}\) min\(^{-1}\) was initiated and continued for 120 min. To maintain euglycaemia during insulin infusion (basal glucose ~6.5±0.3 mmol/l), a variable infusion of 20% glucose was given through the jugular catheter and adjusted according to glycaemic determinations every 5 min. † p<0.05 and †† p<0.01 vs. SAL; ‡‡ p<0.01 vs. OLE; A p<0.1 vs. SAL.
3.4.3 *Ex vivo* studies in rat islets

Glucose stimulated insulin secretion of islets isolated from rats i.v. infused with OLE or OLO was markedly impaired and restored by salicylate (Figure 3.4a,b). OLE or OLO increased phosphorylated IκBα (Figure 3.4c,d) and active nuclear NFκB (Figure 3.4e,f), which were also restored by salicylate.

**Figure 3.4** Insulin secretory response to glucose, phosphorylated IκBα, and active nuclear NFκB of freshly isolated islets of fat-infused rats. Groups are described in Figure 1 legend. *** p<0.001 vs. all at the same glucose concentration; ** p<0.01 vs. all at the same glucose concentration; * p<0.05 vs. all. Panels a, b SAL, n = 16; OLE, n = 14; OLE+SLY, n = 8; OLO, n = 12; OLO+SLY, n = 6; SLY, n = 10. Panels c-f SAL, n = 5-7; OLE, n = 4-6; OLE+SLY, n = 4-5; OLO, n = 5-6; OLO+SLY, n = 5-8; SLY, n = 4-6.
OLE increased islet mRNA of IL-1β, TNFα, MCP-1, IL-1Ra, TGFβ, CD68, and COX-2, and the effect of OLE was prevented by salicylate (Table 3.2). Interestingly, SLY alone decreased these markers. The signal for inducible nitric oxide synthase (iNOS) mRNA was undetectable in all groups. OLE (n=6) increased islet ROS compared to SAL (n=5), however, ROS levels were not restored by combination with SLY (n=8) and SLY alone had no effect (n=4) (Figure 3.5).

Table 3.2 mRNA levels of inflammatory markers in freshly isolated islets of rats infused with Saline (SAL), Oleate (OLE), Oleate + Salicylate (OLE+SLY) or Salicylate alone (SLY)

<table>
<thead>
<tr>
<th>Marker</th>
<th>SAL</th>
<th>OLE</th>
<th>OLE + SLY</th>
<th>SLY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Il1b</td>
<td>0.82 ± 0.17 (n=8)</td>
<td>2.02 ± 0.42†††‡‡‡§§§ (n=8)</td>
<td>0.30 ± 0.12 (n=5)</td>
<td>0.10 ± 0.04† (n=3)</td>
</tr>
<tr>
<td>Tnfa (Tnf)</td>
<td>0.58 ± 0.22 (n=6)</td>
<td>1.97 ± 0.28†§§ (n=7)</td>
<td>0.81 ± 0.46 (n=4)</td>
<td>0.08 ± 0.04 (n=3)</td>
</tr>
<tr>
<td>Mcp1 (Ccl2)</td>
<td>0.76 ± 0.28 (n=6)</td>
<td>5.64 ± 1.89†††‡‡‡§§§ (n=10)</td>
<td>0.52 ± 0.24 (n=5)</td>
<td>0.01 ± 0.00† (n=3)</td>
</tr>
<tr>
<td>Il1ra (Il1rn)</td>
<td>1.00 ± 0.31 (n=5)</td>
<td>15.52 ± 4.67†† (n=10)</td>
<td>2.79 ± 1.34 (n=4)</td>
<td>4.70 ± 2.79 (n=3)</td>
</tr>
<tr>
<td>Tgfb (Tgfb1)</td>
<td>0.85 ± 0.26 (n=7)</td>
<td>2.47 ± 0.51†§§ (n=7)</td>
<td>0.81 ± 0.37 (n=4)</td>
<td>0.54 ± 0.33 (n=4)</td>
</tr>
<tr>
<td>Cd68</td>
<td>0.62 ± 0.14 (n=8)</td>
<td>4.21 ± 1.07†††‡†§ (n=7)</td>
<td>0.54 ± 0.12 (n=4)</td>
<td>0.75 ± 0.37 (n=3)</td>
</tr>
<tr>
<td>Cox2 (Ptgs2)</td>
<td>1.00 ± 0.10 (n=5)</td>
<td>2.66 ± 0.53†††‡†§§§ (n=9)</td>
<td>0.36 ± 0.10† (n=3)</td>
<td>0.33 ± 0.09† (n=4)</td>
</tr>
</tbody>
</table>

Data are mean ± SEM

Rats were infused with: saline; oleate; oleate + salicylate; or salicylate only

Units are normalised to a housekeeping gene

Sample size differed between markers as some samples had expression levels that were either undetectable or outliers according to the Grubb’s test

†p<0.05, ††p<0.01 and †††p<0.001 compared to saline

‡p<0.05, ‡‡p<0.01 and ‡‡‡p<0.001 compared to oleate + salicylate

§p<0.05, §§p<0.01 and §§§p<0.001 compared to salicylate alone
Figure 3.5 Effects of salicylate on ROS levels of freshly isolated islets of fat-infused rats. Rats were treated with Saline (SAL, n=5), Oleate at 1.3 µmol/min (OLE, n=6), Oleate+Salicylate at 0.7 µmol kg⁻¹ min⁻¹ (OLE+SLY, n=8), or Salicylate alone (SLY, n=4). *** p<0.001 oleate infused groups compared to non-oleate infused groups. Representative fluorescent images of individual islets for ROS (200X) (b).

3.4.4 Hyperglycaemic clamps in mice

We used 48h oleate infusion in mice, with or without BMS, which is a much more potent and specific IKKβ inhibitor than salicylate, at a dose previously found to inhibit IKKβ in vivo (490). After the 48h infusion and prior to the hyperglycaemic clamp the groups treated with oleate had higher plasma FFA (OLE: 1.205 ± 0.156 mmol/l; OLE+BMS: 1.177 ± 0.136 mmol/l) than the groups infused with saline (0.761 ± 0.206 mmol/l) or BMS alone (0.689 ± 0.070 mmol/l). The glucose level was raised to 22 mmol/l in all groups (Figure 3.6a). The GINF necessary to maintain
the clamp was lower in OLE but was similar to SAL in OLE+BMS (Figure 3.6b). Different from our oleate model in rats, but similar to our previous studies in mice (266) and our olive oil model in rats, clamp insulin and C-peptide were not lower than control, in oleate-treated mice (Figure 3.6c,d). Accordingly, the sensitivity index M/I (500) was lower in oleate-treated mice (Figure 3.6e). Basal and clamp insulin and C-peptide levels were higher in the groups treated with BMS. BMS had no significant effect on M/I when added to oleate but had an effect by itself in decreasing M/I. DI was decreased with oleate infusion, whereas BMS completely prevented the oleate-induced decrease. BMS alone had no effect on DI (Figure 3.6f).

![Figure 3.6](image.png)

**Figure 3.6** Plasma glucose, GINF, plasma insulin, C-peptide, sensitivity index and disposition index during hyperglycaemic clamps in mice infused with oleate with or without BMS. Mice were treated for 48h with: Saline (SAL, n=7), Oleate at 0.4 μmol/min (OLE, n=6), Oleate+BMS-345541 at 0.12 mmol kg⁻¹ day⁻¹ (OLE+BMS, n=5), BMS alone (n=3). **p<0.01 vs all; †p<0.05 vs SAL; ‡p<0.05 vs OLE.
3.4.5 Studies in ‘beta-cell specific’ IKKβ-deficient (IKKβΔbeta-cell) mice

We also used IKKβΔbeta-cell and littermate floxed control mice to determine whether genetic silencing of IKKβ protects from fat-induced beta-cell dysfunction ex vivo and in vivo. IKKβ deletion in islets of IKKβΔbeta-cell mice was confirmed by immunoblotting (Figure 3.7a) and there was a suggestion of partial deletion in the hypothalamus (Figure 3.7b). There was no significant difference in weight between ~13 week old control (29.7±0.7g, n=14) and IKKβΔbeta-cell (28.5±0.8g, n=13) mice. 48h oleate infusion elevated plasma FFA by ~3 fold (Figure 3.7c). Glucose stimulated insulin secretion of islets isolated from oleate-infused controls was impaired and this impairment was prevented in IKKβΔbeta-cell mice (Figure 3.7d).

Figure 3.7 IKKβ protein expression in islets and hypothalamus of, plasma FFA levels in, and insulin secretory response to glucose in islets of IKKβ-knockout mice. IKKβ protein levels in islets (a) and hypothalamus (b) of IKKβΔbeta-cell mice (IKKβF/F:RIP2-Cre; KO) and floxed controls (IKKβF/F; CON). Each lane represents islets pooled together from 2 mice of the same genotype (a) or one hypothalamus taken from individual mice (b). Plasma FFA (c) and insulin secretory response to glucose of freshly isolated islets of IKKβF/F and IKKβΔbeta-cell mice (d) following 48h oleate (0.4 µmol/min; OLE) or saline (SAL) infusion (CON-SAL, n=8; CON-OLE, n=7; KO-OLE, n=5; KO-SAL, n=9). † p<0.05 vs. CON-SAL.
During the in vivo studies, before hyperglycaemic clamps, FFA were elevated by ~2 fold in the oleate-infused groups but their levels decreased during the clamp as expected (Figure 3.8a). Glucose levels were raised to ~22mM in all groups (Figure 3.8b). The glucose infusion rate did not differ between groups (Figure 3.8c). Basal insulin (Figure 3.8d) and C-peptide (Figure 3.8e) levels did not differ between groups. During the clamp, insulin levels were higher and C-peptide levels tended to be higher in the oleate-infused control mice compared to all other groups. Thus, the M/I was decreased in oleate-infused controls. A decrease in M/I was not observed in IKKβΔbeta-cell mice (Figure 3.8f), which may be explained by partial deletion of hypothalamic IKKβ driven by RIP2. The index of insulin clearance (C-peptide/insulin) tended to decrease (p=0.055) in oleate-infused controls compared to saline infused controls, whereas this decrease did not occur in oleate-infused β-cell specific IKKβ-knockout mice (Figure 3.8g). Importantly, IKKβΔbeta-cell mice were protected from the decrease in DI induced by oleate in controls (Figure 3.8h).
Figure 3.8 FFA levels, plasma glucose, glucose infusion rate (GINF), plasma insulin, plasma C-peptide, sensitivity index (M/I), insulin clearance index, and disposition index (DI) during hyperglycemic clamps in IKKβ knockout mice. IKKβ^FF (CON) and IKKβ^Δbeta-cell (KO) mice were treated with 48h oleate (0.4 μmol/min; OLE) or saline (SAL) infusion (CON-SAL, n=8; CON-OLE, n=7; KO-OLE, n=8; KO-SAL, n=5). § p<0.05 vs. CON-SAL and KO-SAL; * p<0.05 vs. all; ¶¶ p<0.01 vs. CON-SAL and KO-OLE; ‡‡ p<0.01 vs. CON-SAL; || p<0.05 vs. KO-OLE.
3.4.6 *In vitro* studies in islets

To completely rule out that the effect of salicylate or BMS on beta-cell dysfunction was mediated by their systemic effect, we performed *in vitro* studies. Rat islets were cultured for 48h in control or oleate containing medium, with or without salicylate. Oleate decreased glucose stimulated insulin secretion, and salicylate prevented this decrease (Figure 3.9a).

![Graph showing insulin secretion in islets exposed to various conditions](image)

**Figure 3.9** Insulin secretion, IκBα and phosphorylated AMPKα expression in islets exposed for 48h to oleate with or without salicylate and insulin secretion in islets exposed to oleate with or without BMS. Islets were exposed to: a: Control (CON) or oleate (OLE) with/without salicylate (SLY). CON, 0.5% FFA-free BSA in medium, n=7; OLE, 0.4 mmol/l in 0.5% FFA-free BSA, n=14; OLE + SLY, 0.25 mmol/l, n=10; SLY alone in BSA, n=7; b: Same groups as (a). CON, n=7; OLE, n=8; OLE + SLY, n=7; SLY, n=4; c: Same groups as (a). CON, n=8; OLE, n=7; OLE + SLY, n=8; SLY, n=6; d: Insulin secretion in islets exposed to oleate (OLE) for 48h with/without BMS-345541 (BMS). CON, n=7; OLE, n=14; OLE + BMS, 3 μmol/l, n=10; BMS alone in BSA, n=7. ** p<0.01 vs. all; ‡ p<0.05 vs. CON.
Oleate decreased total IκBα and the decrease (marker of IKKβ activity as phosphorylated IκBα is degraded) was prevented with salicylate (Figure 3.9b). Salicylate is known to activate AMPK by preventing its dephosphorylation (501) and AMPK activation can result in IKKβ inhibition (502), although salicylate has also been reported to directly inhibit IKKβ (487). There was a tendency for oleate to decrease phosphorylation of AMPK, which appeared to be prevented with salicylate but there was no significant effect (Figure 3.9c). BMS, which inhibits IKKβ in AMPKα-null cells (503), prevented oleate-induced beta-cell dysfunction similar to salicylate (Figure 3.9d).

Figure 3.10 Serine phosphorylated IRS-1 and PGE2 levels in cultured islets exposed for 48h to oleate (OLE) or vehicle control (CON) with/without salicylate (SLY) and insulin secretion in islets exposed to oleate with or without a COX-2 inhibitor. a: Same groups as (Figure 3.9a), n=5 in all groups; b: Same groups as (Figure 3.9a), n=5-6/group; c: Insulin secretion in CON or OLE with/without the COX-2 inhibitor SC-236. CON, n=11; OLE, n=10; OLE + SC-236, 10 µmol/l, n=9; SC-236 alone in BSA, n=9. ** p<0.01 vs. all; ‡ p<0.05 vs. CON; * p<0.05 vs. all.
The IKKβ/NFκB pathway can mediate oleate-induced beta-cell dysfunction by at least three mechanisms: 1) impairment of beta-cell insulin signalling via serine phosphorylation of IRS (483), 2) increase in COX-2-derived PGE2 (504) and 3) production of nitric oxide (NO) through induction of iNOS (505), however iNOS mRNA was undetectable in our islets, in the ex vivo studies. Oleate increased serine 307-phosphorylated IRS-1 in cultured islets, an effect prevented by salicylate (Figure 3.10a). Oleate also increased PGE2 release in media and this was prevented by salicylate (Figure 3.10b). We also treated islets with the COX-2 inhibitor SC-236, which prevented the secretory defect induced by oleate (Figure 3.10c), whereas the COX-1 inhibitor SC-560 did not (insulin secretion values at 22 mmol/l glucose relative to control: CON = 1.00±0.33, n=4; OLE = 0.59±0.17, n=4; OLE+SC-560 = 0.14±0.06, n=3; SC-560 = 0.51, n=2).
3.5 Discussion

We examined the effects of prolonged FFA exposure with or without IKKβ inhibitors on beta-cell function in vivo, ex vivo, and in vitro. We used our in vivo models of lipotoxicity in rats (265,273) and mice (266). These are models of beta-cell dysfunction, as beta-cell mass is not decreased by 48h fat infusion (266,291) and apoptosis is not increased (506). Importantly, although beta-cell dysfunction is reversible in our models, they allow for the study of mechanisms involved in obesity-mediated diabetes and specifically, the mechanisms by which FFA impair beta-cell function. During hyperglycaemic clamps in vivo, both insulin and C-peptide levels were lower in rats treated with oleate, indicating reduced insulin secretion, and reduced glucose stimulated insulin secretion was also found in isolated islets ex vivo. With olive oil, glucose stimulated insulin secretion ex vivo was reduced but insulin and C-peptide levels (indices of absolute insulin secretion) during hyperglycaemic clamps were unaffected. The different absolute insulin secretion between oleate and olive oil is explained by the effect of olive oil to induce a greater degree of insulin resistance. This was demonstrated using hyperinsulinaemic-euglycaemic clamps, which are the gold standard assessment of insulin sensitivity in vivo. In vivo, the beta-cell compensates for insulin resistance by increasing secretion. In the absence of insulin resistance, absolute insulin secretion corresponds to DI. However, in the presence of insulin resistance, DI rather than absolute insulin secretion should be taken as a measure of beta-cell function, which includes the ability of the beta-cell to compensate for insulin resistance. DI was impaired by both oleate and olive oil, showing a decrease in beta-cell function with both types of fat, consistent with the results ex vivo in islets. The differential effects of oleate and olive oil on insulin sensitivity may be due to the amount of saturated fat in olive oil (16%) and/or to the plasma triacylglycerol elevation induced by olive oil (507).

Our results with either oleate or olive oil indicate that fat-induced beta-cell dysfunction was prevented by salicylate in vivo and ex vivo in isolated islets, suggesting a role for inflammatory pathways involving IKKβ in lipid-induced beta-cell dysfunction.

Inflammatory pathways are known to be activated in beta-cell glucotoxicity (508) and may enhance lipotoxicity (269). Our previous results show that oxidative stress plays a causal role in beta-cell dysfunction induced by monounsaturated fat (265). ROS are known activators of
IKKβ, which in addition to phosphorylating IκBα, thereby activating NFκB, phosphorylates IRS, thus inhibiting insulin signalling. In our *ex vivo* study, salicylate prevented the increase in phosphorylated IκBα and nuclear active NFκB induced by oleate or olive oil, although, interestingly, salicylate alone had no effect at this dose, as previously seen in the liver (493). An increase in ROS was induced by oleate, but salicylate did not prevent this effect. This suggests IKKβ is a downstream effector required for the previously demonstrated (265) effect of oxidative stress to induce beta-cell dysfunction. IKKβ activation could also be unrelated to oxidative stress in the case of olive oil which contains saturated fat. Saturated fatty acids can activate IKKβ via Toll-like receptors (TLR) 2 and 4. TLR4-null mice are protected from beta-cell dysfunction induced by palmitate (55) and TLR2-null mice are protected from beta-cell dysfunction induced by high-fat diet (261). Although saturated fat is believed to exert a more deleterious effect on beta-cells than unsaturated fat, this is mostly based on *in vitro* data, because until recently, palmitate infusion has been a challenge (55).

Salicylate has been previously found to restore glucose stimulated insulin secretion in an *in vitro* glucotoxicity model (484). Although not all studies are concordant (281), the majority report that salicylate improves beta-cell function in humans (509–511). This effect was initially attributed to inhibition of COX-2 (504), a gene controlled by NFκB, and the consequently decreased synthesis of PGE2, a prostaglandin which inhibits insulin secretion. In addition to inhibiting COX-2 transcription via NFκB, salicylate is a direct inhibitor of both COX-1 and COX-2 (512), and an activator of AMPK (501). Salicylate did not likely protect against beta-cell dysfunction through COX-1 inhibition as a COX-1 inhibitor did not prevent oleate-induced beta-cell dysfunction. AMPK phosphorylation, however, did tend to decrease with oleate and this decrease was prevented by salicylate, which raises the question as to whether the protective effect of salicylate was mediated in part by AMPK activation. The effect of AMPK on insulin secretion is generally considered to be inhibitory (513); however, AMPK may also deplete islet triacylglycerol, which accumulates with exposure to high levels of FFA, (514,515) and inhibit IKKβ (502). To further implicate IKKβ in the effect of oleate to decrease beta-cell function, we also used BMS, an inhibitor that to our knowledge has not been reported to activate AMPK, and we obtained the same results on beta-cell function as with salicylate both *in vitro* and *in vivo*. Importantly, the effect of IKKβ to mediate fat-induced beta-cell dysfunction
is also supported by our *ex vivo* and *in vivo* data using a genetic inhibition model, the IKKβ<sub>B</sub>Δbeta-cell mouse.

In mice however, three important differences were noticed with respect to the rat studies. First, 48h exposure to oleate did induce marked insulin resistance in mice in accordance with our previously published data (266). Besides species difference, the reason behind this finding may be the strain of mice, as C57BL/6 mice are very susceptible to fat-induced insulin resistance (516). Oleate infusion which induced insulin resistance in mice did not result in lower absolute insulin secretion during the clamp, but decreased beta-cell function (DI), similar to our results with olive oil in rats. Second, BMS did restore DI but did not affect oleate-induced insulin resistance, as it likely had an effect by itself, in decreasing insulin sensitivity, which resulted in increased plasma insulin and C-peptide during clamps. Decreased insulin sensitivity with an IKKβ inhibitor may be dose-related and due to the inhibition of COX-2-derived prostaglandins that increase insulin action (517). Third, there was no difference in glucose infusion rate between groups in the IKKβ<sub>B</sub>Δbeta-cell studies even though there was a decrease in DI. This was due to the tendency for insulin clearance to decrease, which would increase the level of insulin in the circulation, thereby maintaining the glucose infusion rate, despite a decrease in DI. However, it is not clear whether the decrease in DI is secondary to a decrease in insulin clearance, in which case it may have been due to β-cell rest rather than β-cell dysfunction. Importantly, our *ex vivo* results suggest that there is a direct effect of oleate in the β-cell, prevented by IKKβ deletion, as oleate decreased insulin secretion in control islets and this was prevented in knockout islets. In rats and in mice from the BMS experiments there were no significant differences in insulin clearance between groups (units are nmol/l C-peptide divided by pmol/l insulin; *rats*: SAL, 0.004 ± 0.000; OLE, 0.004 ± 0.000; OLE+SLY, 0.004 ± 0.000; SLY, 0.004 ± 0.001; OLO, 0.003 ± 0.000; OLO+SLY, 0.004 ± 0.000; *mice*: SAL, 0.006 ± 0.001; OLE, 0.005 ± 0.001; OLE+BMS, 0.005 ± 0.001; BMS, 0.005 ± 0.000). The discrepancy between these studies and those in IKKβ<sub>B</sub>Δbeta-cell mice is likely due to higher elevation of FFA in the IKKβ<sub>B</sub>Δbeta-cell studies as FFA-induced changes in insulin clearance require a greater elevation of FFA than do changes in M/I or DI (518).

The mechanisms whereby IKKβ inhibition prevents fat-induced beta-cell dysfunction deserves further study. However, both upregulation of insulin signalling and COX-2 inhibition are plausible mechanisms, as suggested by two lines of evidence. First, salicylate prevented serine
phosphorylation of IRS, which is known to decrease insulin-induced tyrosine phosphorylation (i.e., insulin signalling). Beta-cell insulin signalling is known to be important for beta-cell function (435). Second, salicylate prevented the oleate-induced PGE2 production and a COX-2 inhibitor mimicked the effect of salicylate. Previously, the effect of COX-2 on beta-cell function has mainly been studied in the context of cytokine exposure, with contrasting results, presumably due to COX-1 vs. COX-2 specificity of the inhibitors used (504,519). Also, the effects of exposure to COX-2 products yielded variable results among labs (519,520) but a dose dependent inhibitory effect (519,520) suggests COX-2 may be implicated in decreasing beta-cell function. Since oleate increased IL-1Ra gene expression to a greater extent than that of IL-1β, the increase in COX-2 mRNA expression is likely due to an oxidative stress-induced activation of IKKβ, independent of IL-1β. Nonetheless, salicylate did prevent upregulated gene expression of cytokines and chemokines which may have contributed to beta-cell dysfunction, by further activating IKKβ and possibly other inflammatory pathways.

In summary, we demonstrated that prolonged exposure to fatty acids, which induces oxidative stress in islets, decreases beta-cell function both in vitro and in vivo via activation of IKKβ. The novelty of our findings is the demonstration that IKKβ mediates beta-cell dysfunction induced by FFA selectively, and that the IKKβ/NFκB pathway is a therapeutic target to prevent FFA-induced beta-cell dysfunction in vivo.
Study 2 — β-cell insulin resistance plays a role in fat-induced β-cell dysfunction \textit{in vitro} and \textit{in vivo}

The results of this study are incorporated in a manuscript in revision for Endocrinology and also include data from the PhD thesis of Dr. Andrei Oprescu. They are reproduced in this thesis with permission:


My contributions to the manuscript were performing all the β-cell specific PTEN-knockout experiments, assisting with Western blots in rat islets, as well as drafting the manuscript.
4.1 Abstract

In the classical insulin target tissues liver, muscle and adipose tissue, chronically elevated levels of free fatty acids (FFA) impair insulin signaling. Insulin signaling molecules are also present in β-cells where they play a role in β-cell function. Therefore, inhibition of the insulin/IGF-1 pathway may be involved in fat-induced β-cell dysfunction. To address the role of β-cell insulin resistance in FFA-induced β-cell dysfunction we co-infused bisperoxovanadate (BPV) with oleate or olive oil for 48h in rats. BPV, a tyrosine phosphatase inhibitor, acts as an insulin mimetic and is devoid of any antioxidant effect that could prevent β-cell dysfunction, unlike most insulin sensitizers. Following fat infusion, rats either underwent hyperglycemic clamps for assessment of β-cell function in vivo or islets were isolated for ex vivo assessment of glucose-stimulated insulin secretion (GSIS). We also incubated islets with oleate and BPV for 48h in vitro for assessment of GSIS and Akt phosphorylation. Next, mice with β-cell specific deletion of PTEN (negative regulator of insulin signaling) and littermate controls were infused with oleate for 48h, followed by hyperglycemic clamps or ex vivo evaluation of GSIS. In rat experiments, BPV protected against fat-induced impairment of β-cell function in vivo, ex vivo and in vitro. In mice, β-cell specific deletion of PTEN protected against oleate-induced β-cell dysfunction in vivo and ex vivo. These data support the hypothesis that β-cell insulin resistance plays a role in FFA-induced β-cell dysfunction.
4.2 Introduction

Obesity and type 2 diabetes (T2D) are associated with a chronic elevation of free fatty acids (FFA), which are known to impair β-cell function. The mechanisms involved are not completely understood, but major contributors include oxidative stress, endoplasmic reticulum (ER) stress and inflammation (269,280,282). In insulin target tissues such as muscle, liver and adipose tissue, these pathways are the mechanisms whereby FFA impair insulin signaling (142,230,335,521), due to activation of inflammatory kinases (67,68). Similar mechanisms are involved FFA-induced β-cell dysfunction and it is possible that they lead to insulin resistance in β-cells.

The insulin signaling pathway in β-cells plays important roles in β-cell growth and proinsulin biosynthesis. Inhibition of forkhead box protein O1 (FOXO1) by Akt leads to increased pancreatic and duodenal homeobox 1 (PDX-1)-mediated transcription of the insulin gene (433,434,436).

The effect of insulin on insulin secretion specifically is less clear. Early studies found that incubation of β-cells with insulin stimulated insulin secretion via an IRS-1 dependent mechanism that leads to increased cytosolic calcium (522–524). In contrast, other studies have shown that PI3K inhibits insulin secretion due to opening of KATP channels by PIP3 (442,445,525). In vivo, the prevailing effect of insulin appears to be stimulatory, as β-cell specific insulin receptor knockout (βIRKO) mice show a defect in first phase insulin secretion (435).

In the context of fat exposure, in vitro studies have shown that incubation of INS-1 cells with FFA impairs the insulin-like growth factor (IGF)-1 induced activation of Akt (231,451,452), decreases Akt-mediated mitogenesis (231), and induces β-cell apoptosis (451). Palmitate-induced JNK activation was also found to decrease IRS-1 and IRS-2 phosphorylation and in turn impaired insulin gene transcription (453). Furthermore, adenoviral-mediated overactivation of Akt prevented oleate-induced apoptosis (451). In addition, β-cell specific deletion of the negative regulator of insulin signaling PTEN (phosphatase and tensin homolog), which dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3) to PIP2, protects against obesity-induced diabetes in both dietary (high fat diet) and genetic (db/db mice) models of T2D (437). Therefore, we hypothesized that upregulation of insulin signaling by the insulin mimetic bisperoxovanadate (BPV) as well as by β-cell specific PTEN deletion would protect against β-cell dysfunction induced specifically by elevation of FFA. Vanadium compounds, although tyrosine phosphatase
inhibitors that are nonspecific for the insulin signaling cascade, have the advantage over other insulin sensitizers such as metformin and thiazolidinediones of inducing rather than decreasing oxidative stress (526). These other insulin sensitizers are also AMP kinase activators and thus deplete islets from fat (514). Therefore, a positive effect in preventing lipotoxicity by BPV could not be ascribed to reduction of oxidative stress or islet fat.

In order to address our hypothesis, we intravenously infused rats with oleate or olive oil as in our previous study (527) with and without BPV, followed by hyperglycemic clamps for in vivo assessment of β-cell function or assessment of glucose-stimulated insulin secretion (GSIS) ex vivo in islets. Rat islets were also incubated with oleate and BPV in vitro for assessment of GSIS and Akt phosphorylation. Finally, we infused β-cell specific PTEN-knockout mice with oleate, followed by in vivo hyperglycemic clamps or ex vivo determination of GSIS in islets. In rats, we found that BPV protected against oleate and olive oil-induced β-cell dysfunction in vivo and ex vivo and against oleate-induced β-cell dysfunction in vitro. BPV also increased Akt phosphorylation in rat islets. In mice, β-cell specific deletion of PTEN prevented fat-induced β-cell dysfunction both in vivo and ex vivo.
4.3 Methods

4.3.1 Animals

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. Female Wistar rats (250-300g, Charles River, Canada) were used for BPV experiments. Heterozygous PTEN floxed C57BL6-129J mice were bred with RIP-Cre positive or RIP-Cre negative C57BL/6 mice in order to generate homozygous and heterozygous β-cell specific PTEN knockout mice, homozygous PTEN floxed RIP-Cre negative controls and wildtype PTEN RIP-Cre positive controls. Animals were on a 129J-C57BL/6 genetic background, however, unlike in the study by Wang et al. (437), and similar to the study by Stiles et al. (528), they were not deliberately maintained on this mixed background (only mice with a black coat were used for breeding to minimize genetic heterogeneity). All animals were housed in the University of Toronto’s Department of Medicine, exposed to a 12h light/dark cycle and fed standard rodent chow containing 24% protein, 58% carbohydrate, and 18% fat by calorie content (Teklad Global 2018, Madison, WI).

4.3.2 Cannulation surgeries and intravenous infusions

The jugular vein and carotid artery were cannulated in rats as previously described (137,527). After 3 days of recovery from surgery, rats were randomized and infused for 48 h with either 1) Saline control (SAL); 2) Oleate (OLE, 1.3µmol/min) bound to bovine serum albumin (BSA) and prepared as previously described (265,273,527) or olive oil plus heparin (OLO, 50 U/ml heparin; 5.5 µl/min) to elevate plasma FFA by 1.5 to twofold (527); 3) Oleate plus bisperoxovanadate (BPV, 0.0025 µmol/kg/min, the dose that increased insulin sensitivity in (529)) or OLO plus BPV; or 4) BPV alone. Saline was used as control as we have previously shown that there is no difference compared to BSA infusion in the same experimental models (rats and mice) (266,273). Following the infusions, rats either underwent a 2-step hyperglycemic clamp for in vivo assessment of β-cell function or islets were isolated for ex vivo assessment of GSIS as in (137,265,527).

Controls, either PTEN^F/F (floxed control) or Cre-positive mice, β-cell specific heterozygous PTEN-knockout mice, and β-cell specific homozygous PTEN-knockout mice underwent jugular
vein cannulation for intravenous oleate infusion and hyperglycemic clamps or *ex vivo* assessment of GSIS as described in (137,266,527). 3-5 days after surgery, mice were weighed and infused for 48h at a rate of 0.4 µmol/min with oleate or saline followed by either 2h hour *in vivo* hyperglycemic clamps or islet isolation for *ex vivo* assessment of GSIS.

### 4.3.3 Pancreatic islet isolation

After the 48 h infusion, islets were isolated from the overnight-fasted rats using the Ficoll/Histopaque method, as described previously (265). Another set of islets were isolated from untreated rats using the same method for *in vitro* experiments. Pancreatic islets of mice were isolated after a 4 h fast, following 48 h infusion, as previously described (527,530).

### 4.3.4 Hyperglycemic clamps, insulin sensitivity index, disposition index and insulin clearance index

One-step hyperglycemic clamps at 22 mM glucose in mice and two-step hyperglycemic clamps at 13 mM and 22 mM glucose in rats were performed as previously described (266,527). The sensitivity index (SI) and disposition index (DI) were calculated as previously described (266,527). SI is an index of insulin sensitivity, calculated as Glucose Infusion Rate/[Insulin] (500). DI is an established *in vivo* measure of β-cell function (287,288), calculated as Glucose Infusion Rate/[Insulin] * [C-peptide] (266,527). The insulin clearance index was calculated as [C-peptide]/[Insulin].

### 4.3.5 Ex vivo studies in islets

Isolated islets of *in vivo* infused rats and mice were pre-incubated for 1 h at 37°C in KRBH supplemented with 2.8 mM glucose. Thereafter, five rat islets of approximately the same size were incubated in triplicate at 2.8, 6.5, 13 and 22 mM glucose for 2 h at 37°C. Similarly, ten mouse islets of approximately the same size were incubated in duplicate at 6.5 and 22 mM glucose for 2
h at 37°C. Insulin concentration in the medium was analyzed using a radioimmunoassay kit specific for rat/mouse insulin (Linco, St. Charles, MO) as previously described (137,266,527).

4.3.6 In vitro studies in islets

Islets of untreated rats were cultured for 48h in RPMI 1640 without antioxidants, containing 0.4 mM oleate in 0.5% BSA with or without 4 µM BPV, a dose based on (531). Thereafter, islets were pre-incubated for 1 h at 37°C in KRBH supplemented with 2.8 mM glucose and insulin secretion was measured as in the ex vivo studies or islets were collected and lysed for Western blot analysis of Akt phosphorylation.

4.3.7 Western blots

Western blots were performed in cultured rat islets (Akt) and in freshly isolated mouse islets (PTEN). Following isolation surgery, islets were centrifuged (7000 rpm; 30 seconds), and the pellets (~100 islets per sample) were lysed in an Eppendorf tube containing 100 µL of lysis buffer (RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS), supplemented with 1 mM PMSF, 10 µg/mL aprotinin, and 10 µg/mL leupeptin), and incubated on ice for 45 minutes. The tube was agitated every 5 minutes to promote lysis. The samples were then centrifuged at 13,000 rpm for 10 minutes at 4 degrees Celsius. The supernatant was collected and protein concentration was measured using the BCA protein assay (Pierce). 30 µg of protein from each islet sample was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with primary antibodies against phospho-Akt (Cell Signaling Technology Cat# 9271, RRID:AB_329825), total Akt (Cell Signaling Technology Cat# 9272, RRID:AB_329827), PTEN (Cell Signaling Technology Cat# 9552, RRID:AB_329836) and β-actin (Santa Cruz Biotechnology Cat# sc-47778, RRID:AB_626632). Secondary antibody conjugated to HRP and an enhanced chemiluminescence system (Amersham Biosciences) were used for detection. For phospho-Akt and total Akt, Kodak Imager 4000pro (Carestream, USA) was used to image membranes. The bands obtained from immunoblotting were quantified using ImageJ software (National Institutes of Health). Samples
from the hypothalami of mice used in hyperglycemic clamps were also used for Western blot analysis, to determine PTEN expression.

4.3.8 Plasma assays

Plasma assays were performed as previously described (137,266). Briefly, plasma glucose was measured on a Beckman Analyzer II (Beckman, Fullerton, CA) in rats and on a HemoCue Glucose 201 Analyzer (HemoCue Inc., CA, USA) in mice. Plasma FFA were measured with an enzymatic colorimetric kit (Wako Industries, Neuss, Germany). Radioimmunoassays specific for rat/mouse insulin and C-peptide (Linco, St. Charles, MO) were used to determine their plasma concentrations.

4.3.9 Statistics

Statistics were performed as described in General Methods.
4.4 Results

4.4.1 Studies in rats

4.4.1.1 In vivo hyperglycemic clamps

Throughout the 48h infusion period both oleate and olive oil treated rats had higher plasma FFA than rats that were treated with saline or BPV alone (Table 4.1.A,B).

Table 4.1 (A)

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>FFA (mM)</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>0</td>
<td>0.716±0.093</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.577±0.074</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.608±0.060</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.663±0.059</td>
<td>-</td>
</tr>
<tr>
<td>OLE</td>
<td>0</td>
<td>0.640±0.068</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.810±0.068</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.847±0.087</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.093±0.086</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>OLE+BPV</td>
<td>0</td>
<td>0.889±0.129</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.916±0.148</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>1.263±0.125</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>1.155±0.183</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>BPV</td>
<td>0</td>
<td>0.536±0.088</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.607±0.122</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.477±0.034</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.502±0.076</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 4.1 (B)

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (h)</th>
<th>FFA (mM)</th>
<th>Significance (vs. SAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>0</td>
<td>0.716±0.093</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.577±0.074</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.608±0.060</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.663±0.059</td>
<td>-</td>
</tr>
<tr>
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<td>NS</td>
</tr>
<tr>
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<td>p&lt;0.01</td>
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<tr>
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<td>p&lt;0.01</td>
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<tr>
<td></td>
<td>48</td>
<td>1.225±0.196</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>OLO+BPV</td>
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<td>NS</td>
</tr>
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<td>p&lt;0.01</td>
</tr>
<tr>
<td>BPV</td>
<td>0</td>
<td>0.536±0.088</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.607±0.122</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.477±0.034</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>0.502±0.076</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4.1 Plasma FFA levels during the 48h infusion period of rats infused with oleate or olive oil and with or without BPV. Rats were treated with: 1) Saline alone (SAL, n=12), 2) Oleate alone (OLE, 1.3 µmol/min, n=10), 3) Oleate+Bisperoxovanadate (OLE+BPV, oleate-1.3µmol/min, BPV-0.0025 µmol/kg/ min, n=8), 4) Bisperoxovanadate alone (BPV, n=7). Data are means±SEM (A). Rats were treated with: 1) Saline alone (SAL, n=12), 2) Olive oil alone (OLO, 5.5 µl/min, n=9), 3) Olive oil+Bisperoxovanadate (OLO+BPV, OLO-5.5 µl/min, BPV-0.0025 µmol·kg/min, n=8), 4) Bisperoxovanadate alone (BPV, n=7). Data are means±SEM (B).
Following the 48h infusions, we evaluated β-cell function in vivo using hyperglycemic clamps. As expected, FFA levels decreased during the clamp because of hyperinsulinemia, which stimulates FFA uptake, but remained higher in oleate or olive oil treated rats (Figure 4.1.A,B). Plasma glucose was similar in all groups in the basal state and during the clamp, as per experimental design (Figure 4.1.C,D). The glucose infusion rate (GINF) necessary to maintain the target glucose level was lower in both oleate and olive oil than saline treated group (Figure 4.1.E,F). BPV alone had no effect on GINF. When BPV was added to the oleate infusion, GINF was partially restored during the first step of the hyperglycemic clamp (Figure 4.1.E), but was not affected during the second step of the clamp. When BPV was used in combination with olive oil, GINF was completely restored to control levels (Figure 4.1.F). Basal insulin and C-peptide levels were similar in all groups. During the clamp, plasma insulin and C-peptide were lower than saline and similar to oleate in rats treated with BPV alone or BPV in combination with oleate, but not in the groups treated with olive oil or BPV in combination with olive oil (Figure 4.1.G-J).
Figure 4.1 Plasma FFA, plasma glucose, glucose infusion rate (GINF), plasma insulin and plasma C-peptide during two-step hyperglycemic clamps with/without 48h oleate or olive oil infusion and with/without co-infusion of BPV in rats. Groups are described in the legend of Table 1. Data are means±SEM. †† p<0.01 oleate/olive oil infused groups vs. non-oleate/olive oil infused groups throughout the hyperglycemic clamp (A,B); ** p<0.01 OLE, OLE+BPV, OLO, BPV vs. SAL throughout the hyperglycemic clamp (E-J).
As evidenced by Figure 4.2.A,B and as expected, the M/I showed a tendency to increase with BPV alone, or in combination with oleate, however the increases were only significant during the second step of the hyperglycemic clamp. In the group treated with olive oil M/I was reduced, an effect which was prevented by the addition of BPV. The C-Peptide/Insulin ratio was calculated as an index of insulin clearance, which was expected to increase with BPV as a result of insulin sensitization. Indeed, in the groups exposed to BPV there was a significant increase in this ratio (Figure 4.2.C,D). β-cell function calculated as disposition index (DI) was impaired with both oleate and olive oil infusions, but completely restored with the addition of BPV (Figure 4.2.E,F). BPV alone also had an effect to increase the DI throughout the clamp.
Figure 4.2 Sensitivity index (M/I), insulin clearance index and disposition index (DI) during two-step hyperglycemic clamps with/without 48h olate or olive oil infusion and with/without co-infusion of BPV in rats. Groups are described in the legend of Table 1. Data are means±SEM. ** p<0.01 vs. SAL (A,B,E,F); ‡ p<0.05 BPV and OLE+BPV vs. SAL or OLE (C,D).
4.4.1.2 *Ex vivo* studies in islets

To directly address the effect of *in vivo* infusion of BPV in β-cell function independent of peripheral insulin sensitivity and insulin clearance we evaluated insulin secretion in isolated islets. BPV infusion prevented the GSIS decrease induced by oleate or olive oil infusion at both 13 mM glucose and 22 mM (Figure 4.3.A,B). No significant effect of BPV alone was observed at any glucose concentration.

![Graph](image)

**Figure 4.3** Insulin secretory response to glucose of freshly isolated islets of 12 week old normal female Wistar rats treated for 48h with oleate or olive oil with or without BPV. Rats were treated for 48h with: 1) Saline (SAL, n=16); 2) Oleate alone (OLE; 1.3 µmol/min; n=14); 3) Oleate + Bisperoxovanadate (OLE+BPV; oleate-1.3 µmol/min, BPV-0.0025 µmol/kg/min; n=8); 4) BPV alone (n=10) (A); 1) SAL (n=16); 2) Olive oil alone (OLO; 5.5 µl/min; n=12); 3) OLO+BPV (n=6); 4) BPV alone (n=10) (B). Data are means±SEM. *** p<0.001 OLE or OLO vs. SAL; ** p<0.01 OLE or OLO vs. SAL.
4.4.1.3 *In vitro* studies in islets

We performed *in vitro* studies in cultured islets to investigate whether addition of BPV to the media can prevent oleate-induced β-cell dysfunction directly in islets. Similar to our previous studies (265,527), 0.4 mM oleate in 0.5% BSA decreased GSIS (Figure 4.4.A). Coincubation with 4 µM BPV restored GSIS and BPV alone had no significant effect on insulin secretion.

![Figure 4.4 Insulin secretory response to glucose and phosphorylated Akt in cultured islets exposed for 48h to oleate or vehicle with or without BPV. Islets were exposed to: 1) Bovine serum albumin (BSA, 0.5%; CON; n=7); Oleate (OLE; 0.4 mM in BSA; n=14); 3) Oleate + Bisperoxovanadate (OLE+BPV; oleate-0.4 mM in BSA, BPV-4 µM; n=10); 4) BPV (n=7) (A). Representative blot and quantification of phosphorylated Akt/total Akt expression in cultured islets exposed for 48h to: BSA or OLE with/without BPV (CON, n=5; OLE, n=4; OLE + BPV, n=5; BPV, n=4) (B). Data are means±SEM. § p<0.01 OLE vs. all; ¶ p<0.05 vs. OLE.](image-url)
4.4.1.4 Measurement of Akt phosphorylation

Expression of phosphorylated and total Akt was assessed using Western blot analysis, following 48h incubation of rat islets with or without oleate as above (in the absence of insulin) in the presence or absence of BPV. Oleate did not have any effect on Akt phosphorylation, however co-incubation of oleate with BPV increased phosphorylation of Akt compared to oleate alone (Figure 4.4.B). There was also a strong tendency for BPV alone to increase phospho-Akt compared to BSA control, however this did not reach significance.

4.4.2 Studies in mice

4.4.2.1 Mouse weights and PTEN protein expression

Unexpectedly, there were no significant differences in weight between control (31.5±1.9g), β-cell specific heterozygous PTEN-knockout mice (31.2±1.0g), or β-cell specific homozygous PTEN-knockout mice (30.3±0.9g).

PTEN protein was expressed in control islets and undetectable in islets of β-cell specific homozygous PTEN-knockout mice (Figure 4.5.A). Similarly, PTEN was expressed in hypothalami of control mice and the level of expression in β-cell specific homozygous PTEN-knockout mice was drastically reduced (Figure 4.5.B).
Figure 4.5 PTEN protein expression in islets and hypothalamus of PTEN knockout mice and plasma FFA levels, plasma glucose, and glucose infusion rate (GINF) in PTEN knockout mice during hyperglycemic clamps. 12-wk old β-cell specific homozygous and heterozygous PTEN-knockout mice and littermate controls were infused intravenously for 48h with saline (SAL; Con (n=5), Het (n=7), KO (n=5)) or oleate (OLE; 0.4µmol/min; Con (n=6), Het (n=8), KO (n=5)), which was followed by a 22 mM hyperglycemic clamp. Data are means±SEM. * p<0.05 vs. respective SAL; ** p<0.01 vs. KO-SAL (C); * p<0.05 vs. Con-SAL; ## p<0.01 vs. KO-OLE (E).
4.4.2.2  *In vivo* hyperglycemic clamps

Following 48 hour oleate infusion and prior to the hyperglycemic clamps, plasma FFA levels were higher in oleate infused groups than in their saline infused counterparts, but they decreased during the clamp as expected (Figure 4.5.C).

Glucose levels were raised to ~22mM in all groups (Figure 4.5.D). The glucose infusion rate (GINF) was decreased in oleate-treated control mice compared to saline-treated controls. This decrease was prevented in oleate-treated β-cell specific homozygous and heterozygous PTEN-knockout mice (Figure 4.5.E). Basal insulin (Figure 4.6.A) and C-peptide (Figure 4.6.B) levels did not differ between groups. During the clamp both insulin and C-peptide levels tended to be higher in each oleate-infused group compared to its respective saline-infused group.
Figure 4.6 Plasma insulin, plasma C-peptide, sensitivity index (M/I), insulin clearance index, and disposition index (DI) in PTEN knockout mice during hyperglycemic clamps and insulin secretion in islets of heterozygous PTEN-knockout mice. 12-wk old β-cell specific homozygous and heterozygous PTEN-knockout mice and littermate controls were infused intravenously for 48h with saline (SAL; Con (n=5), Het (n=7), KO (n=5)) or oleate (OLE; 0.4µmol/min; Con (n=6), Het (n=8), KO (n=5)), which was followed by a 22 mM hyperglycemic clamp. Insulin secretion (F) in islets isolated from 12-wk old β-cell specific heterozygous PTEN-knockout mice and littermate controls infused i.v. for 48h with saline (SAL: Con (n=8), Het (n=9)) or oleate (OLE: 0.4µmol/min; Con (n=6), Het (n=6)). Data are means±SEM. * p<0.05 vs. respective SAL; ** p<0.01 vs. respective SAL.
The M/I for each oleate-infused group tended to be decreased compared to its respective saline-infused group, but significance was not reached in homozygous or heterozygous knockout mice (Figure 4.6.C). In contrast, the M/I in the wild-type mice infused with oleate was significantly decreased compared to their saline-infused controls. The index of insulin clearance tended to be decreased in each of the oleate-infused groups compared to its respective saline-infused group, but significance was not reached in any group (Figure 4.6.D). The DI was significantly decreased in oleate-treated control mice compared to saline-treated controls. This decrease was partially prevented in oleate-treated β-cell specific homozygous and heterozygous PTEN-knockout mice (Figure 4.6.E).

4.4.2.3  Ex vivo studies in islets

To directly address the effect of oleate infusion *in vivo* on β-cell function independent of peripheral insulin sensitivity and insulin clearance, we performed *ex vivo* studies in isolated islets. β-cell specific homozygous PTEN-knockout mice were not used for *ex vivo* experiments as *in vivo* studies did not show any differences in β-cell function between the homozygous and heterozygous knockout mice. Islets of control mice infused with oleate had decreased levels of GSIS compared to saline. This decrease was completely prevented in oleate-infused β-cell specific heterozygous PTEN-knockout mice (Figure 4.6.F).
4.5 Discussion

In this study we show that the insulin mimetic tyrosine phosphatase inhibitor bisperoxovanadate (BPV), which stimulated Akt phosphorylation, prevented the impairment in β-cell function induced by prolonged FFA exposure in vivo in rats and ex vivo and in vitro in rat islets. We also show that β-cell specific deletion of an inhibitor of insulin signaling (i.e. PTEN) protects against β-cell dysfunction induced selectively by circulating fat in vivo and ex vivo in mice and mouse islets, respectively. Together, these data support a role of β-cell insulin resistance in fat-induced β-cell dysfunction.

In our in vivo clamp studies in rats both insulin and C-peptide responses to glucose were lower in rats treated with oleate, but not olive oil as in our previous study (527). This is because with olive oil the β-cell attempted to compensate for insulin resistance. The insulin resistance induced by olive oil may be due to the amount (16%) of saturated fat contained in olive oil, as opposed to oleate, which is a monounsaturated fatty acid and also to the effect of the triglycerides of olive oil to decrease insulin sensitivity (527).

As expected, insulin sensitivity was increased by BPV and the olive oil induced insulin resistance was prevented. An even greater effect of BPV was seen on increasing insulin clearance. Since insulin clearance is mainly a liver function and is dependent on insulin receptor tyrosine phosphorylation, these results suggest that liver is a primary target tissue for BPV. When disposition index (DI) was used to assess β-cell function, there was a decrease with both oleate and olive oil infusions. This effect was completely prevented by addition of BPV to both fat infusions. An interesting finding was the effect of BPV alone, which increased β-cell function in vivo, as measured by the DI but did not affect β-cell function ex vivo or in vitro. β-cell function in vivo has to be considered not only in relationship with insulin resistance, but also with insulin clearance. Since BPV alone increased C-peptide/insulin ratio (index of insulin clearance), β-cells presumably had to secrete more to compensate for insulin disappearance. In the oleate treated rats despite normalizing the DI, coinfusion of BPV did not normalize GINF at 22 mM glucose. This is likely because insulin clearance increased in the oleate plus BPV treated group more than the β-cell could compensate for. Insulin clearance did not increase in the olive oil plus BPV group where both GINF and DI were normalized. Since similar signaling mechanisms (i.e. insulin receptor
tyrosine phosphorylation) mediate both insulin clearance and insulin sensitivity, this is consistent with hepatic insulin resistance with olive oil.

To assess β-cell function independent of systemic factors influencing the β-cell response to glucose in vivo (i.e., the prevailing insulin sensitivity and insulin clearance increased by BPV), we examined GSIS ex vivo in isolated islets. Furthermore, to eliminate any indirect effect of in vivo treatments in the 48h preceding islet isolation on GSIS, we exposed islets directly to fat and BPV in vitro. 48h infusion of oleate or olive oil impaired GSIS ex vivo in isolated islets, consistent with our in vivo findings and with our previous study (527). 48h infusion of BPV protected the islets from the FFA-mediated impairment in GSIS. Direct exposure of islets for 48h to oleate decreased the insulin response at high glucose and in vitro exposure to BPV restored GSIS, similar to our ex vivo model. BPV was also found to enhance insulin signaling in islets as it increased Akt phosphorylation. Although Akt phosphorylation did not decrease with oleate, islets were not stimulated by insulin or IGF-1 unlike previous studies that showed impaired Akt activation by FFA (231,451,452). Nonetheless, the effect of BPV to increase Akt phosphorylation implicates upregulation of the insulin signaling pathway in β-cells as a mechanism through which β-cell dysfunction induced by fat may be prevented. Consistent with this notion, we recently demonstrated that exposure to oleate results in IRS-1 serine 307 phosphorylation in islets (527).

Previously, vanadate has been used in vitro in islets of diabetic GK rats where it was found to increase both basal as well as glucose-stimulated insulin secretion. However, it only increased insulin secretion at basal or low stimulatory glucose levels in islets of Wistar control rats, an effect prevented the PI3K inhibitor Wortmannin (531,532). Other authors, using the same dose as our study, showed that 72h treatment with vanadate improved GSIS (533). No study to our knowledge has used vanadate in combination with fatty acids.

Since vanadate is a non-specific tyrosine phosphatase inhibitor and has systemic effects when used in vivo, we used β-cell specific PTEN (negative regulator of insulin signaling) knockout mice for further in vivo studies. As in previous studies the β-cell specific PTEN-knockout mice with Cre promoter expression driven by RIP2 had decreased hypothalamic expression of PTEN, in addition to the deletion of PTEN in islets. In contrast to previous findings (437,528), however, our homozygous knockout mice did not show growth retardation. The discrepant findings between our study and previous studies by some of us (437,534) may be explained in part by the difference in
the genetic mix of C57BL/6 versus 129J in our colony that was not deliberately maintained on a C57BL/6-129J mixed background, unlike that of the previous studies (437,534).

β-cell specific PTEN-knockout mice were reported to have increased insulin signaling in β-cells and this protected against glucose intolerance in high-fat diet-fed and db/db mice and prevented streptozotocin-induced diabetes (437,528). These previous studies support a role for β-cell insulin resistance in fat-induced β-cell dysfunction, but they did not assess the role of β-cell insulin resistance in the β-cell dysfunction induced selectively by elevated circulating levels of fat.

In order to better understand the effect of oleate on β-cell function, and in the context of the ambient insulin sensitivity, insulin and C-peptide levels were assessed during hyperglycemic clamps and used to calculate the M/I and DI. Both insulin and C-peptide levels tended to increase in oleate-infused mice compared to their saline-infused counterparts, which is consistent with the previously demonstrated effect of oleate to induce insulin resistance in mice (similar to olive oil in rats) (266,527) and thereby cause β-cells to increase the level of insulin secretion in an attempt to compensate. In line with this, M/I was decreased in control mice infused with oleate and the decrease was partially prevented in both heterozygous and homozygous β-cell specific PTEN-knockout mice. This finding is not entirely in agreement with previous studies by some of our group that showed that β-cell specific PTEN-knockout mice have enhanced insulin sensitivity and are completely protected against insulin resistance induced by high fat diet (437,534). The discordant results may be due to the greater C57BL/6 genetic mixture in the background of our colony, which would be consistent with the susceptibility of C57BL/6 mice to insulin resistance, to the fact that our mice were not growth-retarded, and/or to differences in age as mice in Nguyen’s study were 8-10 weeks old (late adolescence – early adulthood) (534,535) whereas mice in ours were 11-13 weeks old (mid – late adulthood) (535). Interestingly, the mice studied by Stiles et al., who also found no significant changes in insulin sensitivity in β-cell specific PTEN-knockout mice (528), had similar age to ours and were also not deliberately maintained on a mixed background.

DI was decreased in oleate-infused control mice compared to saline-infused controls and this decrease was partially prevented in both heterozygous and homozygous β-cell specific PTEN-knockout mice. Protection against fat-induced β-cell dysfunction is in line with previous findings (437). In our study, however, according to the DI, mice were only partially protected against fat-induced β-cell dysfunction.
In order to determine the role of β-cell insulin signaling on insulin secretion specifically, independent of effects of insulin sensitivity and insulin clearance, we performed *ex vivo* insulin secretion studies. Insulin secretion was decreased in islets of control mice infused with oleate and this decrease was prevented in islets of β-cell specific heterozygous PTEN-knockout mice. In the *ex vivo* islet studies, prevention of β-cell dysfunction was complete despite the mice being heterozygous, presumably indicating that part of the DI decrease *in vivo* was due to β-cell rest because insulin levels were already elevated by the oleate-induced decrease in insulin clearance. The *in vivo* and *ex vivo* data in this mouse model, taken together, support the hypothesis that upregulation of insulin signaling in β-cells prevents fat-induced β-cell dysfunction.

Although previous *in vitro* studies have yielded conflicting results (442,445,522–525,536), studies performed *in vivo* implicate an essential role of β-cell insulin signaling in β-cell function as βIRKO mice have impaired insulin secretion (435,536). This was attributed to decreased PDX-1 mediated expression of not only the insulin gene, but also of GLUT2 and glucokinase which regulate glucose sensing, an essential step in stimulus secretion coupling (435,536). In humans, exogenous insulin administration, although it decreases concomitant endogenous insulin secretion by a neural reflex (447), has been shown to potentiate subsequent GSIS (448–450).

In summary, we show that both pharmacologic as well as genetic upregulation of insulin signaling in rodents protects against fat-induced β-cell dysfunction *in vitro, ex vivo* and *in vivo*. This supports the hypothesis that β-cell insulin resistance plays an important role in fat-induced β-cell dysfunction.
Study 3 — NOD1 plays a causal role in palmitate induced β-cell dysfunction in vitro and in vivo

The results of this study are incorporated in a manuscript to be resubmitted to Diabetes once experiments in β-cell specific NOD1 knockout mice are performed:


My contributions to the manuscript were performing the majority of the experiments, except for the PCR, and for the hyperglycemic clamps with the NOD1 ligand other members of the lab were also involved. Lastly, I drafted the manuscript.
5.1 Abstract

Studies by our group suggest the intracellular nucleotide-binding oligomerization domain (NOD) receptors of innate immunity play a significant role in metabolism and may contribute to type 2 diabetes (T2D) induced by high fat diet. Saturated fatty acids have been reported to directly activate NODs and deletion of NOD1 protects against glucose intolerance induced by high fat diet. This suggests a β-cell role of NOD1 because measures of glucose tolerance are measures of β-cell performance. Thus, we hypothesized that saturated fatty acid-induced β-cell dysfunction is mediated in part by NOD1 in the β-cell. We sought to determine whether islets express NOD1 and the role it plays in fat-induced β-cell dysfunction. Both mouse and human islets express NOD1 mRNA. The NOD1 activator FK565 decreased β-cell function and NOD1-KO islets were protected from palmitate, but not oleate-induced β-cell dysfunction in vitro. Similarly, in vivo, FK565 decreased β-cell function and NOD1-KO mice were protected from palmitate-induced β-cell dysfunction. These data demonstrate NOD1 is present in mouse and human islets and plays a causal role in saturated fatty acid-induced β-cell dysfunction in vitro and in vivo. We identify NOD1 as a potential therapeutic target to prevent or treat β-cell dysfunction in T2D.
5.2 Introduction

Chronic elevation of free fatty acids (FFA) is known to impair β-cell function and lead to type 2 diabetes (T2D). This is mediated in part by the pattern recognition receptors (PRR) of innate immunity toll-like receptors (TLR) (55).

TLR are membrane PRRs known to mediate fat-induced insulin resistance (537) and β-cell dysfunction (55) in mice. Emerging evidence suggests NOD-like receptors also play an important role in β-cell function. NLRP3 (NOD Leucine-rich Repeat and Pyrin domain containing 3), which is part of the NLRP3 inflammasome that activates interleukin-1β, is involved in fat-induced β-cell dysfunction (538). Our group has implicated the nucleotide-binding oligomerization domain 1 (NOD1) receptor in obesity-induced diabetes (464), however its role in β-cells is unknown.

The bacterial cell wall component γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP) of gram negative bacteria is the endogenous ligand of NOD1, which induces a proinflammatory response through activation of nuclear factor-κB and c-Jun N-terminal kinase (403). High-fat feeding can increase intestinal permeability and thereby enhance the translocation of bacterial products, including NOD1 ligands, into the circulation (131,539). Furthermore, saturated fatty acids directly activate NODs in intestinal epithelial cells (540) and adipocytes (541), suggesting there may be a direct effect of circulating FFA on NODs.

Accordingly, NOD1 is involved in insulin resistance and inflammation induced by high fat diet (464), and NOD1-null mice have better glucose tolerance than controls when fed a high fat diet (131). This implicates a role of NOD1 in fat-induced β-cell dysfunction because glucose tolerance reflects β-cell function as healthy β-cells are able to compensate for changes in insulin sensitivity (288). Furthermore, NOD1 is expressed ubiquitously (403). Therefore, we hypothesized that saturated fat-induced β-cell dysfunction is mediated by NOD1 at the β-cell level.

We show here that the \textit{NOD1} gene is expressed in both mouse and human islets. Importantly, we show for the first time that a NOD1 ligand impairs β-cell function \textit{in vitro} and \textit{in vivo} and that NOD1 deletion protects against palmitate-induced β-cell dysfunction \textit{in vitro} and \textit{in vivo}. 
5.3 Methods

5.3.1 Animals

Procedures are in accordance with the Canadian Council on Animal Care Guidelines and were approved by the Animal Care Committee of the University of Toronto. NOD1-knockout (KO) mice (464) and their littermate controls (on a C57BL/6J background) were used. Another batch of wild-type C57BL/6J mice were used for ligand experiments. Islets of NOD1/2-KO mice (542) were used as negative controls in PCR analysis.

5.3.2 Human islets

Human islets from healthy donors were provided by the IsletCore and Clinical Islet Laboratory (University of Alberta, Canada) and were approved for use by the University of Alberta Health Research Ethics Board. Islets were cultured overnight in low glucose DMEM media (GIBCO, #11885-084) with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine before use.

5.3.3 Microarray and qPCR analyses

Microarray analysis was performed by Dr. Kacey Prentice from the laboratory of Dr. Michael Wheeler as previously described (543) using the Affymetrix Mouse 430 2.0 Gene Chip at the University Health Network microarray center (Toronto, Canada). Quantitative real-time PCR was performed by Charles Maisonneuve from the laboratory of Dr. Dana Philpott as previously described (544).

5.3.4 Studies in cultured islets

Islets of wild-type and NOD1-KO mice were isolated as previously described (266). Wild-type islets were cultured for 48h in RPMI 1640 without antioxidants with or without 0.4 mmol/l oleate
(negative control) or palmitate in 0.5% FFA-free bovine serum albumin (BSA) and with or without the NOD1 ligand DAP (FK565; 5 μg/ml) as in our previous studies (266,464). Islets of NOD1-KO mice were similarly incubated with or without oleate or palmitate.

5.3.5 Glucose stimulated insulin secretion (GSIS) assay

GSIS assays were performed following *in vitro* islet incubation as previously described (266).

5.3.6 Surgeries, injections, and intravenous infusions

Jugular vein cannulation was performed in wild-type and NOD1-KO mice for *in vivo* hyperglycemic clamp experiments as in (266). 4-5 days after surgery, mice were either injected with the NOD1 ligand FK565 (10μg i.p.) or vehicle 6h prior to the clamps (464) or were infused for 48h with ethylpalmitate (0.12 μmol/min) or control (ethanol vehicle or saline as there was no significant difference between the two in any parameters including disposition index [DI], and DI actually tended to be greater in the ethanol vehicle [7.8±0.9] than in the saline group [4.7±0.6]). 48h infusion was followed by 2h hyperglycemic clamps. Palmitate is the most abundant circulating saturated fatty acid but unbound palmitate cannot be infused. Ethylpalmitate is safe for infusion and since rodent blood rapidly hydrolyzes fatty acid ethyl esters to fatty acids and ethanol, this infusion elevates plasma FFA levels (55).

5.3.7 Hyperglycemic clamps, insulin sensitivity index and disposition index

Hyperglycemic clamps were performed as previously described (266). The sensitivity index (SI) was calculated as Glucose Infusion Rate/[Insulin] (266,288). The disposition index (DI), an *in vivo* measure of β-cell function, was calculated as Glucose Infusion Rate/[Insulin] * [C-peptide] (266,288).
5.3.8 Plasma assays

Plasma glucose was measured on a HemoCue Glucose 201 Analyzer using HemoCue Glucose 201 Microcuvettes (HemoCue Inc., CA, USA). Insulin and C-peptide were measured using ELISAs (AIS, Li Ka Shing Faculty of Medicine, Hong Kong University, Hong Kong). FFA were measured with an enzymatic colorimetric kit (Wako Industries, Neuss, Germany).

5.3.9 Statistics

Statistics were performed as described in General Methods.
5.4 Results

5.4.1 NOD1 mRNA expression

NOD1 mRNA expression in islets of wild-type mice was much lower than in macrophages but higher than that in islets of NOD1/2 KO mice (Figure 5.1A) and comparable to that in small intestine organoids (Figure 5.1B). Microarray analysis showed that in human islets NOD1 mRNA expression was similar to that of a known β-cell gene (Figure 5.1C).

Figure 5.1 NOD1 mRNA expression in mouse and human islets. Islets of wild-type (WT; n=6) and NOD1/2 double knockout (DKO; negative control; n=6) C57BL/6J mice and macrophages (positive control; n=3) were assessed for mRNA expression of NOD1, expressed relative to the house-keeping gene RPL19 (A). Islets of wild-type C57BL/6J mice (WT; n=3) and small intestine organoids (positive control; n=2) were assessed for mRNA expression of NOD1, relative to RPL19 (B). Microarray analysis was performed in human islets and expression is shown for NOD1 (NOD1; n=3) and the β-cell marker KCNJ11 (KCNJ11; n=3), relative to the background signal of the microarray (C). Data are means ± SEM. * p<0.001 vs. macrophages; † p<0.05 vs. DKO islets.
5.4.2 The effect of the NOD1 ligand on β-cell function

Incubation of islets in the presence of NOD1 ligand decreased insulin secretion at 22 mmol/l glucose (maximally stimulatory) compared to control (Figure 5.2A). *In vivo*, β-cell function is assessed by the disposition index (DI), which takes into account insulin sensitivity (266,288). DI/Δglucose during hyperglycemic clamps was decreased after NOD1 ligand injection compared to control (Figure 5.2B).

**Figure 5.2 β-cell function in vitro and in vivo in the presence or absence of a NOD1 activator.** Insulin secretion assays were performed in islets of C57BL/6J mice incubated for 18h in the presence (NOD1 activator) or absence (Control) of the NOD1 activator DAP (5μg/ml) (Control, n=12; NOD1 activator, n=7) (A). 2-hour hyperglycemic clamps were performed in C57BL/6J mice injected with a NOD 1 activator (10 μg FK565) or vehicle i.p. 6 h before determination of β-cell function using the disposition index (DI) normalized by the increase in glucose (Δglucose) (Control, n=4; NOD1 activator, n=4) (B). Data are means ± SEM. * p<0.001 vs. Control; † p<0.05 vs. Control.

DI was normalized by the increase in glucose (Δglucose) because injection of NOD1 ligand tended to decrease basal plasma glucose compared to control, as in our previous studies (464), although the levels in both groups were maintained at ~20 mmol/l during the clamps (Figure 5.3A). The glucose infusion rate/Δglucose was similar in both groups (Figure 5.3B). Basal insulin (Figure 5.3C) and C-peptide (Figure 5.3D) did not differ between groups. Insulin (Figure 5.3C) and C-peptide (Figure 5.3D) levels tended to be higher in NOD1 ligand-treated mice compared to controls during the clamp indicating that β-cells attempted to compensate for a tendency of insulin
sensitivity (SI/Δglucose) to be lower than control (Figure 5.3E). The C-peptide/insulin ratio showed that insulin clearance that shares similar molecular mechanisms to those involved in insulin sensitivity, also tended to decrease (Figure 5.3F), maintaining glucose infusion rate/Δglucose unchanged despite decreased DI/Δglucose.

Figure 5.3 Plasma glucose, glucose infusion rate/Δglucose, plasma insulin, plasma C-peptide, sensitivity index/Δglucose, and insulin clearance index during a hyperglycemic clamp following injection of a NOD1 activator or vehicle control 12-wk old C57BL/6J mice were injected with a NOD1 activator (10 μg FK565; n=4) or vehicle control (n=4) i.p. 6 h prior to the clamp. Data are means ± SEM.
5.4.3 The effect of NOD1 deletion on β-cell lipotoxicity

5.4.3.1 In vitro GSIS assays

Insulin secretion in islets of wild-type mice treated with the saturated fatty acid palmitate or the unsaturated fatty acid oleate decreased compared to the insulin secretion in islets treated with vehicle. The palmitate-, but not oleate-induced decrease was prevented in islets of NOD1-KO mice. Deletion of NOD1 alone did not have any effect on insulin secretion (Figure 5.4A,B).

\[\text{Figure 5.4} \text{ Insulin secretion in NOD1KO and wild-type islets in the presence or absence of palmitate or oleate. Glucose-stimulated insulin secretion assays were performed in islets of NOD1 knockout (NOD1KO) and wild-type (WT) littermate C57BL/6J mice incubated for 48h in the presence of palmitate (PAL, 0.4 mmol/l in 0.5% BSA) (A) or oleate (OLE, 0.4 mmol/l in 0.5% BSA) (B) or BSA alone (CON) (A,B). Data are means ± SEM. * p<0.001 vs. WT-CON and NOD1KO-PAL (A); † p<0.05 vs. WT-CON (B). (A): WT-CON, n=7; WT-PAL, n=11; NOD1KO-PAL, n=9; NOD1KO-CON, n=6; (B): WT-CON, n=7; WT-OLE, n=6; NOD1KO-OLE, n=6; NOD1KO-CON, n=7.}\]
5.4.3.2  *In vivo* hyperglycemic clamps

Following 48 hour ethylpalmitate infusion, plasma FFA levels were higher in ethylpalmitate infused groups than in the wild-type control group and decreased during the clamp as expected (266) (Figure 5.5A). Glucose levels were raised to ~20mM in all groups (Figure 5.5B). The glucose infusion rate was decreased in ethylpalmitate-treated wild-type mice compared to all other groups (Figure 5.5C). Basal insulin (Figure 5.5D) and C-peptide (Figure 5.5E) did not differ between groups. Insulin levels tended to be higher in ethylpalmitate-treated mice compared to control mice (Figure 5.5D) and C-peptide levels were higher in the NOD1-KO mice treated with ethylpalmitate compared to wild-type mice treated with ethylpalmitate during the clamp (Figure 5.5E), indicating increased insulin secretion, consistent with a compensatory response to insulin resistance. The SI (Figure 5.5F) and insulin clearance index (Figure 5.5G) in the wild-type ethylpalmitate group tended to be decreased compared to all other groups but the difference did not reach significance. The DI was decreased in ethylpalmitate-treated wild-type mice compared to wild-type controls. This decrease was prevented in ethylpalmitate-treated NOD1-KO mice (Figure 5.5H).
Figure 5.5 Plasma FFA, plasma glucose, glucose infusion rate (GINF), plasma insulin, plasma C-peptide, sensitivity index (M/I), insulin clearance index and disposition index (DI) during hyperglycemic clamps in NOD1KO/WT mice infused with/without ethylpalmitate. 12-wk old NOD1 knockout (NOD1KO) mice and wild-type (WT) littermate controls were infused intravenously for 48h with or without (CON) ethylpalmitate (PAL, 0.12µmol/min). Data are means±SEM. † p<0.05 vs WT-CON (A); ‡ p<0.01 vs. all (C); § p<0.05 vs. WT-PAL (E); ¶ p<0.05 vs. all (H). WT-CON, n=8; WT-PAL, n=8; NOD1KO-PAL, n=10; NOD1KO-CON, n=7.
5.5 Discussion

Our data show NOD1 mRNA is expressed in both mouse and human islets, which is in keeping with previous data, although further studies are required to determine whether NOD1 mRNA is elevated in islets of patients with T2D (545). Islet mRNA expression supports the hypothesis that the improved glucose tolerance in high fat-fed NOD1 knockout mice (131) may indeed be due to a direct effect in islets. Nevertheless, the effect could also be attributed to improved insulin sensitivity (131,464) since glucose tolerance is not a direct measure of β-cell function.

The disposition index (DI) is the gold standard for assessing β-cell function in vivo since it accounts for changes in insulin sensitivity (288). Our results show reduced DI in response to injection of NOD1 ligand, which implicates a role of NOD1 in β-cell dysfunction in vivo. However, this still leaves the possibility of NOD1 in peripheral tissues as an indirect cause of the effect on β-cells. Therefore, we incubated islets with NOD1 ligand in vitro, which decreased insulin secretion, demonstrating a direct role of NOD1 in islets. Using whole islets, however, we cannot exclude the possibility of a role of NOD1 in resident macrophages, especially as it has been shown that NOD1 activation can lead to polarization of macrophages to the proinflammatory M1 phenotype (539). Nonetheless, it is unlikely macrophages are major contributors. In islets, they account for only 0.5% of cells (546) and studies that have shown a macrophage role in β-cell dysfunction have found infiltration of macrophages rather than activation of resident macrophages (55,547). This infiltration cannot occur in vitro, although further studies specifically in β-cells would help exclude any effect of macrophages.

As described above, our group has shown that deletion of NOD1 prevents fat-induced insulin resistance in mice (464). In humans, NOD1 mRNA expression was found to be elevated in the myocardium (548) and monocytes (549) of patients with T2D and in adipocytes (550) and placenta (551) of women with gestational diabetes. Furthermore, a polymorphism in the human NOD1 gene has been linked to predisposition to saturated fat-induced insulin resistance (552). This evidence supports a role of NOD1 in fat-induced diabetes, however these studies do not indicate whether NOD1 is involved in the development of diabetes via an effect on β-cell function.

Our data show that islets of NOD1 knockout mice are indeed protected against palmitate but not oleate-induced β-cell dysfunction. NOD1 has previously been found to be activated by saturated
but not unsaturated fatty acids (540,541). The effect of oleate to decrease insulin secretion likely involves increased oxidative stress independent of NOD1 (266). Regarding palmitate, it is possible that, similar to saturated fat-induced TLR activation, NOD1 activation by saturated fat is mediated by an intermediary molecule (416,417) or by ceramide synthesis (415). The mechanisms remain to be elucidated, however it is clear that palmitate and not oleate impairs β-cell function through NOD1. Therefore, we used NOD1 knockout mice for in vivo infusion experiments with ethylpalmitate and our data showed that NOD1 deletion protects against ethylpalmitate-induced β-cell dysfunction in vivo. However, the SI only tended to be decreased by NOD1 injection or ethylpalmitate. Our previous studies have shown that injection of a NOD1 activator leads to insulin resistance when insulin sensitivity is assessed by the gold-standard hyperinsulinemic-euglycemic clamp (464). NOD1 deletion also protected against insulin resistance (assessed by insulin tolerance test) induced by high fat diet (131). Further studies should address the effect of ethylpalmitate and NOD1 deletion on insulin sensitivity using hyperinsulinemic-euglycemic clamps. These studies would require additional animals as hyperglycemic and hyperinsulinemic-euglycemic clamps cannot both be performed in the same mouse.

In conclusion, we show for the first time that the NOD1 receptor plays a causal role in palmitate-induced β-cell dysfunction in vitro and in vivo in mice. Thus, our studies have identified a novel therapeutic target for the prevention and treatment of β-cell dysfunction in T2D.
General Discussion

6.1 The role of IKKβ in fat-induced β-cell dysfunction

Inflammation has emerged in the last two decades as a defining characteristic of obesity-induced insulin resistance and T2D. The critical role of proinflammatory cytokines in metabolism was first shown in studies that examined the mechanisms involved in fat-induced insulin resistance. Since then, there has been a growing body of evidence implicating inflammatory mediators in the pathogenesis of fat-induced β-cell dysfunction and T2D. In line with this, our studies showed that there was an impairment in β-cell function and an increase in expression of proinflammatory markers in islets of rats infused with fat. In islets of rats co-infused with fat and the IKKβ inhibitor salicylate, β-cell function and expression of proinflammatory markers were not significantly different compared to control islets. These results are in line with a causal role of inflammation and IKKβ in particular in fat-induced β-cell dysfunction, however inhibitors have systemic effects and may have non-specific effects. Therefore, we directly determined whether IKKβ is involved in fat-induced β-cell dysfunction by using a model of β-cell specific deletion of IKKβ.

In this study, β-cell specific IKKβ knockout mice, unlike control mice, did not have a decrease in β-cell function following oleate infusion in vivo, as determined by the DI calculation. This suggests β-cell specific deletion of IKKβ protects against fat-induced β-cell dysfunction. However, in the mouse model we used, β-cell specific deletion of IKKβ was driven by RIP, which is expressed in β-cells as well as in the hypothalamus. Hypothalamic activation of IKKβ has indeed been shown
to have peripheral and systemic effects such as inducing hypertension, impairing muscle and hepatic insulin sensitivity and promoting weight gain (553). In this study, mice with RIP-mediated deletion of IKKβ, unlike control mice, did not become insulin resistant following oleate infusion, which was likely an effect of hypothalamic deletion of IKKβ. It is unlikely that prevention of insulin resistance would have had a protective effect on β-cells. The effect of insulin resistance to impair β-cell function is normally in the long term and due to the chronic increase in stress put on β-cells to produce more insulin. Importantly, in our rat studies, both oleate and olive oil infusions were performed. Since only rats infused with olive oil, and not with oleate, had detectable insulin resistance, the use of both infusion models may help to distinguish between the effects of insulin resistance and of fat itself on β-cell function. In these studies, rats infused with either oleate or olive oil had impaired β-cell function and when salicylate was co-infused with either, there was no impairment in β-cell function, suggesting salicylate protected against fat-induced β-cell dysfunction in both models. Therefore, in our 48h mouse model insulin resistance is most likely not a major factor in the decrease in β-cell function observed in oleate-infused control mice, however, the hypothalamic deletion of IKKβ in knockout mice may still be a confounding factor and make the in vivo results difficult to interpret. Therefore, to directly determine the role of IKKβ in β-cells we also performed ex vivo GSIS experiments in islets isolated from oleate-infused wildtype and β-cell specific IKKβ knockout mice. Islets of control mice infused with oleate had a decrease in GSIS and this decrease was not observed in islets of knockout mice infused with oleate, suggesting IKKβ deletion in β-cells protected against oleate-induced β-cell dysfunction. In ex vivo experiments, islets are directly stimulated by glucose and the resulting level of insulin secretion is indicative of β-cell function. Therefore, the ex vivo data support the interpretation that oleate induced β-cell dysfunction in vivo and that this dysfunction was prevented by deletion of IKKβ specifically in β-cells. This supports the hypothesis that IKKβ in β-cells plays a causal role in fat-induced β-cell dysfunction. Moreover, the decrease in β-cell function observed in vivo in oleate-infused control mice was not likely entirely due to β-cell rest because it was also observed ex vivo.

Still, any effect present during the 48h infusion in vivo is also present ex vivo as the fat infusion is in vivo and hypothalamic deletion of IKKβ may have had a protective effect during this period that would result in improved β-cell secretory function in islets ex vivo. Our in vitro studies with the specific IKKβ inhibitors salicylate and BMS suggest that they protect against fat-induced β-cell dysfunction in islets, however, an entirely in vitro model of fat-induced β-cell dysfunction in islets of β-cell specific IKKβ-knockout mice will be required for future studies.
Many studies have shown that inflammation is involved in β-cell dysfunction in T2D. This inflammation is characterized by immune cell infiltration in islets, expression of cytokines, and inflammatory signaling in β-cells (54,261,269,316,381). Inflammation was first observed to have a detrimental effect on β-cells in a model relevant to T2D when glucose was shown to induce apoptosis through upregulation of IL-1β by β-cells (554). It was further shown that IL-1β impaired β-cell function. Interestingly, under basal conditions in healthy islets, interleukin 1 receptor, type 1 (IL-1R1) is 10-fold higher in β-cells compared to other pancreatic cells and to several other tissues. Expression is approximately 4 fold greater than in liver and the expression level in islets increases with diabetes (269).

Following the studies that demonstrated the effect of glucose and cytokines on β-cell inflammation and function, experiments were performed to determine the effect of fat on cytokine expression in islets and on glucose homeostasis. As mentioned previously, all animal models of T2D have increased markers of inflammation in islets (316,371). Notably, high fat fed mice have improved β-cell function when treated with IL-1Ra (308). The fatty acids oleate, palmitate and stearate have also been shown to directly induce IL-1β secretion and production of proinflammatory mediators in human and rodent islets (269). In human islets, IL-1beta, oleate, palmitate and stearate each increased IL-1β, IL-6 and IL-8, which was prevented by IL-1Ra, and with stearate was also prevented by deletion of MyD88, TLR2 or TLR4 (269). Furthermore, high fat feeding induced glucose intolerance and islet IL-1β expression and this was prevented in mice and islets of mice by TLR2 deletion (261). Similar to IL-1R1, TLR2 is more highly expressed in islets than in most tissues, other than bone marrow (261). Together, these studies demonstrate the ability of inflammation to impair β-cell function and the critical role of cytokines and inflammatory signaling in fat-induced β-cell dysfunction. Furthermore, the combination of fatty acids and moderately elevated glucose levels had a greater cytokine inducing effect than fatty acids alone (269), suggesting that once β-cells are damaged and hyperglycemia ensues in diabetes, there is an accelerated progression of inflammation-mediated β-cell dysfunction. Importantly, although these studies did not measure NF-kB activity, signaling via IL-1β as well as TLRs is through IKKβ and most, if not all metabolic stress signals that result in β-cell dysfunction converge on IKKβ.

In keeping with a causal role of IKKβ activation in the development of β-cell dysfunction, a recent study showed that constitutively active IKKβ specifically in β-cells impairs β-cell function and
glucose homeostasis in mice (555). A number of studies have also shown the role of the IKKβ/NF-κB pathway in cytokine and metabolic stress mediated β-cell dysfunction. β-cell specific expression of a non-degradable IκBα, which prevents NF-κB activation, protected against STZ induced diabetes. Islets from these mice were also protected from IL-1β + IFNγ induced β-cell dysfunction and IL-1β induced β-cell dysfunction and apoptosis (556). Additionally, stimulation of human islets with IL-1β or high glucose induced expression of IL-1β, IL-6 and IL-8, which was partially prevented with an inhibitor of IκBα phosphorylation (370). Similarly, transfection of human islets with non-degradable IκBα prevented IL-1β induced β-cell dysfunction and apoptosis (482). Deletion of IKKβ in β-cells was also shown to protect against IL-1β induced apoptosis, but made islets more susceptible to TNFα induced apoptosis (491).

The role of IKKβ in β-cell dysfunction induced by fat is more controversial. One study using INS-1 cells found that incubation with palmitate for 24h induced β-cell dysfunction and apoptosis. This was associated with NF-κB activation and was inhibited with an IKKβ inhibitor (428). In contrast, another study showed that neither oleate nor palmitate induced NF-κB activation in INS-1 cells (485). The discordant results may be attributed to the difference in the duration of exposure as in the latter study cells were only exposed to FFA for 12h. It may also be due to the cell line as one study showed that incubation of MIN6 cells with palmitate for as little as 14h resulted in IKKβ phosphorylation and activation of NF-κB, which were prevented with an IKKβ inhibitor (55). Notably, studies in human islets showed that incubation with palmitate for 24h or 48h increased IκBα phosphorylation and subsequent IL-1β production (484). Furthermore, an in vivo study in Psammonys obesus showed that an IKKβ inhibitor prevented high-fat diet-induced diabetes and increased pancreatic insulin stores (321).

The results from this study are in keeping with the findings from the majority of the studies described above. Importantly, they are the first to show that mice with deletion of IKKβ specifically in β-cells do not have an impairment in β-cell function when infused with fat in vivo or ex vivo. Studies in our laboratory have shown that a number of treatments that prevent different types of oxidative stress (137,265,266,471), IL-1β-mediated inflammation (557) or JNK activation (unpublished) in islets protect against fat-induced β-cell dysfunction. Studies with our collaborators have shown the same effect with inhibitors of ER stress in humans (365). This may be explained by the reciprocal cause and effect relationship of oxidative stress, ER stress, and
inflammation in which all are required for β-cell dysfunction and the inhibition of one is sufficient to protect against the impairing effect of fat. Oxidative stress and ER stress are known to activate IKKβ directly and the effect of IKKβ to activate NF-κB and transcription of proinflammatory cytokines contributes to oxidative and ER stress (366,558–561), although data from our laboratory do not support a role of ER stress in oleate-induced β-cell dysfunction (unpublished).

As described above, the mechanisms by which IKKβ deletion in β-cells may protect against fat-induced β-cell dysfunction include both the inhibition of NF-κB and subsequent transcription of proinflammatory molecules and prevention of the serine phosphorylation of IRS. Our in vitro studies in rat islets showed that the β-cell dysfunction observed in rat islets treated with oleate was not observed when islets were treated with oleate in the presence a COX-2 inhibitor and that the increase in PGE2 production from islets treated with oleate was not observed when islets were treated with oleate in the presence of salicylate. This is in line with studies which found that inhibitors specific for COX-2, but not inhibitors that target both COX-1 and COX-2 with a predominant effect on COX-1 (519,562), protected against cytokine-induced (504,520,563) and streptozocin-induced (564) β-cell dysfunction. Together, these studies suggest that COX-2 upregulation and subsequent PGE2 production is an important mechanism through which IKKβ impairs β-cell function in the presence of oleate.

The impairing effect of iNOS upregulation in β-cells in response to IL-1β has been well established and iNOS mediated production of NO has also been shown in one study to be required for COX-2-induced β-cell dysfunction (565). However, the role of iNOS in FFA-induced β-cell dysfunction has been disputed. iNOS inhibition in one study did not have any effect on glucose metabolism in ZDF rats, however the inhibitor was given in drinking water and it is not clear to what extent iNOS was inhibited (566). In other studies, the use of different iNOS inhibitors and their administration in vitro, where the effect on islets is more direct, showed that islets of ZDF rats have an increased level of iNOS expression and NO production, which was decreased with the inhibitors. Although iNOS mRNA expression in islets was undetectable following oleate exposure in study 1 of this thesis, iNOS mRNA is difficult to detect and future studies should measure iNOS protein expression. Importantly, iNOS inhibition improved insulin secretion in normal islets exposed to FFA in studies from our laboratory (unpublished) and from others (311,362) and improved insulin secretion in islets of ZDF rats exposed to FFA (311,362). The
mechanisms by which iNOS impairs β-cell function appear to involve impairment of the ETC in the mitochondrion (247) or nitration of tyrosine residues in signaling molecules of the insulin signaling pathway, which prevents their phosphorylation and downregulates the insulin signaling pathway (430,567,568). Therefore, iNOS upregulation by NF-κB may play an important role in the IKKβ mediated decrease in β-cell function caused by FFA.

As in classical insulin target tissues, IRS-1 serine phosphorylation in β-cells impairs β-cell insulin signaling and is another mechanism whereby IKKβ may induce β-cell dysfunction. In our in vitro studies in rat islets, serine phosphorylation of IRS-1 was increased in the presence of oleate. In islets incubated with oleate and treated with salicylate, the level of serine phosphorylation of IRS-1 was the same as that in control islets. This supports the notion that β-cell insulin resistance, caused specifically by IKKβ, is involved in fat-induced β-cell dysfunction both in vitro and in vivo. A number of recent studies have provided further evidence for a causal role of β-cell insulin resistance in fat-induced β-cell dysfunction and this is discussed in greater detail in the following section of this chapter.

The studies described above suggest IKKβ plays a causal role in the development of T2D associated with obesity and this has been studied in humans with the IKKβ inhibitor salicylate and its dimer salsalate. Salicylate, originally derived from willow bark, is one of the oldest drugs in clinical practice and has been used since at least 1500 B.C. to treat inflammation (569). Acetylation of salicylate forms the widely used anti-inflammatory drug aspirin which had been studied for its effects on glucose metabolism and diabetes more than 100 years ago (570). Later studies from the 1980’s suggested that salicylate does not improve and may even impair glucose metabolism (571–573), however interest in salicylate for the treatment of T2D was renewed after a study showed that it could prevent T2D in mice and that this was through IKKβ inhibition (574). This was followed by investigation of its effects on glucose metabolism in humans.

A number of studies showed that salsalate or aspirin improved parameters of glucose metabolism in people with obesity and/or T2D. Oral treatment with 3-7 g/day of salicylate for 1-4 weeks was found to improve fasting blood glucose and increase glucose stimulated insulin secretion (457,509,575–579). However, one study with our collaborators found that treatment with 4.5g/day of sodium salicylate for one week did not prevent the decrease in β-cell function caused by infusion
of intralipid and heparin (IH) (281). This may be due to the experimental protocol as the other studies did not use IH. The effect of oral salicylate may have been overwhelmed by the effect of IH infusion in obese subjects. Nonetheless, it is important to note that the studies that found a beneficial effect of salsalate on glucose tolerance did not directly assess β-cell function using a hyperglycemic clamp or DI calculation (457,509,575–579). In addition, they found decreases in insulin clearance, which is a known but poorly understood effect of salicylate (573,580) and it is possible that the decrease in insulin clearance was responsible for the improvements in glucose tolerance. The improvement in glucose tolerance has been generally attributed to beneficial effects of salicylate on insulin sensitivity, however studies have found discordant results as they report a detrimental (281,571), beneficial (575) or no effect (576) on insulin sensitivity and the differences are likely due to different doses and experimental protocols. Nonetheless, if salicylates improve insulin sensitivity, this may in the long term affect β-cell function as improved sensitivity results in less stress on the β-cell as less insulin is needed to maintain glucose homeostasis. Regardless of the exact mechanism, the primary goal of any treatment for T2D is to improve blood glucose control and larger clinical studies have been initiated in order to determine if the beneficial effects found in the small scale, short-term studies are confirmed in large-scale studies (581).

The TINSAL-T2D (Targeting Inflammation Using Salsalate for T2D) study reported the results of 283 people with T2D treated for 48 weeks with 3.5g/day of salsalate. Most recently, in a parallel clinical trial (TINSAL-CVD [Cardiovascular disease]) completed in 2017, 190 people with obesity and with or without prediabetes were treated for 30 months with 3.5 g/day salsalate. Glycated hemoglobin (HbA1c) was measured to assess long-term glucose control. Both studies found that salsalate significantly decreased HbA1c as well as fasting blood glucose levels (581,582). As in the other studies, it is not clear whether this effect was due to improved insulin sensitivity, decreased insulin clearance or increased β-cell function, however these studies strongly support the potential use of salsalate as a treatment for T2D and the hypothesis that IKKβ activation plays a causal role in obesity-induced diabetes.

Further studies will be required to determine the effect of salsalate in the longer term and on a larger scale. This is especially important for assessing the cardiovascular safety of salsalate, which requires further study (582,583). It will also need to be determined whether salicylate in these studies acts only through inhibition of IKKβ. It has recently been shown that salicylate activates
AMPK (501) and AMPK can activate IKKβ (584) suggesting only a secondary role of IKKβ in the improvement of glucose tolerance achieved with salicylate. However, two studies found that AMPK was not required for salicylate-mediated improvement in glucose metabolism (501,585). Importantly, salicylate is known to activate IKKβ directly (487). Furthermore, BMS-345541 (BMS), which specifically targets and inhibits IKKβ (490) has not been shown to affect AMPK and, as described above, we found that mice co-infused with BMS and oleate did not have the decrease in β-cell function that was observed in mice infused with oleate alone, suggesting that BMS protected against oleate-induced β-cell dysfunction. Therefore, the role of direct inhibition of IKKβ in the studies using salicylate is likely important but will need to be clarified. Further investigation into which tissues salicylate primarily acts on are also needed. It has been shown that salicylate protects against glucose and IL-1β-induced β-cell dysfunction in human islets by inhibiting activation of NF-κB (484) and my study suggests that β-cell IKKβ plays a causal role in β-cell dysfunction induced by fat. However, further investigation into the effect of salicylate to protect against the β-cell dysfunction induced specifically by fat in human islets is needed to better understand the effect of salicylate on glucose metabolism in patients with T2D.

6.2 The role of β-cell insulin resistance in fat-induced β-cell dysfunction

Inflammation has been established as playing a role in fat-induced β-cell dysfunction, however the mechanisms through which an increase in inflammatory signaling leads to β-cell dysfunction are not well understood. In the classical insulin target tissues liver, muscle and adipose tissue, inflammation leads to insulin resistance through inhibition of the insulin signaling pathway, primarily through IRS serine phosphorylation by inflammatory kinases, such as IKKβ (67,68). It is now known that insulin signaling molecules are present in β-cells and inhibition of the insulin/IGF-1 signaling pathway may be involved in fat-induced β-cell dysfunction. It has been established that insulin signaling plays an important role in β-cell growth, insulin gene transcription and proinsulin biosynthesis (435,436,586,587), however the role of insulin signaling in insulin secretion is less clear as some studies have shown a stimulatory effect of insulin (440,522–524,588) while others have shown an inhibitory effect (442,443,445,525,589–591) as described in Chapter 4. With respect to fat-induced β-cell dysfunction it appears that FFA may
induce insulin resistance at the β-cell level, however reports are sparse, as will be discussed below. In our studies in rats, we observed that with oleate or olive treatment there was a decrease in β-cell function in vitro, ex vivo and in vivo and this decrease was not observed when rats or rat islets were treated with the tyrosine phosphatase inhibitor bisperoxovanadate (BPV), suggesting that BPV protected against fat-induced β-cell dysfunction. In islets treated with BPV in vitro, there was also an increase in Akt phosphorylation. As previously described in Chapter 4, vanadium compounds have the advantage over other insulin sensitizers such as metformin and thiazolidinediones of inducing rather than decreasing oxidative stress (526). These other insulin sensitizers are also AMP kinase activators and thus deplete islets from fat (514). Therefore, a protective effect of BPV against FFA-induced β-cell dysfunction would not likely be due to reduction of oxidative stress or islet fat. Nonetheless, the use of a pharmacological agent has its drawbacks which are other nonspecific effects and systemic effects. Therefore, we also wished to determine whether a genetic model of upregulation of insulin signaling in β-cells is protected from the β-cell dysfunction induced specifically by fat.

Control mice treated with oleate had a decrease in β-cell function compared to saline-treated controls. In β-cell specific PTEN knockout mice, which have upregulation of insulin signaling in β-cells, this level of decrease was not observed with oleate treatment, although the disposition index (DI) in oleate-treated β-cell specific PTEN knockout mice tended to be lower than that in saline-treated β-cell specific PTEN knockout mice, suggesting there was partial protection against fat-induced β-cell dysfunction with β-cell specific deletion of PTEN. The mouse model used was similar to that in study 1 in that deletion of PTEN was driven by RIP2, which caused deletion in the hypothalamus, in addition to deletion in β-cells. In β-cell specific PTEN knockout mice treated with oleate, we observed a tendency for insulin sensitivity to decrease and in control mice treated with oleate, there was a significant decrease in insulin sensitivity. This suggests that oleate induced insulin resistance in control mice and that β-cell specific PTEN knockout mice were partially protected from insulin resistance. This protection was likely a result of hypothalamic deletion of PTEN, as in previous studies (437,528,534), and is consistent with the effect of increased hypothalamic PI3K signaling to improve systemic insulin sensitivity (592). The previous studies in β-cell specific PTEN knockout mice, however, found complete protection against high-fat diet induced insulin resistance as well as enhanced baseline insulin sensitivity and growth retardation (437,528,534). The discordant results may be due the different genetic background as two of these
studies maintained mice on a mixed 129J-C57BL6 background (437,534) and we did not. We only used mice with a black coat for breeding as opposed to those with the white coat that results from the 129J background, which may have led to mice with a primarily C57BL6 background. Another group which used β-cell specific PTEN knockout mice and did not maintain them on a mixed background, resulting in an unknown mix between 129J and C57BL6 backgrounds, also observed an effect of PTEN deletion on growth retardation, but found no significant difference in insulin sensitivity between wildtype and PTEN knockout mice (528). Although insulin sensitivity may have been only partially affected by the deletion of PTEN and is therefore not likely to confound the interpretation of the in vivo data in my study, a factor that may be confounding is insulin clearance, which tended to be decreased with oleate in the control group as well as in both heterozygous and homozygous β-cell specific PTEN knockout mice during the hyperglycemic clamps. A decrease in insulin clearance results in a decrease in disposition index, however this does not necessarily indicate a decrease in β-cell function. The increased plasma insulin that results from the decrease in insulin clearance may lead to β-cell rest rather than dysfunction. Therefore, in order to directly assess the effect of oleate infusion on β-cell function, I performed ex vivo studies in islets isolated from oleate or saline infused β-cell specific heterozygous PTEN knockout mice. Only heterozygous mice were used since the in vivo results did not differ between heterozygous and homozygous knockout mice. Islets of control mice treated with oleate had a decrease in β-cell function and this decrease was not observed in islets of knockout mice, suggesting β-cell specific heterozygous deletion of PTEN protects against β-cell dysfunction induced by oleate. Therefore, my results support the hypothesis that β-cell insulin resistance is involved in fat-induced β-cell dysfunction.

Earlier studies have shown that incubation of β-cells with insulin for very short durations (i.e. seconds to minutes) (440,523,524) as well as for 72h (588) stimulated insulin secretion in the presence of both basal and stimulatory concentrations of glucose. These studies found that this was dependent on IRS-1 (523,524,588) and may (523,524) or may not (522) require PI3K activation. The difference in the role of PI3K may be due to the length of incubation with insulin. Notably, the downstream effect of increased insulin signaling was an increase in intracellular calcium levels, independent of a change in plasma membrane potential (440,522–524,588). This suggests the increase in calcium was likely from an intracellular source. Indeed, it was found that insulin signaling inhibited SERCA and thereby prevented uptake of calcium by the ER, resulting in an
increase in cytosolic calcium concentration and in turn insulin granule exocytosis (524,588). This was mediated by IRS-1 binding to SERCA. Furthermore, IRS-1 overexpression was found to decrease gene expression of SERCA (588) and to increase insulin secretion by increasing the intracellular calcium concentration in β-cells (522,588).

As previously described, however, studies that have examined the role of β-cell insulin signaling in insulin secretion have shown discordant results as insulin has also been found to decrease insulin secretion. Inhibition of IRS-1 in islets using an antisense oligonucleotide has been found to increase GSIS, however this may have been due to decreased IRS-1 in α-cells or δ-cells. Indeed, IRS-1 deletion decreased somatostatin mRNA expression and since somatostatin inhibits insulin secretion, a decrease in somatostatin secretion may have contributed to the enhanced GSIS in IRS-1 knockout islets. However, it was not possible to confirm this as amounts of secreted somatostatin are very low and the radioimmunoassay used in the study, which did not detect a decrease in somatostatin protein levels, is unable to detect subtle, yet potentially significant, changes in protein levels (589). Other studies have shown that the PI3K inhibitors wortmannin and LY2942002 (442,445,525,590) or deletion of the regulatory subunit of PI3K, p85α (445), increase insulin secretion. This was attributed to the prevention of PI3K-mediated opening of K_{ATP} channels as PIP3 is known to directly activate these channels (446). However, these results may also have been due to non-specific effects of PI3K inhibitors. The discrepancy with the results of the previously mentioned studies may be explained by different doses used between studies (440,442,523,588), different culture media (440,442,443,445,522–524,588,590) or exposure of β-cells and islets to glucose concentrations of only 10 mM rather than maximally stimulatory concentrations (442,525).

In order to better understand the role of insulin in the β-cell insulin signaling pathway, studies have examined the role of β-cell insulin signaling in more long term and in vivo models. The presence of the insulin receptor in β-cells has been shown to be essential for first phase insulin secretion as β-cell specific insulin receptor knockout (βIRKO) mice have a relative impairment in first phase insulin secretion which is unrelated to the changes in β-cell mass also found in these mice (435). Another study showed that second phase insulin secretion was decreased in 7 month-old βIRKO mice and this was associated with decreased gene expression of GLUT2 and glucokinase (536), suggesting β-cell insulin signaling is essential for glucose sensing and in turn insulin secretion.
Deletion of the IGF-1 receptor in β-cells has also been shown to decrease insulin secretion and to be associated with a decrease in GLUT2 and glucokinase, although the decrease in secretion was less than that seen in βIRKO mice (593). The combined deletion of the insulin and IGF-1 receptors in β-cells has an additive effect, suggesting possible compensation between the receptors in the single gene knockout models, and resulted in impaired β-cell function by 3 weeks of age, which became progressively worse with age (593). However, deletion of the insulin receptor in the β-cells of these mice was achieved using RIP, resulting in hypothalamic deletion of the insulin and IGF-1 receptors. In order to exclude a role of the hypothalamus, in vitro studies were performed and the results were the same as in vivo (593). In healthy humans, exogenous insulin administration decreases concomitant endogenous insulin secretion, which was shown to be due to a neural reflex in the pancreas as denervation was found to prevent the decrease in secretion (447). However, insulin administration has also been shown to potentiate subsequent GSIS (448–450). Furthermore, human islets with the common IRS1 Gly972 → Arg amino acid polymorphism, which is associated with insulin resistance and T2D, have decreased insulin secretion. This was associated with a greater number of immature insulin granules and a smaller number of mature granules as well as an increased proinsulin:insulin ratio (591), which may be due in part to insulin resistance in β-cells. Insulin signaling in β-cells stimulates PDX-1 binding via Akt mediated phosphorylation and inhibition of FOXO-1, which prevents FOXO-1 translocation to the nucleus, allowing for the translocation and binding of PDX-1 (231,594,595). This may in turn increase expression of the prohormone convertase 1/3 (596), which may have been downregulated in these islets, leading to a decrease in insulin secretion and content and to an increase proinsulin:insulin ratio.

The role of insulin signaling in fat-induced β-cell dysfunction has not been studied extensively, however a number of studies have provided evidence to suggest β-cell insulin resistance is involved in fat-induced β-cell dysfunction. In vitro studies have shown that incubation of INS-1 cells with FFA, such as oleate (231,451) or palmitate (452), impairs the IGF-1 induced activation of Akt (231,451,452) as well as insulin signaling and induces β-cell apoptosis (451). Furthermore, adenoviral-mediated overactivation of Akt prevented the oleate-induced apoptosis (451). Notably, one study showed directly that palmitate-induced impairment of glucose stimulated insulin gene transcription was due to JNK1 activation and its serine phosphorylation of IRS-1 and IRS-2 (453). This implicates a causal role of inflammation-induced β-cell insulin resistance in fat-induced β-
cell dysfunction. Additionally, in vivo studies in mice with genetic or dietary obesity, which have increased PTEN expression, possibly as a result of insulin resistance (437), have shown that deletion of PTEN protects against diabetes. This was associated with increased PI3K signaling as well as expression of PDX-1 and GLUT2 (437), in accordance with the converse findings in βIRKO mice (536,593). Although mice with PTEN deletion in β-cells did not have a significant decrease in β-cell function in the presence of fat in our study and were protected against β-cell dysfunction induced by high fat diet in the study by Wang et al. (437), PTEN also plays a number of other roles in the cell.

PTEN is well known as a regulator of cell cycle progression and apoptosis (597). PTEN increases activation of the cell cycle inhibitors p27 and p21, likely by decreasing Akt activity which in turn decreases phosphorylation and therefore inactivation of p27 and p21. Furthermore, it has been shown that PTEN deletion decreases p27 expression, which in combination with an increase in S6K activity that results from the PTEN deletion, enhances cell cycle progression and in β-cells may lead to proliferation (528,598–600). PTEN could also promote apoptosis by preventing Akt-mediated phosphorylation of the proapoptotic protein Bcl-2-associated death promoter (BAD), thereby allowing for its activation as well as for Bcl-2-associated X protein (BAX)-mediated cytochrome c release from mitochondria, which initiates caspase-induced cell death (601–603). In addition, PTEN has been shown to promote apoptosis by localizing to the ER and decreasing phosphorylation of IP3 receptors of the ER, possibly through direct protein dephosphorylation (604). The decreased phosphorylation increases calcium release, resulting in increased cytosolic and mitochondrial calcium (604) and increased sensitivity to calcium induced apoptosis (605). Therefore, deletion of PTEN in β-cells may result in a decrease in cytosolic and mitochondrial calcium and an increase in β-cell mass in addition to an improvement in β-cell secretory function. In our study, it is unlikely that any effect on β-cell mass played a role as we used a 48h model, in which there is not likely sufficient time for a significant change in β-cell mass and importantly, in previous studies in our laboratory, we observed no changes in β-cell mass in this model (266). Notably, although PTEN is an important tumor suppressor and inhibition of its function is well known to cause cancer in various tissues (606), its effects are tissue and context specific and studies have shown that PTEN deletion in β-cells does not induce tumorigenesis (437,528).
Overall, these studies support the hypothesis that β-cell insulin resistance plays an important role in fat-induced β-cell dysfunction and thus, the development of diabetes. They identify the insulin signaling pathway in β-cells as a potential therapeutic target. In line with this, early studies using vanadium compounds in humans found that they improved insulin sensitivity and glucose tolerance, however they also frequently led to gastrointestinal side-effects including nausea, vomiting and diarrhea (607–610), making them unsuitable for T2D treatment. Targeting PTEN in particular may also be a nonviable therapeutic option due to its role as a tumor suppressor. Indeed, it has been shown that PTEN haploinsufficiency in humans improves insulin sensitivity but increases the risk of cancer (611). Identifying other parts of the insulin signaling pathway as therapeutic targets for T2D may also be of value. Furthermore, since inflammation is known to play a critical role in β-cell dysfunction and can induce insulin resistance, further studies that draw a direct link between fat, inflammation and insulin resistance in β-cells may help to identify the most effective therapeutic targets, whether they are molecules of the insulin signaling pathway or inflammatory kinases that impair this pathway when activated by fat.

6.3 The role of NOD1 in fat-induced β-cell dysfunction

Downstream intracellular inflammatory signaling molecules and the mechanisms by which they impair β-cell function in response to fat have been studied extensively. Although these remain incompletely understood, investigation of the upstream mediators of these pathways has only recently gained interest. The activation of the proinflammatory TLRs, activated by bacterial products, have been shown to play an important role in fat-induced β-cell dysfunction (55,420). The role of NOD receptors have also been implicated as NOD1 deletion has been shown to protect against fat-induced glucose intolerance (131), which is reflective of β-cell dysfunction, however the role of NOD1 in fat-induced β-cell dysfunction had not been investigated directly. The final objective of this thesis was to determine whether NOD1 is expressed in islets, whether a NOD1 activator has an effect on β-cell function and whether fat-induced β-cell dysfunction is impacted by NOD1.

My study showed that NOD1 mRNA is expressed in both mouse and human islets. It also showed that islets treated with the NOD1 activator FK565 had decreased insulin secretion compared to
control islets in vitro and mice treated with the NOD1 activator had a decreased disposition index compared to control mice in vivo. These results suggest that NOD1 impairs β-cell function in vitro and in vivo. In order to determine whether this is relevant in a model related to T2D, I used NOD1KO mice for in vivo studies and islets from these mice for in vitro studies that examined the role of NOD1 in fat-induced β-cell dysfunction. In vitro, wild-type islets had decreased insulin secretion in the presence of oleate or palmitate. In NOD1KO islets, there was a decrease in insulin secretion in the presence of oleate, but not in the presence of palmitate, suggesting they were protected from palmitate-, but not oleate-induced β-cell dysfunction. This would be consistent with the role of saturated, but not unsaturated fatty acids to lead to NOD1 activation (540). In vivo, wild-type mice infused with ethylpalmitate had impaired β-cell function and NOD1KO mice did not, suggesting NOD1KO mice were protected from β-cell dysfunction induced by palmitate and that this was a result of NOD1 deletion. These data are in line with previous studies that implicated a causal role of NOD1 in glucose intolerance induced by high fat feeding (131).

Studies in humans have shown that NOD1 expression is elevated in the myocardium (548) and monocytes (549) of patients with type 2 diabetes and that gestational diabetes is associated with elevated expression of NOD1 in adipocytes (550) as well as in the placenta (551). The increase in NOD1 expression was associated with T2D, which included both hyperglycemia and elevated circulating FFA, and BMI-matched normoglycemic controls did not have this increase in NOD1 expression, suggesting that hyperglycemia may be the cause of the increase. However, although fat may not contribute to NOD1 expression, it is still possible that fat led or contributed to NOD1 activity also in humans. Interestingly, a polymorphism in the human NOD1 gene that appears to affect the response to lipid intake has been linked to an increased predisposition to saturated fat induced-insulin resistance (552). In line with this, NOD1 has previously been shown to be activated by saturated, but not unsaturated fatty acids in human intestinal epithelial cells (540) and adipocytes (541).

The effect of saturated fat to stimulate activation of NOD1 likely depends on the ability of saturated fatty acids to induce oligomerization of NOD1 receptors (540). It is possible that, similar to saturated-fat induced TLR activation, saturated fat activates NOD1 through an intermediary molecule rather than through direct binding of NOD1 (417). Alternatively, NOD1 activation may be mediated by the metabolite of saturated fat, ceramide, which is known to activate TLR
Importantly, palmitate is the most prominent circulating saturated fatty acid and is known to play a causal role in the development of type 2 diabetes (466). These studies support the notion that NOD1 may mediate this effect.

It is also important to note that high fat feeding is known to increase intestinal permeability and thereby increase the translocation of bacterial cell wall products into the circulation, which can activate NOD1 (612,613). It has also been shown that adipose tissue from high fat-fed mice contain live bacteria, translocated from the intestine, that cause low-grade chronic inflammation (131). This inflammation has been shown to be dependent on the co-receptor of TLR4, CD14, and NOD1. NOD1 or CD14 deletion prevented high fat diet-induced glucose intolerance, which was associated with decreased inflammation and bacterial translocation in the intestine (131). This suggests that endogenous NOD1 ligands (i.e. DAP from bacterial cell walls) play a role in high fat diet-induced glucose intolerance. Nonetheless, the focus of my study was to gain an understanding of the role of NOD1 receptors in β-cell dysfunction induced selectively by circulating fat and my study suggests that fat-induced β-cell dysfunction is indeed mediated by NOD1. The relative contribution to β-cell dysfunction by endogenous NOD1 ligands and activation of NOD1 by circulating fat itself will require further investigation.

In addition to NOD1 receptors, TLRs have been shown to be involved in fat-induced β-cell dysfunction. TLR4 activation has been shown to inhibit insulin secretion in rat islets (419) and human β-cells (132). In line with this, islets of high-fat fed mice and of db/db mice have elevated TLR4 and deletion of TLR4 significantly reduces blood glucose in these mice (261,319,320,420). A recent in vivo study also showed that infusion of ethylpalmitate for 72h in mice impaired insulin secretion and this was prevented by deletion of either TLR4 or MyD88 (55). TLRs have similar downstream effectors as NOD receptors, most notably NF-κB and JNK, and cross-talk between TLRs and NODs is involved in the proinflammatory response to infection (614–616). One study showed that NOD1 activation enhances TLR2-mediated T-cell activation (615) and another showed that TLR4 upregulated NOD1 in response to LPS (617). TLR4-mediated bacterial clearance has also been shown to be dependent on NOD1 activation (407). There is not yet sufficient in vivo evidence to implicate interdependence between NOD1 and TLRs in the activation of proinflammatory signaling, however these studies suggest that they do depend on each other, at least for full activation. This cross-talk must be further investigated in the context of fat-induced
diabetes and β-cell dysfunction and it may explain the ability of the deletion of either TLR4 or NOD1 to protect against fat-induced β-cell dysfunction.

Interestingly, it is also known that there is cross-talk between NOD1 and the insulin signaling pathway as NOD1 can stimulate the phosphorylation of Akt. This may have implications for understanding the role of β-cell insulin resistance in fat-induced β-cell dysfunction. However, it is primarily in immune cells that NOD1 leads to Akt phosphorylation and this contributes to the survival and differentiation of these cells (618,619). In contrast, studies in metabolic tissues such as hepatocytes (464) and adipocytes (620) found that stimulation with a NOD1 ligand decreased Akt phosphorylation and glucose uptake (adipocytes). In β-cells exposed to fat, it is likely that Akt phosphorylation is decreased, as seen in previous studies (437), and that NOD1 activation is increased, as suggested by our present study and that both contribute to β-cell dysfunction. Nonetheless, further studies will be required to understand the interaction between NOD1 and the insulin signaling pathway at the level of the β-cell and particularly in the context of lipotoxicity.

As mentioned above, the primary mechanism of action of NOD1 receptors is the activation of IKKβ and JNK (404–406), which as described previously, are known to induce insulin resistance (67,68). Therefore, my three studies suggest a novel model of fat-induced β-cell dysfunction whereby chronically elevated levels of saturated fat lead to activation of NOD1 and subsequent activation of IKKβ, resulting in β-cell insulin resistance, and in turn a decrease in β-cell insulin secretion. A direct link between these processes in β-cells exposed chronically to fat will need to be investigated further, however there is now evidence to suggest that each of them (i.e. NOD1, IKKβ, and β-cell insulin resistance) plays a role in fat-induced β-cell dysfunction. The identification of NOD1 as a potential mediator of fat-induced β-cell dysfunction may serve to develop novel therapeutic approaches for T2D. There currently exist inhibitors of NOD1 (621–623) and the docking protein RIP2 which is downstream of NOD1 (623), however the specificity of these inhibitors and their effects in vivo will need to be clearly defined before they can be applied clinically and specifically to patients with T2D or at risk of developing T2D.
7 Summary and conclusions

7.1 Summary of the studies

Our first study investigated the role of β-cell IKKβ in fat-induced β-cell dysfunction. Previous studies have examined the effect of β-cell specific IKKβ deletion in cytokine-induced β-cell dysfunction (370,482,491,556) and of the IKKβ inhibitors aspirin, sodium salicylate and a proprietary IKKβ inhibitor in high fat diet-induced β-cell dysfunction in vivo (321,574). We have investigated the effect of IKKβ inhibitors in the β-cell dysfunction induced selectively by elevation of FFA levels, which suggested that IKKβ was involved and led us to further investigate this effect using a highly specific model of IKKβ inhibition. Our studies are the first to directly investigate the role of IKKβ specifically in β-cells in the β-cell dysfunction induced selectively by fat. We show that mice with β-cell specific IKKβ deletion do not have a decrease in β-cell function in the presence of oleate in vivo. However, in the control oleate group there was a discrepancy between the glucose infusion rate and the disposition index which was due to a tendency for the insulin clearance to decrease. Additionally, in the β-cell specific IKKβ-knockout mice there was no insulin resistance in the presence of oleate, unlike in the control mice. In order to exclude any confounding effect of insulin clearance or insulin sensitivity, we also performed ex vivo studies which showed that in islets of β-cell specific IKKβ knockout mice, there was no decrease in β-cell function following oleate infusion. Together, these data suggest that IKKβ specifically in β-cells plays a
role in fat-induced β-cell dysfunction. This supports the potential use of IKKβ inhibition as a therapeutic approach in the prevention or treatment of T2D.

In our second study, we investigated the role of β-cell insulin resistance in fat-induced β-cell dysfunction as this is a possible downstream mechanism of IKKβ-mediated β-cell dysfunction. Previous studies have shown conflicting results regarding the role of insulin signaling in insulin secretion (440,442,443,445,522–525,588–591), however the most recent in vivo data suggest that insulin signaling is required for maintaining β-cell function and insulin secretion in response to fat (437). However, these studies examined the role of β-cell insulin resistance in high fat-fed models which have expansion of adipose tissue and changes in incretin hormone secretion and intestinal permeability. In order to investigate the role of β-cell insulin resistance in fat-induced β-cell dysfunction, we performed initial studies in a model of selective elevation of FFA in which the tyrosine phosphatase inhibitor BPV was used to increase insulin signaling in β-cells. In rats and rat islets treated with fat, there was a decrease in β-cell function in vitro, ex vivo and in vivo. When rats and rat islets with treated with fat in the presence of BPV, we found no decrease in β-cell function in vitro, ex vivo or in vivo. This was the first step taken by our laboratory to determine the role of β-cell insulin signaling in fat-induced β-cell dysfunction and it warranted further investigation in another model. This model is the first to demonstrate that in mice and mouse islets with β-cell specific deletion of PTEN, which results in increased β-cell insulin signaling (437), β-cell function is not significantly decreased in the presence of selective elevation of FFA. We show that in the presence of oleate there is only a tendency for β-cell function to decrease in both heterozygous and homozygous β-cell specific PTEN knockout mice, suggesting they are partially protected from oleate-induced β-cell dysfunction in vivo, however there was also a decrease in insulin clearance in these mice in the presence of oleate. This led to increased circulating insulin levels and may have led to β-cell rest. Therefore, we also performed ex vivo studies in islets of heterozygous β-cell specific PTEN-knockout mice and found that they did not have a decrease in β-cell function following oleate infusion, suggesting they were completely protected from oleate-induced β-cell dysfunction and that the tendency for the disposition index to be decreased in the knockout mice in vivo was likely due to β-cell rest. This study suggests a role of β-cell insulin resistance in fat-induced β-cell dysfunction and therefore further suggests the insulin signaling pathway in β-cells may be an important target for the prevention and treatment of T2D.
The final study in this thesis investigated the role of the NOD1 receptor in fat-induced β-cell dysfunction since NOD1 is upstream of IKKβ and may be a major mechanism through which it is activated in response to elevated FFA levels and since recent studies have shown that NOD1 plays an important role in high fat diet-induced glucose intolerance (131). We show that NOD1 is expressed in both mouse and human islets. We also show for the first time that islets treated with the NOD1 activator FK565 had decreased insulin secretion compared to control islets in vitro, assessed using glucose-stimulated insulin secretion assays, and that mice treated with the NOD1 activator had a decreased disposition index compared to control mice in vivo, assessed using hyperglycemic clamps. These results suggest that NOD1 impairs β-cell function in vitro and in vivo. In order to determine whether this is relevant in a model related to T2D, I used NOD1KO mice for in vivo studies and islets from these mice for in vitro studies. In vitro, wild-type islets had decreased insulin secretion in the presence of oleate or palmitate. In NOD1KO islets, there was a decrease in insulin secretion in the presence of oleate, but not in the presence of palmitate, suggesting they were protected from palmitate-, but not oleate-induced β-cell dysfunction. In vivo, wild-type mice infused with ethylpalmitate had impaired β-cell function and NOD1KO mice infused with ethylpalmitate did not, suggesting they were protected from β-cell dysfunction induced by palmitate and that this was a result of NOD1 deletion. This is relevant to T2D as palmitate is the most abundant circulating saturated fat in obesity mediated T2D (466). Additionally, and in contrast to the fat infusion experiments in the first two studies, there was no drastic change in insulin clearance between groups in the ethylpalmitate studies, likely due to a lesser elevation of FFA compared to the first two studies and to previous studies from our laboratory (137,265,266,291,293), and thus the glucose infusion rate and disposition index results corresponded to each other. Overall, this suggests a role of NOD1 in fat-induced β-cell dysfunction and identifies NOD1 as a potential novel therapeutic target for the prevention or treatment of T2D.

7.2 Conclusions

The studies in this thesis have shown that the β-cell dysfunction that occurs with 48h exposure to FFA likely involves IKKβ in β-cells, may be prevented with increased β-cell insulin signaling and when induced by palmitate, may be mediated by NOD1. This provides a novel working model of fat-induced β-cell dysfunction whereby exposure to saturated fat leads to stimulation of NOD1 and
possible activation of IKKβ in β-cells which leads to β-cell insulin resistance that in turn decreases insulin secretion. The potential sequential nature of these three processes and the potential role of each of them in fat-induced β-cell dysfunction requires further study, however the studies in this thesis suggest any or all of these may have the potential to serve as therapeutic targets for the prevention and treatment of T2D.
Limitations and future directions

The studies performed in this thesis used a selective model of elevation of FFA. This model provides mechanistic insights into the effect of fat in particular on β-cell function, however findings that are made through the use of this model do not necessarily translate directly to T2D. First, the model is relatively short-term compared to the months or even years it takes to develop T2D in humans (624). Second, in T2D and the progression towards it, there are a number of metabolic perturbations other than elevated FFA, such as elevated glucose levels (although this becomes evident only when impaired glucose tolerance has already developed) or changes in the secretion of incretin hormones, which contribute to β-cell dysfunction (190). Nonetheless, our model is designed for the study of mechanisms involved in fat-induced β-cell dysfunction and the mechanistic insights it provides about the effects of fat on β-cell function are an important piece of the puzzle of T2D. This model may serve as a first step of in vivo studies needed to better understand the pathogenesis of T2D, but should be complemented in future studies with models of T2D such as high fat feeding or genetic obesity, as well as eventual studies in humans with obesity or T2D.

It is also important to recognize that the intervention strategy used in our models is preventative rather than therapeutic or curative. We used mouse models that had deletion of the genes we hypothesized to be involved in fat-induced β-cell dysfunction, however these genetic mutations were present prior to the exposure of these mice to elevated levels of FFA. Although mice with deletion of these genes did not have decreased β-cell function in the presence of FFA, thereby suggesting that the proteins encoded by these genes are involved in the development of fat-induced
β-cell dysfunction and T2D, these models do not show whether inhibition of the proteins in question would be beneficial in the treatment of existing T2D. Therefore, further studies will be needed to determine whether the inhibition of these proteins can treat existing T2D. Experiments using high fat-fed animals could be performed in inducible knockout models or models in which treatment with inhibitors is not initiated until diabetes develops in order to determine whether the diabetes can be reversed.

Complementary studies examining the effect of interventions at different stages in the development of T2D would help to develop effective therapeutic strategies. This would involve: 1) prevention studies in people with obesity who are at risk of T2D; 2) studies to determine the ability of therapies to reverse glucose intolerance or prevent its progression to T2D; 3) studies to reverse prediabetes or prevent its progression to T2D; and 4) studies to determine the effect of interventions on the treatment of existing T2D, which could potentially be further divided into T2D treated with diet and exercise, with oral medications, or with insulin. Our studies represent the first category (i.e. prevention of the development of any fat-induced impairment in glucose metabolism). We would expect that the mechanisms involved in the progression from a state of elevated FFA levels and normal glucose tolerance to β-cell dysfunction, as in our studies, are also important in the progression of glucose intolerance, prediabetes and T2D and could be used as therapeutic targets for patients who already have β-cell dysfunction. However, it is certainly possible that the mechanisms at later stages are distinct, especially in the last stage when β-cells are destroyed. At this stage, β-cell apoptosis is likely a greater contributing factor than is β-cell dysfunction and the degree of β-cell dysfunction in remaining β-cells is more severe. It is also important to note that the studies in this thesis and many others from our laboratory were performed in rodents, which have less metabolic stability than do humans. This is an evolutionary adaptation to the high variability of access to resources encountered by mice (625) and may result in metabolic changes in mice that would not occur in humans in response to various treatments that affect metabolic parameters such as insulin sensitivity and secretion. Therefore, treatments that are effective in preventing β-cell dysfunction induced by fat in mice may not be effective in humans.

Nonetheless, as described previously, the IKKβ inhibitor salsalate improves glucose metabolism in humans with existing T2D, suggesting that inhibition of IKKβ may improve β-cell function in β-cells that have already been damaged (457,509,575–579,581). The roles of β-cell insulin
resistance and NOD1 activation, which may be related to IKKβ, have not been studied in humans as therapeutic targets for T2D. Importantly, the potential of these treatments to ameliorate T2D would likely depend on the stage of T2D. If the disease reaches a stage where a significant proportion of β-cells have already been lost, it would be unlikely that treatment with agents that target these pathways could restore glucose homeostasis and more intensive therapy, such as insulin injection, would likely be required.

In our model, β-cell dysfunction was assessed in vivo using the disposition index calculation. This is the gold standard assessment of β-cell function in vivo as it takes into account insulin sensitivity in order to assess relative rather than absolute insulin secretion (287,288,624). However, it does not take into account changes in insulin clearance, which are present in our model. Insulin clearance is mediated primarily by hepatocytes. Once the insulin receptor is activated by insulin, it phosphorylates and activates carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), which initiates endocytosis of the insulin-insulin receptor complex and leads to insulin degradation by insulin degrading enzyme (IDE) in endosomes (626,627). The kidneys also play a role in insulin clearance through degradation of insulin in a similar manner (628). Importantly, elevated FFA levels can decrease insulin clearance by inducing hepatic insulin resistance (518,629). This effect of FFA could lead to an elevated or diminished DI as a result of changes in insulin clearance, which lead to changes in circulating insulin levels, rather than of changes in β-cell secretory function. Nonetheless, one of the factors used to calculate the DI is the glucose infusion rate (GINF) and the GINF is a representation of glucose tolerance that takes into account insulin secretion, insulin sensitivity as well as insulin clearance. The use of the GINF, which takes into account all three factors, but does not distinguish between them, and the DI together, in addition to measuring the index of insulin clearance (i.e. C-peptide/insulin) helps to give a clearer picture of β-cell function in vivo and to distinguish between changes in β-cell secretory capacity and changes in insulin clearance. Therefore, both the GINF and the DI are taken into account in order to assess β-cell function in vivo in all of our studies. Importantly, ex vivo and in vitro studies were also performed in order to directly assess β-cell function in response to fat exposure as these experiments exclude the effect of insulin clearance or any other peripheral effect present during the clamps. Importantly, it has been shown that the impairing effect of fat on insulin secretion persists for at least 6 hours (259), which is greater than the combined duration of the islet isolation procedure (~30 minutes) and the islet recovery period (3 hours).
The specific model used in the first study served to examine the role of IKKβ in β-cells in fat-induced β-cell dysfunction. As described previously, the model we studied uses the RIP2 promoter to drive Cre recombinase expression, which is present in the hypothalamus in addition to in β-cells. This confounds the interpretation of the effect of IKKβ in β-cells. Previous studies have shown that hypothalamic IKKβ activation is involved in metabolic homeostasis (94,119,553) and our studies found that unlike in control mice, IKKβ knockout mice were not insulin resistant following fat infusion, suggesting they were protected from fat-induced insulin resistance. Although our in vitro studies with salicylate and BMS and our ex vivo studies in β-cell specific IKKβ knockout mice support a role of β-cell specific IKKβ as a mediator of fat-induced β-cell dysfunction, more specific models of β-cell specific deletion of IKKβ may be needed. To this end, we are currently breeding mouse insulin promoter (MIP)-Cre mice which have been shown to have less hypothalamic expression of Cre and provide a more suitable β-cell specific model. Future studies should also use Pdx1-Cre mice. Although these mice have Cre expression in both β-cells and pancreatic cells other than β-cells, they do not have Cre expression in the hypothalamus (630). Therefore, the use of these different models together would more accurately address the role of IKKβ in β-cells. Another limitation of our model is the use of oleate rather than palmitate or a physiological 2:1 oleate:palmitate infusion. When our studies were performed, there was no known method of in vivo palmitate infusion. With the recent development of the ethylpalmitate infusion model (55), it is now possible to determine the role of β-cell specific IKKβ in saturated fat-induced β-cell dysfunction and in the β-cell dysfunction induced by a physiological mix of saturated and unsaturated FFA, which may be used in future studies. Finally, and as previously described, IKKβ may impair β-cell function by serine phosphorylating and inhibiting IRS-1 mediated insulin signaling or through NF-κB mediated transcription of proinflammatory genes. To better distinguish between these two effects, future studies should use mouse models of selective inactivation of NF-κB. This model uses a β-cell specific degradation-resistant IκBα, known as the superrepressor, which is a selective and potent inhibitor of NF-κB as it prevents its translocation to the nucleus, but still allows for activation of IKKβ (491).

Similar to the first study, the second, which examined the role of β-cell insulin signaling in fat-induced β-cell dysfunction, used a β-cell specific model of PTEN deletion that also has hypothalamic deletion of PTEN and only used oleate infusion rather than palmitate or a physiological combination. Future studies using in vitro experiments, MIP-Cre and Pdx1-Cre
mice, and palmitate infusion will help to determine whether β-cell insulin resistance is involved in fat-induced β-cell dysfunction and whether this applies to saturated fat. Additionally, another model of upregulation of insulin signaling should be used in future studies. Downregulation of PTEN results in overactivation of PI3K, which is a common mediator of receptor pathways other than insulin/IGF-1 (631–633) and is downstream of IRS-1, the serine phosphorylation of which is the main mechanism through which fat induces insulin resistance. Therefore, an alternative strategy would be to use an IRS-1 Ser → Ala construct (634) ligated to a β-cell specific promoter for the generation of β-cell specific IRS-1 Ser → Ala mice. This would prevent the serine phosphorylation of IRS-1 that is induced by fat and would therefore determine with more certainty the importance of IRS-1 serine phosphorylation and β-cell insulin resistance in fat-induced β-cell dysfunction.

The final study in this thesis examined the role of NOD1 in fat-induced β-cell dysfunction. Since this was the first study to assess the role of NOD1 in β-cell function in vivo, we used whole body NOD1KO mice. Although this study suggests NOD1 plays a role in fat-induced β-cell dysfunction, it did not examine the role of NOD1 in β-cells specifically. It is possible that NOD1 activation in immune cells led to an inflammatory response that in turn damaged β-cells in our study. Fatty acids have been shown to polarize macrophages towards the M1 proinflammatory phenotype and increase secretion of inflammatory mediators via NOD1 (539) and this may have contributed to β-cell dysfunction in our study. NOD1 has also been shown to induce inflammation in other cells, such as adipocytes, in response to high fat feeding (131). Therefore, we performed in vitro experiments and in NOD1KO islets, unlike in wild-type islets, we found that there was no decrease in β-cell function in the presence of palmitate.

Although there is still the possibility of a role of resident macrophages, we do not expect that macrophages are the primary contributors. In one study, Eguchi et al. performed macrophage depletion experiments with clodronate liposomes in ethylpalmitate-infused mice. Islet mRNA levels of PDX-1 and insulin were restored and mRNA levels of inflammatory genes were decreased in clodronate-treated mice compared to controls, however insulin secretion was not assessed. Furthermore, they performed similar experiments in db/db mice, in which macrophage depletion was only partially protective. Peak glucose concentrations during oral glucose tolerance tests were ~600 mg/dl in mice treated with clodronate, which was significantly lower than the ~800
mg/dl in vehicle-treated mice, but was far greater than the ~150 mg/dl seen in control db/+ mice (55). These experiments implicate an important role of macrophages, but do not demonstrate that they are essential for fat-induced β-cell dysfunction. As described previously, macrophages account for only 0.5% of islet cells (546) and it is macrophage infiltration rather than activation of resident macrophages that leads to macrophage-mediated β-cell dysfunction (55,547). This infiltration cannot occur in vitro. Nonetheless, our laboratory aims to directly determine the role of β-cell specific NOD1 activation in fat-induced β-cell dysfunction and to accomplish this, we are currently breeding MIP-Cre NOD1-floxed mice. Equally, our laboratory plans to study macrophage-specific NOD1-knockout mice.

It will also be important to assess β-cell apoptosis induced by palmitate in vitro. Although oleate does not induce apoptosis with 48h exposure, palmitate is known to do so (144,361) and therefore may have contributed to the decrease in insulin secretion in our study by decreasing the number of β-cells rather than the secretory capacity of each β-cell. Nonetheless, our experiments do demonstrate that NOD1 is involved in the palmitate-induced decrease in overall insulin secretion. Further studies will be required to assess β-cell apoptosis and/or viability in order to determine the mechanisms involved and to distinguish between these two possibilities. In addition, future studies should be performed to exclude a role of NOD1 in β-cell dysfunction induced by oleate in vivo. Our in vitro data suggest that it does not play a role as NOD1 deletion did not protect against oleate-induced β-cell dysfunction in vitro, however this should be confirmed in vivo. It would also be interesting to investigate the role of NOD1 in β-cell dysfunction induced by a physiological mixture of palmitate and oleate to better understand the potential role of NOD1 in obesity associated T2D before moving onto studying the role of β-cell NOD1 activation in a chronic model of high fat diet-induced diabetes. Additionally, future studies in germ-free mice would help to distinguish between the role of fat and the role of bacterial products in the NOD1-mediated impairment of β-cell function.
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