p38 Mitogen Activated Protein Kinase Mediates Free Fatty Acid Induced Hepatic Insulin Resistance In Vivo

by

Wen Qin Yu

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Physiology
University of Toronto

© Copyright by Wen Qin Yu 2013
Abstract

Elevated free fatty acids (FFA) contribute to the development of insulin resistance but the mechanisms are not completely understood. We have shown that p38 mitogen activated protein kinase (p38 MAPK) is activated in the liver by lipid infusion and we wished to determine whether it plays a causal role in hepatic insulin resistance after prolonged lipid exposure as shown in isolated hepatocytes. Intralipid and heparin (IH) infusion for 48 hours impaired insulin’s ability to suppress glucose production and tended to impair glucose utilization. Co-infusion of p38 MAPK inhibitor SB239063 with IH restored the insulin-induced suppression of glucose production but did not improve peripheral glucose utilization. IH-induced hepatic insulin resistance was accompanied by increased phosphorylated activating transcription factor 2 (ATF2), a p38 MAPK substrate and this was prevented by co-infusion of SB239063. Our results suggest that p38 MAPK activation mediates hepatic insulin resistance induced by prolonged exposure to FFA in vivo.
Acknowledgments

Foremost I would like to thank my supervisor Dr. Adria Giacca for giving me this incredible opportunity to participate in diabetes research, for her patience and guidance throughout my studies.

I would also like to thank my supervisory committee members, Dr. Carol Greenwood and Dr. Tony Lam, for their helpful feedback and support. In addition I would like to give thanks to other members on my examination committee, Dr. George Fantus, Dr. Tianru Jin, and Dr. Amira Klip for their time and assistance.

I am grateful to Loretta Lam for her technical expertise and other members of the Giacca laboratory for their help: Simon Chiang, Tejas Desai, Cristina Dirlea, June Guo, Saniun Haque, Alex Ivovic, Andrew Ko, Dr. Khajag Koulaian, Tavleen Malhi, Dr. Yusaku Mori, Austin Paul, Dr. Sandra Pereira, and Kaitai Ye.

I also want to take a moment to thank the administrative staff at Department of Physiology for their contributions and thank OGS and BBDC for their financial support.

Lastly I want to thank my family and friends for believing in me and for encouraging me through the tough times.
Table of Contents

Acknowledgments................................................................................................................................. iii

Table of Contents.................................................................................................................................... iv

List of Abbreviations .............................................................................................................................. vi

List of Tables ........................................................................................................................................... ix

List of Figures .......................................................................................................................................... x

Chapter 1 – Introduction ............................................................................................................................ 1
  1.1 Obesity, Insulin Resistance, Type 2 Diabetes....................................................................................... 1
    1.1.1 Obesity......................................................................................................................................... 1
    1.1.2 Type 2 Diabetes .......................................................................................................................... 1
    1.1.3 Insulin Resistance Links Obesity to Type 2 Diabetes................................................................... 2
  1.2 Adipose Tissue-Derived Factors and Insulin Action.......................................................................... 2
    1.2.1 Adipokines .................................................................................................................................. 5
      1.2.1.1 Adiponectin .......................................................................................................................... 5
      1.2.1.2 Leptin .................................................................................................................................... 6
      1.2.1.3 Resistin .................................................................................................................................. 7
      1.2.1.4 TNF-α ................................................................................................................................. 7
      1.2.1.5 IL-6 ....................................................................................................................................... 8
    1.2.2 Free Fatty Acids ......................................................................................................................... 9
  1.3 Free Fatty Acids and Hepatic Glucose Production ............................................................................ 11
  1.4 Free Fatty Acids and Hepatic Insulin Action ...................................................................................... 13
    1.4.1 Insulin Signaling ........................................................................................................................ 14
      1.4.1.1 Glucose Fatty-acid Cycle Hypothesis .................................................................................... 16
      1.4.1.2 Lipid Metabolites Hypothesis .............................................................................................. 17
      1.4.1.3 Inflammation Hypothesis ..................................................................................................... 20
      1.4.1.4 Endoplasmic Reticulum Stress Hypothesis .......................................................................... 22
      1.4.1.5 Oxidative Stress Hypothesis ............................................................................................... 25
      1.4.1.6 p38 MAPK Hypothesis ....................................................................................................... 26
  1.5 Summary of Previous Findings from Our laboratory on Fat-Induced Hepatic Insulin Resistance..... 27
  1.6 Study Rationale .................................................................................................................................. 29
  1.7 Hypothesis ......................................................................................................................................... 29

Chapter 2 – Methods .................................................................................................................................. 31
  2.1 Animals ............................................................................................................................................. 31
    2.1.1 Rats ........................................................................................................................................... 31
    2.1.2 Surgery ....................................................................................................................................... 31
    2.1.3 Infusion Groups and Sampling ................................................................................................. 32
    2.1.4 Hyperinsulinemic-euglycemic Clamp ....................................................................................... 32
    2.1.5 Tissue Collection ...................................................................................................................... 34
  2.2 Plasma assays ..................................................................................................................................... 34
    2.2.1 Glucose ....................................................................................................................................... 34
    2.2.2 Tracer .......................................................................................................................................... 35
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1C</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AdipoR1</td>
<td>Adiponectin receptor 1</td>
</tr>
<tr>
<td>AdipoR2</td>
<td>Adiponectin receptor 2</td>
</tr>
<tr>
<td>AdipoRs</td>
<td>Adiponectin receptor 1 and 2</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B (PKB)</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APPL1</td>
<td>Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1</td>
</tr>
<tr>
<td>ATF</td>
<td>Activating Transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>C/EBP</td>
<td>Ccaat-enhancer-binding protein</td>
</tr>
<tr>
<td>CAP1</td>
<td>Adenylyl cyclase-associated protein 1</td>
</tr>
<tr>
<td>CHOP</td>
<td>Ccaat-enhancer-binding protein homologous protein</td>
</tr>
<tr>
<td>CK2</td>
<td>Casein kinase 2</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase I</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CRTC2</td>
<td>CREB regulated transcription coactivator 2, also known as TORC2</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DGAT</td>
<td>Diglyceride acyltransferase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGP</td>
<td>Endogenous glucose production</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic initiation factor 2 alpha</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic-ribosome-associated protein degradation</td>
</tr>
<tr>
<td>Erp46</td>
<td>Endoplasmic reticulum protein 46</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>4E-BP1</td>
<td>Eukaryotic translation initiation factor 4E-binding protein 1</td>
</tr>
<tr>
<td>FABPm</td>
<td>Plasma membrane fatty acid-binding protein</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>Fatty acid translocase/cluster of differentiation 36</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
</tr>
<tr>
<td>FBP</td>
<td>Fructose-1,6-bisphosphatase</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1</td>
</tr>
<tr>
<td>FPG</td>
<td>Fasting plasma glucose</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>G6Pase/G6pc</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanyl-nucleotide exchange factor</td>
</tr>
</tbody>
</table>
Grb2 Growth factor receptor-bound protein 2
GSK Glycogen synthase kinase
H$_2$O$_2$ Hydrogen peroxide
HDL High-density lipoprotein
HGP Hepatic glucose production
HMW High-molecular-weight
HNF4α Hepatocyte nuclear factor 4 alpha
IDL Intermediate-density lipoprotein
IH Intralipid and heparin
IKK Inhibitor of kappa B kinase
IL-6 Interleukin 6
IRE1 Inositol-requiring enzyme 1
IRS Insulin receptor substrate
IkBα Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
JAK Janus kinase
JNK c-Jun N-terminal kinase
LCFA Long-chain fatty acid
LDL Low-density lipoprotein
LMW Low-molecular-weight
LPL Lipoprotein lipase
MAPK Mitogen-activated protein kinase
MCP1 Monocyte chemoattractant protein 1
MMW Medium-molecular-weight
MRS Magnetic resonance spectroscopy
mTOR Mammalian target of rapamycin
NAC N-Acetyl-L-cysteine, antioxidant
NAD+/NADH Nicotinamide adenine dinucleotide
NADPH Nicotinamide adenine dinucleotide phosphate
NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells
O$_2$ Oxygen
OGTT Oral glucose tolerance test
PBA 4-Phenyl-3-butenoic acid, chemical chaperone
PC Pyruvate carboxylase
Pck1 Phosphoenolpyruvate carboxykinase 1
PDH Pyruvate dehydrogenase
PDK1 Phosphoinositide-dependent kinase-1
PEPCK Phosphoenolpyruvate carboxykinase
PERK Protein kinase RNA-like endoplasmic reticulum kinase
PGC1α Peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PHLPP PH domain and leucine rich repeat protein phosphatase
PI3K Phosphoinositide 3-kinase
PIP3 Phosphatidylinositol (3,4,5)-trisphosphate
PKB/Akt Protein kinase B
PKC Protein kinase C
PP2A Protein phosphatase 2
PPARα Peroxisome proliferator-activated receptor alpha
PPRE  Peroxisome proliferator response element
PTB  Phosphotyrosine binding
PTEN  Phosphatase and tensin homolog
PTP1B  Protein-tyrosine phosphatase 1B
RACK1  Receptor for activated C kinase 1
RIP2  Receptor interacting protein 2
ROS  Reactive oxygen species
RXR  Retinoid X receptor
S6K1  Ribosomal S6 kinase 1
SAT  Subcutaneous adipose tissue
SB  SB239063, p38 MAPK inhibitor
SEM  Standard error of the mean
SERCA  Sarco/endoplasmic reticulum Ca2+ ATPase
SH  Src-homology
SHIP2  SH2 domain containing inositol 5-phosphatase 2
siRNA  Small interfering RNA
SOCS3  Suppressor of cytokine signaling 3
SOS  Son of sevenless, a GEF
SPSS  Statistical product and service solutions
STAT3  Signal transducer and activator of transcription 3
T1D  Type 1 diabetes
T2D  Type 2 diabetes
TACE  TNF-α convertizing enzyme
TAG  Triglyceride
TLR  Toll-like receptor
7TM  7 Transmembrane
TNFR  TNF-α receptor
TNFα  Tumor necrosis factor alpha
TORC2  CREB regulated transcription coactivator 2
TRADD  Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF2  TNF receptor-associated factor 2
TRB3  Tribbles homolog 3
TSC  Tuberous sclerosis protein
TUDCA  Tauroursodeoxycholic acid, chemical chaperone
UCP1  Uncoupling protein 1
UPR  Unfolded protein response
USD  United States dollars
VAT  Visceral adipose tissue
VLDL  Very low-density lipoprotein
WAT  White adipose tissue
XBP1  X-box binding protein 1
List of Tables

Table 1. Summary of results on mechanisms of fat-induced hepatic insulin resistance studied in our laboratory. ............................................................................................................................... 30

Table 2. Plasma levels of free fatty acids, insulin, and glucose before and during the hyperinsulinemic-euglycemic clamp. ............................................................................................................................... 45

Table 3. IC50 values for SB239063 from in vitro experiments ............................................................................................................................... 57
List of Figures

Figure 1. The experimental protocol .......................................................................................... 41
Figure 2. Whole-body insulin sensitivity .................................................................................. 46
Figure 3. Peripheral insulin sensitivity .................................................................................. 47
Figure 4. Hepatic insulin sensitivity ...................................................................................... 48
Figure 5. Western blot analysis for p38 MAPK ....................................................................... 49
Figure 6. Western blot analysis for ATF2 .............................................................................. 50
Chapter 1 – Introduction

1.1 Obesity, Insulin Resistance, Type 2 Diabetes

1.1.1 Obesity

Obesity is a condition of excessive fat accumulation that may impair health. Adults are classified as obese when their body mass index (BMI), which is measured as weight in kg divided by square height in meters, is equal to or greater than 30 kg/m² (1). The prevalence of global obesity has doubled from 1980 to 2008, from 6% to 12% of world population or half a billion adults (2). In 2005, Canadian obesity prevalence was estimated to be 24.2% and the total cost of obesity and related chronic conditions was estimated to be $ 4.39 billion (3). Obesity is a major risk factor for many chronic illnesses including diabetes, cardiovascular diseases (CVD) and some types of cancer (3).

1.1.2 Type 2 Diabetes

Diabetes mellitus is a disorder characterized by hyperglycemia which is associated with many chronic complications affecting the eyes, kidneys, nerves, and increased risk of CVD (1). Patients are diagnosed when they have one or more of the following: a fasting plasma glucose (FPG) equal or greater than 7.0 mmol/l, a plasma glucose level equal or greater than 11.1 mmol/l 2 hours after receiving a 75g oral glucose tolerance test (OGTT), a glycated hemoglobin (A1C) equal to or greater than 6.5%, or a random plasma glucose equal or greater than 11.1 mmol/l(1). Type 1 diabetes (T1D) generally develops early in life and occurs when the pancreas cannot produce insulin due to autoimmune destruction of β cells (1). In contrast, type 2 diabetes (T2D) which affects 90% of people with diabetes mellitus (4), usually develops later in life and occurs
as a result of the combination of insulin resistance and β cell failure (1). Insulin resistance is present in patients for many years and initially pancreatic β cells compensates by increasing insulin production but when these cells cannot sustain the demand for higher insulin secretion T2D develops. Worldwide more than 371 million people or 8.3% of world population have diabetes and more than 471 billion United States dollars (USD) were spent on healthcare for diabetes in 2012 (5).

1.1.3 Insulin Resistance Links Obesity to Type 2 Diabetes

Evidence from several studies has linked obesity to T2D, here on referred to as diabetes. Weight gain is strongly associated with increased risk of diabetes (6-8). One study demonstrated that for every kg of weight gain, the risk of diabetes increases by 4.5% which is a conservative estimate (8). In contrast weight loss was associated with reduced risk of diabetes (7, 8). The majority of people with diabetes, around 80%, are overweight or obese (1). However 20% of patients with type 2 diabetes are not overweight or obese (1) and the majority of obese patients do not develop diabetes (9). This suggests that although obesity is a strong predictor of diabetes it is not enough to cause diabetes which means other factors are involved such as genetic factors (9). At the molecular level, insulin resistance is the common link between obesity and T2D.

1.2 Adipose Tissue-Derived Factors and Insulin Action

Obese individuals have increased adipose tissue mass. There are many different cell types in adipose tissue including adipocytes, preadipocytes, immune cells, neural cells, vascular cells, fibroblasts and connective tissue matrix (10). The parenchymal cells of adipose tissue are adipocytes, of which there are three types, white, brown and the recently discovered beige adipocytes.
For a long time brown adipose tissue was thought to exist in human babies but not in adults. Using positron emission tomography paired with a radioactive glucose analogue it was found that regions surrounding the neck and shoulder had high uptake. When combined with computer tomography which allows the visualization of tissue density, the area was later identified to be brown adipose tissue (BAT) (11). The distinct brown color of BAT is due to the appearance of many mitochondria. BAT is known for their ability to generate heat via the mitochondrial protein uncoupling protein 1 (UCP1) which is unique to brown adipocytes. Interestingly classical brown adipocytes are derived from the same progenitor cell that gives rise to muscle cells (12). Recently a new type of adipocytes has been identified as beige or “brown-like” adipocytes. These adipocytes are capable of expressing UCP1 but they are derived from progenitors of white adipocytes (13). There is an emerging interest in the therapeutic potential of these non-white adipocytes because mice that overexpress these adipocytes are protected from obesity and diabetes (14).

The classical role of white adipose tissue (WAT) is to store energy in the form of triglyceride and release free fatty acids (15). WAT distribution is mainly separated into subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) (10). VAT refers to the fat surrounding organs with the majority located in the abdominal cavity whereas SAT refers to body fat elsewhere in the body (10). Excess VAT in patients takes the form of a protruding abdomen which is synonymous with the “apple” body type (10). In contrast patients with “pear” body shape have big hips which are mostly SAT (10). Although VAT only accounts for about 20% of total body fat (16), it is more closely associated with insulin resistance than total body fat due to its anatomical and physiological differences.
One important anatomical difference is that VAT drains into the liver via the portal vein whereas SAT drains into systemic veins (10). Due to proximity, the liver is directly exposed to secretory products like free fatty acids which promote fat storage in the liver and hepatic insulin resistance. In terms of physiology, VAT is more lipolytically active and the increased release of FFA impairs insulin sensitivity (17). This higher activity can be explained by different receptor expression on tissues that favors lipolysis. For example VAT has elevated levels of glucocorticoid receptor (18) but reduced levels of estrogen receptor (19). In contrast SAT has less lipolytic (β3) adrenergic receptors but more anti-lipolytic alpha adrenergic receptor (20). Lipolysis in VAT is further enhanced by its resistance to insulin’s antilipolytic action. Despite the increased lipolysis of VAT, fat is able to accumulate in the visceral region due to increased lipid synthesis (17).

WAT has recently been recognized to be an endocrine and paracrine organ that can secrete a number of bioactive polypeptides known as adipokines (15) which have been shown to play a role in the development of insulin resistance. Studies have shown that obese humans have a different plasma adipokine profile than lean individuals as exemplified by lower adiponectin (21), higher leptin (22) and resistin (23) levels. Furthermore, adipose tissue from obese humans shows greater secretion of pro-inflammatory cytokines TNFα and IL-6 (24). VAT secretes more adipokines including IL-6 (25) which may also explain the closer association between VAT and insulin resistance. It is interesting to note that the majority of adipokines, with the exception of adiponectin and leptin, is secreted from non-fat cells in WAT (25). Macrophages, accumulated in adipose tissue as a result of obesity, contribute to a large portion of the adipokines secreted (26). I will first review evidence in the literature that demonstrates a link between adipokines and insulin resistance and then I will focus on the effects of FFA on insulin resistance.
1.2.1 Adipokines

1.2.1.1 Adiponectin

Adiponectin is a 30 kDa peptide exclusively secreted by adipose tissue (27). The full length protein contains a collagen domain and a globular domain (27). The collagen domain joins to form homo-oligomers, of which three types are found in the plasma: low-molecular-weight (LMW) trimer, medium-molecular-weight (MMW) hexamer, and high-molecular-weight (HMW) 12- to 18-mer (28). HMW is the most potent form (28). There are three adiponectin receptors, two 7-transmembrane domain (7TM) receptors AdipoR1 and AdipoR2 (AdipoRs), and the cell surface protein T-cadherin (29). AdipoR1 is most abundantly expressed in skeletal muscle and AdipoR2 receptor most abundantly expressed in the liver (30). T-cadherin is highly expressed in the vasculature (31).

Plasma adiponectin levels are lower in conditions of obesity (21), insulin resistance (32) and diabetes (33). Adiponectin administration in rodents lowered plasma glucose (34), enhanced insulin sensitivity (35), and prevented diet-induced weight gain (36). Furthermore overexpression of AdipoR1 in skeletal muscle improved insulin-stimulated glucose uptake (37). In contrast, adiponectin deficiency in mice induced insulin resistance and accelerated the onset of diet-induced obesity (38, 39). Disruption of AdipoRs in knockout mice resulted in glucose intolerance and insulin resistance (40).

The insulin-sensitizing effect of adiponectin is predominantly mediated by AMPK and PPARα activation. In the liver, AMPK activation via AdipoR1 reduced glucose production by decreasing expression of gluconeogenic enzymes (40). AMPK promotes phosphorylation of the CREB coactivator TORC2 which prevents its transport into the nucleus. Therefore TORC2
cannot bind CREB to stimulate gluconeogenesis (41). Activation of PPAR-alpha pathway via AdipoR2 in the liver increased fatty acid oxidation and decreased tissue triglyceride (40). In C1C12 myocytes, in addition to AMPK and PPARα, p38 MAPK also appeared to mediate the effect of adiponectin on glucose uptake and fatty acid oxidation (40). Several adaptor proteins that directly bind to AdipoRs have been identified including APPL1, CK2, RACK1, and Erp46 (42) but the link between these adaptor proteins and downstream signaling molecules are areas that require further investigation. Independent of AMPK activation, AdipoRs have been reported to improve insulin sensitivity by enhancing ceramidase activity and thus reduce hepatic ceramide levels (see Lipid Metabolites Hypothesis) (43). Adiponectin has been reported to stimulate IL-6 production in macrophages with mostly beneficial effect on the liver (44) (See section on IL-6 below for the controversial effects of IL-6 on hepatic insulin sensitivity).

1.2.1.2 Leptin

Leptin is a 16 kDa protein that is primarily secreted from the adipose tissue (45). Mutation of the ob gene, which encodes for leptin, in ob/ob mice results in an obese and diabetic phenotype. Another animal model of the obesity is the db/db mice which is missing the db gene that encodes the receptor for leptin. There are several splice variants for the leptin receptor and they are expressed ubiquitously (46). Most isoforms mediate Janus Kinase (JAK) binding. Upon leptin binding to the extracellular portion of the leptin receptor, JAK2 is recruited and activated JAK2 in turn phosphorylates multiple tyrosines on the leptin receptor. Phosphorylated leptin receptor serves as binding sites for downstream protein targets such as STAT3 (47). Plasma leptin is positively correlated with BMI in rodents and humans and is reduced after weight loss (22). The central effects of leptin are to decrease food intake and body weight, and increase energy expenditure (48). The effect of leptin on insulin signaling in the liver is complex and
lacks consensus. Acute intravenous leptin administration in rodents enhanced insulin’s inhibition of hepatic glucose production (49, 50) but paradoxically increased gluconeogenesis. In contrast, mice with disrupted hepatic signaling were protected from diet-induced glucose intolerance (51). Intracerebroventricular administration of leptin, which did not increase plasma leptin, enhanced insulin action in skeletal muscle and adipose tissue, suggesting that the effect of leptin on insulin action is mainly via leptin receptors in the brain (49).

1.2.1.3 Resistin

Resistin is a 12.5 kDa cysteine rich protein that is synthesized and secreted by adipocytes in rodents (52) and predominantly secreted by macrophages in humans (53). Recently a number of receptors has been proposed (54-56). Circulating resistin levels are increased in animal models of diet-induced and genetic form of obesity (57) and in insulin resistant humans (58, 59). Injection of recombinant resistin in wildtype mice impaired glucose tolerance and insulin action (57). Infusion of recombinant resistin in rats impaired whole-body insulin sensitivity by increasing glucose production without affecting glucose disposal (60). The effect of resistin on glucose production was due to an increase in gluconeogenesis and glycogenolysis (60). Mouse models of resistin deficiency show improved glucose tolerance on a high fat diet because of decreased glucose production and gluconeogenesis likely as a result of increased AMPK activity (57, 61). Although animal studies have shown convincing evidence for a role of resistin in insulin resistance, data in humans are unclear and require further investigation (52, 62).

1.2.1.4 TNF-α

TNF-α is a 26 kDa trimetric transmembrane pro-protein with N-terminal facing intracellularly and C-terminal facing outside the cell (63). It is cleaved by the TNF-α
convertizing enzyme (TACE) to form the 17 kDa soluble mature trimer (64). Macrophages account for the majority of TNF-α expression (26). Obese animals (65) and humans had higher serum TNF-α and TNF-Rs and as compared with lean subjects (66, 67). There are two TNF-α receptors and they transduce TNF-α signaling by binding cytoplasmic adaptor proteins (68). Acute TNF-α infusion in humans reduced insulin-stimulated glucose uptake (67). Neutralizing TNF-α in fa/fa rats improved the response to insulin by increasing peripheral glucose uptake (65). TNFR1 receptor knockout mice (69) and rats that were treated with TNFR1 blocking peptide (70) were protected from diet-induced obesity. Mice lacking TNFR2 receptor were reported to be protected from diet-induced obesity and insulin resistance (71). The exact role of each TNF receptor in TNF alpha mediated insulin resistance is not clear but it is believed that TNFR1 is responsible for the majority of the TNF alpha biological activities (72). Following activation, TNFR1 recruit intracellular adaptor proteins TRADD, TRAF2 and RIP2 to activate IKK, JNK, or p38 MAPK to induce insulin resistance. TNFR1 can also activate the neutral sphingomyelinase which catalyzes the formation of ceramides to impair insulin action (73).

1.2.1.5 IL-6

Within adipose tissue, IL-6 is predominantly produced from resident macrophages (26). The IL-6 receptor is widely expressed and consists of a ligand-binding chain known as IL-6R and a signal-transducing chain known as gp130 (74). Receptor activation recruits JAK2 which is activated by autotransphosphorylation. JAK2 in turn phosphorylates gp130 tails which serves as docking sites for STAT3. Phosphorylated STAT3 dimerizes and enters the nucleus to upregulate transcription of genes such as those encoding SOCS3 proteins which provide negative-feedback inhibition (75).
Plasma IL-6 concentrations were positively correlated with adiposity and insulin resistance (76). Systemic deletion of IL-6 in mice resulted in mature onset diabetes (77). However the role of IL-6 in the development of obesity and insulin resistance appears to be tissue-specific and is still under debate (78). In the liver, chronic exposure of IL-6 induced insulin resistance (79) and depletion of IL-6 in ob/ob mice improved liver insulin action. IL-6 causes insulin resistance through induction of SOCS3 which interferes with IRS1 tyrosine phosphorylation and signal transduction (80). However, IL-6 can also have beneficial effects in the liver. It has been reported that insulin’s ability to suppress glucose production was mediated by hepatic IL-6 secretion as a result of insulin action in the brain (81). The discrepancy in response could be explained by differences in level and duration of IL-6 exposure. In skeletal muscle, increased IL-6 expression during exercise improved glucose uptake (82). This finding was reinforced by another study in healthy humans where infusion of IL-6 was shown to increase glucose disposal (83). The mechanism by which IL-6 increases glucose uptake is partly accounted for by AMPK activation (84). Alternatively, activated IL-6 receptor complex can recruit IRS-1 and induce IRS-1 serine phosphorylation on Ser-318 in cultured skeletal muscle cells (85). This phosphorylation improved insulin-stimulated Ak phosphorylation and glucose uptake (85).

1.2.2 Free Fatty Acids

The predominant forms of fatty acids in the plasma are esterified to glycerol in the form of triglycerides in lipoproteins. The remaining fatty acids are unesterified, bound to albumin, and originate from adipose tissue lipolysis. In this thesis, FFA refers to unesterified fatty acids. Lipoproteins transport dietary fat or fat produced by the liver. Dietary fat in the form of triglyceride is emulsified by bile acids and hydrolyzed to fatty acids, monoacylglycerol, and
glycerol in the small intestine by pancreatic lipase. After entering enterocytes via diffusion or fat transporters such as FAT/CD36 and FATP4 (86), FFA, glycerol, and monoglycerol are re-esterified to triglycerides and packaged into chylomicrons which are secreted into the lymphatic system and drained into the systemic circulation via the thoracic duct. Chylomicrons deliver triglycerides to tissues such as skeletal and heart muscle for energy production and adipose tissue for energy storage. Lipoprotein lipase (LPL) that resides on endothelial cells of tissues hydrolyzes the triglyceride in chylomicrons to promote FFA uptake into tissues via diffusion or transporters such as FATP, FABPm and FAT/CD36 (87). Inside the cell, chylomicron derived FFA are esterified to fatty acyl-CoA and can enter mitochondria for energy generation (muscle) or be used to form triglycerides (adipose tissue). After lipolysis by LPL, chylomicron remnants are taken up by the liver. During fasting, adipose tissue lipolysis occurs and FFA are used in muscle. The excess FFA enters the liver and are re-esterified to triglycerides and delivered to the rest of the body in very-low-density lipoproteins (VLDL) which is also a source of FFA for peripheral tissues via LPL. In the liver fatty acids are also synthesized from glucose.

Circulating FFA levels are elevated in obese humans (88) and in people with type 2 diabetes (89). There are several mechanisms that account for this observed FFA elevation. First, expanded adipose tissue releases more FFA due to its large mass. Second, VAT in obese individuals has increased rate of lipolysis (17). Third, insulin resistant fat cells are less sensitive to the antilipolytic effect of insulin and therefore release more FFA which are taken up by peripheral tissues. Plasma FFA elevation induces insulin resistance after 2 hours in healthy humans (90). The effect of FFA on insulin-stimulated glucose disposal and insulin signaling is dose dependent (91). Furthermore, lowering plasma FFA levels in patients with chronic elevated plasma FFA improves insulin resistance (92).
1.3 Free Fatty Acids and Hepatic Glucose Production

Liver generates glucose from glycogen breakdown (glycogenolysis) and de novo synthesis of glucose (gluconeogenesis) from precursors such as pyruvate, lactate, glycerol, and certain amino acids. After an overnight fast, liver is the major contributor to glucose production with kidney playing a minor role (93). However the contribution of hepatic glucose production (HGP) to endogenous glucose production (EGP) is reduced after prolonged fasting. Gluconeogenesis accounts for about 50% of glucose production during 4 to 13 hours of fasting (post-absorptive state) but progressively increases with duration of fasting (94).

There are four key reactions in gluconeogenesis that bypass the irreversible reactions in glycolysis which are catalyzed in order by pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-biphosphatase (FBP), and glucose-6-phosphatase (G6Pase) (95). It is important to note that G6Pase which is responsible for hydrolyzing glucose-6-phosphate to free glucose is also shared by glycogenolysis. Unlike PC and FBP which are mainly regulated by post-transcriptional mechanisms, PEPCK and G6Pase are regulated at the transcriptional level by various hormones including insulin and glucagon (95). Several transcription factors and co-activators control gluconeogenesis including CREB, FOXO1, HNF4α, PGC1α, and C/EBP (95).

FFA have been shown to increase hepatic gluconeogenesis in vitro (96-98) and vivo (99-101). The effect of FFA on gluconeogenesis can be achieved through several mechanisms. FFA is oxidized in the mitochondria to generate products including acetyl-CoA, NADH and ATP. First, acetyl-CoA can increase gluconeogenesis by allosterically activating PC. Second, NADH produced by fatty acid oxidation facilitates an intermediate reaction step in the gluconeogenic
pathway by increasing formation of glyceraldehyde-3-phosphate from 1,3-bisphosphoglycerate. Third, ATP and citrate generated from acetyl-CoA and oxaloacetate, inhibit phosphofructokinase 1, a glycolytic enzyme, to promote reciprocal regulation of gluconeogenesis. Allosteric effects of FFA on G6Pase have also been described (102). FFA also increase transcription of gluconeogenic genes.

The mechanisms underlying the stimulatory effect of FFA on transcriptional control of gluconeogenesis are not clear. Short-term incubation of hepatocytes in FFA increased PEPCK and G6Pase expression which was attributed to p38 MAPK mediated increase in PGC-1 alpha expression and phosphorylated CREB (96). In another study, incubation of hepatocytes and H4IIE hepatoma cells in short-chain fatty acids (SCFA) increased expression of G6Pase via HNF-4α (97). Long-chain fatty acids (LCFA) were found to recruit a number of transcription factors to the G6Pase promoter to increase gene transcription (98). FFA, saturated and polyunsaturated, are endogenous ligands for PPAR (103). PPAR form heterodimers with RXR which bind to peroxisome proliferator responsive element (PPRE) to regulate transcription of genes including those involved in gluconeogenesis (103). PPAR alpha which is mainly expressed in the liver has been shown to directly bind PPRE in the promoter region of G6Pase to increase glucose production (104). In vivo data in this area are lacking and, which warrants future investigation.

The effect of FFA on post-absorptive hepatic glucose production is controversial. FFA increased glucose production in certain studies but had no effects in other studies (100, 101). Roden et al. found that FFA by lipid infusion increased gluconeogenesis but did not change endogenous glucose production in healthy volunteers (101). In another study plasma FFA elevation achieved through discontinuation of nicotinic acid also increased gluconeogenesis
without changing EGP in type 2 diabetic patients (100). In contrast Staehr et al. found that lipid infusion increased gluconeogenesis and endogenous glucose production in type 1 diabetic patients (105). Similarly another study also found that lipid infusion increased endogenous glucose production in healthy humans which is evident after prolonged fasting duration as well as in diabetes (106). The discrepancies between these studies can be in part explained by hepatic autoregulation.

Hepatic autoregulation occurs when FFA induced gluconeogenesis does not result in increased glucose production because of a compensatory reduction in glycogenolysis (107). FFA-stimulated decrease in glycogenolysis can be achieved through intrahepatic or extrahepatic mechanisms (107). Intrahepatic mechanisms occur through 1) glucose-6-phosphate from gluconeogenesis, which stimulates glycogen synthase (synthesis) and inhibits glycogen phosphorylase (breakdown) and 2) ATP from fat oxidation, which inhibits glycogen phosphorylase. Extrahepatic mechanisms depend on FFA-induced secretion of insulin by the pancreas. Insulin can directly act on the liver to decrease glycogenolysis (107). Alternatively, FFA can enter the brain and activate $K_{ATP}$ channels in the mediobasal hypothalamus to increase vagal input to the liver (108). This central innervation results in reduced glycogenolysis. Healthy humans have intact hepatic autoregulation which may be absent in diabetic patients because of insulin deficiency/resistance (106). The breakdown in hepatic autoregulation can also occur as a result of glycogen depletion which is present after prolonged fasting as well as in diabetes.

1.4 Free Fatty Acids and Hepatic Insulin Action

As stated above, FFA do not always increase hepatic glucose production, despite increasing gluconeogenesis. However, FFA have been shown to impair insulin’s ability to
suppress glucose production (hepatic insulin resistance) which results in increased hepatic glucose production in the presence of postprandial insulin levels (109-111). The molecular mechanisms underlying FFA-induced hepatic insulin resistance are not completely understood. Several hypotheses have been proposed including increased lipid metabolites, inflammation, endoplasmic reticulum (ER) stress and oxidative stress. I will review these in details below after examining normal insulin signaling.

1.4.1 Insulin Signaling

Insulin binding to the extracellular domain of insulin receptor changes the receptor conformation to activate the intracellular tyrosine kinase. The receptor transphosphorylates itself on critical tyrosine residues (112). Phosphorylated tyrosine recruits the insulin receptor substrate (IRS) through the phosphotyrosine binding (PTB) domain and phosphorylates IRS (113). There are several IRS isoforms. Studies of IRS knockout models show that IRS1 and 2 are particularly important in mediating the metabolic actions of insulin (114, 115). Phosphorylated tyrosines on IRS serve as scaffolds for proteins that contain Src-homology (SH) 2 domain (116). One of the proteins is Grb2 which forms a complex with the guanyl-nucleotide exchange factor (GEF) SOS. When activated by IRS, Grb-SOS subsequently activates the GTPase Ras and the downstream mitogen-activated protein kinase (MAPK) cascade (117). This pathway largely regulates expression of genes that are involved in cell growth and differentiation (117). The other protein that binds to IRS via the SH2 domain is the p85 regulatory unit of phosphatidylinositol-3-kinase (PI3K) which is involved in glucose metabolism (118). The catalytic p110 subunit of PI3K phosphorylates membrane lipids to form phosphatidylinositol 3- phosphate (PIP₃) (117). PIP₃ then attracts protein kinase B (PKB) or Akt to the membrane via the pleckstrin homology (PH) domain (117). For full activation, Akt requires two residues to be phosphorylated, one by
phosphoinositide-dependent kinase-1 (PDK1) (119, 120) and the other by a complex between mammalian target of rapamycin (mTOR) and the rapamycin-insensitive companion of mTOR (RICTOR) (121). There are also different isoforms of Akt. Knockout models show that Akt2 is the key isoform that mediates metabolic actions of insulin (122).

Activated Akt has several targets. In muscle and adipose tissue, it can phosphorylate and inactivate the GTPase activating protein (GAP) Akt substrate of 160 kDa (AS160), therefore remove inhibition on Rab GTPase which mediates GLUT4 translocation to increase glucose uptake (117). In the liver and muscle, Akt can also phosphorylate and inactivate glycogen synthase kinase (GSK) 3 (123). Unphosphorylated GSK3 inhibits glycogen synthase, therefore GSK3 phosphorylation increases glycogen synthesis. In addition, Akt can phosphorylate and inactivate tuberous sclerosis complex 1 and 2 (TSC1/2) (117). TSC2 in the TSC1/2 heterodimer functions as a GTPase-activating protein (GAP) on the GTPase Rheb (124). Inactivation of TSC2 removes inhibition on Rheb. Rheb can therefore activate mTORC1 and its effectors ribosomal protein S6 kinase (S6K1) and eukaryotic translation initiation factor 4E binding protein-1 (4E-BP1) to increase protein synthesis (124). In the liver, Akt also phosphorylates the transcription factor forkhead box O1 (FOXO1) (125). This traps it in the cytoplasm and prevents its translocation to the nucleus and transcription of gluconeogenic genes.

In contrast, phosphatases remove phosphate from the proteins above to downregulate insulin signalling at several steps. For example, protein tyrosine phosphatase (PTP) 1B removes phosphate from tyrosine residues on insulin receptor and IRS (117). Membrane phospholipid phosphatases such as phosphatase and tensin homologue (PTEN) and Src-homology-2 domain-containing inositol phosphatase-2 (SHIP2) dephosphorylate PIP₃ (117). Lastly phosphatase-2A
(PP2A) and PH-domain leucine-rich-repeat protein phosphatase (PHLPP) dephosphorylate and inactivate Akt (117).

1.4.1.1 Glucose Fatty-acid Cycle Hypothesis

The glucose fatty-acid cycle, proposed by Randle and colleagues, attempts to explain how fatty acids from adipose tissue can impair insulin-stimulated glucose uptake in muscle and adipose tissue (126). Increased amount of fatty acids leads to increased fat oxidation in mitochondria. Higher ratios of acetyl coenzyme A (CoA) to CoA and NADH to NAD+, inhibit pyruvate dehydrogenase (PDH) and therefore glucose oxidation. Acetyl CoA from fat oxidation shuttles into the Krebs cycle but during energy excess NADH and ATP inhibits Krebs cycle enzymes and causes citrate to accumulate. Excess citrate exits the mitochondria and inhibits phosphofructokinase and therefore increases glucose-6-phosphate (G6P). Increased G6P inhibits hexokinase which results in glucose accumulation and decreased glucose uptake. Randle and his colleagues showed that incubation of rat heart in fatty acids increased G6P and glucose concentration, in agreement with their hypothesis (126). However these results could not be replicated in human skeletal muscle. Using 13-carbon and 31-phosphate magnetic resonance spectroscopy (MRS), human studies found lower G6P concentrations and reduced glycogen synthesis in people with diabetes compared to controls (94). Lower levels of muscle G6P and glycogen were also found in healthy, insulin-sensitive patients given Intralipid and heparin infusion to raise plasma FFA levels (127). Hence insulin resistance is more complex than substrate competition between glucose and fatty acids. Instead, muscle insulin resistance is likely caused by defective insulin signaling and glucose transport.
1.4.1.2 Lipid Metabolites Hypothesis

Lipid metabolites were thought to be involved in FFA induced insulin resistance. Elevated FFA levels due to expanded adipose tissue lead to accumulation of triglycerides and lipid metabolites in the liver and muscle (128). Excess FFA could be channeled into the fatty acid esterification pathway instead of fatty acid oxidation. This would generate lipid metabolites such as long chain fatty acyl-CoA (LCFA-CoA) and diacylglycerol (DAG). As described in the previous section, in situation of energy excess, the products of FFA oxidation inactivate Krebs cycle enzymes. As a result citrate accumulates and exits the mitochondria to provide cytosolic acetyl-CoA and activates acetyl-CoA carboxylase (ACC). Malonyl-CoA which is the product of ACC can then allosterically inhibit carnitine palmitoyl transferase I (CPTI) to prevent transport of fatty acids across mitochondria for β-oxidation. This results in accumulation of LCFA which can be esterified to form DAG or ceramides and both have been implicated in insulin resistance (128).

A number of studies have demonstrated that DAG is detrimental to insulin signaling. ACC knockout in mice decreased intramyocellular concentration of DAG and improved muscle insulin sensitivity (129). The enzyme diglyceride acyltransferase (DGAT) catalyzes the formation of triacylglyceride (TAG) from DAG. Decreasing DAG by overexpressing DGAT in mouse skeletal muscle protected mice from insulin resistance caused by high fat diet (130). Unexpectedly, DGAT deficient mice which should have increased DAG were also protected from diet-induced obesity and insulin resistance (131). However the tissue level of DAG did not increase and the improvement in phenotype could be attributed to decreased PPAR expression which is involved in gluconeogenesis (132). Furthermore, in healthy humans, following reversal
Both LCFA-CoA and DAG can induce protein kinase C (PKC) activation which can mediate or more often inhibit insulin action (134). PKC are categorized into conventional (α, β and γ) novel (δ, ε, η and θ), and atypical PKC (ζ and ι/λ). Conventional and novel PKC require calcium and DAG for activation, while atypical PKC are independent of both (134). In vitro studies suggest that DAG containing unsaturated fatty acids are better activators of PKC (135).

PKC isoform activation is dependent on species and tissue types. In humans, short-term fat infusion activates PKC-β and PKC-δ in muscle (136). Liver samples from obese patients showed increased expression of PKC-δ and PKC-ε compared to lean controls (137). In rodent muscle, insulin resistance induced by high fat diet enhanced PKC-θ and PKC-ε activity (138). In rodent liver, short-term lipid infusion activated PKC-δ (109, 139) and high fat feeding activated PKC-ε (140)

Whole-body knockout of PKC-θ protected mice from muscle insulin resistance induced by lipid infusion (138). However, PKC-θ knockout mice showed insulin resistance after exposure to high fat diet (141). Inhibition of PKC-ε with antisense oligonucleotide protected rats from hepatic insulin resistance due to short-term high fat diet (142). Global PKC-ε knockout mice were also protected from glucose intolerance induced by prolonged high fat diet but this improvement was due to enhanced circulating insulin rather than improved insulin sensitivity (143). Thus, the effect of PKC-θ and PKC-ε in fat-induced insulin resistance is still controversial. Whole-body knockout PKC-δ mice have improved insulin sensitivity and better
glucose tolerance on normal diet (137) and after high fat diet (143). Liver-specific PKC-δ knockout in mice resulted in improved glucose tolerance and insulin signaling after high fat diet (143) whereas liver-specific overexpression of PKC-δ in mice impaired glucose tolerance (137). Our lab showed that inhibition of PKC-δ using antisense oligonucleotides prevented hepatic insulin resistance after acute lipid infusion suggesting that PKC-δ plays a causal role in fat-induced liver insulin resistance (unpublished data).

In response to insulin, phosphorylated PKCζ by PDK1 facilitates glucose uptake in skeletal muscle and adipocytes (144). However PKCζ has also been implicated in insulin resistance caused by ceramides (see below). Novel PKC have been mainly implicated in insulin resistance. Activated PKC may inhibit insulin signaling directly by phosphorylating serine or threonine residues on insulin receptor and insulin receptor substrate (IRS). This phosphorylation interferes with insulin receptor tyrosine kinase activity and downstream signaling. Also, activated PKC may indirectly inhibit insulin signaling via other kinases such as inhibitor of nuclear factor kappa-B kinase (IKKβ), c-Jun N-terminal kinase (JNK), or through oxidative stress which is upstream of IKKβ and JNK.

Ceramide is another lipid metabolite that has been linked to insulin resistance. In healthy humans, Intralipid infusion decreased insulin sensitivity and increased muscle ceramide contents (145). In rodents, infusion of lard-oil (mostly saturated fat) decreased insulin sensitivity in association with accumulation of ceramides in the liver and skeletal muscle (146). Myriocin, which blocks ceramide synthesis, prevented ceramide accumulation and insulin resistance due to lard-oil (146). The study also found that infusion of soy-oil which is similar to Intralipid (mostly unsaturated fat) induced insulin resistance due to increased levels of DAG but not ceramide
This suggests that ceramides play a more important role in the regulation of insulin signaling by saturated fatty acids. Ceramides can induce insulin resistance by several mechanisms. First, ceramides activate PKCζ to increase phosphorylation of Akt at a site that decreases its affinity for membrane phosphoinositides. Second, ceramides activate phosphatase PP2A which dephosphorylate Akt (147). Third, ceramides can activate JNK via MAPK kinase kinases, TAK1 (MAPKKK) (148) and ASK1 (149) to impair insulin action (see Endoplasmic Reticulum Stress Hypothesis).

1.4.1.3 Inflammation Hypothesis

Obesity is now considered a state of chronic low-grade inflammation marked by high levels of proinflammatory cytokines including tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) in plasma and tissues. Adipose tissue is recognized as not only a storage depot but also as a highly active, metabolic and endocrine organ that secretes hormones such as leptin, resistin, and cytokines, although recently the discovery of macrophages in adipose tissue of obese individuals indicates that these macrophages contribute to the production of inflammatory factors (26). For example TNF and IL-6 are mainly produced by macrophages (26).

Saturated FFA can directly activate IKKβ, a pro-inflammatory kinase, through Toll-like receptors 4 (TLR-4) (150) and both saturated and unsaturated FFA can do it via oxidative stress. The TLR receptors recognize pathogens and activate the innate immune response. This suggests that nutrient and pathogen sensing may use the same signalling pathway. In fat cells, FFA activated IKKβ was shown to be PKC dependent (151). Hence DAG and PKC may be upstream of IKKβ. PKC also links the lipid metabolite and inflammation hypothesis.
Similar to PKC activation, activated IKKβ can phosphorylate insulin receptor and IRS on serine or threonine residues. Activated IKKβ also phosphorylates IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, alpha) which causes its degradation. IκBα normally inhibits NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) which is a transcription factor for pro-inflammatory mediators such as TNFα and IL-6, which decrease insulin action via activating pro-inflammatory kinases (152) and increasing suppressors of cytokine signaling (SOCS) (153). Therefore IKKβ impairs insulin signaling through activation of inflammatory and stress-activated pathways.

In human skeletal muscle, acute plasma FFA elevation has been shown to activate NFkB (136). In rats, acute plasma FFA elevation increased hepatic mRNA expression of proinflammatory cytokines and plasma MCP1 protein level which may be involved in recruiting monocytes to adipose tissue (139). In vitro, human adipocytes treated with conjugated linoleic acid showed increased IL-6 secretion 6 hours after treatment which plateaued after 24 hours, transient TNF-α gene expression which peaked at 3 hours but no protein secretion was detected (154). As well, cultured adipocytes that are treated with linoleic acid for 3 hours had lower IκBα and higher NF-κB protein levels (154).

The majority of studies suggest that IKKβ activation in muscle and liver plays a role in fat induced insulin resistance. Inhibition of IKKβ improved insulin resistance induced by lipid infusion (155, 156) and high fat diet (157). However there are also studies that show that inhibition of IKKβ had no effect on fat-induced insulin resistance (158, 159). The majority of the studies were on whole-body or peripheral insulin action. The role of IKKβ on hepatic insulin resistance has been less investigated. However, liver specific IKKβ knockout mice were protected from insulin resistance induced by high fat diet (157). This may be due to cytokines
rather than fat per se, as supported by our findings that IKKβ inhibition prevented fat induced hepatic insulin resistance after 7 hours (110) of lipid infusion but not 48 hours (111).

1.4.1.4 Endoplasmic Reticulum Stress Hypothesis

The ER is an important organelle responsible for protein synthesis, folding, and maturation, Ca\(^{2+}\) storage, and lipid synthesis (160). ER stress refers to a disruption of ER homeostasis which causes accumulation of unfolded or misfolded proteins in ER (160). In response to this, the unfolded protein response (UPR) is activated, which attempts to reduce ER protein load and increase production of protein chaperones that assist protein folding (160).

ER stress can be induced by free cholesterol which increases the ordering of the phospholipid membrane, thereby inhibiting the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) (161). Normally SERCA transfers Ca\(^{2+}\) from the cytosol into ER lumen. Therefore inhibition of SERCA leads to a loss of Ca\(^{2+}\) in the ER. In addition oxidative stress can induce ER stress by depleting ER calcium stores via SERCA (162). It is thought that ER stress in adipose tissue is due to increased synthesis of structural proteins required to store excess fat (163).

There are three ER stress transducers in the ER membrane: inositol-requiring 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6). In the absence of ER stress, these ER stress transducers are bound to chaperones such as immunoglobulin heavy chain binding protein (BiP) (160). In the presence of ER stress, BiP chaperone molecules bind to misfolded proteins in the lumen and thereby release and activate the ER stress transducers.
Activated IRE1 splices X-box binding protein 1 (XBP1) mRNA to form the spliced XBP-1 mRNA which codes for the transcription factor XBP1 (160). XBP1 upregulates genes that code for ER chaperones, endoplasmic reticulum associated protein degradation (ERAD), and phospholipid biosynthesis (160). ER chaperones assist protein folding and ERAD components ubiquitinate misfolded proteins for degradation. IRE1 can also degrade mRNA non-specifically (160). In addition IRE1 can activate JNK and IKKβ through tumor necrosis factor receptor-associated factor 2 (TRAF2) (160). Unexpectedly, XBP1 can lower hepatic glucose output by targeting FOXO1 for proteasomal degradation (164).

Activated PERK phosphorylates eukaryotic translation initiation factor 2 (eIF2α) and reduces global protein translation but facilitates the translation of transcription factor ATF4 (160). ATF4 increases expression of CHOP, another transcription factor (160) which can bind to the TRB3 promoter to increase TRB3 transcription (165). TRB3 can then inhibit Akt/PKB and reduce metabolic actions of insulin (166). Substantial eIF2 phosphorylation can also activate NFκB though translational inhibition of the inhibitor IκBα (167).

Lastly, activated ATF6 enters the Golgi apparatus where it is processed and activates transcription of genes that code for ERAD, lipid biosynthesis, and ER chaperones (160). ATF6 has been shown to lower glucose output by disrupting the interaction (160) between TORC2 and CREB complex (168).

Administration of chemical chaperones PBA and TUDCA, which reduces ER stress, have been shown to improve insulin action in obese and diabetic mice (169). Both PBA and TUDCA are approved by FDA for human use. Obese humans treated with TUDCA demonstrated improved insulin sensitivity (170). Similarly PBA treatment improved insulin resistance caused
by lipid infusion (171). Clinical trials are currently being performed to determine the effect of PBA and TUDCA in obese subjects.

ER stress can activate JNK as mentioned above. JNK can also be activated by other factors including FFA, TNFα, and oxidative stress (172). JNK is a pro-inflammatory kinase and its activation via phosphorylation may also play a role in fat-induced insulin resistance (152, 173-175). For example, JNK activation was found in the liver, fat, and muscle in mice fed a high fat diet (173). JNK activation was also found in mouse hepatocytes exposed to FFA (152). Reduced JNK expression through genetic manipulation show reduced insulin resistance. For instance, JNK1 null mice were protected from insulin resistance induced by high-fat diet (173). Knockdown of JNK in liver of obese mice via siRNA improved insulin sensitivity (175) as did dominant-negative JNK overexpression (174). Sabio et al. investigated the different roles of JNK in different tissues using diet-induced insulin resistant mice. They found that JNK1 in muscle plays a role in peripheral insulin resistance (176), in agreement with the previous literature. However, mice with hepatocyte-specific ablation of JNK1 exhibit insulin resistance (177) in contrast to its prevention discussed in the literature. Furthermore, adipose tissue-specific ablation of JNK1 prevented hepatic insulin resistance (178). This suggests that JNK1 activation in adipose tissue cause liver insulin resistance and not JNK1 activation in hepatocytes. It is believed that JNK1 expression in adipose tissue promotes IL-6 secretion which in turn increases liver protein SOCS3 and subsequently hepatic insulin resistance. Similar to PKC and IKKβ, activated JNK can increase phosphorylated IRS on serine or threonine residues (179).
1.4.1.5 Oxidative Stress Hypothesis

Oxidative stress also plays a role in fat-induced insulin resistance. Oxidative stress refers to a build-up of highly ROS or reduced antioxidant defenses. ROS directly damage cells by oxidizing macromolecules such as DNA, protein and lipid. They can also indirectly damage cells by activating stress-sensitive signalling pathways including IKKβ, JNK, and p38 MAPK. In healthy humans, fat infusion increased plasma FFA levels and plasma free-radical concentrations which is indicative of oxidative stress (180), and intravenous administration of the antioxidant glutathione improved insulin sensitivity (180). Oral administration of the antioxidant taurine in humans also ameliorated insulin resistance induced by lipid infusion (181). Muscle insulin resistance induced by lipid infusion was improved by the antioxidant NAC (182) and we have shown that NAC also prevents hepatic insulin resistance induced by 7 hour- but not 48 hour-lipid infusion (unpublished data).

FFA can induce production of ROS by peroxisomal and mitochondrial oxidation or by inhibiting antioxidant enzymes (183, 184). The electron transport chain (ETC) normally generates water as the final product. Incomplete reduction of oxygen to water produces the superoxide radical O$_2^-$ which is not very reactive. However the protonated superoxide, hydroperoxyl HO$_2^-$ can inactivate enzymes or initiate lipid peroxidation. Normally only 2 to 3% of O$_2$ from the ETC is converted to ROS (185). In states of energy excess, more ROS is produced. In addition to mitochondria, long chain fatty acid oxidation also occurs in peroxisomes. Oxidation is not coupled to ATP production but electrons are transferred to O$_2$ to produce H$_2$O$_2$, another ROS (185).
More importantly, FFA may increase ROS via PKC which activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Evidence shows an increase in NADPH oxidase activity in liver of Zucker rats on high-fat diet (186). NADPH oxidase activity also increased in liver of rats on high fat diet (187) and cultured adipocytes exposed to FFA (188). Our data in the 7 hour-infusion model showed that apocynin, an NADPH oxidase inhibitor, prevented hepatic insulin resistance (unpublished).

1.4.1.6 p38 MAPK Hypothesis

p38 MAPK is a stress activated kinase activated by cellular stresses such as oxidative stress (189) and inflammatory cytokines (TNFα, IL-1) (190). Dual phosphorylation by MAPK kinases (MEK3, MEK6) on threonine and tyrosine residues is required for p38 MAPK activation (191). There are four isoforms of p38 MAPK: p38α, p38β, p38γ, and p38 δ. They are expressed in all tissues, although p38β, p38γ, and p38 δ expression appear to be higher in specific tissues. In general, p38β is abundant in brain, p38γ in skeletal muscle, and p38δ in endocrine glands (192). p38 MAPK usually refers to p38α which has been widely studied. Targets of all MAPK-isoforms include activated protein kinase (MK) 2 and transcription factor ATF2 (191).

The literature suggests that stress-activated kinase p38 MAPK has opposing roles in the liver and peripheral tissues (skeletal muscle and adipose tissue). There are contrasting reports about whether p38 MAPK is activated by insulin in peripheral tissues and whether SB203580, a p38 MAPK inhibitor decreases glucose uptake via or independent of p38 MAPK (193, 194). p38 MAPK inhibition with SB203580, which is specific to p38α MAPK and p38β MAPK, reduced insulin-stimulated glucose uptake without affecting GLUT4 translocation (193). However p38β MAPK loss did not affect insulin-stimulated glucose uptake in muscle and adipocytes (194).
Therefore p38α MAPK may be involved in GLUT4 activation. It has been suggested that SB203580 may directly impair GLUT4 function. Inhibition of p38 MAPK by expressing the dominant negative form or using small interfering RNA had no effect on insulin-stimulated glucose uptake in L6 myotubes (195). Furthermore SB203580 decreased insulin-stimulated glucose uptake in L6 myotubes that express SB203580-resistant form of p38 MAPK (195).

Liver-specific overexpression of dominant negative p38α MAPK via adenovirus in ob/ob mice, which is a genetic model of obesity and diabetes, improved insulin sensitivity, and overexpression of wild type p38α MAPK in lean mice increased insulin resistance (196). However there is also evidence showing that activation of p38 by expression of constitutively active p38 MAPK kinase MKK6 in the liver of ob/ob mice enhanced insulin sensitivity by reducing endoplasmic reticulum (ER) stress (197). In vitro, SB203580 as well as genetic inhibition of p38α MAPK prevented insulin resistance in primary hepatocytes with prolonged exposure to oleate (198) and blocked FFA-induced expression of gluconeogenic genes (96).

1.5 Summary of Previous Findings from Our laboratory on Fat-Induced Hepatic Insulin Resistance

To circumvent the multitude of effects observed in genetic and diet-induced models of obesity, our lab focuses on the selective effect of fat elevation by infusing animals with Intralipid and heparin. Intralipid is a triglyceride emulsion containing largely unsaturated fatty acids and co-administration of heparin stimulates lipoprotein lipase which hydrolyzes triglyceride to release FFA. This model is advantages for studying the effect of FFA elevation on insulin resistance because unlike genetic and diet-induced insulin resistance, the animals do not gain substantial fat mass which can release adipokines and impair insulin action. In addition, infusing
fat avoids changes in gastrointestinal hormones, autonomic activity, and gut flora, associated with ingestion of fat. Intralipid can be used in humans, thus allowing comparison between animal and human studies. We infuse fat for 7 or 48 hours (h) to compare acute and more prolonged effects of fat elevation. Acute lipid infusion is a better model of short-term changes in lipolysis such as during fasting or stress whereas prolonged-lipid infusion better mimics the chronic elevation in circulating FFA found in obesity.

In our 7h model (Table 1), FFA-induced hepatic insulin resistance was prevented by IKKβ inhibitor sodium salicylate (110), the antioxidant NAC, and antisense oligonucleotide against PKCδ (unpublished data). These studies established IKKβ, oxidative stress, PKCδ, and ER stress as causal factors in short-term FFA-induced hepatic insulin resistance in vivo. By analyzing liver tissues, we found that NAC co-infusion prevented FFA-induced IKKβ activation but did not change PKCδ activation. This suggests that a likely pathway of FFA induced hepatic insulin resistance is: FFA → PKCδ → oxidative stress → IKKβ. JNK and p38 MAPK are activated after 7h of lipid infusion but they play a causal role is not clear. To identify the sources of oxidative stress, we looked at NADPH oxidase because it was activated after 7h lipid infusion. We found that NADPH oxidase was involved in our model because apocyin, which is an NADPH oxidase inhibitor, prevented short-term hepatic insulin resistance. We also found that the chemical chaperones PBA and TUDCA (unpublished data) improved lipid-induced hepatic insulin resistance. However, interestingly, lipid infusion did not increase markers of ER stress in the liver. We suspect that lipid infusion may activate ER stress in extra-hepatic tissues, as it does in the 48h model (see next paragraph), to cause insulin resistance in the liver.

In our 48h model (Table 1), IKKβ is not activated and oxidative stress markers are not elevated. Accordingly, sodium salicylate (111) and NAC did not prevent FFA-induced hepatic
insulin resistance. Co-infusion of the chemical chaperones PBA and TUDCA partially improved lipid-induced hepatic insulin resistance (unpublished data). As in the 7 hour model, lipid infusion for 48 hours did not elevate ER stress makers in the liver. However it did elevate ER stress markers in fat and brain, suggesting cross-talk mechanisms in the modulation of hepatic glucose production in this model. These studies suggest that the duration of FFA elevation is an important determinant of the mechanisms of fat-induced hepatic insulin resistance. Since the mechanisms underlying hepatic insulin resistance in the prolonged lipid infusion model are still unclear, we wished to address the role of other potential candidates.

1.6 Study Rationale

We have shown that the stress kinase p38 MAPK is activated by lipid infusion after 7 hours. In vitro studies suggest that p38 MAPK plays a role in insulin resistance in hepatocytes with prolonged exposure to oleate, as oleate impaired insulin signaling via PTEN (198). Also oleate increased transcription of gluconeogenic genes in isolated hepatocytes via p38 MAPK (96). However the role of p38 MAPK in fat-induced hepatic insulin resistance remains to be investigated in vivo. Therefore, I will focus on the role of p38 MAPK in the 48h lipid infusion model, using SB239063 which is a chemical inhibitor selective for p38α MAPK and p38β MAPK.

1.7 Hypothesis

p38 MAPK plays a causal role in hepatic insulin resistance induced by prolonged fat exposure in vivo.
Table 1. Summary of results on mechanisms of fat-induced hepatic insulin resistance studied in our laboratory.

<table>
<thead>
<tr>
<th>Inhibition of</th>
<th>7h lipid infusion</th>
<th>48h lipid infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCδ</td>
<td>√ (activated)</td>
<td></td>
</tr>
<tr>
<td>Ox stress</td>
<td>√</td>
<td>x</td>
</tr>
<tr>
<td>ER stress</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>IKKβ</td>
<td>√</td>
<td>x</td>
</tr>
<tr>
<td>JNK (activated)</td>
<td></td>
<td>?</td>
</tr>
<tr>
<td>p38MAPK (activated)</td>
<td></td>
<td>?</td>
</tr>
</tbody>
</table>

In the 7 hour model, various inflammatory and stress mediators are involved but it is not clear which ones are playing a role after 48 hours of lipid infusion. We have shown that lipid activates p38 MAPK after 7 hours of lipid infusion but we wished to investigate its role in the 48 hour model. Ox – oxidative. ER – endoplasmic reticulum. √ - effective. x – not effective. ? – to be investigated. (activated) – on Western blots.
Chapter 2 – Methods

2.1 Animals

2.1.1 Rats

The protocol for animal use was approved by the Animal Care Committee at University of Toronto. Female Wistar rats weighing between 250 and 300 g were ordered from Charles River (Quebec, Canada). Animals were housed in the Department of Comparative Medicine, Medical Sciences Building at University of Toronto. Rats were fed a rodent diet that consists of 58% calories from carbohydrate, 18% calories from fat, and 24% calories from protein (Teklad Global Diet 2018, Harland Laboratories). Animals were placed in 12 hours of light-dark cycle and given 1 week to acclimatize to the facility before undergoing cannulation surgery.

2.1.2 Surgery

Neck vessel surgery was performed under anesthesia using isofluorane. Rats were cannulated in the left jugular vein and the right carotid artery. Catheters consisted of a variable length of polyethylene tubing (PE-50, BD Intramedic) connected with 2.7 cm of silastic laboratory tubing (0.51mm internal diameter, Dow Corning), both beveled to 45 degrees. Catheters were filled with heparinized saline (4U/ml) to prevent clotting. Indwelling catheters were externalized subcutaneously through a metal button located behind the head and the ends were capped with metal pins. Buprenorphine (0.03mg/ml/kg) was given during surgery for analgesia. Animals were given 3 to 6 days to recover before experiments.
2.1.3 Infusion Groups and Sampling

After recovery, rats were placed into metabolic cages (Harvard Apparatus, Quebec, Canada) allowing unrestricted movements. Catheters were covered by a metal tether which is connected to the metal button placed in the animal to prevent catheter kinking and chewing by the animal. Rats were given one hour to acclimatize to the cage and during this time, saline (5µl/min) was infused into the venous catheter. After acclimatization, one of the 4 treatments, randomly chosen, was infused into the venous catheter for 48 hours. Treatment groups include: saline (5.5µl/min), lipid (20% Intralipid + 20U/ml heparin (IH), 5.5µl/min, lipid + p38 MAPK inhibitor (IH + SB239063, 2.25mg/kg/h for the 1st hour + 0.55mg/kg/h), and p38 MAPK inhibitor (SB239063, 2.25 mg/kg/h for the 1st hour + 0.55mg/kg/h). SB239063 infusion rate was derived from another study (199). Heparinized saline (4U/ml, 5µl/min) was infused into the arterial catheter to maintain patency. Blood samples were collected from the arterial catheter at several time points throughout 48 hours. Plasma was collected and red blood cells were diluted in heparinized saline (4U/ml) in 1:1 ratio and reinfused into the animal. Animals were given 1g pebble of food prior to fasting overnight for around 17 hours before tracer infusion.

2.1.4 Hyperinsulinemic-euglycemic Clamp

Fourty-four hours into the treatment infusion, 3-³H-glucose or tracer was infused into the venous catheter to assess endogenous glucose production (EGP) via the tracer dilution method. An initial bolus of 8 uCi was given followed by a constant infusion of 0.15uCi/min. One and a half hours into the infusion of titrated glucose (45.5 hours into the treatment), basal blood samples were collected at 10-minute intervals for 30 minutes to measure plasma glucose, insulin, FFA, and tritiated glucose specific activity. The hyperinsulinemic euglycemic clamp was
performed during the last two hours of treatment. After the last blood sample (46 hours into the treatment), insulin (Humulin R, Eli Lilly Canada) infusion was started (5 mU/kg/min) and lasted 2 hours while variable labeled glucose was infused to maintain the basal glucose level, depending on blood glucose measured every 5 to 10 minutes. During the last 30 minutes, clamp blood samples were collected also at 10-minute intervals and for the same parameters as basal samples.

Insulin was diluted in 0.1% fat-free bovine serum albumin (BSA). The labeled glucose infusate was prepared by adding $^{3-\text{H}}$-glucose to a 50% glucose solution (48 $\mu$Ci/g) to maintain constant plasma specific activity which is important for obtaining accurate values for glucose production.

Three types of blood samples were collected during the basal and clamp period. First, around 30µl of blood was collected in a small cuvette for plasma glucose assay (see 3.2.1). Second, 0.2 ml of blood was collected in heparinized Eppendorf tubes (50U/ml) for plasma tracer assay (see 3.2.2). Third, 0.2 ml of blood was collected in Eppendorf tubes containing EDTA + Trasylol (10% of blood volume) and lipase inhibitor for plasma FFA and insulin assays (see 3.2.3 and 3.2.4). Blood samples were centrifuged and plasma in the supernatant was collected for direct measurement of glucose or transferred to clean and labeled Eppendorf tubes. The remaining red blood cells (RBC) were diluted in heparinized saline (2U/ml) in 1:1 ratio and reinfused into the animal. Collected plasma samples were stored at -20°C.
2.1.5 Tissue Collection

At the end of the clamp, animals were deeply anesthetized with a mixture of ketamine, xylazine, and acepromazine (87:1.7:0.43mg/ml), diluted with heparinized saline (2U/ml) in a ratio of 1:4. Liver tissue was collected by flash freeze with pre-cooled aluminum tongs and submerged in liquid nitrogen. Thereafter left and right soleus skeletal muscle and retroperitoneal fat tissue was collected. Animals were euthanized by anesthetic overdose after tissue collection.

2.2 Plasma assays

2.2.1 Glucose

Two methods were used to measure glucose concentration. 1) For convenience, plasma glucose in blood samples collected from 0h to 44h of treatment infusion was measured with OneTouch UltraMini Meter and Code25 OneTouch Ultra Blue Test Strips. It only requires 1ul of blood and the meter is calibrated to provide the plasma value. 2) From 44h to 48h of treatment infusion, plasma glucose was measured with the Analox GM9/D Analyzer which is more accurate than a glucose meter. On the day of clamp, the glucose analyzer was calibrated with a standard glucose solution with a concentration of 144.1 mg/dl. To measure glucose concentration, 10ul of plasma was injected directly into the analyzer. In the presence of oxygen the glucose in the plasma is oxidized by glucose oxidase (GO). The chemical reaction is summarized as follows:

\[
\text{Glucose} + \text{O}_2 \xrightarrow{\text{GO}} \text{Gluconic acid} + \text{H}_2\text{O}_2
\]

Oxygen consumption which is directly proportional to glucose concentration is monitored using a Clark oxygen electrode.
2.2.2 Tracer

3-^3^H-glucose radioactivity was measured in tracer, labeled glucose infusate, and plasma samples. Samples were deproteinized by adding Ba(OH)\textsubscript{2} and ZnSO\textsubscript{4} and centrifuged at 4\textdegree C. The supernatant was collected and evaporated overnight to remove tritiated water. Scintillation cocktail was added next morning and beta radioactivity was measured using a liquid scintillation counter. Each sample was measured in duplicates and the average radioactivity was accepted if the difference between the two values was within 10%.

2.2.3 Free Fatty Acid Levels

Plasma FFA were determined using a kit from Wako Diagnostics (Osaka, Japan). The method employs enzymatic reactions (see below) and colorimetric analysis. FFA are first converted to acyl-CoA by acyl-CoA synthetase (ACS). Next, acyl-CoA is oxidized by acyl-CoA oxidase (ACO) to generate hydrogen peroxide. In the presence of hydrogen peroxide, 4-aminoantipyrine and 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) is condensed by peroxidase (PO) to produce a chemical that absorbs light maximally at 550nm. Sample absorbance was measured using a spectrophotometer and the corresponding FFA concentration was extrapolated from a standard curve generated from known FFA values.

\[
\begin{align*}
1) & \quad \text{FFA} + \text{ATP} + \text{CoA} \quad \xrightarrow{\text{ACS}} \quad \text{Acyl-CoA} + \text{AMP} + \text{PPi} \\
2) & \quad \text{Acyl-CoA} + \text{O}_2 \quad \xrightarrow{\text{ACO}} \quad 2,3\text{-trans-Enoyl-CoA} + \text{H}_2\text{O}_2 \\
3) & \quad \text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + \text{MEHA} \quad \xrightarrow{\text{PO}} \quad \text{chemical absorbant} + \text{H}_2\text{O}
\end{align*}
\]
2.2.4 Insulin

Plasma concentration was assessed using a Rat Insulin Radioimmunoassay (RIA) kit (Millipore) with 100% cross reactivity with human insulin. For this radioimmunoassay, a fixed concentration of $^{125}$I labeled insulin tracer is incubated with a constant amount of rat insulin antibody. Unlabeled insulin added from standard and plasma samples competes with labeled insulin for antibody and reduces the amount of tracer bound. The mixture was incubated overnight at 4°C for the above reaction to equilibrate. Precipitating agent was added and the mixture was centrifuged to separate free tracer in the supernatant from antibody-bound tracer in the pellet. Pellets were saved and gamma radiation was counted in a gamma counter. Duplicates of standard and plasma samples were averaged and accepted if the difference was within 10%. The tracer bound to antibody in each standard or sample ($B_S$) was expressed as a percentage of the total binding which is the tracer bound to antibody in absence of standard or sample ($B_T$).

$$\% \frac{B_S}{B_T} = \left( \frac{\text{Standard or Sample}}{\text{Total Binding}} \right) \times 100$$

A standard curve was generated by plotting percentage of standard binding on the y-axis and the concentration of known insulin concentration on the x-axis using logarithmic scale. The insulin concentration in the plasma samples was interpolated from the standard curve.

2.3 Protein Immunoblot

2.3.1 Liver Fractionation

Liver samples were fractionated to separate cytosolic from membrane proteins. 150mg of frozen liver was added to Buffer A (50 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA, 1 mM NaHCO$_3$, 5 mM MgCl$_2$ (hexahydrate), 1 mM Na$_3$VO$_4$, 1 mM NaF, 1 $\mu$g/ml aprotinin, 1 $\mu$g/ml
leupeptin, 1 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 μM okadaic acid) and homogenized by hand using a glass homogenizer. The homogenates were centrifuged at 100 000 g and 4°C for 1 hour. The supernatant, containing cytosolic proteins, was collected and stored at -80°C. The pellet was dispersed in Buffer B (Buffer A + 1% Triton X-100) using a plastic homogenizer and passaged through a 25 gauge needle. The new homogenates were centrifuged at 100 000 g and 4°C for 1 hour. The supernatant which contains membrane proteins was collected and stored at -80°C.

2.3.2 Protein Preparation

Protein concentration was measured using the Bradford method with dye reagent from Bio-Rad (Bio-Rad Laboratories, Mississauga, ON, Canada) and bovine serum albumin as the standard. Protein samples were evaporated and denatured by adding 1x Laemmli Sample Buffer (2% SDS, 10% glycerol, 0.0625 M Tris-HCl (pH 6.8), 0.1% bromophenol blue, and 5% β-mercaptoethanol) and boiling.

2.3.3 Western Blot

Fifty μg of protein and the PageRuler™ Prestained Protein Ladder (Fermentas) were loaded onto an SDS-PAGE gel (10% polyacrylamide). Gels were run at 100V for an hour followed by 150V until the dye front reached the bottom of the gel. Proteins were then transferred from the gel onto a Polyvinylidene Fluoride (PVDF) membrane. After transferring, the membrane was incubated in a blocking solution. The membrane was then incubated in primary antibody overnight at 4°C on a shaker. On the following day, primary antibody solution was removed and the membrane was washed with Tris-buffered saline-Tween (TBST) for 3 times to remove residual primary antibody. The membrane was then incubated with matching secondary antibody
linked to horseradish peroxidase (HRP) for 1 hour on a shaker at room temperature. To remove residual secondary antibody, the membrane was washed again with TBST for three times. Following incubation with enhanced chemiluminescent (ECL) substrate (SuperSignal West Pico, Thermo Scientific) the membrane was visualized by developing the film in the dark room. For removal of antibodies, the same membrane was stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) for 1 hour. Following washing with TBST for 3 times, the membrane was blocked and reprobed for another protein as described above. Phosphorylated proteins were probed on fresh membranes, total proteins were probed on membranes that were stripped once and actin (for loading control) was probed on membranes that were stripped twice. Blocking solution was prepared as 5% milk dissolved in TBST. Primary antibody was prepared in 5% BSA and secondary antibody was prepared in 5% milk, both dissolved in TBST. Primary antibodies used and their dilutions are listed: phosphorylated p38 MAPK (Cell Signaling, 1:200), total p38 MAPK (Cell Signaling, 1:500), phosphorylated ATF2 (Cell Signaling, 1:1000), total ATF2 (Santa Cruz, 1:4000), beta actin (Abcam, 1:6000). Corresponding secondary antibodies used and their dilutions were: anti-rabbit (Santa Cruz, 1:2000), anti-goat (Santacruz, 1:6000). Densitometry for protein bands on films was performed using ImageJ software (NIH).

2.4 Calculations

During the basal (fasting) steady state, the rate of glucose appearance (Ra) due to endogenous glucose production (EGP), equals rate of glucose disappearance (Rd). During the (hyperinsulinemic) clamp steady state, Ra due to exogenous glucose infusion and EGP equals Rd. EGP can therefore be calculated as Ra – glucose infusion.
Average values were calculated for glucose infusion rate during the clamp period (Figure 1). Average values were also calculated for EGP and Rd during the basal and clamp periods (Figure 1). EGP and Rd were determined from glucose concentration and $3^\text{H}$-glucose radioactivity using modified Steele’s equation by Finegood et al. (1987) to account for non-steady-state conditions (in round brackets) and the tracer included in the glucose infusion [in square brackets].

$$\frac{EGP}{SA_p} = \frac{I}{SA_p} + (-V \frac{G \cdot dSA_p}{SA_p} / dt) + \left[ \frac{SA_g \cdot GINF}{SA_p} \right] - GINF, \quad Rd = Ra + (-V \cdot dG / dt)$$

Ra = glucose appearance rate (mg/min/kg)

I = tracer infusion rate (uCi/min/kg)

$SA_p$ = specific activity of plasma (uCi/mg)

$V = pV_T$, fraction of glucose pool that is effectively mixed from the total distribution volume of glucose (dl/kg)

$G =$ plasma glucose concentration (mg/dl)

$SA_g =$ specific activity of exogenous glucose infusate (uCi/mg)

$GINF =$ exogenous glucose infusion rate (mg/min/kg)

Rd = glucose disappearance rate (mg/min/kg)
2.5 Statistical Analysis

Data are means ± SEM (standard error of the mean). Data were normally distributed.

One-way ANOVA (analysis of variance) and Tukey’s post-hoc test was used to compare differences between treatments. Statistical calculations were performed using Statistical Package for the Social Sciences (SPSS, IBM) and significance was accepted at p<0.05.
Figure 1. The experimental protocol.

48 hour-treatment infusion and 2 hour-hyperinsulinemic-euglycemic clamp. Black bars indicate infusion timeline while gray bars indicate timeline for blood sample collection. Tissues were collected at the end of the clamp. * Blood glucose was measured every 5 to 10 minutes to determine rate of labeled variable glucose infusion.
Chapter 3 – Results

Table 2 shows concentration of plasma free fatty acids, insulin, and glucose in rats that were infused with different treatments for 48 hours, before and after the hyperinsulinemic-euglycemic clamp. Prolonged lipid infusion increased plasma FFA levels by approximately 2 fold during the basal period. These results validate our FFA elevation model. The p38 MAPK inhibitor co-infused with lipid or alone had no effect as expected. Insulin infusion during clamp lowered FFA levels across all treatments, in agreement with the antilypolytic effect of insulin. FFA remained elevated in the IH groups during the clamp due to lipid infusion. Plasma insulin levels were not statistically different between treatments during the basal or clamp period. Insulin infusion at a constant rate of 5mU/kg/min increased plasma insulin levels by 4-6 fold. Plasma glucose levels were comparable between different treatment groups during both basal and clamp period, as per euglycemic clamp.

Whole-body insulin sensitivity is a reflection of glucose infusion rate during the last 30 minutes of the hyperinsulinemic-euglycemic clamp. In response to exogenous insulin infusion, were glucose not clamped, it would decrease due to glucose uptake and reduced glucose production. Thus, more glucose infusion is needed to maintain euglycemia in rats sensitive to insulin. 48 hours of lipid infusion decreased glucose infusion rate, i.e. induced whole-body insulin resistance (Figure 2). Co-infusion of p38 MAPK inhibitor did not prevent the effect of lipid to lower glucose infusion rate (Figure 2). p38 MAPK inhibitor alone showed a strong tendency to lower glucose infusion rate but this did not reach statistical significance (Figure 2).

Glucose utilization was assessed during the basal fasting state and during the hyperinsulinemic clamp period. Glucose utilization was not significantly different between
treatment groups during the basal period (Figure 3A). Co-infusion decreased glucose utilization during the clamp period (Figure 3A). Lipid infusion showed a tendency to decrease insulin-stimulated augmentation in glucose utilization, which is an indicator of peripheral insulin sensitivity (Figure 3B). Co-infusion of p38 MAPK inhibitor with lipid did not seem to improve peripheral insulin sensitivity (Figure 3B). p38 MAPK inhibitor alone showed a trend to decrease insulin-stimulated augmentation in glucose utilization (Figure 3B).

EGP was also assessed during basal fasting state and during the hyperinsulinemic clamp period. Lipid infusion showed a trend to increase EGP during the basal fasting period (Figure 4A) and this trend disappeared with co-infusion of p38 MAPK inhibitor (Figure 4A). p38 MAPK inhibitor alone had no effect on basal EGP (Figure 4A). Lipid infusion significantly increased EGP during the clamp period (Figure 4A), which was prevented by co-infusion of p38 MAPK inhibitor (Figure 4A). p38 MAPK inhibitor alone had no effect on clamp EGP (Figure 4A). Lipid infusion reduced insulin-induced suppression of EGP (Figure 4B) which is consistent with hepatic insulin resistance. Co-infusion of p38 MAPK inhibitor restored insulin’s ability to suppress EGP (Figure 4B). p38 MAPK inhibitor alone had no effect on insulin-stimulated suppression of EGP (Figure 4B).

To avoid possible confounding effect of hyperinsulinemia on p38 MAPK activation in liver samples obtained at the end of the hyperinsulinemic-euglycemic clamp, treatment was infused in rats for 48 hours without performing the hyperinsulinemic-euglycemic clamp and collected liver tissues to determine p38 MAPK activation. Lipid infusion showed a trend to increase phosphorylated p38 MAPK which was not prevented by co-infusion of p38 MAPK inhibitor (Figure 5). p38 MAPK inhibitor alone did not affect p38 MAPK phosphorylation status (Figure 5). Total p38 MAPK levels were similar between treatments (Figure 5).
To confirm that p38 MAPK activity was indeed increased by lipid infusion and determine whether the inhibitor was effective at inhibiting p38 MAPK activity, a direct substrate of p38 MAPK, namely ATF2 was examined. Lipid infusion increased phosphorylation of ATF2 and this increase was prevented by co-infusion of p38 MAPK inhibitor, which demonstrates that the inhibitor was effective (Figure 6). Total ATF2 levels were not different between treatments (Figure 6).
Table 2. Plasma levels of free fatty acids, insulin, and glucose before and during the hyperinsulinemic-euglycemic clamp.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAL (5)</td>
<td>IH (6)</td>
</tr>
<tr>
<td>FFA (μEq/l)</td>
<td>448±36</td>
<td>922±66*</td>
</tr>
<tr>
<td>INS (pM)</td>
<td>75±16</td>
<td>100±29</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>119±4</td>
<td>112±5</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM. Basal refers to values measured during the fasting state before the start of insulin infusion whereas clamp refers to the last 30 minutes of the 2-hour hyperinsulinemic-euglycemic clamp. * p<0.05 vs. saline. FFA – free fatty acids, INS – insulin, SAL – saline, IH – Intralipid and heparin, SB – p38 MAPK inhibitor SB239063.
Figure 2. Whole-body insulin sensitivity.

48 hour lipid infusion reduced glucose infusion rate during the clamp consistent with induction of whole-body insulin resistance which was not ameliorated by co-infusion of p38 MAPK inhibitor. Data are mean ± SEM. * p<0.05 vs. saline. SAL – saline, IH – Intralipid plus heparin, SB – p38 MAPK inhibitor SB239063.
48 hour lipid infusion and p38 MAPK inhibitor reduced glucose utilization during the clamp. Lipid infusion tended to impair (p = 0.06) insulin-stimulated augmentation in glucose utilization, i.e. peripheral insulin sensitivity, and co-infusion of p38 MAPK inhibitor did not seem to improve peripheral insulin sensitivity. **A.** Glucose utilization before and after insulin infusion. **B.** Percentage augmentation in glucose utilization. Data are mean ± SEM. * p<0.05 vs. saline. SAL – saline, IH – Intralipid plus heparin, SB – p38 MAPK inhibitor SB239063.
48 hour lipid infusion improved the insulin induced suppression of endogenous glucose production (EGP), i.e. induced hepatic insulin resistance which was prevented by p38 MAPK inhibitor co-infusion. A. EGP before and after insulin infusion. B. Percentage suppression of EGP. Data are mean ± SEM. * p<0.05 vs. saline.
Figure 5. Western blot analysis for p38 MAPK.

Lipid infusion showed a trend to increase p38 MAPK phosphorylation. Representative Western blots and ratio of phosphorylated (p-p38 MAPK) to total p38 MAPK in the liver of rats that did not receive hyperinsulinemic euglycemic clamp. Data are mean ± SEM. SAL – saline, IH – Intralipid plus heparin, SB – p38 MAPK inhibitor SB239063.
Figure 6. Western blot analysis for ATF2.

Lipid infusion significantly increased phosphorylation of p38 MAPK substrate ATF2 which was reversed by co-infusion of p38 MAPK inhibitor. Representative Western blots and ratio of phosphorylated (p-ATF2) to total ATF2 in the liver of rats that did not receive hyperinsulinemic euglycemic clamp. Data are mean ± SEM. * p<0.05 vs. saline. SAL – saline, IH – Intralipid plus heparin, SB – p38 MAPK inhibitor SB239063.
Chapter 4 - Discussion

In this study, prolonged lipid infusion for 48 hours induced whole-body and liver insulin resistance while showing a tendency to impair peripheral insulin sensitivity. This is in agreement with previous published animal data from our lab (109-111) showing that lipid infusion induced insulin resistance. Co-infusion of p38 MAPK inhibitor did not ameliorate lipid-induced whole-body insulin resistance or improve peripheral insulin sensitivity but prevented hepatic insulin resistance. Furthermore this improvement was associated with inhibition of lipid-induced p38 MAPK activity in the liver as confirmed by phosphorylation status of p38 MAPK substrate ATF2. This suggests that p38 MAPK mediates hepatic insulin resistance in our model and it has no beneficial effect in the periphery.

We analyzed liver tissues from animals that did not undergo the hyperinsulinemic clamp because insulin has been shown to increase phosphorylation of p38 MAPK (193). We found lipid infusion showed a trend to increase p38 MAPK phosphorylation but significantly increased phosphorylation of p38 MAPK substrate ATF2. Our results suggest that the phosphorylation status of p38 MAPK may not be the best indicator of p38 MAPK activation. Although activation of p38 MAPK is mainly thought to be regulated by phosphorylation and dephosphorylation of the dual residues (Thr180 and Tyr182) in the activation loop, additional mechanisms have been proposed. For example in T cells, p38α MAPK is activated by phosphorylation of Tyr 323 and not the classical dual residues (200). Co-infusion of the p38 MAPK inhibitor SB239063 did not appear to have any effect on p38 MAPK phosphorylation but prevented lipid-induced ATF2 phosphorylation. This suggests that SB239063 inhibits the p38 MAPK activity without inhibiting the phosphorylation of p38 MAPK by upstream kinases. The mechanism of SB239063 is deduced from a related pyridinylimidazole compound SB203580. It has been shown that the
binding of SB203580 in the ATP binding pocket of p38 MAPK does not prevent MKK6 from phosphorylating p38 MAPK (201). SB239063 is a relative specific inhibitor for the alpha and beta isoforms of p38 MAPK as compared to other isoforms and kinases (Table 3). Our results are in agreement with the study by Hemi et al., where overexpression of dominant negative p38α MAPK in the liver of ob/ob mice improved insulin sensitivity as demonstrated by lowered fasting insulin levels and improved glucose tolerance (196). Overexpression of p38α MAPK in the liver of wild type mice had the opposite effect of attenuating insulin sensitivity, reflected by increased fasting insulin levels in parallel with increased IRS1 serine phosphorylation, reduced IRS1 tyrosine phosphorylation and Akt phosphorylation (196). However p38 MAPK activation is not always associated with insulin resistance in the liver. A study done by Lee et al. found that liver-specific expression of constitutively active MAPK kinase 6 (MKK6) in ob/ob mice activated p38 MAPK but enhanced glucose tolerance, lowered blood glucose and plasma insulin levels (197). This improvement in insulin action was associated with reduced ER stress markers. However it cannot be excluded that in addition to p38 MAPK, MKK6 can activate other yet unidentified substrates, to improve insulin sensitivity. For example one study found that another type of constitutively active MKK6 downregulated oxidative stress by inhibiting NADPH dependent production of reactive oxygen species (202). Since p38 MAPK activation has been associated with both beneficial and detrimental effects on insulin resistance, it is important to determine what is occurring downstream of p38 MAPK activation.

Fat-induced p38 MAPK can impair insulin signaling by increasing PTEN protein level (198). In hepatocytes, this occurs without changes in PTEN mRNA. PTEN is a negative regulator of insulin signaling because it dephosphorylates PIP3 to PIP2 which is the reverse reaction to PI3K. Phosphorylation of PTEN at certain serine and threonine residues of C-terminal
has been shown to stabilize the protein (203) and caseine kinase (CK2) and glycogen synthase kinase 3 beta (GSK3beta) but not p38 MAPK have been shown to participate in direct phosphorylation (204). Thus, the mechanisms of the effect of p38 MAPK to increase PTEN protein level is not clear. Also, in endothelial cells, ATF2 which is a transcription factor and a direct substrate of p38 MAPK can regulate PTEN at the level of transcription by binding to PTEN promoter (205). Although PTEN may explain for reduced Akt phosphorylation observed in liver of mice overexpressing p38α MAPK, other mechanisms are responsible for changes in tyrosine and serine phosphorylation of IRS1 which is upstream of PI3K/PTEN. One potential link between p38 MAPK and IRS1 tyrosine phosphorylation is the protein-tyrosine phosphatase SHP1 (Src homology-2 domain containing phosphatase-1) which has been reported to be activated by p38α MAPK in cultured pericytes (206). However the mechanisms underlying the regulation of SHP-1 by p38 MAPK have not been reported. The role of SHP1 in insulin resistance is reinforced by a study that demonstrates hepatocyte-specific Shp1 knockout mice were protected from liver insulin resistance despite an obese phenotype (206). No study has been able to demonstrate a direct phosphorylation of IRS1 by p38 MAPK to my knowledge. However p38 MAPK can indirectly regulate IRS1 serine phosphorylation through IKKβ activation (207) or transactivation of receptors such as ErbB (196). p38 MAPK has been suggested to sustain phosphorylation of ErbB1 via neutralization of tyrosine phosphatases, activation of tyrosine kinases, although the mechanism has not been established. In addition, p38 MAPK mediates activation of metalloproteases that process the pro-peptide for heparin-binding EGF-like growth factor (HB EGF) which can bind and activate ErbB receptor.

While fat-induced p38 MAPK can regulate gluconeogenesis by blunting insulin suppression of gluconeogenesis, it can also directly promote gluconeogenesis at the
transcriptional level. P38 MAPK has been shown to upregulate expression of gluconeogenic transcripts PEPCK and G6Pase by increasing levels of PGC-1 alpha transcripts (96). PGC-1alpha is a transcription co-activator that binds and activate gluconeogenic transcription factors such forkhead box O1 (FOXO1) which goes on to bind promoter regions of gluconeogenic genes to increase their transcription (208). In cultured myocytes, p38 MAPK-mediated increase in PGC-1 alpha promoter activity was dependent on transcription factor ATF2 (209). Although p38 MAPK can directly phosphorylate PGC-1 in cells at three amino acid residues to increase protein stability (210), the exact mechanism for protein stabilization is not clear and its role in vivo has not been demonstrated. In addition to PGC-1 alpha, free fatty acids increased phosphorylated CREB in hepatocytes in a p38 MAPK-dependent manner (96). CREB is a transcription factor that binds cAMP response element in the promoter of PEPCK which is a key enzyme that controls gluconeogenesis. It is known that p38 MAPK does not phosphorylate CREB directly but via other substrates (96). p38 MAPK has also been shown to stimulate phosphorylation of C/EBPalpha (CCAAT/enhancer-binding protein alpha) at serine 21 to increase PEPCK transcription in hepatoma cells (211).

Our results suggest that p38 MAPK has no significant effects in peripheral tissues. The β isoform does not seem to play a role in insulin signaling as demonstrated by p38β MAPK knockout mouse which showed normal glucose tolerance, plasma insulin and glucose compared to wild type controls (194). Insulin increased p38 MAPK activation in L6 myotubes (193) but not in primary adipocytes and isolated soleus muscle from wild-type mice (193, 194). Despite this inconsistency, p38 MAPK inhibition with SB203580, which is relatively specific for p38α MAPK and p38β MAPK isoforms, reduced glucose uptake in insulin exposed L6 myotubes, primary adipocytes, and isolated soleus muscle without affecting GLUT4 translocation (193,
194). It is controversial whether SB203580 decreases glucose uptake via or independent of p38α MAPK. Our study cannot rule out the possibility that SB239063 can bind GLUT4 transporter and inhibit its glucose uptake which has been suggested by in vitro studies (195, 212). Further in vivo investigation is warranted. P38 MAPK mediates phosphorylation of PGC1 as described in the above paragraph and phosphorylated PGC1 in cultured myotubes has been shown to increase cellular respiration via increased expression of mitochondrial genes involved in respiration (210). Increasing energy expenditure is beneficial as it reduces ectopic fat accumulation and therefore improves insulin sensitivity. Since the effect of inhibiting p38 MAPK in the liver is beneficial but the effect is not clear in the periphery, it would be better to selectively target the liver for therapeutics. Thus, the oral route of administration would be preferable to parental routes. There is an oral p38 MAPK inhibitor available and it has been used in patients with chronic obstructive pulmonary disease (213). Its metabolic effects have not been investigated to my knowledge.

The mechanism of fat-induced p38 MAPK activation in the liver in our model remains to be determined. Another graduate student in our laboratory has shown that after prolonged lipid infusion, the IKKβ inhibitor salicylate (111) and the antioxidant NAC (unpublished data) did not prevent hepatic insulin resistance. These studies suggest that IKK beta and oxidative stress are not playing a role after 48 hours of lipid infusion. Therefore, it is unlikely that oxidative stress activates p38 MAPK in our model, although we cannot exclude that p38 MAPK remains activated after oxidative stress, which is present after 7 hour of lipid infusion, is no longer present. It is unlikely that ER stress activates p38 MAPK in our model. The chemical chaperone 4-PBA partially prevented lipid-induced hepatic insulin resistance but we failed to find markers of ER stress in the liver and therefore it is unlikely that ER stress activates p38 MAPK in our model. Interestingly, we found that lipid infusion increased ER stress markers in fat and
hypothalamus, thus PBA might be increasing hepatic insulin sensitivity by its action in fat and brain. p38 MAPK has been reported to decrease markers of ER stress by phosphorylating XBP1 which increase its nuclear translocation. Thus, we cannot dismiss the possibility that p38 MAPK activation is the mechanism for normalizing ER stress markers in the liver.

To identify the molecular mechanisms of lipid-induced hepatic insulin resistance, we analyzed liver tissues from animals that underwent the hyperinsulinemic-euglycemic clamp. Although not statistically significant, we found that lipid infusion increased mRNA for PEPCK by 2 fold (111). However, lipid infusion did not change IRS-1 phosphorylation on Ser 307, a proximal insulin signaling molecule, which suggests that kinases responsible for Ser 307 phosphorylation like JNK and IKK are not involved (111). Lipid infusion decreased Akt phosphorylation on Ser 473 and increased PKC-δ protein translocation (111). This suggests that lipid may have activated PKC-delta to impair insulin sensitivity in the liver. Notably, PKCδ inhibition blocked oleate-induced p38 MAPK activation in primary hepatocytes (96) which suggests that PKCδ may be an upstream activator of p38 MAPK. In light of these findings it is possible that the sequence of events that occur after prolonged lipid infusion in the liver is as follows: 1) FFA → PKCδ → p38 MAPK→ increased gluconeogenesis or 2) FFA → PKCδ → p38 MAPK→ impaired insulin sensitivity → increased glucose production. The first pathway would result in increased basal glucose production in the absence of autoregulation, as may be expected after prolonged fasting. Lipid infusion showed a tendency to increase basal glucose production and this positive trend was significant in another study (111). The finding that co-infusion of salicylate restored Akt phosphorylation (insulin signaling) but not glucose production suggests that inhibition of both pathways (with p38 MAPK inhibitor) may be required.
Table 3. IC50 values for SB239063 from in vitro experiments.

<table>
<thead>
<tr>
<th>Inhibition of enzymes</th>
<th>IC50 for SB239063 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p38α/β MAPK</td>
<td>~0.044</td>
</tr>
<tr>
<td>p38δ/γ MAPK</td>
<td>&gt;10</td>
</tr>
<tr>
<td>MEK</td>
<td>&gt;10</td>
</tr>
<tr>
<td>ERK</td>
<td>&gt;10</td>
</tr>
<tr>
<td>JNK-1</td>
<td>&gt;10</td>
</tr>
<tr>
<td>MK2</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

SB23963 is a relative specific inhibitor for the alpha and beta isoform of p38 MAPK. Table adapted from Barone et al., 2001(199). IC – inhibitory concentration.
Study Limitations

1) It is important to note that Intralipid infused in our model is a triglyceride emulsion and co-administration of heparin facilitates the lipolysis of triglyceride to achieve elevated FFA with glycerol as a byproduct. Since glycerol is a substrate for gluconeogenesis, it may contribute to the endogenous glucose production. However, this is unlikely in this study because IH did not significantly increase fasting EGP. Furthermore, our lab has shown previously that glycerol infusion to match the levels found after 7 hour of IH infusion did not affect insulin suppression of EGP (109). We are currently performing 48 hour glycerol infusion to confirm that this is also true after 48 hours.

2) The triglyceride in Intralipid contains mainly of ω-6 polyunsaturated fatty acids. However the predominant circulating free fatty acid in the human plasma is the monosaturated fatty acid oleate followed by the saturated fatty acid palmitate in a ratio of 2:1 (214). The Western diet, which is associated with increased risk of insulin resistance, is characterized by increased consumption of saturated fatty acids (215). It is important to consider the effect of individual fatty acids because studies have shown that unsaturated and saturated fatty acids may mediate insulin resistance through different pathways (147).

3) The pharmacologic inhibitor SB239063 may have non-specific effects. It is important to selectively target p38 MAPK using an alternative method in a tissue specific manner since my study suggests that p38 MAPK mediates liver insulin resistance but its role in peripheral tissues is not clear.

4) Although the two-fold plasma FFA elevation in my study models differences in FFA between obese/diabetic and healthy humans, our method of FFA elevation is not physiological.
A more physiological model of fat elevation would be using a high fat diet. In addition my study only focuses on one animal model using rats which may display a different response compared to humans.

5) We found that hepatic expression of adipokines TNFα and IL-6 was not affected by prolonged Intralipid infusion (111). Although circulating cytokines (216) and adiponectin were not affected, we also found that circulating IL-6 was increased and may be the link between fat ER stress and hepatic insulin resistance. Since IKKβ and IRS1 phosphorylation on Ser 307 were not affected in our model, it is possible that IL-6 may affect insulin signaling via SOCS3 which remains to be determined.

6) Another study found that acute infusion of soy/lard-oil elevated hepatic contents of lipid metabolites DAG and ceramides (146). We also found an increase in liver DAG contents after 7 hours of lipid infusion but ceramides were not elevated and both remain to be determined after 48 hours. DAG impairs insulin sensitivity via PKC and ceramides may affect Akt independent of IRS, which would be relevant to our model.

**Future Directions**

1) Verify the molecular mechanism downstream and upstream of p38 MAPK in lipid-induced hepatic insulin resistance:

   a) It remains to be identified if and how lipid-induced p38 MAPK activation impaired insulin signaling. Since we found that prolonged lipid infusion decreased Akt phosphorylation in the liver (111), we will perform Western blots to determine the effect of p38 MAPK inhibition on Akt phosphorylation and then PTEN which is lipid
phosphatase that inhibits insulin signaling at the level of PI3K by dephosphorylating PIP3 to PIP2.

b) Although IH increased mRNA level of gluconeogenic enzyme PEPCK (96), it is not known whether p38 MAPK inhibitor decreased PEPCK or another gluconeogenic enzyme in our model which can be verified using RT-PCR. After verifying the effect of lipid-induced p38 MAPK on gluconeogenic enzyme mRNA, we can establish the link by looking at phosphorylation and expression of transcription co-activator PGC-1 alpha and transcription factor CREB, because FFA-stimulated PEPCK promoter activity in murine hepatoma cells was dependent on phosphorylated PGC1-alpha and CREB (96).

c) We found that prolonged lipid infusion increased PKCδ translocation in the liver (111). Notably, PKCδ inhibition blocked oleate-induced p38 MAPK activation in primary hepatocytes (96) which suggests that PKCδ is an upstream activator of p38 MAPK. To test for this link, we will measure PKCδ translocation (marker of activation) by comparing its membrane to cytosolic fraction ratio in the liver samples. If PKCδ is indeed upstream of p38 MAPK, then its inhibition should not affect PKCδ activity. To further establish the link between hepatic PKCδ and p38 MAPK, we could inhibit PKCδ and determine whether this decreases p38 MAPK activity and glucose production. This can be done by administering antisense oligonucleotides for PKC-δ, using adenovirus mediated transfer of kinase dead PKC-δ, or liver-specific PKCδ-knockout mice (137). Conversely, overexpression of PKC-δ should increase p38 MAPK activity and glucose production. This can be achieved using adenovirus-mediated gene transfer.
2) Use a more selective model to confirm the role of p38 MAPK in lipid-induced hepatic insulin resistance. This can be achieved using an adenoviral-mediated knockdown of p38α MAPK, which preferentially targets the liver. Alternatively, liver-specific p38α MAPK knockout mice, which can be generated from p38α MAPK floxed mice (commercially available), can be used. Overexpression of wild type p38α MAPK can serve as a positive control. The main advantage of using adenoviral vectors instead of the liver-specific knockout mice is that inhibition is acute and thus, chronic compensations are avoided. The disadvantages of using the adenovirus method are that it targets not only hepatocytes but also other cells including adipocytes and that it induces non-specific inflammation. Adenovirus also targets Kupffer cells which are resident macrophages in the liver. This may be an advantage and a disadvantage. Regarding p38 MAPK, in vitro studies report it plays a role in hepatocytes, however a role in macrophages is also possible.

3) To selectively study the role of the saturated fatty acid on lipid-mediated hepatic insulin resistance, individual ethyl-fatty acids can be infused in rodents using ethanol treated mice as control. This method has been newly found to elevated palmitate levels in a non-toxic manner, because ethyl-FFA is converted to FFA in the rodent circulation (217). This is advantageous since studies suggest that the FFA type is important and their effects on insulin resistance are through different mediators (146). Preliminary data from our lab show that infusion of ethyl-oleate and plamitate mixture in a ratio of 2:1, as in physiology, impairs insulin action in the liver compared to ethanol controls. The effect of individual ethyl-FFA and their mechanisms remain to be investigated.
References


98. Xu C, Chakravarty K, Kong X, Tuy TT, Arinze IJ, Bone F, Massillon D: Several transcription factors are recruited to the glucose-6-phosphatase gene promoter in response to palmitate in rat hepatocytes and H4IIE cells. *J Nutr.* 137:554-559, 2007


159. Meex RC, Phielix E, Moonen-Kornips E, Schrauwen P, Hesselink MK: Stimulation of human whole-body energy expenditure by salsalate is fueled by higher lipid oxidation under fasting conditions and by higher oxidative glucose disposal under insulin-stimulated conditions. J Clin Endocrinol Metab. 96:1415-1423, 2011


