# THE MECHANISMS UNDERLYING FREE FATTY ACID-INDUCED HEPATIC INSULIN RESISTANCE

By

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# General Abstract

Elevated circulating free fatty acids (FFA) cause hepatic insulin resistance; however, the mechanisms for this process are incompletely understood. The objective of the studies in the thesis was to examine whether protein kinase C (PKC)-delta (δ), oxidative stress, and the serine kinase IκBα kinase (IKK) β are causally involved in FFA-induced hepatic insulin resistance. To test this, we infused rats with lipid with or without inhibitors of the aforementioned factors for 7h, during the last 2h of which a hyperinsulinemic-euglycemic clamp was performed. In Study 1, inhibition of hepatic PKC-δ using antisense oligonucleotide prevented FFA-induced membrane translocation of PKC-δ, which is a marker of its activation, in parallel with prevention of lipid-induced hepatic insulin resistance, without affecting lipid-induced peripheral insulin resistance. These results implicate PKC-δ as a causal mediator of FFA-induced hepatic insulin resistance. In Study 2, the antioxidant N-acetyl-L-cysteine (NAC) prevented lipid-induced hepatic insulin resistance in conjunction with reversal of lipid-induced increase in markers of IKKβ and c-Jun $NH_2$-terminal kinase 1 (JNK1) activation, and of impairment of insulin signaling, without affecting PKC-δ membrane translocation and increase in phosphorylated p38 mitogen-activated protein kinase (MAPK) induced by lipid infusion. These findings suggested that oxidative stress is a causal mediator of lipid-induced hepatic insulin resistance upstream of IKKβ and JNK1, and potentially

downstream of PKC-δ and p38 MAPK. In Study 3, sodium salicylate, an IKKβ inhibitor, prevented FFA-induced hepatic insulin resistance via restoration of hepatic insulin signaling, thus implicating IKKβ as a causal factor in the process. Together, the results from these studies demonstrate that PKC-δ, oxidative stress, and IKKβ are causally involved in FFA-induced hepatic insulin resistance and suggest that the sequence for the process is: FFA $\rightarrow$ PKC-δ $\rightarrow$ oxidative stress $\rightarrow$ IKKβ $\rightarrow$ impaired hepatic insulin signaling.

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# List of Abbreviations

| | |
|---|---|
| **aPKC** | atypical protein kinase C |
| **ACC** | Acetyl-CoA carboxylase |
| **ALT** | Alanine aminotransferase |
| **AMPK** | Adenosine monophosphate-activated protein kinase |
| **ANOVA** | Analysis of variance |
| **APPL** | Adaptor protein containing pleckstrin homology domain |
| **ASO** | Antisense oligonucleotides |
| **AST** | Aspartate aminotransferase |
| **BMI** | Body mass index |
| **CON** | Control |
| **COX** | Cyclooxygenase |
| **CPT** | Carnitine palmitoyltransferase |
| **CREBP** | Cyclic AMP-response element-binding protein |
| **DAG** | Diacylglycerol |
| **DNPH** | Dinitrophenylhydrazine |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **EGP** | Endogenous glucose production |
| **eIF** | Eukaryotic initiation factor |
| **eNOS** | Endothelium nitric oxide synthase |
| **ER** | Endoplasmic reticulum |
| **ERK** | Extracellular signal-regulated kinase |
| **FAS** | Fatty acid synthase |
| **FFA** | Free fatty acids |
| **FOXO** | Forkhead transcription factor |
| **G6Pase** | Glucose-6-phosphatase |
| **GIR** | Glucose infusion rate |
| **GLUT** | Glucose transporter |
| **GSK** | Glycogen synthase kinase |
| **GU** | Glucose utilization |
| **HGP** | Hepatic glucose production |
| **IH** | Intralipid plus heparin |
| **IκBα** | Inhibitor of κB α |
| **IKKβ** | IκBα kinase β |
| **IL** | Interleukin |
| **IRS** | Insulin receptor substrate |
| **JAK** | Janus kinase |
| **JNK** | c-Jun $NH_2$-terminal kinase |
| **LCFA-CoA** | Long-chain fatty acyl coenzyme A |
| **LPS** | Lipopolysaccharide |
| **MAPK** | Mitogen-activated protein kinase |
| **MCP** | Macrophage chemoattractant protein |
| **mTOR** | Mammalian target of rapamycin |
| **NAC** | N-acetyl-L-cysteine |

| | |
|---|---|
| **NAFLD** | Non-alcoholic fatty liver disease |
| **NASH** | Non-alcoholic steatohepatitis |
| **NFκB** | Nuclear factor κ B |
| **ORP** | Oxygen-regulated protein |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PE** | Polyethylene |
| **PEPCK** | Phosphoenolpyruvate carboxykinase |
| **PGC** | Peroxisome proliferator-activated receptor γ coreceptor |
| **PH** | Pleckstrin homology |
| **PI3K** | Phosphatidylinositol 3 kinase |
| **$PIP_3$** | Phosphatidylinositol-3,4,5-triphosphate |
| **PKC** | Protein kinase C |
| **PPAR** | Peroxisome proliferator-activated receptor |
| **PS** | Phosphatidyl serine |
| **PTB** | Phosphotyrosine binding |
| **PTEN** | Phosphatase and tensin homologue |
| **PTP** | Protein tyrosine phosphatase |
| **PUFA** | Polyunsaturated fatty acids |
| **RIA** | Radioimmunoassay |
| **RSV** | Resveratrol |
| **SAL** | Saline |
| **SDS** | Sodium dodecyl sulphate |
| **Ser** | Serine |
| **S6K1** | S6 kinase 1 |
| **SH2** | Src homology 2 |
| **SHP** | SH2-containing intracellular protein tyrosine phosphatase |
| **SOCS** | Suppressor of cytokine signaling |
| **SOD** | Superoxide dismutase |
| **SREBP** | Sterol regulatory element-binding protein |
| **SS** | Sodium salicylae |
| **STAT** | Signal transducers and activators of transcription |
| **TBST** | Tris-buffered saline tween |
| **TCA** | Trichloroacetic acid |
| **TG** | Triglyceride |
| **Thr** | Threonine |
| **TLR** | Toll-like receptor |
| **TNF** | Tumor necrosis factor |
| **Tyr** | Tyrosine |

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# Published Manuscript Arising from Completion of this Thesis

Park E, Wong V, Guan X, Oprescu AI, Giacca A. Salicylate Prevents Hepatic Insulin Resistance Caused by Short-Term Elevation of Free Fatty Acids In Vivo. *Journal of Endocrinology* 2007 Nov;195(2):323-31.

# 1

# Introduction

## 1.1 Obesity, Insulin Resistance, and Type 2 Diabetes

### 1.1.1 Diabetes Mellitus

Diabetes mellitus is the most common metabolic disorder with such signs and symptoms as glycosuria, polyuria, and polydipsia. The hallmark of the disease is hyperglycemia, which leads to various vascular and neurological complications, such as retinopathy, cardiovascular diseases, neuropathy, and nephropathy.

There are two main types of diabetes. The onset of type 1 diabetes usually occurs in adolescence and is characterized by a lack of insulin production, which is believed to be caused by an autoimmune destruction of insulin-secreting beta (β) cells of the pancreas. Type 2 diabetes, on the other hand, is characterized by resistance of the insulin target tissues to insulin and by the failure of increased insulin secretion to compensate for the insulin resistance.

It is estimated that more than two million Canadians currently have diabetes and that by 2010, this number will reach three million (1). It is also expected that by the year 2020, approximately 250 million people will be affected by type 2 diabetes worldwide (2). The prevalence of diabetes is growing, mainly due to a rapid rise in type 2 diabetes.

The increase in prevalence of type 2 diabetes is explained by a similarly sharp rise in obesity, which is a primary risk factor for development of type 2 diabetes. Diabetes is responsible for a significant rate of morbidity and mortality, and is associated with staggering health care costs.

## 1.1.2 Obesity and Type 2 Diabetes

### 1.1.2.1 Obesity

Obesity is clinically defined as a body mass index (BMI) of greater than or equal to 30 kg/m$^2$. It is estimated that over 1 billion people are currently overweight (BMI = 25-29.9 kg/m$^2$) (3) and over 300 million adults are obese worldwide (4). Obesity is considered to be a serious health problem in both industrialized and developing countries around the world and, due to numerous major health risks associated with obesity such as diabetes, cardiovascular diseases, and cancer, obesity is responsible for astronomical health care costs. Alarmingly, the prevalence of obesity is increasing dramatically among children. With the prevalence of obesity and the associated morbidity and mortality rising worldwide, the need for better understanding of the mechanisms underlying the etiology and pathogenesis of obesity and obesity-related diseases is paramount for development of prevention and treatment strategies.

### 1.1.2.2 Obesity and Type 2 Diabetes

More than 90% of individuals with type 2 diabetes are obese. Furthermore, people with type 2 diabetes tend to have more centrally distributed body fat. However, obesity is neither a requisite nor a sufficient condition for type 2 diabetes, as lean individuals can

develop type 2 diabetes and a high prevalence of obesity is associated with a low prevalence of type 2 diabetes in certain populations (5). Indeed, the majority of obese people do not develop type 2 diabetes. Therefore, it appears that, among obese individuals, only those who are genetically predisposed develop type 2 diabetes. Nonetheless, numerous epidemiological studies have established obesity as the major risk factor for type 2 diabetes and that obesity plays a direct role in the pathogenesis of diabetes (6-9). Based on these studies, the main factor that links obesity and type 2 diabetes was found to be insulin resistance, which in obesity is mainly caused by elevated levels of circulating factors that are released by expanded adipose tissue. The following section describes the potential role of various adipocyte-derived substances in obesity-related insulin resistance.

### 1.1.3 Obesity and Insulin Resistance

Insulin resistance in obesity is due to various adipocyte-derived hormones, metabolites, and cytokines released from the expanded adipose tissue. These substances regulate insulin action not only locally in adipose tissue but also in skeletal muscle and liver, the other major target organs of insulin. Recent studies have unequivocally illustrated that adipose tissue is an active and dynamic endocrine organ rather than merely a fat storage depot. The following briefly describes factors released by adipose tissue that play an important role in the regulation of insulin action in obesity.

#### 1.1.3.1 Adipocyte-Derived Mediators of Insulin Action and Resistance

#### *1.1.3.1a Leptin*

Leptin, a product of the *ob* gene, is an adipocyte-derived hormone secreted in proportion to the degree of obesity (10) and is strongly correlated with insulin sensitivity. Leptin binds to OB-R receptors, which belong to the class 1 cytokine receptor family and are ubiquitously distributed. Binding of leptin to its receptor activates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signal transduction pathway, leading to its numerous functions. There is considerable amount of crosstalk between the leptin signaling and other signaling pathways, including insulin-stimulated phosphatidylinositol 3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signaling (11).

Humans with rare leptin deficiency or leptin receptor mutations are severely obese (12). Similarly, two of the most studied animal models of obesity, *ob/ob* and *db/db* mice, have a genetic defect in leptin hormone and leptin receptor, respectively. These mice become obese due to excessive food intake and reduced energy expenditure. As a result, they develop severe insulin resistance and eventually diabetes (13). In addition to leptin's role as a satiety hormone that regulates food intake and energy balance via its actions in ventromedial hypothalamus (14;15), leptin also has direct insulin-sensitizing effects on peripheral tissues (16-18).

#### *1.1.3.1b Adiponectin*

Adiponectin (Acrp 30) is a 30kDa hormone secreted mainly by adipocytes. Two receptor forms have been cloned for adiponectin: AdipoR1, which is a high affinity

receptor for a putative proteolytic fragment of adiponectin containing globular terminal domain but with very low affinity for full-length adiponectin and AdipoR2, which has intermediate affinity for both forms of adiponectin (19). AdipoR1 is abundantly expressed in skeletal muscle and at moderate levels in other tissues, whereas AdipoR2 is predominantly expressed in the liver. The complete signaling pathway for adiponectin remains to be elucidated but a docking protein called adaptor protein containing PH domain 1 mediates adiponetin-induced activation of AMP-activated protein kinase (AMPK), leading to increased fat oxidation in skeletal muscle (20). Adiponectin also stimulates the activation of p38 MAPK and peroxisome proliferator-activated receptor α (PPARα) (21).

Expression and circulating levels of adiponectin are decreased in obese humans and mice, and correlate strongly with insulin sensitivity (22). Mice deficient in adiponectin are insulin resistant (23) and, conversely, an adiponectin administration to obese, insulin resistant mice improves insulin sensitivity (24-26).

Adiponectin increases glucose utilization by fat and muscle, where AdipoR1 is predominantly expressed (27). In the liver where AdipoR2 is mainly expressed, adiponectin acts to enhance suppression of hepatic glucose production (24). Adiponectin also appears to have central effects on the regulation of body weight, as intracerebroventricular administration of the hormone increases energy expenditure and decreases body weight of mice (28).

#### *1.1.3.1c Resistin*

Resistin is another adipocyte-derived hormone that influences insulin action. Resistin belongs to a family of cysteine-rich proteins and is primarily synthesized by adipose tissue in rodents (29) and by macrophages and adipocytes in humans (30). Presently, physiological roles and the receptor for resistin are yet to be identified.

Resistin decreases insulin-stimulated glucose transport in skeletal muscle *in vitro* (31) and increases hepatic glucose production *in vivo* in rodents (29;32). The latter effect may be closely associated with the increased expression of gluconeogenic enzymes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (33). In resistin knockout mice, AMPK activity is markedly increased, suggesting that resistin may exert its anti-insulin effects on hepatic glucose production at least partly by inhibiting AMPK (33), which has insulin-sensitizing effects by promoting intracellular fat oxidation (see below). The physiological relevance of resistin in human is, however, unclear.

#### *1.1.3.1d Cytokines: Tumor Necrosis Factor α and Interleukin-6*

Tumor necrosis factor (TNF) α is a pro-inflammatory cytokine involved in a wide array of physiological processes, including inflammation, proliferation, and apoptosis. TNFα exerts its functions through its two main receptors, p50 and p75, with subsequent activation of serine kinases, such as IκBα kinase β (IKKβ), c-Jun $NH_2$-terminal kinase (JNK), and p38 MAPK, and increased transcription and expression of various pro-inflammatory mediators (34). In obese individuals and in animal models of obesity, TNFα expression is increased in abdominal fat and skeletal muscle (35;36). Furthermore,

circulating levels of TNFα are elevated in obesity and decrease with weight reduction (36). A landmark study by Hotamisligil et al. (35) first identified the increased plasma levels of TNFα as a causal mediator of insulin resistance in obese mice. Since then, TNFα has been shown to impair insulin signaling by decreasing tyrosine phosphorylation of insulin receptor and insulin receptor substrates (IRS) (37;38), which is likely due to their serine phosphorylation by the serine kinase JNK1 (see below for more details).

Like TNFα, interleukin (IL)-6 is a pro-inflammatory cytokine that is expressed in and released by adipose tissue and whose circulating levels increase with obesity (39). IL-6 binds to its receptor composed of two subunits, a subunit that determines ligand specificity and glycoprotein 130 that is shared by other cytokines in the IL-6 family. IL-6 binding to the receptor activates either Ras-mediated signaling or JAK/STAT pathway (40). The current evidence on the role of IL-6 on insulin action is controversial. Several *in vitro* and *in vivo* studies have demonstrated that IL-6 can impair hepatic insulin signaling, likely via the action of suppressor of cytokine signaling (SOCS) proteins to promote degradation of IRS (41-45). However, there exists evidence supporting a beneficial effect of IL-6 released during and following exercise on insulin action (46;47). This topic is described in more detail in a later section.

#### *1.1.3.1e Free Fatty Acids*

Free fatty acids (FFA) are elevated in obesity mainly due to increased lipolysis from the expanded adipose tissue stores, particularly visceral fat, and due to resistance of adipose tissue to the anti-lipolytic action of insulin and increased sensitivity to lipolytic

hormones. FFA enter the cell via passive diffusion (48) or by binding to receptors, for instance the G protein-coupled receptor 40 (49) and fatty acid translocase (50), and are distributed by fatty acid-binding proteins (51) to be metabolized or esterified into triglycerides (TG). In situations of nutrient excess, which can be simulated using lipid infusion or high-fat feeding experimental models, lipid metabolites such as long-chain fatty acyl (LCFA)-CoA and diacylglycerol (DAG) may accumulate in the cell. Numerous studies have demonstrated that increased levels of circulating free fatty acids and/or tissue accumulation of lipid metabolites in the liver or skeletal muscle cause insulin resistance in humans and animal models (52-60). The exact mechanisms by which fat causes insulin resistance are not completely understood, but the evidence from *in vitro* and animal studies indicates that elevated levels of FFA or intracellular fat accumulation activate serine/threonine kinases that can phosphorylate insulin receptor and/or IRS, thereby inhibiting insulin signaling. The potential mechanisms for the FFA-induced hepatic insulin resistance are described in detail in the following sections.

## 1.2 Free Fatty Acids and Hepatic Glucose Metabolism

### 1.2.1 Free Fatty Acids and Hepatic Glucose Production

Liver is the primary organ for endogenous glucose production (EGP), which occurs via glycogenolysis and gluconeogenesis. Glycogenolysis is the breakdown of stored liver glycogen into individual glucose molecules whereas gluconeogensis refers to *de novo* synthesis of glucose from such substrates as glycerol, alanine, and lactate. The

enzymes G6Pase and glucokinase regulate the final pathways of hepatic glucose production (HGP) common to glycogenolysis and gluconeogenesis, and glucose uptake, respectively. G6Pase removes a phosphate group from glucose-6-phosphate to convert it to glucose and glucokinase phosphorylates glucose to glucose-6-phosphate. Accordingly, the relative activities of these enzymes are a rate-determining factor of hepatic glucose balance between HGP and glucose uptake (61).

Hepatic glucose flux *in vivo* is determined using tracer dilution method (61). The rate of HGP can be assessed with tritiated glucose labeled at carbon 3 or 6, which lose tritium at different stages of glycolysis and thus requires different method of tracer quantification. The use of tritiated glucose is advantageous over $^{14}$C-labeled glucose as $^{14}$C released from the metabolized tracer is reincorporated into newly released glucose, thus resulting in underestimation of glucose turnover, whereas tritiated glucose is not recycled (62).

Intravenous infusion of TG emulsion is a standard method of elevating plasma FFA *in vivo*. For instance, Intralipid 20% (Baxter healthcare corporation, Deerfield, IL) is a TG emulsion commonly given with heparin that activates endogenous lipoprotein lipase on peripheral endothelium, which hydrolyses TG into FFA and glycerol. Such preparation and administration of the lipid is important as direct infusion of FFA causes hemolysis because of the surfactant effect of high concentrations of FFA on membranes. Intralipid consists of soybean oil and is 50% linoleic acid (18:2), 26% oleic acid (18:1), 10% palmitic acid, 9% linolenic acid, and 3.5% stearic acid.

Since Intralipid is a TG emulsion that is broken down to non-esterified fatty acids and glycerol *in vivo* by heparin-induced activation of lipoprotein lipase, it is possible that

glycerol derived from TG by itself affects EGP measured in the present study. However, a previous study in our laboratory (58) has shown that glycerol infusion (5mg/kg/min) resulting in plasma glycerol levels similar to that achieved by 7 hours of Intralipid plus heparin infusion has no effect on EGP when compared with saline infusion either in the basal fasting state or during the hyperinsulinemic-euglycemic clamp.

Liposyin II and Liposyn III 20% (Abbott laboratories) are other commercially available TG emulsion products that are used to elevate plasma FFA levels *in vivo*. Liposyin III 20% and Intralipid 20% are composed of essentially same types and proportions of fatty acids while Liposyin II 20% is made up of 10% soybean and 10% safflower oils, and contains slightly more linolenic acid and less oleic acid than Intralipid. All of these TG products use egg-yolk phospholipids as an emulsifying agent. In this thesis, Intralipid 20% plus heparin was used to study the effects of short-term (7h) FFA elevation on hepatic insulin resistance in the fasting state and during a hyperinsulinemic-euglycemic clamp (see General Methods for description).

Elevated levels of circulating FFA can increase HGP via their stimulatory effect on hepatic gluconeogenesis and by attenuating the ability of insulin to decrease glucose production in the liver. Elevated FFA levels increase hepatic gluconeogenesis both *in vitro* and *in vivo* (63;64). A mechanism similar to Randle's theory of substrate competition has been suggested to explain FFA-induced stimulation of gluconeogenesis (65). Firstly, FFA are converted to LCFA-CoA, which is then transported into mitochondria and converted to acetyl CoA. Acetyl-CoA allosterically inhibits pyruvate dehydrogenase, resulting in pyruvate accumulation and inhibition of glucose oxidation. Acetyl--CoA also allosterically activates pyruvate carboxylase, which converts pyruvate

to oxaloacetate, which in turn can form phosphoenolpyruvate in the cytosol to increase gluconeogenesis. Secondly, in the liver, NADH produced from FFA oxidation is used for formation of glyceraldehyde-3-phosphate from 1,3-bisphosphoglycerate, leading to increased gluconeogenesis. Thirdly, citrate (product of acetyl-CoA derived from FFA oxidation and oxaloacetate) inhibits phosphofuctokinase 1, leading to increased gluconeogenesis in the perfused rat liver and in isolated hepatocytes exposed to FFA (66-68).

Under conditions of predominant gluconeogenesis such as those of prolonged fasting, FFA increase HGP, which is contributed by allosteric stimulation of key gluconeogenic/glycogenolytic enzymes, such as G6Pase, by FFA (69).

### 1.2.2 Free Fatty Acids and Hepatic Glucose Autoregulation

Increased gluconeogenesis caused by short-term FFA elevation, as after overnight fasting, does not always result in increased HGP. This is due to hepatic glucose autoregulation, which is a process under basal physiological conditions whereby HGP is maintained constant despite the FFA–induced increase in gluconeogenesis, owing to a compensatory reduction in glycogenolysis (70;71). This regulation of glucose production occurs via extrahepatic and intrahepatic mechanisms. Extrahepatic autoregulation occurs via FFA-induced increase in insulin secretion and thus in circulating insulin levels that inhibit glycogenolysis in the liver (72). Intrahepatic mechanism, on the other hand, is independent of insulin and involves activation of glycogen synthase and inactivation of

glycogen phosphorylase by increased glucose-6-phophate from gluconeogenesis (73) and inhibition of glycogen phosphorylase by ATP derived from FFA oxidation (74).

The brain also plays an important role in the regulation of hepatic glucose metabolism and autoregulation. Increasing hypothalamic levels of LCFA-CoA markedly decreases hepatic glycogenolysis via hepatic vagus nerve input, which suggests that brain is a key modulator of hepatic glucose autoregulation in the presence of elevated systemic lipid levels (75).

In certain situations, the hepatic autoregulation of glucose breaks down and increased HGP ensues. Increased basal HGP after high-fat feeding has been reported in rats whose liver glycogen was depleted (76). In this case, glycogenolysis is limited by glycogen depletion (76;77) and may not further decrease to provide hepatic autoregulation of basal HGP in the presence of FFA-stimulated gluconeogenesis. Furthermore, in type 2 diabetic patients, defective hepatic autoregulation has been reported and may contribute significantly to increased HGP observed in these individuals in the presence of chronically elevated FFA levels (53). Although it is unclear why hepatic autoregulation does not function properly in type 2 diabetes, it may, at least in part, be explained by a defect in insulin secretion and/or hepatic insulin resistance present in the affected individuals. Furthermore, liver glycogen content, which is reduced in type 2 diabetes, may also affect hepatic autoregulation (78).

### 1.2.3 Free Fatty Acids and Hepatic Gluconeogenic and Lipogenic Gene Regulation

By impairing insulin signaling, FFA indirectly influence insulin-mediated regulation of gluconeogenic gene expression as insulin inhibits the expression of the key gluconeogenic enzymes at the transcription level (79) via Akt (80;81). In addition, FFA have direct effects on expression of gluconeogenic enzymes. FFA increase gluconeogenesis in primary hepatocytes in association with FFA-induced increase in gene transcripts of PEPCK and G6Pase via phosphorylation of peroxisome proliferator-activated receptor gamma coactivator (PGC) 1α and cAMP-response element-binding protein (CREBP) (82). Moreover, elevating plasma FFA by lipid infusion (83) or high-fat feeding (84) results in a marked increase in mRNA and protein levels of the catalytic subunit of G6Pase in the rat liver. However, the degree of saturation appears to affect whether a particular fat activates or inhibits G6Pase activity, as polyunsaturated fatty acids (PUFA) actually decreases G6Pase activity (85;86) whereas saturated fatty acids increase it (84).

FFA can also directly influence lipogenic gene expression by modulating the activity of transcription factors, including PPAR and sterol regulatory element-binding protein (SREBP)-1 (87). PUFA are strong activators of PPARα and a diet high in unsaturated fat increases PPARα gene expression in the liver (88). PPARα increases gene expression of enzymes involved in peroxisomal and mitochondrial FFA oxidation (87;89). PUFA suppress the expression of SREBP-1 and thus the expression of lipogenic enzymes, such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (90-94). The consequence of suppression of ACC is a decrease in malonyl-coA, which not only results in a reduction in TG accumulation but also an increase in FFA oxidation as malonyl-coA inhibits carnitine palmitoyltransferase (CPT)-1. PUFA suppress the

expression of SREBP-1 (both 1a and 1c) by decreasing mRNA stability (89) and via post-translational modification (95). The overall effect of PUFA regulation of gene expression is a control of hepatic TG accumulation as an adaptation to excess FFA supply via increased FFA oxidation and decreased generation of TG.

## 1.3 Insulin Signaling and Action

Insulin signaling at target tissues results in a wide array of biological outcomes. These include cellular growth and development as well as homeostasis of glucose, fat, and protein metabolism (96). In adipocytes, insulin promotes energy storage by increasing glucose and free fatty acid uptake, and stimulating lipogenesis while inhibiting lipolysis. In skeletal muscle, insulin enhances glucose and amino acid uptake and stimulates glycogen and protein synthesis. In the liver, insulin increases glycogen synthesis and suppresses HGP.

Insulin signaling involves two major pathways – one involving the MAP kinase, which is important for cellular growth and development, and the other involving PI3K, which is crucial for metabolic functions. For the purpose of this thesis, only the PI3K pathway involved in insulin's metabolic functions is described in detail. However, it should be noted that each pathway may in certain situations activate the other.

Insulin receptor belongs to a highly-conserved family of growth factor receptors with intrinsic tyrosine kinase activity (97). The receptor is a tetramer with an extracellular domain containing $\alpha$ subunits and a transmembrane domain containing $\beta$ subunits ($\alpha 2 \beta 2$ configuration). Insulin binds to $\alpha$ domain of its receptor, which initiates a

rapid conformational change in the receptor that leads to phosphorylation of specific tyrosine residues of the β subunits. This event, in turn, enables insulin receptor to phosphorylate docking proteins such as IRS on tyrosine residues, which serve as docking sites for downstream effectors. This phosphorylation of IRS by insulin receptor is considered to be a critical step in insulin signaling.

There are many subtypes of IRS (IRS1-6) expressed in insulin-responsive tissues, with IRS-1 and –2 being the main IRS involved in insulin-mediated metabolic functions in the liver. Although the relative importance of IRS-1 and –2 in regulation of metabolic functions is unclear, a recent study demonstrated that IRS-1 is important for Akt1 activation and glucose transporter 4 (GLUT4) translocation while IRS-2 contributes to extracellular signal-regulated kinase (ERK) signaling in L6 myotubes (98). Moreover, IRS-2 may at least partly compensate for absence of IRS-1, as IRS-1 null mice are only mildly insulin resistant (99) whereas IRS-2 knockout mice are not only insulin resistant but also develop diabetes (100). IRS contain a conserved pleckstrin homology (PH) domain that serves to localize the IRS proteins to the membrane in close proximity to the receptor and a phosphotyrosine binding (PTB) domain that functions as a binding site to the NPXY motif of the juxtamembrane region of the insulin receptor to promote insulin receptor/IRS interactions. The poorly conserved carboxy terminal of IRS contains many tyrosine phosphorylation motifs that serve as docking sites for Src homology 2 (SH2) domain-containing proteins, such as the p85α regulatory subunit of PI3K (101).

Phosphorylated IRS is then able to associate with the p85/p110 complex of PI3K, a lipid kinase with an SH2 domain that phosphorylates phosphotidylinositol-4,5-bisphosphate to phosphotidylinositol-3,4,5-triphosphate ($PIP_3$). By binding to its PH

domains, $PIP_3$ activates phosphoinositide-dependent kinase (PDK)–1, which in turn phosphorylates and activates Akt/PKB. PDK-1 also activates atypical protein kinase C (aPKC) isoforms ζ and λ. Akt and aPKCs are essential for insulin-stimulated translocation of glucose transporters, such as GLUT4 expressed mainly in muscle and fat, from intracellular pools to the cell surface (96). Akt also increases glycogen synthesis and decreases glycogenolysis via inactivation of glycogen synthase kinase (GSK)-3 (102) and activation of protein phosphatase 1 (103). In addition, Akt promotes protein synthesis by reversing GSK-3-induced inactivation of protein synthesis eukaryotic initiation factor (eIF)-2B (104). Akt also activates mammalian target of rapamycin (mTOR), which promotes protein synthesis through p70 ribosomal S6 kinase (S6K1) and inhibition of eIF-4E binding protein (105).

In addition to promotion of glycogen synthesis and inhibition of glycogen breakdown, insulin suppresses gluconeogenesis by inhibiting the expression of rate-limiting enzymes for gluconeogenesis, PEPCK and G6Pase, at the transcriptional level in a PI3K- and Akt-dependent manner (80;106). The role of Akt in the insulin-mediated inhibition of gluconeogenic gene expression is via phosphorylation-dependent regulation of forkhead transcription factor (FOXO) (107;108) and PGC-1 (109;110). FOXO family of transcription factors are capable of binding to the promoters of genes for gluconeogenesis and initiating transcription; however, phosphorylation of FOXO proteins by Akt results in transcriptional inactivation, nuclear export, and inhibition of target gene expression (111). Accordingly, a number of studies have shown that FOXO proteins are involved in insulin-dependent regulation of gluconeogenic gene expression and insulin resistance (108;111;112).

Insulin via Akt phosphorylates and thus inhibits transcriptional coactivator PGC-1α, resulting in decreased gluconeogensis in primary hepatocytes (106). The expression of PGC-1 is upregulated in fasting mice and in insulin-resistant mice with increased HGP (110). In the same study, adenoviral mediated expression of PGC-1 in cultured hepatocytes or *in vivo* increased expression of key gluconeogenic genes, PEPCK and G6Pase, with resultant increase in glucose output. Interestingly, FOXO1 and PGC-1α appear to interact to promote expression of the gluconeogenic genes that are regulated by insulin/Akt-mediated phosphorylation. PGC-1α binds and co-activates FOXO1 while FOXO1 function is required for robust induction of gluconeogenic genes by PGC-1α in the hepatocytes and in mouse liver (110;112).

Growing evidence suggests that a major cause of insulin resistance is inhibition of insulin signaling via phosphorylation of insulin receptor and/or IRS on certain serine/threonine residues. In particular, phosphorylation of the IRS molecules on specific serine/threonine residues, for instance serine 307, by certain kinases results in decreased ability of insulin receptor to phosphorylate tyrosine residues of IRS and eventually in degradation of the IRS (110;113-116), thereby impairing insulin signaling (55;117-121). Multiple hormones and metabolites have been implicated in this process. For instance, TNFα and FFA, the levels of which are increased in obesity, have been shown to impair insulin signaling via serine/threonine phosphorylation of insulin receptor and/or IRS (60;121;122) (This topic is covered in more depth in the following sections). Furthermore, increased activity of protein tyrosine phosphatase (PTP) 1B, which decreases tyrosine phosphorylation of insulin receptor and IRS (123-125) and are important for negative-feedback downregulation of insulin signaling, are also implicated

in insulin resistance. Mice lacking the PTP1B gene show increased insulin sensitivity and are protected against insulin resistance caused by a high-fat diet (126). Similarly, *in vivo* knockdown of phosphatase and tensin homologue (PTEN), another phosphatase that inactivates lipid products of PI3K, using antisense oligonucleotides improved glycemic control in diabetic *ob/ob* and *db/db* mice (127). Finally, mice deficient in SH2-containing intracellular protein tyrosine phosphatase 1 (SHP-1) protein are markedly more insulin sensitive due to enhanced insulin signaling compared to the wild type littermates, providing further evidence that protein tyrosine phosphatase plays a key role in insulin resistance and type 2 diabetes (128).

## 1.4 Free Fatty Acids and Hepatic Insulin Action

### 1.4.1 Hepatic Insulin Resistance

Numerous studies have demonstrated that intrahepatic fat accumulation achieved via intravenous infusion of fat or high-fat diet impair insulin-induced suppression of HGP (54;58;59;129-133). This is, at least in part, attributed to disruption of hepatic insulin signaling by fat. Intracellular accumulation of lipid metabolites due to increased FFA flux and/or release from intracellular fat stores in the liver may activate various intracellular signals, such as serine/theronine kinases, that can interfere with normal insulin signaling and thus attenuate hepatic insulin action. Such potential intrahepatic signals activated by FFA include PKC-δ and -ε, oxidative stress, IKKβ/nuclear factor κ B

(NFκB) inflammatory pathway, and JNK stress-activated pathway, which are described in detail in the following sections.

#### 1.4.1.1 Free Fatty Acids, Lipid Accumulation, and Insulin Resistance

Accumulation of intracellular lipid metabolites in insulin sensitive tissues plays an important role in insulin resistance of obesity and type 2 diabetes (134). For instance, muscle TG levels are increased in type 2 diabetic patients (135) and correlate with insulin resistance in non-diabetic individuals (136;137). Similarly, accumulation of lipid in the liver (hepatic steatosis) is associated with hepatic insulin resistance (138-143).

With energy excess, as in the case of elevated FFA and glucose levels, there is inhibition of Krebs cycle enzymes by NADH and ATP, and exit of citrate from the mitochondria. Citrate activates ACC with consequent increase in malonyl-CoA and inhibition of CPT1 (144). This prevents FFA oxidation, resulting in accumulation of cytosolic LCFA-CoA and diversion of their metabolism toward esterification (145). This leads to accumulation of fat esterification products, which have been implicated in insulin resistance (65).

With energy deficit, on the other hand, there is preferential oxidation of fat over synthesis. An important regulator of energy metabolism is AMPK, which is activated in response to increased AMP/ATP ratio (146) and inhibits ACC (147-149), thus increasing fat oxidation and decreasing fat accumulation. Accordingly, AMPK activators, such as 5-aminoimidazole-4-carboxamide-1-ß-D-ribofuranoside (19;129;150), thiazolidinedione

(151-153), and adiponectin (19;150), decrease intrahepatic lipid content and improve hepatic insulin action.

The molecular mechanism by which lipid accumulation causes hepatic insulin resistance likely involves activation of one or more serine/threonine kinases, such as PKC, IKKβ, and JNK1, which may directly disrupt hepatic insulin signaling via serine/threonine phosphorylation of insulin receptor and/or IRS. Indeed, in fatless mice (154), in mice with liver lipoprotein lipase over-expression (155), and in high-fat fed rats (131), intrahepatic fat accumulation has been associated with impaired insulin signaling via serine/threonine phosphorylation of IRS-1 and -2.

Both LCFA-CoA and DAG can stimulate PKC, various isoforms of which have been linked to insulin resistance (see below) (156). LCFA-CoA (palmitoyl CoA in particular) can also form ceramides, which can activate JNK1(157;158) and have been implicated in fat-induced insulin resistance *in vitro* (159) and in muscle insulin resistance of obese individuals (160). Ceramide buildup inhibits insulin-stimulated activation of Akt in skeletal muscles cells *in vitro* (161;162).

#### 1.4.1.2 Effects of Different Types of Fat on Hepatic Lipid Metabolism and Insulin Sensitivity

Monounsaturated and saturated fatty acids do not suppress the expression of SREBP-1 and only stimulate PPARα, thus resulting in greater hepatic fat accumulation than PUFA (89). Intracellular lipid accumulation and insulin resistance are closely related and current evidence indicates that saturated fat causes more severe insulin resistance

compared to monounsaturated and polyunsaturated fatty acids (163-165). Fish oil high in polyunsaturated omega-3 fatty acids even prevents insulin resistance induced by high saturated fat diet both in the liver and peripheral tissues (164;166). Medium-chain fatty acids, which are more easily oxidized, result in less severe insulin resistance than long chain fatty acids (167).

### 1.4.1.3 Free Fatty Acids, PKC, and Insulin Resistance

#### *1.4.1.3.a PKC Isoforms*

PKC comprise a family of isoenzymes that play a key role in cell functions (168). PKC are classified into three major subgroups: 1) 'conventional' PKCs (α, βI, βII, and γ), which require phosphatidylserine (PS) and are activated by calcium and DAG; 2) 'novel' PKCs (δ, ε, μ, η, θ) which require PS and DAG but not calcium for activation; and 3) 'atypical' PKCs (ζ, λ), which are calcium- and DAG-independent but PS-dependent (169). When PKC is activated, the enzyme translocates from the cytosol to the plasma membrane. Therefore, the determination of PKC content in the cytosol and the membrane fractions using Western blot analysis is a common method of assessing PKC membrane translocation and a generally accepted indicator of PKC activation.

PKC isoforms are ubiquitously expressed. In the major insulin-responsive tissues, i.e. liver, skeletal muscle, and adipose tissue, PKC isoforms from each of the three categories are expressed to varying degrees (170). For instance, PKC-θ is expressed

predominantly in the skeletal muscle (171) but is not highly expressed in the liver. In contrast, PKC-δ is expressed robustly in all major insulin-responsive tissues (172).

Selective activation of individual PKC isoforms by physiological stimuli provides one explanation for isoform-selective responses (173). Novel PKCs are selectively activated by DAG produced from phospholipase D pathway, which does not produce a calcium transient (174) whereas the calcium from phosphoinositide hydrolysis may be required for full activation of conventional PKC isoforms (175). The subcellular localization of PKCs also determines isoform-dependent functions (173).

#### *1.4.1.3.b PKC and Insulin Signaling*

PKC, in particular novel isoforms, are known to phosphorylate a number of serine/threonine residues of the insulin receptor and IRS (176-178). This diminishes insulin receptor tyrosine kinase activity and interferes with tyrosine phosphorylation of the IRS, thereby impairing insulin signaling. For instance, Greene et al. have shown that activated PKC-δ can directly phosphorylate specific serine residues of human IRS-1, including serine 307, 323, 573, that diminishes the ability of insulin receptor to tyrosine phosphorylate IRS-1 *in vitro* (177). Moreover, PKC-θ decreases tyrosine phosphorylation of IRS-1 by phosphorylating serine 1101 of IRS-1 in cultured murine C2C12 myocytes and 3T3 L1 fibroblasts (179). These results point to a potentially direct role of PKC in impairment of insulin signaling.

### *1.4.1.3.c PKC and Insulin Resistance*

Activation of PKC isoforms, both conventional and novel, is associated with various animal models of insulin resistance. In addition, elevated PKC activity has been observed in muscle tissue from individuals with type 2 diabetes and in liver of humans with type 2 diabetes (180). Therefore, PKC activation is likely a key mediator of insulin resistance, particularly in the context of elevated plasma FFA and tissue fat accumulation.

*PKC and Skeletal Muscle Insulin Resistance*

The role of PKC in insulin resistance appears to be species- and tissue-specific. In human skeletal muscle, the activation of a conventional PKC isoform βII and a novel isoform δ is involved in insulin resistance caused by lipid infusion (56). Peripheral insulin resistance caused by lipid infusion in rats was shown to occur in conjunction with an accumulation of lipid metabolites, membrane translocation of PKC-θ, and an impairment of insulin signaling (55;60). The same group also demonstrated that mice lacking PKC-θ expression are protected from peripheral insulin resistance caused by short-term lipid infusion (57). Furthermore, rats subject to a high-fat diet exhibited peripheral insulin resistance in parallel with increased membrane translocation of PKC-θ and –ε (181). These results indicate that PKC is a causal mediator of insulin resistance in skeletal muscle; however, the type of PKC involved in the process likely depends on the species and the context in which insulin resistance occurs.

*PKC and Hepatic Insulin Resistance*

The precise role of PKC in fat-induced hepatic insulin resistance is unclear but novel isoforms PKC-δ and -ε likely play an important role. FFA enhance membrane translocation of PKC in hepatocytes (182;183) and hepatic PKC activity is greater in obese hypertriglyceridemic diabetic rats compared to lean rats (184). Our laboratory (58), as well as others (54), have found that a short-term intravenous infusion of triglyceride emulsion plus heparin leads to plasma membrane translocation of PKC-δ in association with hepatic insulin resistance in rats. However, whether PKC-δ activation is necessary for, or is only a marker associated with, the FFA-induced defect is unknown. In addition, it is unclear whether PKC directly interferes with insulin signaling or mediates activation of downstream signals such as IKKβ or JNK1 pathway. *In vitro* studies have provided evidence, as described above, that PKC is capable of phosphorylating serine/threonine residues of the insulin receptor and IRS that blunts insulin signaling (176;178;185). However, whether this direct inhibitory effect of PKC on insulin signaling molecules is relevant *in vivo* is unclear, since serine 307 phosphorylation of IRS-1 has been linked to IKKβ and JNK1, but not PKC, in established rodent models of insulin resistance. Another possibility involves PKC-mediated activation of downstream serine kinases IKKβ or JNK1 either directly, although whether PKC can activate IKKβ or JNK directly *in vivo* is unknown, or via generation of oxidative stress (see below). The latter possibility that PKC impairs insulin signaling indirectly is consistent with the findings that fat-induced insulin resistance is prevented in IKKβ and JNK1 knockout mice (186;187).

Inhibition of PKC-ε was recently shown to prevent hepatic steatosis and insulin resistance in rats fed high-fat diet (132), indicating that activation of PKC-ε may be critical in hepatic insulin resistance caused by prolonged fat elevation. As described above, PKC-ε was also found to be activated in skeletal muscle after a high-fat diet (181). Therefore, it appears that PKC isoforms involved in fat-induced hepatic insulin resistance may depend on the duration of fat elevation.

#### 1.4.1.4 Free Fatty Acids, Oxidative Stress, and Insulin Resistance

Oxidative stress is defined as a persistent imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses (188). ROS are highly reactive atoms and molecules that cause tissue damage by altering the structure of proteins, lipids, and DNA. In addition to inducing macromolecular damage, ROS can function as signaling molecules to activate various cellular stress-sensitive pathways. Oxidative stress has been suggested to play a causal role in the pathogenesis (189) as well as complications (190;191) of type 2 diabetes based on the evidence that ROS is elevated in diabetic patients (190;192) and that antioxidants can prevent toxic effects of high glucose and lipid levels on insulin sensitivity and β-cell function (193-197).

FFA may produce ROS via peroxisomal and mitochondrial oxidation, arachidonic acid metabolism, ceramides, and possibly through hexosamine pathway, and may have direct effect on antioxidant enzymes. For example, a diet rich in PUFA decreased superoxide dismutase (SOD) and catalase activities in rodent liver (198;199).

However, in other studies SOD and catalase activities were found to be increased, which suggests a compensatory response to oxidative stress (200;201).

Another mechanism by which FFA can increase oxidative stress is via ceramides, which can be synthesized *de novo* from LCFA-CoA. Ceramides increase ROS production by the mitochondria (202-204) and via activation of NADPH oxidase (205). Oxidative stress conversely can activate sphingomyelinase, which converts sphingomyelin into ceramides (202;204). Ceramides cause insulin resistance by impairing activation of Akt and thus insulin signaling in skeletal muscle cells *in vitro* (161;162). Ceramides can also activate JNK1 (206), which has been implicated in insulin resistance (207-209).

FFA can also activate in tissue-specific manner certain protein kinase C isoforms that induce oxidative stress, which in turn may cause insulin resistance via activation of serine kinases IKKβ, JNK1, or p38 MAPK. For instance, PKC can increase ROS via activation of NADPH oxidase (210-212), a potentially major source of $O_2^-$ in the liver. PKC activates NADPH oxidase by phosphorylation of subunits $p47^{phox}$, $p67^{phox}$, and/or Rac (212), as was shown in a study where FFA increased ROS through PKC-dependent activation of NADPH oxidase in cultured vascular smooth muscle cells and endothelial cells (210). In particular, PKC-δ activates NADPH oxidase via serine phosphorylation or membrane translocation of $p47^{phox}$ (213), which results in elevated intracellular ROS production in mouse hepatocytes (212). Support for a potential role of NADPH oxidase in hepatic insulin resistance *in vivo* comes from a study in which oxidative stress and NADPH oxidase activity were elevated in the liver of *fa/fa* rats fed on high-fat diet (214). Thus, NADPH oxidase may be an important source of oxidative stress in FFA-induced hepatic insulin resistance.

The molecular mechanism by which oxidative stress causes insulin resistance likely involves activation of other intracellular signals. For instance, *in vitro* studies have shown that ROS can activate multiple serine kinase cascades (215), which are capable of phosphorylating the insulin receptor and/or IRS at critical serine sites, such as serine 307 of IRS-1, to attenuate insulin action (121). One such serine kinase is JNK1, which may be an important mediator of insulin resistance in the liver (see below for more on the role of JNK1 in insulin resistance). Oxidative stress-dependent activation of JNK1 increases serine 307 phosphorylation of IRS-1 in Chinese hamster ovary cells (117). Another possible downstream target of oxidative stress is serine kinase IKKβ, which activates pro-inflammatory transcription factor NFκB. Kamata et al. (216) demonstrated that $H_2O_2$ has stimulatory effects on NFκB through the activation of IKKβ, which is capable of interfering with insulin signaling by phosphorylating insulin receptor and IRS on serine residues, such as serine 307 of IRS-1 (see the section on the role of inflammatory pathway in hepatic insulin resistance for more details) (119). Finally, $H_2O_2$-mediated inhibition of insulin-stimulated glucose transport is accompanied by activation of p38 MAPK by $H_2O_2$ in L6 muscle cells, suggesting a potential role of p38 MAPK in insulin resistance caused by oxidative stress (217). However, p38 MAPK does not appear to directly phosphorylate IRS and its inhibitory effect on insulin signaling may be downstream of Akt (217).

Oxidative stress can also directly target the key transcription factors that regulate the expression of gluconeogenic enzymes, such as FOXO and PGC1α. ROS phosphorylate (218) or acetylate FOXO (219), both of which allow it to translocate into the nucleus and regulate transcription of its target genes. Oxidative stress can also

increase the nuclear content of PGC1α, which increases expression of gluconeogenic enzymes (82;220).

Consistent with a causal role of oxidative stress in insulin resistance, the use of antioxidants has been successful against insulin resistance in rodent models. Previous studies in our laboratory have demonstrated that the antioxidants NAC and taurine prevent hyperglycemia-induced insulin resistance in rats (193). Moreover, another antioxidant, α-lipoic acid, was shown to partially protect against oxidative stress-induced defect in GLUT4 translocation and Akt activation in 3T3-L1 adipocytes (221). Similar results were observed in cultured rat L6 muscle cells in which lipoic acid pre-treatment prevented an impairment in insulin-stimulated glucose transport caused by oxidative stress (196). Moreover, α-lipoic acid improved insulin-stimulated glucose uptake in skeletal muscle of obese, insulin resistant Zucker fatty rats (222) and of diabetic Goko-Kakizaki rats (223).

Similarly, antioxidants alleviate insulin resistance in humans. Intravenous infusion of glutathione partly prevented FFA-induced whole-body insulin resistance in humans (224). Moreover, vitamin E, a dietary antioxidant important for free radical scavenging (225), improved insulin resistance in type 2 diabetic patients (226). Vitamin E is also effective as a treatment for non-alcoholic fatty liver disease (NAFLD), a common liver disease closely associated with hepatic insulin resistance. Daily vitamin E treatment normalized serum aminotransferase and alkaline phosphatase levels in obese children with non-alcoholic steatohepatitis (NASH) (227). In another study, vitamin E treatment improved fibrosis with no change in inflammation in the liver of individuals with NASH (228). Vitamin E alone was not effective in another study; however, a

combination therapy with pioglitazone improved histological parameters associated with NASH (229).

#### 1.4.1.5 Free Fatty Acids, Inflammatory Pathways, and Insulin Resistance

Epidemiological studies first established a strong correlation between circulating levels of markers and/or mediators of inflammation (fibrinogen, C-reactive protein, IL-6, plasminogen activator inhibitor-1) and obesity or type 2 diabetes (230). Obesity is now recognized as a state of low-grade, chronic inflammation whereby increased levels of circulating pro-inflammatory cytokines (e.g. TNFα, IL-6) and FFA released from expanded adipose tissue cause insulin resistance via activation of inflammatory pathways. Furthermore, type 2 diabetes is closely associated with increased levels of circulating pro-inflammatory cytokines. Evidence from both human and animals studies point to inflammatory pathways as an important causal link between adipocyte-derived products and insulin resistance.

##### *1.4.1.5a IKKβ / NFκB*

The earliest observations linking inflammation to type 2 diabetes came from anecdotal reports from physicians who noted anti-diabetic effects of aspirin or its derivative, sodium salicylate, in the late part of the 1800s and early part of the 1900s (230). Since then, numerous *in vivo* and *in vitro* studies have demonstrated that activation of the IKKβ/NFκB inflammatory pathway is causally involved in insulin resistance

(187;231-233). When activated, IKKβ phosphorylates IκBα, the constitutive inhibitor of pro-inflammatory transcription factor NFκB, on specific serine residues, after which IκBα is targeted for degradation and NFκB is liberated to translocate into the nucleus to initiate transcription of numerous pro-inflammatory cytokines, including TNFα, IL-1β, and IL-6, that can impair insulin signaling via a feed-forward mechanism (230). IKKβ is also capable of phosphorylating insulin receptor and IRS on serine/threonine residues, such as serine 307 (119), which interferes with their tyrosine phosphorylation and thus insulin signaling (187;231-233).

Sodium salicylate is an inhibitor of IKKβ that has been utilized in many recent studies to identify IKKβ as a causal mediator of insulin resistance. Administration of salicylate at high dose prevented insulin resistance caused by lipid infusion in rat skeletal muscle (233). In this study, this effect of salicylate was attributed to its reversal of lipid-induced decrease in tyrosine phosphorlyation of IRS-1 and in PI3K activity in skeletal muscle, suggesting that salicylate prevented serine phosphorylation of IRS-1 by IKKβ or by downstream pro-inflammatory products of NFκB. The results of this study also suggested that acutely raising plasma FFA via intravenous lipid infusion leads to IKKβ activation, thereby revealing IKKβ pathway as an intracellular mechanism by which FFA cause insulin resistance. Around the same time, Yuan et al. demonstrated that high-dose aspirin and salicylate dramatically improve insulin sensitivity in Zucker fatty rats, a commonly used rodent model of non-diabetic, obesity-related insulin resistance (187). Again, the effects of aspirin and salicylate were attributed to reversal of impaired insulin signaling. The same two groups also reported that heterozygous deletion of IKKβ protects mice from developing insulin resistance due to lipid infusion (233), high-fat

feeding, or genetic (*ob/ob*) obesity (187), a finding which is consistent with the causal involvement of IKKβ in insulin resistance. Furthermore, FFA decreased insulin-induced glucose uptake in 3T3-L1 adipocytes via activation of serine kinases, including IKKβ, in association with increased serine 307 phosphorylation of IRS-1 (120).

In human skeletal muscle, insulin resistance caused by short-term lipid infusion is associated with decreased muscle IκBα, which is an indication of increased IKKβ activity since, as previously mentioned, IκBα is targeted for degradation upon phosphorylation by activated IKKβ (56). In another human study, daily aspirin treatment (7g/day) over a two-week period resulted in a reduction of basal rates of hepatic glucose production and in increased insulin-stimulated peripheral glucose uptake during hyperglycemic-euglycemic clamp in type 2 diabetic patients (234). These studies suggest that IKKβ/ NFκB inflammatory pathway is also causally involved in insulin resistance in humans. It should be noted, however, that the side effects associated with high-dose salicylate treatment, such as gastrointestinal damage, that is required to effectively inhibit IKKβ precludes it from being a safe, routine treatment of insulin resistance at the present time. Pharmacological intervention that targets IKKβ more specifically would be desirable.

IKKβ/NFκB pathway is implicated in hepatic insulin resistance as well. Hepatocyte-specific deletion of IKKβ protected mice from obesity or high-fat diet-induced hepatic insulin resistance but not peripheral insulin resistance (231). In another study, treatment with high-dose salicylate reversed both hepatic and peripheral insulin resistance in mice with hepatic over-expression of IKKβ (232). In the latter study, it was proposed that increased activation of IKKβ/NFκB pathway in the liver results in

overproduction of inflammatory cytokines by the liver that cause not only hepatic insulin resistance but circulate into the periphery to cause peripheral insulin resistance. In both studies, increased IKKβ activity was associated with decreased tyrosine phosphorylation of IRS-2 as well as IRS-associated PI3K activity in the liver. Boden et al. also showed that hepatic insulin resistance caused by a short-term lipid infusion is associated with activation of the IKKβ/ NFκB inflammatory pathway in rats (54).

The molecular mechanism that links elevated FFA and IKKβ in hepatic insulin resistance is unclear. One possibility is that elevated FFA results in increased accumulation of lipid metabolites which activates IKKβ through oxidative stress. ROS has been shown to activate IKKβ pathway *in vitro* (216); however, whether oxidative stress links FFA to IKKβ activation *in vivo* is unknown.

As discussed above, a wealth of evidence exists to support a causal role of IKKβ/ NFκB pathway in hepatic insulin resistance. However, a question remains as to whether IKKβ as a serine/threonine kinase is directly involved in impairment of hepatic insulin signaling or whether it participates indirectly by allowing NFκB to upregulate expression of various pro-inflammatory mediators that exert their effects locally to induce hepatic insulin resistance. Likely candidates for these factors are pro-inflammatory cytokines IL-1β, IL-6, and TNFα (the latter two cytokines are discussed in depth below), as their mRNA levels are markedly elevated in mice over-expressing hepatic IKKβ (232).

#### *1.4.1.5b Cytokines (TNFα, IL6)*

The knowledge that adipose tissue is an active endocrine organ that produces a number of hormones and cytokines (together termed adipokines) that are implicated in obesity-related insulin resistance is well documented. However, other tissues such as liver and skeletal muscle, as well as macrophages are also capable of producing significant amounts of cytokines in obesity. The mechanisms of their action in the target tissues are currently the topic of intense investigation.

The finding that TNFα expression is elevated in the adipose tissue and skeletal muscle of obese individuals (36;235-239) and in animal models of obesity (35;240) suggested the possibility that pro-inflammatory cytokines and their activation of inflammatory pathways play an important role in insulin resistance. The causal relationship between TNFα and insulin resistance was first established in studies in which neutralization of TNFα using a soluble TNF-receptor IgG fusion protein improved insulin sensitivity in obese mice (241) and in obese Zucker (*fa/fa*) rats (242). Furthermore, obese mice deficient in TNFα gene or have mutations in TNFα receptors p50 and p75 were more insulin sensitive compared to their obese counterparts with intact TNFα gene or functional TNFα receptors (240). However, the results of using an inhibitor against TNFα to treat insulin resistance in humans have not been as positive as in rodents (243).

TNFα causes insulin resistance by interfering with autophosphorylation of the insulin receptor and tyrosine phosphorylation of IRS (37), likely via TNFα-induced serine phosphorylation (38). This finding raised the possibility that the effect of TNFα on insulin signaling occurs through activation of serine/threonine kinases (38). One kinase identified in this capacity is JNK1. TNFα is capable of activating JNK1 in multiple cell types and its induction leads to a decrease in glucose transport in association with a

decrease in tyrosine phosphorylation of insulin receptor and IRS-1 in 3T3-L1 adipocytes (244). Moreover, TNFα-induced serine 307 phosphorylation of IRS-1 was prevented with treatment with a JNK inhibitor in HEK 239 cell line (245). Another serine kinase implicated in TNFα-mediated insulin resistance is IKKβ. TNFα-induced activation of IKKβ results in impaired insulin signaling pathway via serine phosphorylation of IRS-1 at 307, whereas salicylate, an IKKβ inhibitor, prevents this effect in primary neonatal rat myotubes (122). This finding was corroborated in another study in which serine 307 phosphorlyation of IRS-1 following TNFα treatment was reversed with aspirin in 3T3-L1 and Hep G2 cells (246).

The effect of IL-6 on insulin action is a controversial topic. Recent evidence indicates that IL-6 has both pro-inflammatory and anti-inflammatory properties depending on the circumstance under which IL-6 is released. IL-6 is one of several adipocyte-derived inflammatory cytokines whose levels are elevated in obesity (247) and is closely associated with insulin resistance and type 2 diabetes (248;249). Acute IL-6 treatment can blunt the ability of insulin to suppress HGP (250) and impair hepatic insulin signaling (251;252). Furthermore, in mice over-expressing IKKβ in the liver, IL-6 is produced excessively and its neutralization is effective in reversing systemic insulin resistance (232). These effects of IL-6 are likely via activation of SOCS proteins, which participate in negative feedback inhibition of cytokine signaling through JAK/STAT pathway (44;45;251). In particular, SOCS-3 can suppress insulin-stimulated receptor autophosphorylation and IRS-1 tyrosine phosphorylation in the liver (43), possibly by binding to tyrosine (960) residue of insulin receptor and interfering with the association of IRS with the insulin receptor (45). SOCS can also mediate specific degradation of

IRS-1 and 2 proteins via the elongin BC ubiquitin-ligase complex in multiple cell types including hepatocytes (253).

However, there has been emerging evidence in support for a protective role of IL-6 against insulin resistance. While adipose tissue is a major source of IL-6 production in obesity, a significant amount of IL-6 is released from the skeletal muscle during and after exercise in an intensity- and duration-dependent manner (254). Exercise-induced IL-6 production by skeletal muscle promotes lipolysis and hepatic glucose production. IL-6 increases AMPK activity in L6 myotubes (255) and IL-6-null mice show diminished levels of phosphorylated AMPK both at the basal state and following exercise (256). Furthermore, acute IL-6 infusion increased fatty acid oxidation and insulin-stimulated glucose uptake in humans (257). Perhaps the most striking evidence in support of the positive effect of IL-6 on insulin action is a recent finding that IL-6 knockout mice at 9 months of age become obese, hypertriglyceridemic, and glucose intolerant, which is likely attributed to diminished AMPK activity in skeletal muscle (47).

#### *1.4.1.5c Macrophage Chemoattractant Protein 1/Macrophages*

Obesity is closely associated with macrophage infiltration of adipose tissue in both animals and humans (258;259). Macrophage accumulation in adipose tissue is induced by release of chemotactic factors by expanding adipose tissue that attracts macrophages, such as macrophage chemoattractant protein (MCP)-1 (260). Over-expression of MCP-1 in adipose tissue results in increased macrophage infiltration into adipose tissue in obese mice or mice fed high-fat diet (261). Interestingly, in the same

study, over-expression of MCP-1 in adipocytes exacerbated, whereas MCP-1 knockout mice showed alleviated, hepatic steatosis and insulin resistance caused by high-fat diet.

Recently, it was shown that absence of IKKβ signaling in myeloid cells, which are precursors of macrophages and neutrophils, protects against systemic insulin resistance caused by obesity or high-fat diet in mice (231). This finding suggests that activation of inflammatory pathways and subsequent release of inflammatory mediators by myeloid cells play an important role in causing insulin resistance in various insulin-responsive tissues. Of particular interest with regard to hepatic insulin resistance is the potential involvement of resident macrophages, Kupffer cells, in the liver. Kupffer cells can be activated by hepatocytes and may release pro-inflammatory factors that propagate inflammatory pathway and impair insulin action in hepatocytes (232) .

#### *1.4.1.5d Toll-Like Receptor Pathway*

Toll-like receptors (TLR) are a family of pattern-recognition receptors that bind to lipopolysaccharide (LPS) and activate inflammatory pathway (262). TLR4 binds to LPS of gram-negative bacterial cell walls and, when associated with co-receptors CD14 and MD-2, interacts with the adaptor protein myeloid differentiation factor 88, triggering a downstream activation of the NFκB pathway. TLR are capable of initiating intracellular signals that activate NFκB, JNK, and SOCS pathways, all of which have been implicated in insulin resistance. The findings that FFA are capable of eliciting inflammatory pathways and that the medium-chain fatty acid lauric acid, a major component of LPS, can activate TLR4 signaling (263;264) raised the possibility that TLR4 mediate FFA-

induced insulin resistance in obesity and type 2 diabetes. This concept was recently examined in a study in which increased circulating FFA following 5h lipid infusion or high-fat diet activated TLR4 signaling leading to NFκB activation in adipocytes and macrophages, and to whole body insulin resistance (265). TLR4 deficiency prevented these adverse effects of FFA in association with prevention of NFκB activation.

#### 1.4.1.6 Free Fatty Acids, Stress-Activated Pathways, and Insulin Resistance

Over the last decade, it has become clear that stress-activated pathways, in addition to or in conjunction with IKKβ/NFκB, play an important role in the development of insulin resistance in obesity. In particular, two members of the MAPK family, JNK and p38 MAPK, that respond to various stress signals have been implicated in insulin resistance. The following sections describe the current evidence on the mechanism by which these kinases participate in the process.

##### *1.4.1.6a JNK1 Pathway*

JNK is a member of stress-activated kinase family that responds to such signal as inflammatory cytokines and oxidative stress and phosphorylates c-Jun. c-Jun is the transactivation component of the heterodimeric transcription factor activation protein 1, which regulates cellular proliferation and apoptosis. In mammals there are three JNK genes, which can give rise to at least 10 different JNK proteins via alternate splicing. JNK1 and JNK2 are broadly distributed while JNK3 is predominantly expressed in

neurons. Of the three isoforms, only JNK1 has been linked to insulin resistance and type 2 diabetes.

Numerous studies have implicated JNK in the development of insulin resistance and type 2 diabetes (120;207;209;244). Elevated JNK activity was observed in the liver, fat, and skeletal muscle of high-fat-fed or genetically obese (*ob/ob*) mice which were insulin resistant. Interestingly, the increase in total JNK activity was attributed mainly to an increase in JNK1 activity. In the same study, mice lacking JNK1 expression were protected from insulin resistance due to high-fat diet whereas those lacking JNK2 expression were not (207). Similarly, genetically obese (*ob/ob*) mice with mutations in the JNK1 gene exhibited significantly diminished hyperglycemia, hyperinsulinemia, and weight gain compared to obese mice without JNK1 mutation. In addition, intraperitoneal administration of cell-permeable JNK-inhibitory peptide markedly improved insulin resistance and ameliorated glucose tolerance in obese diabetic mice (266).

Two adipocyte-derived factors whose levels are elevated in obesity and cause insulin resistance via JNK pathway are TNFα and FFA. TNFα, an inflammatory cytokine which is overproduced in obesity and causes insulin resistance, is a potent stimulator of JNK1 (267-269). Hepatocytes treated with TNFα show increased serine 307 phosphorylation of IRS-1 and decreased tyrosine phosphorylation of IRS-1, and these effects were reversed with co-treatment with JNK1 inhibitor, SP600125 (186). Moreover, increased serine 307 phosphorylation of IRS-1 observed in the livers of obese mice was absent in the livers of $JNK1^{-/-}$ mice, which also exhibited enhanced insulin-stimulated tyrosine phosphorylation of IRS-1 in the liver compared to the wild-type mice.

JNK1 has been shown to also mediate FFA-induced insulin resistance in 3T3-L1 adipocytes (120) and in hepatocytes (209). In 3T3-L1 adipocytes, FFA-induced activation of JNK resulted in a decrease in insulin-stimulated GLUT 4 translocation and glucose uptake while RNAi-mediated inhibition of JNK activity reversed these effects (244). Furthermore, treatment of primary mouse hepatocytes with saturated fatty acid palmitic acid for 2h led to sustained JNK activation in association with increased IRS-1 serine 307 phosphorylation and decreased insulin-stimulated Akt activation. Expectedly, hepatocytes isolated from $JNK1^{-/-}$ mice did not exhibit FFA-induced IRS-1 serine 307 phosphorylation or inhibition of insulin stimulated PI3K or Akt activation (209). The results from these *in vitro* studies demonstrate that JNK1 is a causal link between FFA and insulin resistance, particularly in the liver.

Evidence for a causal role of JNK in hepatic insulin resistance *in vivo* comes from a study in which over-expression of dominant negative JNK in the liver of obese, diabetic *(db/db)* mice drastically improved hepatic insulin sensitivity whereas wild-type JNK expressed in the liver of normal mice diminished hepatic insulin sensitivity (270). In this study, wild-type or dominant negative JNK expressing adenovirus was delivered to 8 week-old c57BL6 mice. The mice which received the wild type JNK adenovirus showed increased hepatic JNK activity, impaired glucose tolerance, and hepatic insulin resistance during hyperinsulinemic-euglycemic clamp in conjunction with a decrease in tyrosine phosphorylation of IRS-1, an increase in serine 307 phosphorylation of IRS-1, and a decrease in Akt serine 473 phosphorylation. In contrast, the obese, diabetic mice which received dominant negative JNK adenovirus exhibited improved glucose tolerance and hepatic insulin sensitivity in parallel with reversal of impaired insulin signaling observed

in the mice with hepatic JNK over-expression and in the control mice. This finding is consistent with the effect of JNK-inhibitory peptide to increase tyrosine phosphorylation and decrease serine 307 phosphorylation of IRS-1 in the major target tissues of insulin in obese, diabetic *(db/db)* mice and of mice fed on a high-fat, high-sucrose diet (266).

Normal rats subject to a short-term high-fat diet show hepatic steatosis and hepatic insulin resistance in association with JNK1 activation in the liver (271;272). Furthermore, JNK1-null mice are resistant to high-fat diet-induced steatohepatitis (272). However, whether JNK1 is a causal mediator of hepatic insulin resistance induced by short-term lipid infusion is unknown.

#### *1.4.1.6b p38 MAPK Pathway*

p38 MAPK belongs to a family of mitogen-activated protein kinases. In addition to responding to various stress stimuli, p38 MAPK is activated by insulin in L6 myotubes, (273) and may be important for the insulin-stimulated glucose uptake (273-275), although the data are controversial (276).

The precise role of p38 MAPK in insulin resistance and type 2 diabetes is unknown. Basal phosphorylation status of p38 MAPK is increased in the skeletal muscle of type 2 diabetic patients compared to non-diabetic individuals (277;278). Furthermore, phosphorylation of p38 MAPK in the liver of diabetic *ob/ob* mice is increased but treatment with antisense oligonucleotide against PTP1B lowers the phosphorylation of p38 MAPK in association with improvement of hyperglycemia and hyperinsulinemia (279).

It appears that TNFα causes insulin resistance partly through p38 MAPK. In fetal brown adipocytes, the TNFα-induced decrease in glucose uptake was abolished by treatment with a p38 MAPK inhibitor, PD169316 (280). Similarly, pre-treatment of primary neonatal myotubes with the p38 MAPK inhibitor prevented TNFα-induced decrease in glucose uptake and GLUT4 translocation as well as TNFα-induced impairment in insulin signaling (122). Interestingly, TNFα activated IKKβ in these cells in a p38 MAPK-dependent manner, indicating that p38 MAPK regulates the activation of downstream serine kinases causally involved in insulin resistance rather than interfering with insulin signaling directly. Recently, p38 MAPK was found to mediate TNFα-induced impairment of insulin signaling in bovine aortic endothelial cells; however, the serine kinase responsible for the TNFα-induced increase in serine phosphorylation of IRS-1 was not identified (281).

p38 MAPK may also mediate the effect of resistin to impair insulin signaling in human endothelial cells (282). In this study, resistin impaired the activation of Akt and endothelium nitric oxide synthase (eNOS) in parallel with an upregulation of PTEN expression. PTEN is implicated in insulin resistance as it can reverse phosphorylation of $PIP_2$ to $PIP_3$ by PI3K (283). Impaired activation of Akt and eNOS, and expression of PTEN by resistin were prevented by treatment with siRNA against p38 MAPK, suggesting that p38 MAPK is an important mediator of resistin-induced insulin resistance in endothelial cells. It would be of interest to investigate whether p38 MAPK mediates PTEN-induced impairment of insulin signaling in the major target tissues of insulin.

While *in vivo* evidence for a causal role of p38 MAPK in FFA-induced insulin resistance is lacking, recent studies showed that both saturated and unsaturated fatty acids

stimulate the phosphorylation of p38 MAPK (82;284) and that prolonged treatement of primary hepatocytes with oleate blunts insulin signaling in association with p38 MAPK activation via PKC-δ (82). Moreover, there are reports that p38 MAPK is a key signaling component of the pathway for FFA-induced increase in hepatic gluconeogenesis. As described previously, FFA increase hepatic gluconeogenesis, which contributes significantly to fasting and postprandial hyperglycemia observed in type 2 diabetes. Several studies have shown that p38 MAPK mediates FFA-induced increase in hepatic gluconeogenesis (82;284). For instance, in isolated hepatocytes, p38 MAPK mediates FFA-induced increase in transcription of hepatic gluconeogenic genes, PEPCK and G6Pase (82). In this study, phosphorylaton of PGC-1α and CREBP by p38 MAPK was necessary for FFA-induced activation of the PEPCK promoter. Furthermore, in the aforementioned study, prolonged oleate treatment prevented insulin-induced inhibition of gluconeogenesis in association with activation of p38 MAPK, which increased protein expression of PTEN, in primary hepatocytes (284). These findings indicate that p38 MAPK, potentially via PTEN, plays an important role in FFA-induced increase in HGP; however, a potentially direct impact of p38 MAPK on hepatic insulin signaling cannot be ruled out.

#### 1.4.1.7 Other Potential Pathways of Hepatic Insulin Resistance

##### *1.4.1.7a Endoplasmic Reticulum Stress*

The endoplasmic reticulum (ER) is an organelle responsible for processing of secretory and membrane-bound proteins. Certain stress signals can disrupt ER homeostasis and lead to an accumulation of unfolded or misfolded proteins in the ER lumen. This process is termed 'ER stress', to which the cell responds by mounting an unfolded protein response (285;286). An example of such signals is obesity, as obesity is associated with excess lipid accumulation and with abnormalities in energy fluxes (287). ER stress is increased in animal models of obesity (287) and growing evidence suggests that ER stress is a key link between insulin resistance and obesity (288;289).

Various *in vitro* and *in vivo* studies using molecular chaperones to reduce ER stress have linked ER stress with obesity-associated insulin resistance. For example, hepatic over-expression of oxygen-regulated protein 150 (ORP150), a molecular chaperone found in the ER, alleviates ER stress and reverses hepatic insulin resistance whereas treatment with antisense ORP150 in the liver causes increased hepatic glucose production in association with an increase in the expression of gluconeogenic enzymes and with impairment of hepatic insulin signaling in diabetic (*db/db*) mice (290). Similarly, genetically-induced systemic expression of ORP150 improves insulin sensitivity in diabetic (Akita) mice (291). These results are consistent with those from Ozcan et al (292), who demonstrated that oral treatment of obese, diabetic mice with chemical chaperones 4-phenyl butyric acid and taurine-conjugated ursodeoxycholic acid reduced ER stress and improved glucose tolerance and insulin sensitivity .

The mechanism by which ER stress participates in insulin resistance involves activation of JNK1 (293) and subsequent serine phosphorylation of IRS (287). In high-fat-fed mice and genetically obese mice (*ob/ob*), the levels of markers of ER stress,

phosphorylated PKR-like kinase and translation initiation factor 2 subunit α, were increased in association with increased JNK activity in the liver (287). The same results were obtained in the adipose tissue of these animals, but not in skeletal muscle. Furthermore, pre-treatment of Fao liver cells with agents that induce ER stress markedly increased serine phosphorylation and decreased tyrosine phosphorylation of IRS-1, and decreased insulin-induced Akt phosphorylation (287). These effects induced by ER stress were prevented when the cells were treated with SP600125, a JNK inhibitor, suggesting that JNK mediates ER stress-induced impairment of hepatic insulin signaling.

#### *1.4.1.7b mTOR/p70 S6 Kinase*

An emerging area of investigation on the etiology of insulin resistance is a potential role of the ribosomal kinase S6K1, which is a downstream effector of the mTOR pathway. mTOR plays a crucial role in integrating insulin and nutrient signals (294). mTOR pathway is particularly sensitive to amino acid availability and is integral for regulation of protein synthesis via activation of downstream translational modulators S6K1 and 4E binding protein 1 (295). mTOR and subsequently S6K1 are also activated by insulin via the IRS-1/PI3K/Akt pathway and are involved in the negative regulation of insulin signaling via serine phosphorylation of IRS-1 on specific residues in adipose tissue and skeletal muscle.

Several *in vitro* studies have shown that chronic stimulation of mTOR/S6K1 pathway by insulin or amino acids leads to insulin resistance via serine phosphorylation and degradation of IRS-1 in fat and muscle cells (296-298). The link between

mTOR/S6K1 and obesity-related insulin resistance was, however, established by *in vivo* studies, which showed that S6K1 activity is upregulated in animal models of obesity. In S6K1-deficient mice, high-fat diet fails to cause insulin resistance, whereas genetically obese mice exhibit insulin resistance in association with markedly increased S6K1 activity and phosphorylation of IRS-1 on serine 307 and 636/639 in adipose tissue (299). These results suggest that nutrient- or obesity-induced activation of S6K1 suppresses insulin signaling via serine phosphorylation of IRS-1 on specific sites and that S6K1 is another serine kinase causally involved in insulin resistance. These results are consistent with the finding that over-activation of mTOR/S6K1 pathway as observed in diet-induced obesity is responsible for impairment of hepatic insulin signaling via phosphorylation of IRS-1 on serine 636 and 639 (300).

## 1.5 Summary

Insulin resistance is a major causal factor of not only type 2 diabetes but also other diseases, including atherosclerosis and certain types of cancer. Obesity is a primary risk factor for insulin resistance due to altered release of various circulating factors, such as cytokines, adiponectin, resistin, leptin, and FFA, released from the expanded adipose tissue. Hepatic insulin resistance is a major defect found in type 2 diabetes and contributes significantly to hyperglycemia. Numerous studies have demonstrated that elevated levels of plasma FFA, which are common in obesity, and fat accumulation due to energy excess and altered adipokine secretion in obese individuals cause hepatic insulin resistance. The mechanisms underlying fat-induced hepatic insulin resistance are

not fully understood and are likely multiple. Current evidence indicates that one important mechanism may be activation of novel PKC isoforms δ and ε by accumulation of fat metabolites. These PKC isoforms can act directly upon key upstream signaling molecules of insulin action or activate other downstream mediators, such as oxidative stress, IKKβ, and JNK1, to impair hepatic insulin signaling.

## 1.6 Rationale for the Studies

Hepatic insulin resistance and the resultant increase in HGP is the primary cause of hyperglycemia in obesity-associated type 2 diabetes. Thus, efforts to better understand the etiology of hepatic insulin resistance are paramount for development of treatment strategies. As described in the Introduction, various circulating factors released from adipocytes are potentially involved in obesity-related hepatic insulin resistance. In particular, numerous studies have established that elevated levels of circulating FFA or intrahepatic fat accumulation cause hepatic insulin resistance. However, identification of molecular mechanisms underlying fat-induced hepatic insulin resistance has been elusive.

Previous studies in our laboratory (58), as well as by others (54), had implicated PKC-δ membrane translocation, an indication of its activation, in hepatic insulin resistance induced by lipid infusion in rats; however, it is unknown whether PKC-δ activation is a necessary event or merely an associated marker of hepatic insulin resistance caused by the acute elevation of plasma FFA. Oxidative stress is another potential mediator of FFA-induced hepatic insulin resistance. FFA can induce oxidative stress via PKC (210), and oxidative stress may cause insulin resistance by activating downstream mediators such the serine kinases IKKβ and JNK1 (117;210). It is unknown whether oxidative stress is a causal mediator of hepatic insulin resistance caused by an acute FFA elevation. Furthermore, although current evidence demonstrates that IKKβ is causally involved in hepatic insulin resistance in rodent models of obesity, it remains to be elucidated whether IKKβ mediates hepatic insulin resistance caused by acute elevation of circulating FFA. Therefore, the three studies in this thesis were performed to

specifically address whether PKC-δ, oxidative stress, and IKKβ are causally involved and whether they are sequentially linked in FFA-induced hepatic insulin resistance by using specific inhibitors: antisense oligonucleotide (ASO) against PKC-δ, the antioxidants N-acetyl-L-cysteine (NAC) and resveratrol (RSV), and sodium salicylate, respectively.

To do this, we utilized an *in vivo* experimental model of short-term (7h) intravenous infusion of Intralipid plus heparin (IH) in normal rats in all three studies. We used rats as it allowed us to utilize in Study 1 the ASO against PKC-δ, whose effectiveness was characterized in rats, and to assess insulin signaling and various molecular markers in the insulin target tissues. Although acute FFA elevation via the lipid infusion does not reproduce the chronic FFA elevation of obesity, it does facilitate the investigation of the effect of fat *per se* before the other factors present in obesity, for instance elevated adipokine levels, come into play. Intralipid is a standardized, sterile triglyceride emulsion that is commercially available and is safe to infuse intravenously, thus bypassing the oral route of fat administration, which may itself affect HGP by vagal afference. Finally, IH has been commonly used in studies involving human subjects to investigate the effect of fat on insulin action; therefore, we can compare the results of our studies to those of human studies which have used the IH infusion model and extend the non-invasive studies of inhibitors to humans in the future.

## 1.7 General Hypothesis

The pathway for FFA-induced hepatic insulin resistance involves FFA activating PKC-δ, which leads to increased oxidative stress and activation of inflammatory pathways that impair hepatic insulin signaling (Figure 1).

## 1.8 Specific Aims

This thesis consists of three studies that examined the potentially causal role of each factor (PKC-δ, oxidative stress, and inflammatory pathways) and allowed us to draw preliminary conclusions regarding how these factors are linked in the pathway of FFA-induced hepatic insulin resistance in rats

**Study 1.** To examine whether PKC-δ mediates FFA-induced hepatic insulin resistance.

**Study 2.** To investigate whether oxidative stress is causally involved in FFA-induced hepatic insulin resistance.

**Study 3.** To examine whether IKKβ is a causal mediator of FFA-induced hepatic insulin resistance.

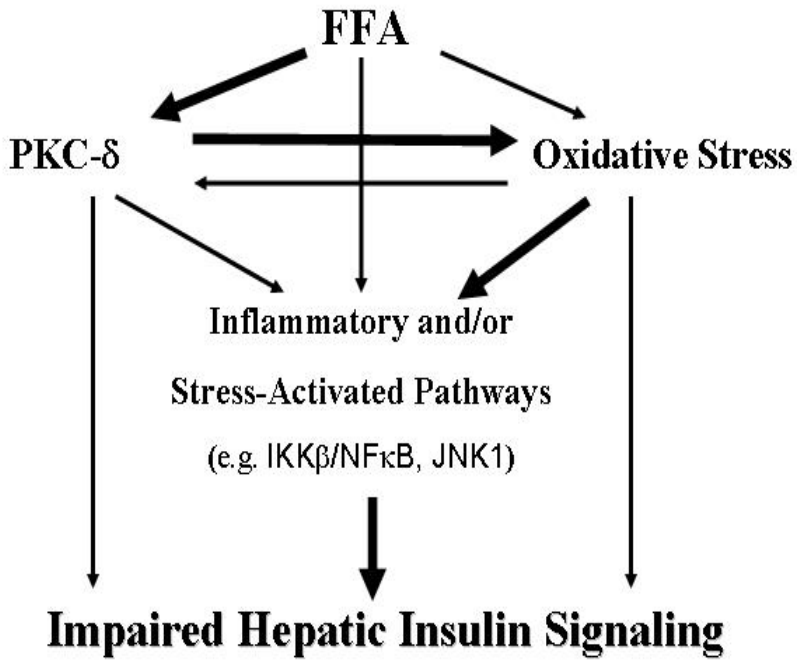



**Figure 1. The Hypothesized Relationships between PKC, oxidative stress, and inflammatory pathways in the mechanism of hepatic insulin resistance caused by acute FFA elevation**. FFA: Free Fatty Acids; PKC: Protein Kinase C; IKKβ: IκBα Kinase β; JNK1: c-Jun $NH_2$-Terminal Kinase 1. The figure is not meant to be all-inclusive.

# 2

# General Methods

## 2.1 Experimental Animal Model and Surgical Procedures

### 2.1.1 Animals

For all studies, normal Female Wistar rats (Charles River, Quebec, Canada) weighing 250-300g were used for experiments. The rats were housed in the University of Toronto's Department of Comparative Medicine. They were exposed to a 12h light/dark cycle. The rats used in Study 2 were fed a different rat chow (28% protein, 60% carbohydrate, and 12% fat; Purina 5001, Ralston Purina, St Louis, MO) than the new diet used for Studies 1 and 3 (Teklad Global 21% Protein Diet with 64% carbohydrate and 14% fat; Harland Teklad Global Diets, Madison, WI).

### 2.1.2 Surgery

After a week of adaptation to the facility, rats were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg/ml, 1 μl/g of body weight; Study 2) or

with isofluorane (Studies 1 and 3), and indwelling catheters were inserted into the right internal jugular vein for infusions and the left carotid artery for sampling. Polyethylene catheters (PE-50; Cay Adams, Boston, MA), each extended with a segment of silastic tubing (length of 3 cm, internal diameter of 0.02 inches; Dow Corning, Midland, MI), were used. The venous catheter was extended to the level of the right atrium, and the arterial catheter was advanced to the level of the aortic arch. Both catheters were tunneled subcutaneously and exteriorized. The catheters were filled with a mixture of 60% polyvinylpyrrolidone and heparin (1,000 U/ml) to maintain patency and were closed at the end with a metal pin The rats were allowed a minimum 3-4 days period of post-surgery recovery before experiments. The animal care committee of the University of Toronto approved all procedures.

### 2.1.3 Infusion and Hyperinsulinemic-Euglycemic Clamp

After overnight fasting, the rats were subject to a 7-hour intravenous infusion of either saline (SAL), Intralipid plus heparin (IH; 20% Intralipid + 20 U/ml heparin at 5.5 μl/min) with or without specific inhibitor, or inhibitor alone (see the Methods section of the individual studies for further details.

In Studies 1 and 3, infusion of [3-$^{3}$H] glucose (8 μCi bolus followed by constant infusion at 0.15 μCi/min) was initiated at the 3h point of 7h infusion period and hyperinsulinemic-euglycemic clamp was performed during the last 2h of the infusion (Figure 2). During 30 minutes preceding the clamp ("Basal Period"), measurements were taken at 10-minute interval for plasma glucose, insulin, FFA, and [3-$^{3}$H] glucose specific

activity. [3-$^{3}$H] was used in Studies 1 and 3 as it is a simpler tracer to use and we wished to obtain basal and clamp measurements from same rats. Since same rats were used for both basal and clamp measurements, glucose specific activity was maintained constant during the clamp to minimize error on calculation of glucose production. In Study 2, separate rats were used for basal and clamp experiments and plasma samples were taken during the last 30 min of the experiment. For both basal and clamp experiments, infusion of [6-$^{3}$H] glucose (20 μCi bolus followed by constant infusion at 0.4 μCi/min) was initiated at the 5h point of the 7h infusion period. [6-$^{3}$H] was used in Study 2 in order to compare the results from a previous study in our laboratory (58). In all three studies, clamp was performed during the last 2h of the 7h infusion period to assess hepatic and peripheral insulin sensitivity. At the onset of the clamp, an infusion of porcine insulin at 5 mU/kg/min, resulting in plasma insulin levels in the postprandial range, was initiated. To maintain euglycemia during insulin infusion, a variable infusion of 20% glucose was given through the jugular catheter and adjusted according to glycemic determinations every 5 minutes. The glucose infusate was radiolabelled with 48 μCi/g [3-$^{3}$H] glucose (Studies 1 and 3) or 15 μCi/g [6-$^{3}$H] glucose (Study 3) to maintain plasma glucose specific activity constant during the clamp at basal levels in Studies 1 and 3 (not necessary in Study 2). The total volume of blood withdrawn was ~3.8 ml. After plasma separation, red blood cells were diluted at 1:1 ratio in heparinized saline (4 U/ml) and re-infused into the rats. At the end of the experiments, the rats were anesthetized with intravenous administration of ketamine:xylazine:acepromazine cocktail (87:1.7:0.4 mg/ml), immediately after which liver was freeze-clamped with pre-cooled aluminum

tongs while infusions were maintained through the jugular vein and stored in -80°C for immunoprecipitation and Western blotting studies.

## 2.2 Laboratory Methods

### 2.2.1 Plasma Glucose Determination

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

$$\text{D-glucose} + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2$$

In the reaction, oxygen is used at the same rate of glucose to form gluconic acid. A polarographic oxygen sensor is used to detect oxygen consumption, which is directly proportional to the glucose concentration in the sample. Results are obtainable within 30 seconds following sample addition. Plasma sample was re-analyzed until repeated measurements were within a difference of 3 mg/dl. The analyzer was calibrated before use and frequently during the experiment with the 150/50 glucose/urea nitrogen standard (Beckman, Fullerton, USA) that accompanied each kit.

### 2.2.2 Plasma Glucose Tracer Specific Activity

Plasma radioactivity from [6-$^3$H] glucose was determined after deproteinization with $Ba(OH)_2$ and $ZnSO_4$, passage through ion exchange columns to eliminate ionized 3-carbon metabolites, and subsequent evaporation (Study 2). Plasma radioactivity from [3-$^3$H] glucose was determined after deproteinization with $Ba(OH)_2$ and $ZnSO_4$, and subsequent evaporation to dryness to remove tritiated water. In both cases, aliquots of the [6-$^3$H] glucose or [3-$^3$H] glucose and of the tritiated glucose infusate were assayed together with the plasma samples. The intra-assay coefficient of variation was 2.5% and the interassay coefficient of variation was 6.5%.

### 2.2.3 Plasma Free Fatty Acids Assay

Plasma levels of FFA were analyzed using a colorimetric kit under enzymatic reaction from Wako Industrials (Neuss, Germany). The method relies upon the acylation of coenzyme A by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA produced is oxidized by adding acyl-CoA oxidase (ACOD), which generates $H_2O_2$. $H_2O_2$, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(B-hydroxyethl)-aniline (MEHA) with 4-aminophenazone to form the final reaction product, which is a purple colored adduct. This can be measured colorimetrically at 550 nm. The results are correct to within 1.1%. The reactions of this assay is listed below:

*ACS*

FFA + ATP + CoA ------> Acyl-CoA + AMP + PPi

*ACOD*

Acyl-CoA + $O_2$ -----> 2,3-trans-Enoly-CoA + $H_2O_2$

*POD*

2 $H_2O_2$ + 4-aminoantipyrine + MEHA -----> Final Reaction Product + 3 $H_2O$

### 2.2.4 Plasma Triglyceride Assay

A colorimetric enzymatic kit from Roche Diagnostics GmbH (Mannheim, Germany) corrected for free glycerol concentration was used to determine the plasma triglyceride levels. (A) The reaction first removed free glycerol from the sample prior to the hydrolysis of triglyceride. (B) Then, with the use of lipase and 4-aminophenazone, triglycerol was hydrolyzed to glycerol. The reactions were as follows:

*Glycerol kinase*

(A) Free glycerol + ATP ----------> Glycerol 3-phosphate + ADP

(B)

*Glycerol 3-phosphate oxidase*

Glycerol 3-phosphate + $O_2$ ----------> Dihydroxyacetonephosphate + $H_2O_2$

*Peroxidase*

H2O2 + 4-chlorophenol ----------> oxidation product

(does not react with 4-aminophenazone)

*Lipase*

(C) Triglyceride + 3 $H_2O$ ----------> Glycerol + fatty acids

*Glycerol Kinase*

Glycerol + ATP ----------> Glycerol-3-phosphate + ADP

*Glycerol 3-phosphate oxidase*

Glyceol-3-phosphate + $O_2$ ----------> Dihydroxyacetone phosphate + $H_2O_2$

*Peroxidase*

$H_2O_2$ + 4-aminophenazone + 4-chlorophenol --------> 4-(p-benzoquilone-monoiminol)-phenazone (4-PBMP) + $2H_2O$ + HCl

The absorbance of 4-PBMP at 550 nm determined the amount of glycerol liberated from triglyceride. The coefficient of variation for this assay was ~ 1.4%.

### 2.2.5 Plasma Insulin Assay

Radioimmunoassay (RIA) kit specific for rat insulin from Linco Research Inc. (St. Charles, MO, USA) was used to determine plasma insulin concentrations. Insulin in the plasma sample competes with a fixed amount of $^{125}$I-labelled insulin for the binding sites on the specific antibodies. A standard curve was determined using insulin standards from 2.24 tp 22.4 μU/ml in duplicate. $^{125}$I-insulin and rat insulin antibody were mixed with plasma sample. The tubes were then vortexed and incubated overnight at 4°C. Precipitating reagent was added to all tubes followed by vortexing and incubating for 20 minutes at 4°C. The tubes then were spun at 2000-3000 g for 20 min. The supernatant was aspirated and the radioactivity in the pellet was counted for 4 min in a gamma counter (Beckman Instruments, Fullerton Ca, USA). The counts (B) for each of the standards and unknowns were expressed as a % of the mean counts of the "0=standard" (Bo):

| % Activity Bound = B (Standard or sample) / Bo x 100% |
|---|

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

### 2.2.6 Plasma C-Peptide Assay

Rat C-peptide RIA kit was used to determine the plasma C-peptide levels. The kit uses an antibody specific for rat C-peptide (Linco Research, Inc, St. Charles, MO, USA). The principle is the same as insulin RIA as described above. The procedures are the same as insulin RIA with the exception of one extra day. In the first day, only rat C-peptide antibody was added followed by an overnight incubation at 4°C. In the second day, $^{125}$I-rat C-peptide was added followed by vortexing and overnight incubation at 4°C. In the last day, precipitating reagent was added followed by vortexing and incubation for 20 min at 4°C. Then, the tubes were centrifuged at 2000-3000 g for 40 min. The supernatant was then aspirated and the radioactivity in the pellet was counted for 1 min in a gamma counter. The % activity bound was calculated in the same manner as insulin RIA. The % activity bound for each standard was plotted against the known concentration to obtain standard curve. The concentrations of the unknown samples were determined by interpolation with a coefficient of inter-assay variation determined on reference plasma less than 10%.

### 2.2.6 Protein Carbonyl Assay

Protein carbonyl is the most commonly assessed marker of protein oxidation. This assay measures protein-hydrazone, which is a product formed by a reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. Liver tissue was homogenized using hand-held glass homogenizer at 4 ºC in a solution containing HEPES (10 mmol/L; pH 7.4), NaCl (137 mmol/L), KCl (4.6 mmol/L), $KH_2PO_4$ (1.1 mmol/L), $MgSO_4$ (0.6 mmol/L), ethylenediaminetetraacetic acid (EDTA) (1.1 mmol/L), leupeptin (0.5 μg/mL),

pepstatin (0.7 μg/mL), phenylmethylsulfonyl fluoride (40 μg/mL), and aprotinin (0.5 μg/mL) to solubilize protein and cell debris was removed by centrifugation. Protein concentrations were determined as described above. Oxidative protein damage, assessed by the formation of carbonyl groups, was measured as described by Levine et al (301). In order to remove nucleic acids, samples were incubated with 10% streptomycin sulfate until a final concentration of 1% streptomycin sulfate was reached. The test tubes were left at room temperature for 15 minutes and then centrifuged at 6000 g for 10 minutes in a tabletop centrifuge. The supernatant was separated from the pellet and discarded. One mg of protein was precipitated by addition of 20% trichloroacetic acid (TCA) and centrifuged (8500 g) for 3 minutes, after which the supernatant was discarded. Protein pellets were incubated with and without DNPH (10 mM in 2M HCl) and were allowed to stand at room temperature for 1 hour, during which time the mixture was vortexed every 15 minutes. Following incubation, protein was re-precipitated using 20% TCA and the pellet was obtained by centrifugation (8500 g) for 3 min. The pellet was washed 3 times with ethanol-ethyl acetate (1:1) to remove free DNPH, allowing the samples to stand for 10 minutes each time before discarding the supernatant. The pellet was then re-dissolved in guanidine solution (6M with 20mM potassium phosphate, adjusted to pH 2.3 with trifluoroacetic acid) for 1 hour at 37ºC. Insoluble material was removed by centrifugation (8500 g) for 3 minutes. Carbonyl content was calculated from the sample absorbance at 365 nm compared to their complementary HCl-treated blanks, using a molar absorption coefficient of 22000 $M^{-1}cm^{-1}$.

### 2.2.7 Tissue Homogenization

Liver samples (150 mg) were homogenized by hand-held glass homogenizer in buffer A (50mM Tris-HCl pH 7.5, 10 mM ethylene glycol tetraacetic acid, 2 mM EDTA, 1 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM $Na_3VO_4$, 1 mM NaF, 1 μg/ml aprotinin, leupeptin, pepstatin, 0.1 mM phenylmethylsulphonyl fluoride, 1 μM microcystin). The homogenates were centrifuged at 100,000 x g for 1 h at 4$^{o}$C, and the supernatants were retained as the cytosolic fraction. The pellet was resuspended in buffer B (buffer A + 1% Triton X-100), homogenized by passing through a 23-gauge needle three times, incubated for 15 min on ice, and centrifuged at 100,000 x g for 1 h at 4$^{o}$C. The supernatant provided the solubilized membrane fraction. The purity of the cytosolic and membrane fractions was assessed by assaying glucose-6-phosphate dehydrogenase (Sigma, St Louis, MO) and 5'-nucleotidase activities (Sigma, St Louis, MO), respectively. Homogenization of muscle (302) and fat (303) samples was performed as described previously. The results showed that the index of purity of both fractions were >90%.

### 2.2.8 Immunoprecipitation

Studies 2 and 3: For detection of tyrosine phosphorylation of IRS-1 and IRS-2 in the liver, proteins were extracted from rat liver tissue as described previously (304). Briefly, 1 ml of lysis buffer (20 mM Tris·HCl (pH 7.4), 1 % Triton X-100, 10 % glycerol, 150 mM NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 20 μM leupeptin, 1 mM benzamidine, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 1μM microcystin) was added to approximately 50 mg of frozen liver sample. The liver sample was disrupted by sonication and resulting homogenates were rocked for 40 min at 4°C.

Detergent-insoluble material was precipitated by centrifugation at 12,000 x g for 10 min at 4°C and lipid layers were removed. Protein content from the supernatant was measured by detergent-compatible modified Lowry microassay. Liver lysates containing equal amounts of protein (1 mg) were subject to immunoprecipitation overnight at 4 °C with agarose-conjugated anti-IRS-1 antibody (Upstate) or with anti-IRS-2 antibody (Upstate) followed by 2h incubation with Protein A/G PLUS-agarose immunoprecipitation reagent (Santa Cruz) at room temperature. Immune complexes were collected by brief centrifugation (12,000 rpm) and washed four times with ice cold PBS. The equivalent amount of protein samples was then resuspended in 1X Laemmli sample buffer (2% sodium dodecyl sulphate (SDS), 10% glycerol, 62.5 mM Tris (pH 6.8), 0.1% bromophenol blue, 5% ß-mercaptoethanol), boiled for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide) under reducing conditions, as described below. Note that immunoblotting of serine 473 phosphorylated Akt in the rat liver was performed using these methods for liver homogenization and SDS-PAGE.

### 2.2.9 Western Blot Analysis

The protein concentration in all samples was determined by the detergent-compatible modified Lowry microassay (BioRad), using serum albumin as the standard. Fifty µg of protein in all samples were mixed with equal volumes of 3 x sample-loading buffer (6.86 M urea, 4.29% (SDS), 300 mM dithiothreitol, 43 mM Tris-HCl pH 6.8) and left at room temperature for 30 min. The mixture was then vortexed and subjected to

SDS-PAGE (10% polyacrylamide). Following electrophoretic separation, protein was transferred to polyvinylidene fluoride membranes. The membranes were then incubated for 1 hour at room temperature in Tris-buffered saline-Tween (TBST) containing 5% non-fat dried milk, pH 7.4. After the blocking step, membranes were washed in rinsing solution (TBST, pH 7.4) and then incubated overnight with the primary antibody dissolved in the blocking solution (See Methods for each study for the concentrations of antibodies used). Note that for tyrosine phosphorylated IRS-1 and IRS-2, bovine serum albumin was used instead of non-fat dried milk at same concentration for preparation of blocking solution. After three washes with TBST (20 min each), membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham, Baie d'Urfe, Quebec) at 1:4000 dilution, unless indicated otherwise. The membranes were then washed several times with TBST and developed using enhanced chemiluminescence (Zymed Laboratories, San Francisco, CA). The bands obtained from immunoblotting were quantified by scanning laser densitometry.

## 2.3 Calculations

Glucose turnover (rate of appearance of glucose, Ra, determined with [6-$^3$H] glucose for Study 2 and [3-$^3$H] glucose for Studies 1 and 3) was calculated using Steele's non-steady-state equation (305), taking into account the extra tracer infused with the glucose infusate (306).

In the basal fasting state, the total rate of glucose appearance (Ra) corresponds to the endogenous glucose production (EGP). During the clamp, EGP was calculated by

subtracting the exogenous glucose infusion rate from the total rate of glucose appearance. At steady state, glucose disappearance corresponds to the rate of glucose appearance and, at euglycemia, glucose disappearance corresponds to tissue glucose utilization as renal glucose clearance is zero. Data are presented as average values of the samples that were taken during the last 30 min of the experiment.

## 2.4 Statistical Analysis

Data are presented as means $\pm$ SEM (Standard Error of Mean). Repeated measures analysis of variance (ANOVA), where appropriate, followed by Tukey's post hoc analysis was used to compare the results between treatments. See the Methods section of each study for the details on statistical analyses. Statistical calculations were performed using SAS software (Statistical Analysis System, Cary, NC) or Statistica software (Statistical Analysis System, Cary, NC). Significance was accepted at $P<0.05$.

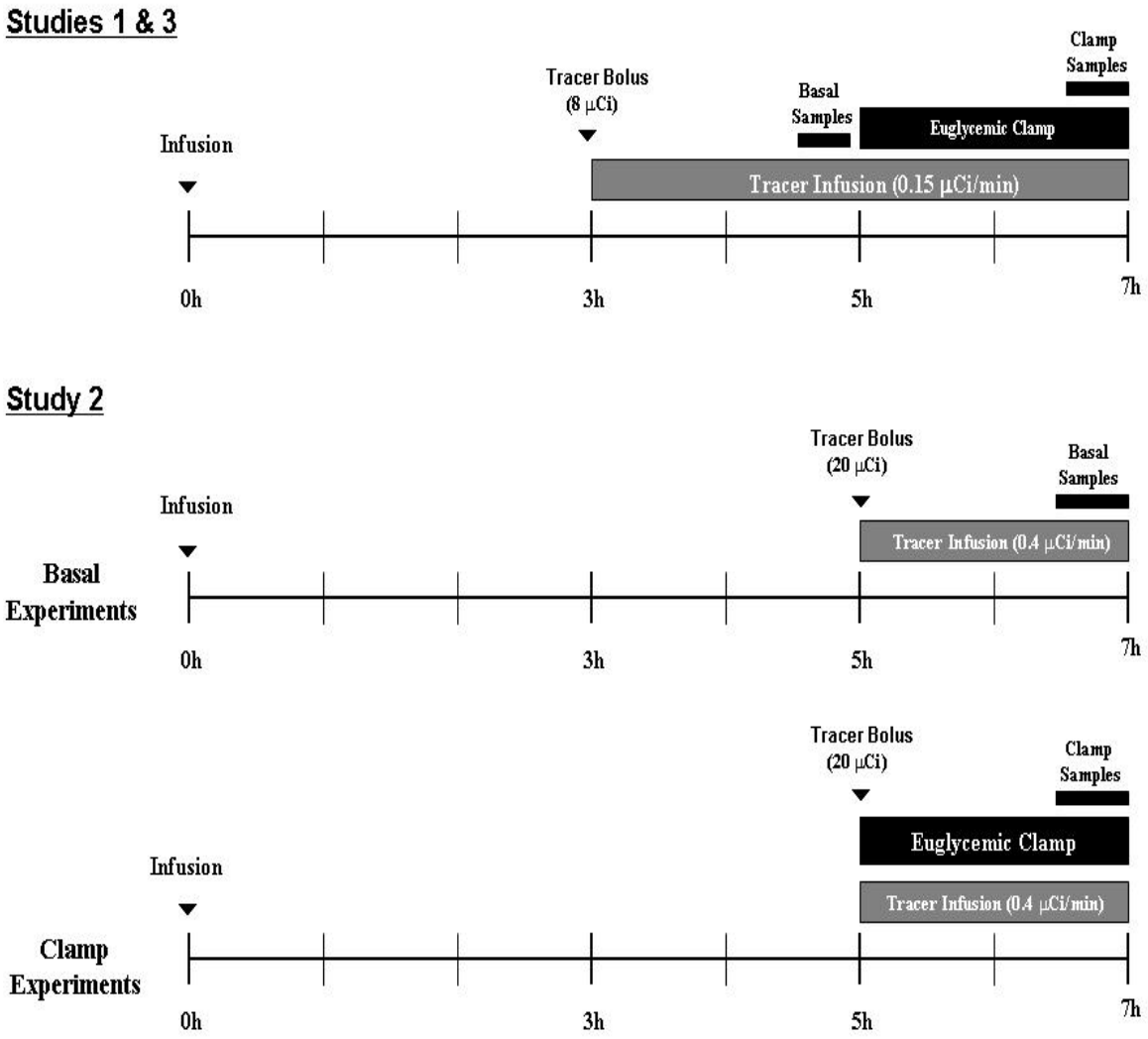



**Figure 2. A scheme illustrating the different infusion and clamp protocols for the studies in this thesis.**

# 3

# Study 1

## *Acute Inhibition of PKC-δ Prevents Hepatic Insulin Resistance Caused by Short-Term Fat Infusion*

## 3.1 Abstract

Our previous studies have found an association between PKC-δ membrane translocation and hepatic insulin resistance induced by short-term (7h) lipid infusion; however, it is unknown whether PKC-δ is a causal mediator in the process. In the present study, we utilized a specific antisense oligonucleotide against PKC-δ to determine whether inhibiting liver PKC-δ protein synthesis abolishes FFA-induced hepatic insulin resistance. Wistar rats were injected i.p. with either antisense oligonucleotide against PKC-δ (PKC-δ ASO) or control antisense oligonucleotide (CON ASO) at a dose of 20 mg/kg, 3 times per week, for 2 weeks. In preliminary studies, we found that this dose of PKC-δ ASO decreased liver PKC-δ protein expression by ~ 50%. After the last injection, the rats were overnight fasted and subjected to a 7h intravenous infusion of either saline or Intralipid plus 20U/ml heparin, during the last 2 hours of which a hyperinsulinemic-euglycemic clamp with tracer infusion was performed to test hepatic and peripheral insulin sensitivity. Whole body insulin sensitivity, as indicated by glucose infusion rate during the clamp, was decreased with IH infusion compared to SAL infusion in CON ASO treated rats. Treatment with PKC-δ ASO partially prevented the IH-induced decrease in whole body insulin sensitivity. Furthermore, treatment with PKC-δ ASO completely prevented the effect of IH infusion to decrease insulin-stimulated suppression of hepatic glucose production without affecting IH-induced decrease in peripheral glucose utilization. Administration of CON ASO did not affect peripheral or hepatic insulin sensitivity. These results provide evidence for a potentially causal role of PKC-δ in hepatic insulin resistance caused by a short-term (7h) lipid infusion and point to PKC-δ as a potential therapeutic target for treatment of hepatic insulin resistance.

## 3.2 Introduction

Circulating free fatty acids (FFA) are often elevated in obesity, which is a major risk factor for type 2 diabetes, mainly due to increased lipolysis in the expanded adipose tissue, particularly visceral fat. Numerous studies have demonstrated that FFA cause hepatic insulin resistance (52;54;58;69;130;307), which is the primary contributor to increased hepatic glucose production and overt hyperglycemia in type 2 diabetes. Thus, FFA represent a key link between obesity and type 2 diabetes. However, the exact mechanism by which FFA cause insulin resistance in the liver is not completely understood.

Protein kinase C (PKC) is a serine/threonine kinase involved in numerous cellular functions. PKC is classified into three major subgroups: 1) 'conventional' PKCs (α, βI, βII, and γ), which require phosphatidylserine (PS) and are activated by calcium and DAG; 2) 'novel' PKCs (δ, ε, μ, η, θ) which require PS and DAG but not calcium for activation; and 3) 'atypical' PKCs (ζ, λ), which are calcium- and DAG-independent but PS-dependent (169).

PKC is implicated in various models of insulin resistance in a species- and tissue-specific manner. For instance, lipid infusion causes insulin resistance in association with membrane translocation of PKC-βII and δ, which indicates their activation, in human skeletal muscle (56). In contrast, PKC-θ and -ε are linked to fat-induced insulin resistance in rodent skeletal muscle (57;60;181).

Previous studies in our laboratory (58), as well as those by Boden et al. (54), have established an association between membrane translocation of PKC-δ and FFA-induced hepatic insulin resistance. However, it is unknown whether PKC-δ activation is a causal event or merely an association in the mechanism of FFA-induced hepatic insulin resistance. We hypothesized that PKC-δ is a causal mediator of hepatic insulin resistance caused by a short-term lipid infusion in rats.

To test this hypothesis, we acutely inhibited hepatic PKC-δ protein expression by administering antisense oligonucleotide (ASO) against PKC-δ in rats and tested hepatic and peripheral insulin sensitivity using hyperinsulinemic-euglycemic clamp during the last 2h of 7h infusion of saline control or Intralipid plus heparin. The results from the present study show that PKC-δ ASO, which reduced hepatic PKC-δ levels by approximately 50%, was effective in preventing FFA-induced decrease in insulin-stimulated suppression of hepatic glucose production (HGP) without affecting FFA-induced decrease in insulin-stimulated peripheral glucose utilization. It is also shown that PKC-δ ASO prevents IH-induced increased in serine phosphorylated $p47^{phox}$ subunit of NADPH oxidase, a potentially important source of oxidative stress that can cause insulin resistance (211;212). These findings suggest that PKC-δ is a causal mediator of FFA-induced hepatic insulin resistance, potentially via NADPH oxidase-induced oxidative stress, and thus PKC-δ represents a potential target for treatment of hepatic insulin resistance.

## 3.2 Materials and Methods

### 3.2.1 Animals

Female Wistar rats (250-300g) were ordered from Charles River (Quebec, Canada) and maintained in the animal facility under the conditions described in the General Methods section. They were fed rodent chow (Teklad Global 21% Protein Diet with 64% carbohydrate and 14% fat; Harland Teklad Global Diets, Madison, WI).

### 3.2.2 Dose-Response Characterization

We obtained three different types of antisense oligonucleotides (ASO) against PKC-δ (PKC-δ ASO; ASO1, ASO2, and ASO3) and a control ASO (CON ASO), which contains a scrambled sequence, from ISIS Pharmaceuticals. ISIS had previously demonstrated that these three PKC-δ ASOs are able to reduce PKC-δ protein expression by ~80% in rat primary hepatocytes (unpublished data). Female Wistar rats (n=4/group) received intraperitoneal injection of PKC-δ ASO or CON ASO at a dose of 20 mg/kg, every other day for 6 times. An additional group of rats (n=4) received saline injection at the same time. One day after the last injection, liver was excised and freeze-clamped under anesthesia in order to perform Western blot to measure cytosolic and membrane contents of PKC-δ. Moreover, liver and spleen weights were measured, and plasma was collected to determine aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme levels. Additional studies were also performed using higher doses at 50 and 100 mg/kg.

### 3.2.3 Selection of Lead PKC-δ ASO

Based on dose-response characterization, the most potent ASO was chosen: ASO3, with the following sequence: 5'-TGGTCCATAGAGTCGATGAG-3'. The control ASO had a scrambled sequence and is not complementary to any known gene in public databases. The first 2 bases and last 2 bases of chimeric ASOs have a 2'-*O*-(2-methoxy)-ethyl modification, and the ASOs also have a phosphorothioate backbone. This chimeric design has been shown to provide both increased nuclease resistance and mRNA affinity, while maintaining the robust RNase H terminating mechanism used by these types of ASOs (308).

### 3.2.4 Experimental Design for Clamp Studies

Female Wistar rats (250-300g) were injected with either PKC-δ ASO or CON ASO intraperitoneally at a dose of 20 mg/kg, 3 times per week, for 2 weeks. Cannulation was performed between the $4^{th}$ and the $5^{th}$ injections. Under anesthesia using isoflourane, indwelling catheters were inserted into the right internal jugular vein for infusion and the left carotid artery for blood sampling, as described in the General Methods section of the thesis.

After the last injection, the rats were overnight fasted then subject to 7h intravenous infusion of either saline or Intralipid plus heparin (IH; 20% Intralipid + 20 U/ml heparin at 5.5 μl/min). At the 3h point of infusion, [3-$^{3}$H] glucose was initiated (8

μCi bolus followed by constant infusion at 0.15 μCi/min) to assess peripheral glucose utilization and endogenous glucose production. Hyperinsulinemic-euglycemic clamp was performed with tracer infusion during the last 2h of the 7h infusion period to assess hepatic and peripheral insulin sensitivity. Refer to the General Methods section for details regarding the clamp procedure.

### 3.2.5 Plasma FFA and Insulin Levels

Assays for plasma FFA and insulin were performed as described in the General Methods section of the thesis.

### 3.2.6 Western Blot Analysis

The translocation of the DAG-sensitive isoforms of PKC, PKC- δ and -ε, from cytosol to membrane reflects their activation and was assessed by comparing immunoblots of the cytosolic- and membrane-associated fractions of hepatocytes. The polyclonal antibodies specific for PKC- δ and -ε were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Measurement of serine phosphorylated $p47^{phox}$ was done in the membrane fraction using a polyclonal antibody (provided by Dr. El-Benna) directed against phospho-Ser345 by using as antigen the peptide QARPGPQ[pS]PGSPLEEER ($p47^{phox}$ amino acids 338-354). Refer to the Western Blotting part of the General Methods section for detailed protocol.

### 3.2.7 Statistical Analysis

For the various measurements in the dose-response study, one-way ANOVA was used. To determine statistical differences between the treatment groups for plasma insulin and FFA; PKC-δ and PKC-ε protein expression in the liver; EGP; and peripheral glucose utilization, two-way ANOVA with Tukey's post hoc test was used. For the results pertaining to whole body insulin sensitivity, percentatge suppression of EGP, insulin-stimulated peripheral glucose utilization, and serine phosphorylated $p47^{phox}$, one-way ANOVA was used. Statistical calculations were performed using Statistica software (Statistical Analysis System, Cary, NC). Significance was accepted at $P<0.05$.

## 3.3 Results

### 3.3.1 PKC-δ ASO Dose-Response Studies

Prior to the clamp studies, we performed a dose-response study using the three PKC-δ ASOs provided to us by ISIS Pharmaceuticals to select the lead ASO and ascertain the optimal dose of the PKC-δ ASO for *in vivo* inhibition of PKC-δ expression in the liver. After 6 injections at 20 mg/kg dose, ASO2 and ASO3 reduced the cytosolic PKC-δ protein expression by ~50%, with the latter yielding a slightly greater suppression, whereas ASO1 had no effect, when compared to CON ASO (Figure 1A). This dose and injection schedule was not associated with an increase in serum AST or ALT levels or

enlargement of spleen (Table). Control ASO on its own had no effect on PKC-δ protein expression in the liver. With the 50mg/kg and 100mg/kg doses, we did not observe significantly greater reduction in PKC-δ protein expression in the liver but these doses were associated with gastrointestinal inflammation (Data not shown). Based on these results, we selected ASO3 as the lead PKC-δ ASO and decided to use the 20 mg/kg dose for our subsequent clamp studies.

### 3.3.2 Plasma Insulin and FFA Levels

Plasma FFA levels were markedly elevated in the groups that received IH infusion (Figure 2; P<.0.01 vs SAL). In all groups, plasma FFA levels decreased during the hyperinsulinemic-euglycemic clamp from the basal levels, consistent with anti-lipolytic effect of insulin (P<0.01 vs Basal Period). Basal plasma insulin levels increased modestly (not statistically significant) following IH infusion in the CON ASO group, which was absent in the PKC-δ ASO group that received IH infusion (Figure 3). As expected, plasma insulin levels were markedly elevated during the clamp and were not different between the treatment groups.

### 3.3.3 PKC-δ and –ε Protein Expression and Membrane Translocation in the Liver

Figure 4 illustrates the PKC-δ (A) and PKC-ε (B) protein expression in the cytosolic and membrane fractions of liver. PKC-δ ASO administration resulted in approximately 50% reduction in cytosolic PKC-δ protein levels (P<0.01) without

affecting the membrane PKC-δ levels in the liver (Figure 4A). PKC-ε protein expression in the liver were not affected by PKC-δ ASO administration (Figure 4B).

In the CON ASO groups, IH infusion decreased cytosolic PKC-δ (not statistically significant) and increased membrane PKC-δ ($P<0.01$) compared with SAL infusion, indicating IH-induced PKC-δ membrane translocation. In the PKC-δ ASO groups, this effect of IH infusion was prevented in rats, as IH infusion neither decreased cytosolic PKC-δ nor increased membrane PKC-δ. PKC-ε did not translocate from the cytosol to the membrane in response to IH infusion in either groups.

### 3.3.4 Hyperinsulinemic-Euglycemic Clamp Studies

***Glucose Infusion Rate*** Glucose infusion rate (GIR) during the hyperinsulinemic-euglycemic clamp is an indication of whole body insulin sensitivity. The data represent averages of GIR during the last 30 min of the clamp studies. In CON ASO groups, IH infusion when compared with SAL infusion significantly decreased GIR during the clamp ($P<0.001$, Figure 5). PKC-δ ASO, however, partially prevented IH-induced decrease in GIR (PKC ASO+IH vs CON ASO+IH; $P<0.05$).

***Hepatic Glucose Production*** Basal rate of HGP was not different between the treatment groups (Figure 6). Infusion of IH markedly decreased insulin-stimulated suppression of HGP in rats that received CON ASO, which is consistent with results of previous studies (54;58) (CON ASO + SAL: 62±4% vs CON ASO + IH: 22±7%; $P<0.01$). In contrast, PKC-δ ASO completely prevented IH-induced decrease in insulin-stimulated suppression

of HGP (PKC-δ ASO + IH: 47±10% vs PKC-δ ASO + SAL: 53±2%; N.S.). Administration of CON ASO did not affect hepatic insulin sensitivity.

***Peripheral Glucose Utilization*** Basal rate of peripheral glucose utilization was not different between the treatment groups (Figure 7A). IH infusion markedly reduced insulin-stimulated increase in peripheral glucose utilization in CON ASO groups, as expected (Figure 7A & 7B). PKC-δ ASO treatment did not affect IH-induced decrease in insulin-stimulated peripheral glucose utilization. Moreover, CON ASO did not affect peripheral insulin sensitivity.

### 3.3.5 Serine Phosphorylated $p47^{phox}$ Subunit of NADPH Oxidase

IH infusion resulted in a significant increase ($P<0.05$) in serine 345 phosphorylated $p47^{phox}$ subunit of NADPH oxidase in the CON ASO groups (Figure 8). PKC-δ ASO treatment, however, prevented the IH-induced increase in serine phosphorylated $p47^{phox}$.

## 3.4 Discussion

The mechanism underlying FFA-induced hepatic insulin resistance has not been completely elucidated. Previous studies have implicated novel PKC isoform δ in the process (58;309); however, it is unknown whether PKC-δ activation is a causal event. The findings of the present study show that an acute inhibition of liver PKC-δ using ASO

specific against PKC-δ prevents hepatic insulin resistance caused by a short-term lipid infusion, suggesting a causal role of PKC-δ in FFA-induced hepatic insulin resistance.

Based on the previous dose-response characterization, we injected rats with PKC-δ ASO 6 times over the course of 2 weeks at a dose of 20mg/kg per injection, which resulted in approximately 50% reduction in cytosolic PKC-δ protein levels in the liver. Despite the moderate reduction in hepatic PKC-δ protein expression, the ASO completely prevented hepatic insulin resistance caused by Intralipid plus heparin infusion. This may be explained by the fact that PKC-δ ASO prevented FFA-induced membrane translocation of PKC-δ, which is an essential process in its activation.

Another novel PKC isoform, epsilon, is causally linked to hepatic insulin resistance caused by high-fat diet (131;132), indicating that chronically elevated circulating or tissue lipid levels may cause insulin resistance via a different mechanism. We measured the expression and membrane translocation of PKC-ε and found that PKC-ε protein expression was not reduced by PKC-δ ASO and that an acute lipid infusion does not lead to membrane translocation of PKC-ε. These findings illustrate that PKC-δ ASO used in the study was specific for PKC-δ isoform and that FFA activate PKC in a tissue- and time-course-dependent manner.

Glucose infusion rate (GIR) during the hyperinsulinemic-euglycemic clamp is an indicator of whole body insulin sensitivity. PKC-δ ASO partially restored the decrease in GIR caused by lipid infusion, indicating that the effect of PKC-δ ASO was localized to either liver or peripheral insulin-target tissues, or was partial in both, as whole body insulin sensitivity comprises insulin action at both sites. In order to determine the site of PKC-δ ASO action, we used tracer infusion during the clamp to separately assess hepatic

and peripheral insulin sensitivity. The results showed that PKC-δ ASO completely prevented FFA-induced decrease in insulin-stimulated suppression of HGP without affecting FFA-induced decrease in insulin-stimulated peripheral glucose utilization. These findings suggest that the inhibitory effect of PKC-δ ASO on protein expression was specific to the liver and that either PKC-δ ASO uptake in the skeletal muscle was minimal or PKC-δ does not play a causal role in FFA-induced insulin resistance in rat skeletal muscle. Determination of PKC-δ protein in the peripheral tissues is required to investigate this. Based on other studies that used ASO administration *in vivo*, we expect that PKC-δ ASO uptake in the skeletal muscle was not appreciable. On the other hand, there may have been appreciable uptake of PKC-δ ASO in the adipose tissue, as has been reported in studies employing multiple intraperitoneal ASO injections (131;310). Although adipose tissue does not contribute substantially to glucose utilization during a hyperinsulinemic-euglycemic clamp, we cannot rule out the possibility that reduction of PKC-δ in the adipose tissue contributed indirectly to improvement in FFA-induced hepatic insulin resistance in our study, potentially via lowering production of adipokines such as TNFα and IL-6.

The precise role of PKC in impairment of insulin signaling is not clear. PKC can disrupt insulin signaling by directly phosphorylating serine/threonine sites of insulin receptor and IRS *in vitro* (177;178). Moreover, several *in vivo* studies have established an association between PKC activation and decreased tyrosine phosphorylation and/or increased serine phosphorylation of IRS (57;60;132;271); however, it is unknown whether PKC is directly responsible for the phosphorylation of IRS molecules or it mediates activation of other downstream factors that can cause insulin resistance. One

potential factor is oxidative stress, which is closely linked to insulin resistance. In particular, PKC-δ has been shown to increase oxidative stress via NADPH oxidase (211;212). Our finding that PKC-δ ASO treatment prevents lipid-induced serine phosphorylation of p47phox subunit, which is essential for NADPH oxidase activation, raises a possibility that PKC-δ participates in FFA-induced hepatic insulin resistance via upregulation of NADPH oxidase activity and associated oxidative stress. However, it remains to be examined whether PKC-δ activates NADPH oxidase directly or indirectly via another serine kinase.

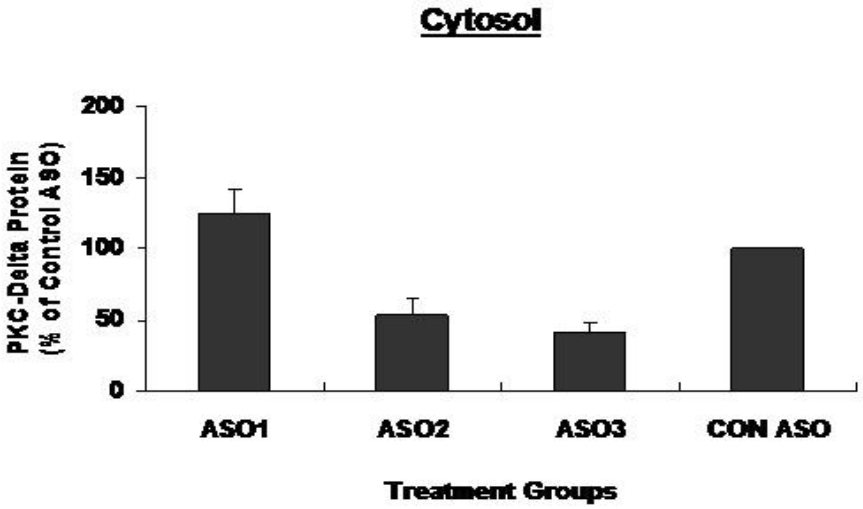
PKC-δ may also activate downstream serine/threonine kinases, such as IKKβ and JNK1, which also impair insulin signaling (186;187;233). To address this, it should be examined whether the inhibition of PKC-δ leads to reduced activities of IKKβ and/or JNK1, which would indicate that PKC-δ lies upstream of the pro-inflammatory serine kinases in the pathway of FFA-induced hepatic insulin resistance.

In summary, we show that an acute inhibition of hepatic PKC-δ using *in vivo* ASO administration prevents hepatic insulin resistance caused by a short-term lipid infusion in rats. This finding suggests that PKC-δ is a causal mediator of FFA-induced hepatic insulin resistance, potentially via NADPH oxidase-mediated oxidative stress, and identifies PKC-δ as a potential pharmacological target for the treatment of hepatic insulin resistance.

| Groups | Liver Weight / Body Weight | Spleen Weight / Body Weight | ALT | AST |
|---|---|---|---|---|
| ASO1 | 0.047 ± 0.0007 | 0.0056 ± 0.0002 | 39.3 ± 3.9 | 84.5 ± 22 |
| ASO2 | 0.044 ± 0.002 | 0.0041 ± 0.0001 | 38.3 ± 6.5 | 79.8 ± 26 |
| ASO3 | 0.047 ± 0.002 | 0.0054 ± 0.0005 | 40.3 ± 6.5 | 96.8 ± 8.4 |
| CON ASO | 0.038 ± 0.003 | 0.0034 ± 0.0003 | 36.0 ± 5.4 | 56.8 ± 7.1 |
| Saline | 0.036 ± 0.003 | 0.0026 ± 0.0004 | 52.0 ± 17 | 86.0 ± 9.0 |

**Table.** Liver weight, spleen weight, and serum liver enzyme levels following PKC-δ antisense oligonucleotide treatment (20mg/kg, 6 injections, 2 weeks) in Wistar rats (n=4/group). ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase

A




B

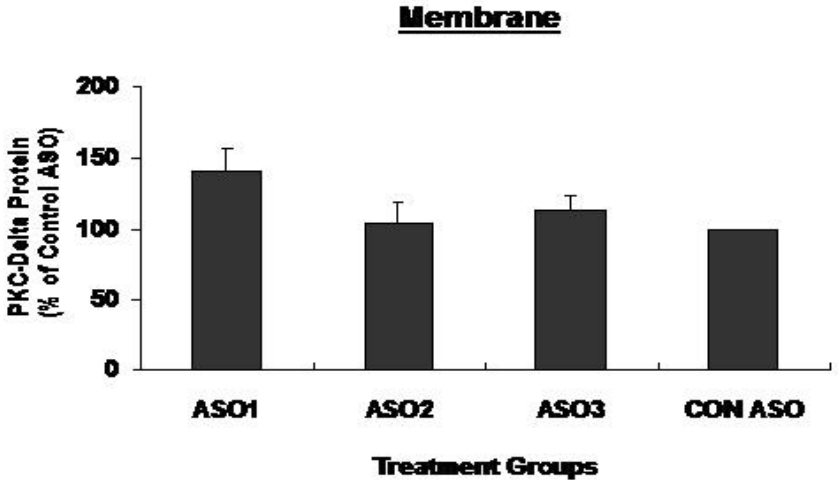



**Figure 1. The effect of PKC-δ antisense oligonucleotide (ASO) treatment on PKC-δ protein expression in the cytosol (A) and membrane (B) of rat liver (n=5-6/group).** Data are mean ± SEM. CON: Control . *: P<0.01 vs CON ASO

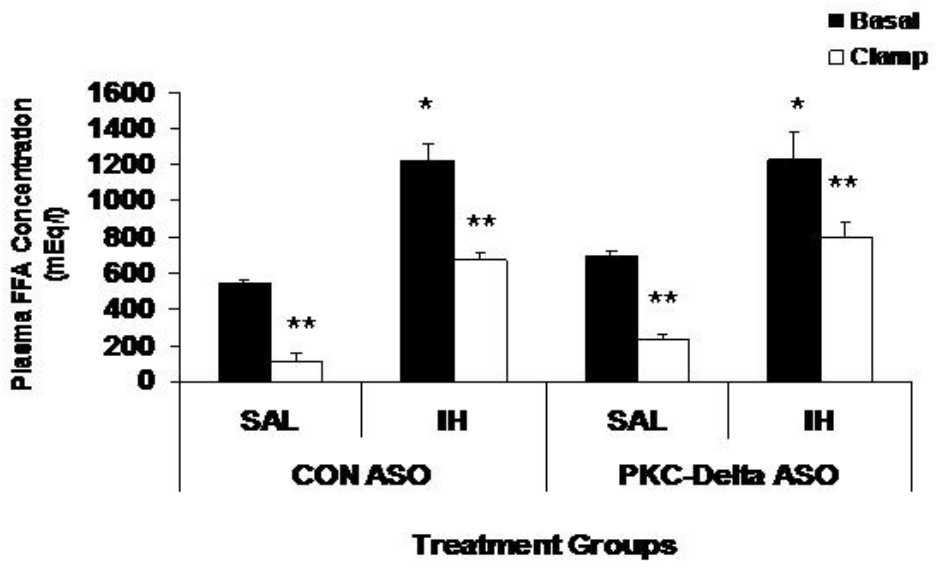



**Figure 2. Plasma free fatty acid concentrations (n=5-6/group).** Data are mean ± SEM. SAL: Saline. IH: Intralipid plus heparin. CON: Control. *: P<0.01 vs SAL **: P<0.01 vs Basal Period

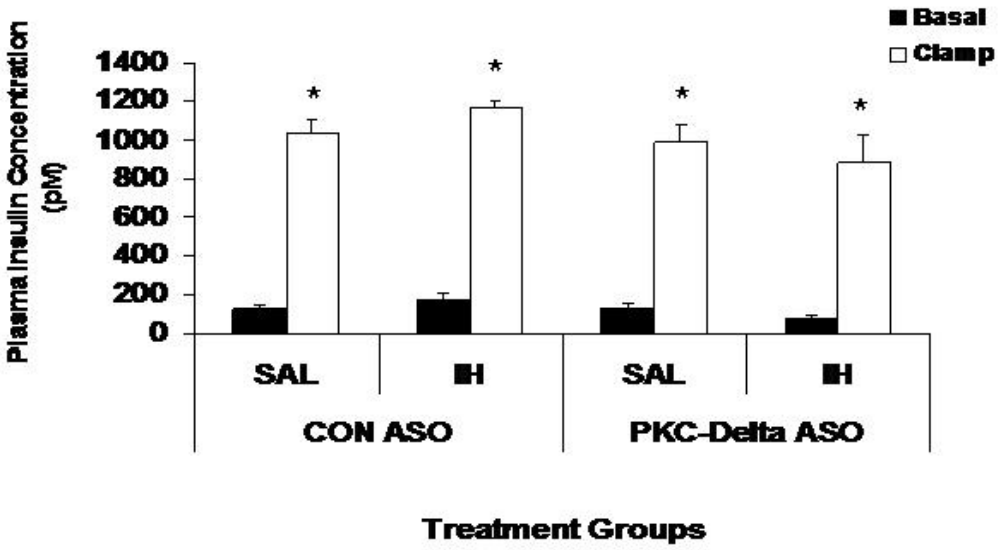
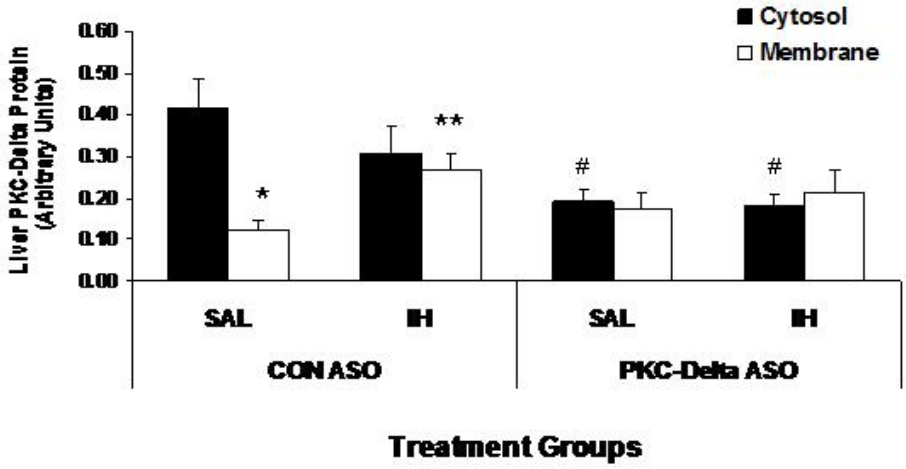



**Figure 3. Plasma insulin concentrations (n=5-6/group).** Data are mean ± SEM. SAL: Saline. IH: Intralipid plus heparin. CON: Control. *: P<0.001 vs Basal Period

A

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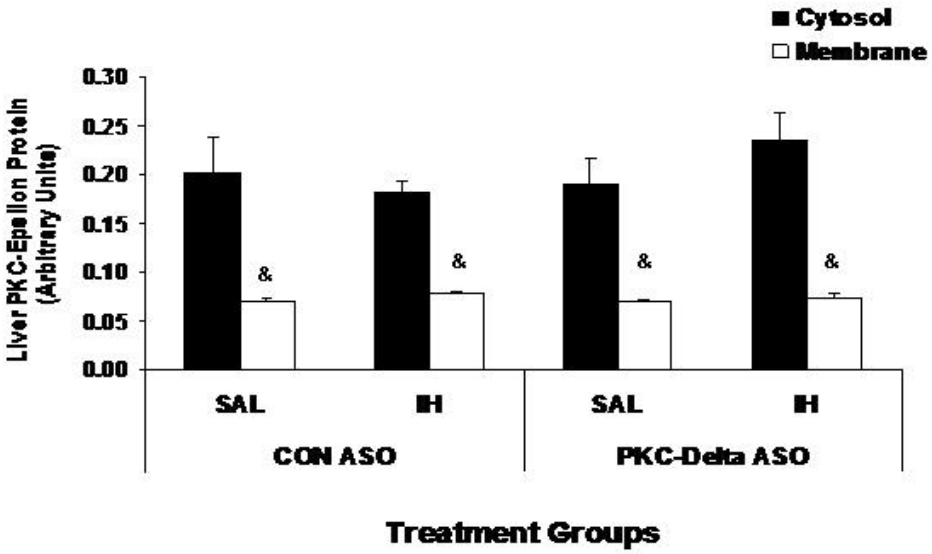



**Figure 4. The effect of PKC-δ antisense oligonucleotide (ASO) treatment on PKC-δ (A) and PKC-ε (B) expression in the cytosol and membrane of liver (n=5-6/group).** Data are mean ± SEM. SAL: S aline. IH: Intralipid plus heparin. CON: Control. *: P<0.001 vs Cytosolic PKC-δ **: P<0.01 vs CON ASO + SAL #: P<0.01 vs CON ASO + SAL &: P<0.001 vs Cytosolic PKC-ε

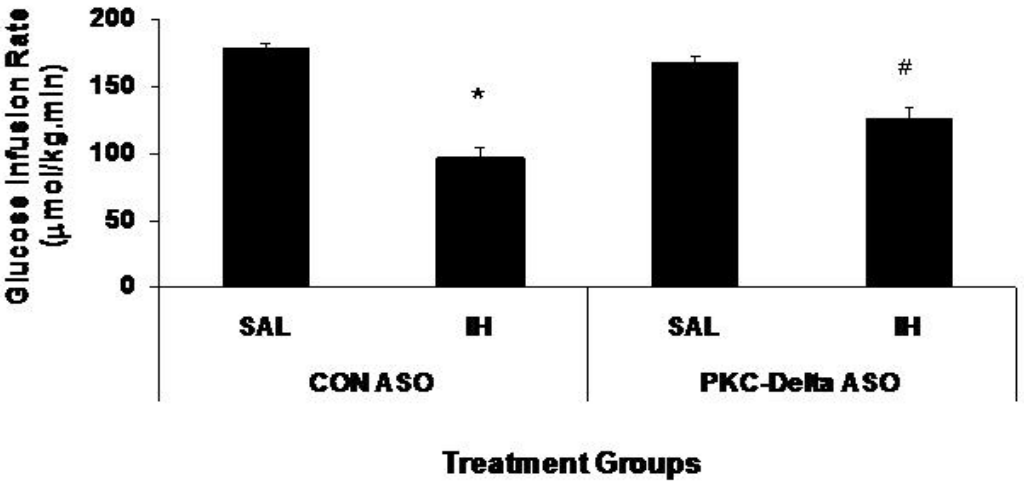
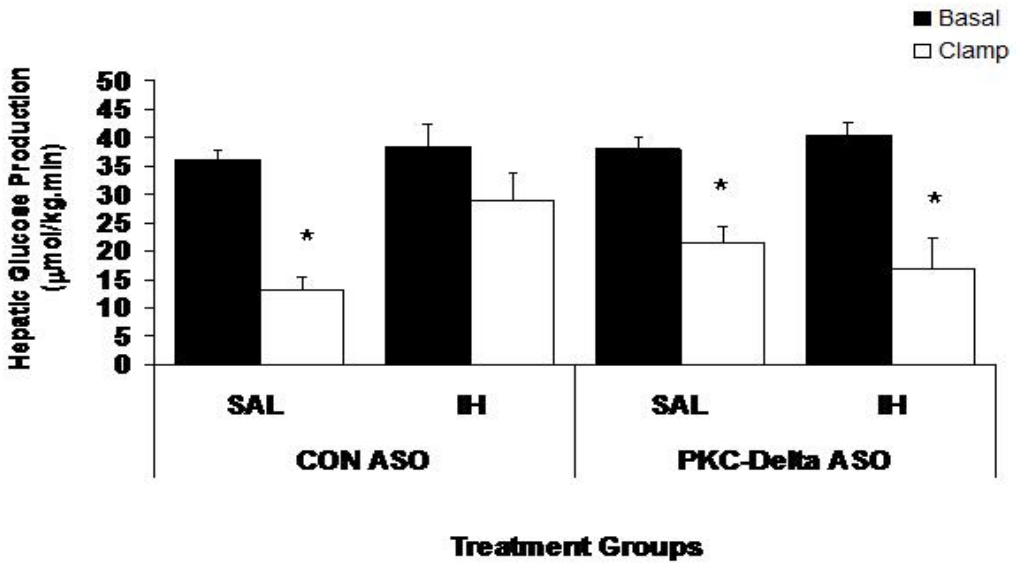



**Figure 5. The effect of PKC-δ antisense oligonucleotide (ASO) treatment on whole body insulin sensitivity as determined by glucose infusion rate during the last 30 min of 2h hyperinsulinemic-euglycemic clamp (n=5-6/group).** Data are mean ± SEM. SAL: Saline. IH: Intralipid plus heparin. CON: Control. *: P<0.001 vs CON ASO + SAL

#: P<0.05 vs CON ASO + IH

A

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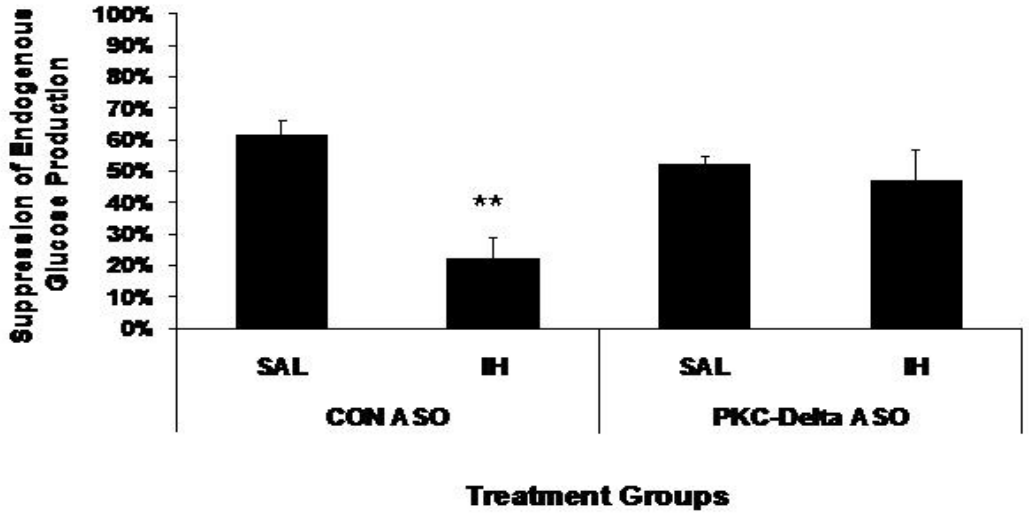
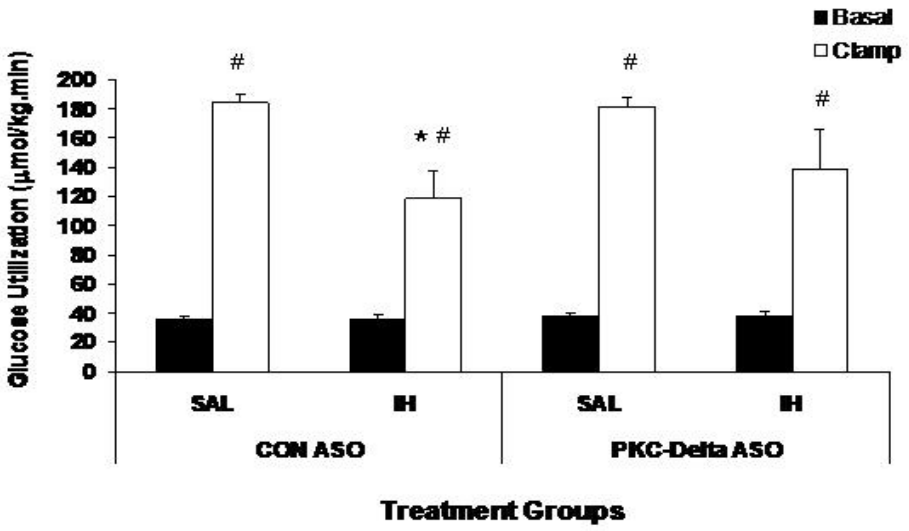



**Figure 6. The effect of PKC-δ antisense oligonucleotide (ASO) treatment on hepatic glucose production (HGP) during the basal period and the last 30 min of the clamp (A), as well as insulin-stimulated suppression of HGP during the last 30 min of the clamp (B) (n=5-6/group).** Data are mean ± SEM. SAL: Saline. CON: Control. IH: Intralipid plus heparin. *: P<0.01 vs Basal Period. **: P<0.05 vs other groups

A

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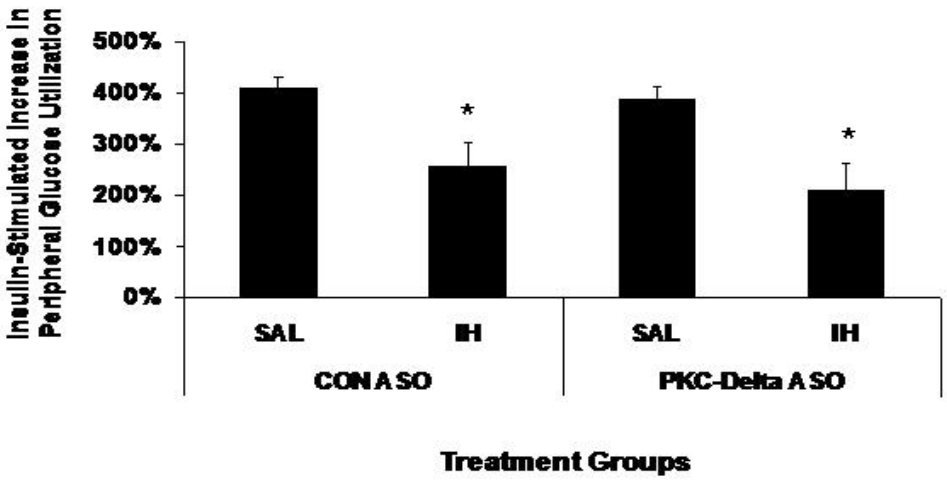



**Figure 7. The effect of PKC-δ antisense oligonucleotide (ASO) treatment on peripheral glucose utilization (A) and insulin-stimulated increase in peripheral glucose uptake (B) during the last 30 min of the clamp (n=5-6/group).** Data are mean ± SEM. SAL: Saline. IH: Intralipid plus heparin. CON: Control. *: P<0.05 vs SAL #: P<0.01 vs Basal Period

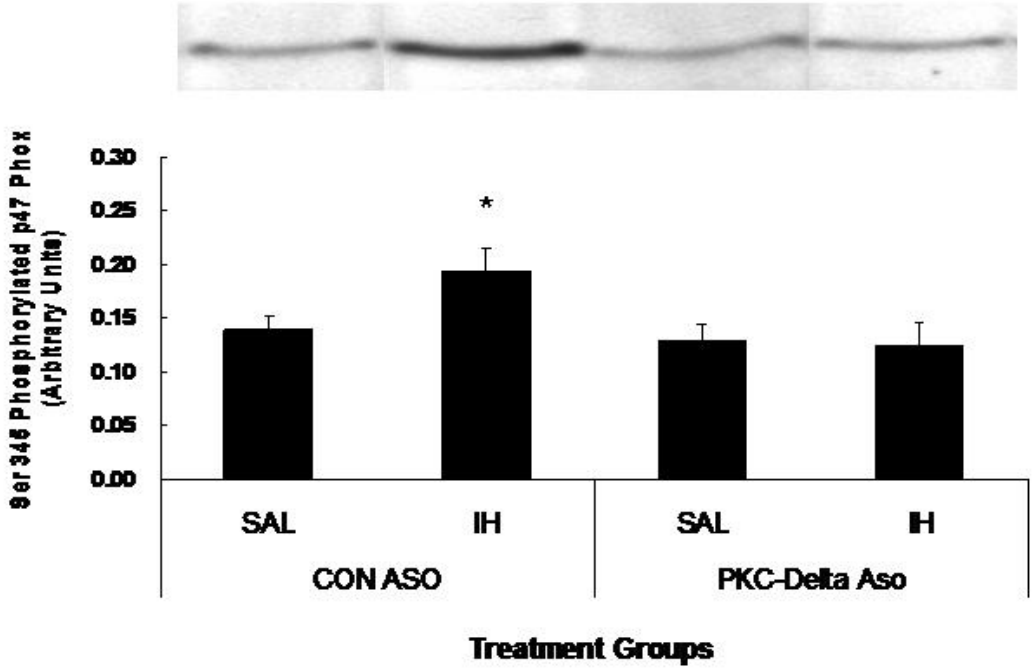



**Figure 8. The effect of PKC-δ antisense oligonucleotide (ASO) treatment on serine 345 phosphorylated $p47^{phox}$ (n=5-6/group).** Data are mean ± SEM. SAL: Saline. IH: Intralipid plus heparin. CON: Control. *: P<0.05 vs other treatment groups

# 4

# Study 2

***Oxidative Stress is a Causal Mediator of Free Fatty Acid-Induced Hepatic Insulin Resistance***

## 4.1 Abstract

Although activation of PKC and inflammatory pathways has been implicated in free fatty acid (FFA)-induced hepatic insulin resistance, the sequence of events leading to impaired insulin signaling is unknown. In the present study, we investigated the role of oxidative stress in this sequence and its relationship with PKC delta, pro-inflammatory and stress-activated kinases IκBα kinase β (IKKβ) and JNK1. Wistar rats were infused intravenously for 7 hours with saline or Intralipid plus heparin to elevate plasma FFA with or without an antioxidant, N-acetyl-L-cysteine (NAC). Insulin-induced suppression of endogenous glucose production during hyperinsulinemic-euglycemic clamp was measured using tracer methods. NAC co-infusion prevented FFA-induced hepatic insulin resistance in association with prevention of FFA-induced oxidative stress and phosphorylation of NADPH oxidase $p47^{phox}$ subunit. Furthermore, NAC prevented FFA-induced decrease in IκBα content, increase in serine phosphorylated JNK1, and impaired hepatic insulin signaling without affecting FFA-induced hepatic PKC-δ membrane translocation and increase in p38 mitogen-activated protein kinase (MAPK) phosphorylation. These results demonstrate that oxidative stress is a causal factor in the mechanism of FFA-induced hepatic insulin resistance and suggest that the pathway of FFA-induced hepatic insulin resistance *in vivo* is FFA → PKC-δ → NADPH oxidase and oxidative stress → IKKβ/JNK1 → impaired insulin signaling.

## 4.2 Introduction

Free fatty acids (FFA), which are often elevated in obese individuals (311;312), play a causal role in the association between obesity and insulin resistance (72;313-315) together with adipokines. Insulin resistance is characterized by defect(s) in insulin signal transduction whereby insulin binding to its receptor leads to tyrosine phosphorylation of insulin receptor substrates (IRS) and subsequent activation of PI3 kinase and Akt. In particular, recent evidence indicates that FFA impair insulin signaling via serine/threonine kinase(s)-mediated phosphorylation of IRS that impedes their tyrosine phosphorylation by the insulin receptor (54;57;233).

In skeletal muscle, FFA appear to induce insulin resistance in association with intracellular accumulation of lipid metabolites (e.g. diacylglycerol and long chain fatty acyl coA) and activation of serine (Ser)/threonine (Thr) kinases such as PKC (isoforms β and δ in humans, ε and θ in rodents) and/or IκBα kinase β (IKKβ) (56;57). However, the exact sequence of events in the FFA-induced defect is unknown. Furthermore, the mechanisms of FFA-induced impairment of hepatic insulin action and glucose metabolism have not been extensively investigated.

Our previous studies (58) have demonstrated that FFA induce hepatic insulin resistance in parallel with PKC-δ membrane translocation, which indicates its activation. This link has recently been confirmed by another study (54). PKC is known to impair tyrosine phosphorylation of the insulin receptor and IRS by phosphorylating serine/threonine residues (316-318). Reactive oxygen species (ROS) (319) can stimulate PKC and PKC may be the common mediator of FFA and ROS-induced insulin resistance.

Other important players that are activated by oxidative stress and have been implicated in insulin resistance are IKKβ and JNK1. IKKβ, in addition to activating pro-inflammatory transcription factor nuclear factor kappa B (NFκB), phosphorylates serine/threonine residues of IRS and thus impairs insulin action (119). High dose treatment with the IKKβ inhibitor salicylate has been shown to prevent muscle insulin resistance induced by lipid infusion in rats (233). Similarly, IKKβ$^{+/-}$ mice were protected from muscle insulin resistance induced by lipid infusion, high fat diet, or obesity (187). In humans, FFA-induced muscle insulin resistance is associated with increased PKC-δ and -βII membrane translocation, and decreased IκBα (a constitutive inhibitor of NFκB) in the skeletal muscle (56). Recently, it was shown that FFA-induced hepatic insulin resistance and PKC-δ membrane translocation are associated with IKKβ activation in rats (54). Furthermore, JNK1 is upregulated in various animal models of obesity and insulin resistance (120;207;244) and, like IKKβ, is capable of inhibiting insulin signaling via serine phosphorylation of IRS (118;120;207).

Consistent with the findings that oxidative stress leads to insulin resistance, intravenous infusion of glutathione, an antioxidant, partly prevented FFA-induced whole-body insulin resistance (224) while treatment with an antioxidant, N-acetyl-L-cysteine (NAC), improved insulin sensitivity in hyperinsulinemic patients with polycystic ovary syndrome (320). We have demonstrated that treatment with the antioxidant NAC or taurine prevents hyperglycemia-induced insulin resistance in rats (193). Recently, NAC was shown to partially prevent the defect in insulin-mediated glucose uptake caused by TNFα or dexamethasone in 3T3-L1 adipocytes (321). These studies together point to a potentially important role of oxidative stress in insulin resistance; however, it remains

unknown whether oxidative stress is a causal factor in the mechanism of FFA-induced hepatic insulin resistance *in vivo*.

The objectives of the present study were to determine whether 1) NAC prevents FFA-induced hepatic insulin resistance; 2) FFA-induced hepatic insulin resistance is associated with PKC-δ activation, oxidative stress, IKKβ/JNK1 activation, and impairment of insulin signaling; and 3) NAC can prevent the FFA-induced changes in any of these parameters. To this end, normal Wistar rats were intravenously infused with either saline, Intralipid plus heparin to elevate plasma FFA levels with or without NAC, or NAC alone for 7 hours. During the last 2 hours of the infusion period, we gave glucose tracer under basal fasting conditions or during hyperinsulinemic-euglycemic clamp to measure basal glucose production and its suppression by insulin. Liver tissue was obtained at the end of each experiment for signaling studies.

## 4.3 Materials and Methods

### 4.3.1 Animals

Female Wistar rats (Charles River, Quebec, Canada), weighing 250-300g, were used for experiments. The rats were fed rat chow (28% protein, 60% carbohydrate, and 12% fat; Purina 5001, Ralston Purina, St Louis, MO) and water *ad libitum*, and were maintained as described in the General Methods section of the thesis.

### 4.3.2 Surgery

After 3-5 days of adaptation to the facility, rats were anesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg/ml, 1 μl/g of body weight), and indwelling catheters were inserted into the right internal jugular vein for infusion and the left carotid artery for blood sampling, as described in the General Methods section.

### 4.3.3 Experimental Design

The rats were fasted overnight and randomized to four infusion groups (n=6-9/group): 1) Saline control (SAL; 5.5 μl/min); 2) Intralipid plus heparin (IH) (20% Intralipid + 20 U/ml heparin; 5.5 μl/min); 3) IH plus NAC (0.35 mg/kg/min); and 4) NAC alone. The duration of infusion was 7 hours and experimental determinations were made in the basal fasting state and during hyperinsulinemic-euglycemic clamp (insulin infusion: 5mU/kg/min) using separate animals. We tested another antioxidant, resveratrol, using the same experimental design as above. Note that, for this part of the study, infusion of [3-$^{3}$H] glucose was used to determine hepatic glucose production during the basal period and during the clamp, as was the case for Studies 1 and 3.

For the basal experiment, during the last two hours of the experiment, [6-$^{3}$H] glucose (20 μCi bolus + 0.4 μCi/min infusion) was given to assess the glucose utilization and endogenous glucose production. Details of hyperinsulinemic-euglycemic clamp can be found in the General Methods section. Since we have previously reported that IH decreases insulin clearance in this model (58), we experimentally matched the insulin

levels in the IH group by lowering the insulin infusion rate to 2.5 mU/kg/min. In the IH+NAC and NAC alone groups, we used the full dose of insulin. The glucose infusate was radiolabelled with 15 μCi/g [6-$^3$H] glucose to avoid variability in plasma glucose specific activity due to changes in the rate of the cold glucose infusate.

Blood samples for glucose, insulin, FFA, C-peptide, and [6-$^3$H] glucose specific activity were taken during the last 30 minutes (every 10 minutes) of basal or clamp experiment. The hepatic tissue determinations reported in the Results section refer to the hepatic tissue samples taken at the end of the basal experiments.

### 4.3.4 Plasma Assays

Plasma glucose was measured with a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Plasma radioactivity from [6-$^3$H] glucose was determined after deproteinization with $Ba(OH)_2$ and $ZnSO_4$, passage through ion exchange columns, and subsequent evaporation. Aliquots of the [6-$^3$H] glucose and of the tritiated glucose infusate were assayed together with the plasma samples. The intra-assay coefficient of variation was 2.5% and the interassay coefficient of variation was 6.5%. Insulin and C-peptide levels in plasma were determined by radioimmunoassay (RIA) using kits specific for rat insulin (but with 100% cross reactivity with porcine insulin used for infusion) and C-peptide from Linco Research (St. Charles, MO). The coefficients of variation were less than 9 and 10.5% for insulin and C-peptide, respectively. Plasma FFA levels were measured using a colorimetric kit from Wako Industrials (Neuss, Germany). Refer to the General Methods section for the details of these procedures.

### 4.3.5 Insulin Challenge For Insulin Signaling Studies

Following 7h infusion of reagents, rats were challenged with an acute intra-portal insulin bolus to evaluate the insulin signaling in the liver. Rats were anaesthetized with ketamine:xylazine:acepromazine (87:1.7:0.4 mg/ml), the abdominal wall was dissected, and the portal vein was exposed and ligated distally. Insulin at 2 U/kg (Humulin R, Lilly) was then injected over a period of one minute while separate groups of rats were injected with saline. A portion of the medial lobe of the liver was immediately harvested, snap frozen in liquid nitrogen, and kept at -80°C for subsequent analysis.

### 4.3.6 Immunoprecipitation and Western Blot Analysis

For determination of tyrosine phosphorylation, IRS-1 and IRS-2 were immunoprecipitated and then immunoblotted with phosphotyrosine antibody (Santa Cruz; 1:1000 dilution) and goat anti-mouse secondary antibody (Santa Cruz; 1:2000 dilution), as described in the General Methods section. Serine 473 phosphorylated Akt (Antibody purchased from Cell Signaling; 1:1000 dilution) was measured by Western blotting using the same liver lysates that were used for immunoprecipitation of IRS-1 and IRS-2.

Detailed procedures for Western blot are described in the General Methods section. The translocation of the DAG-sensitive isoform of PKC-δ from cytosol to membrane reflects its activation and was assessed by comparing immunoblots of the cytosolic and membrane-associated fractions using an affinity-purified polyclonal

antibody specific for PKC-δ (Sigma, St. Louis, MO) at a concentration of 1/2000. The same protocol was used to measure tyrosine (311) phosphorylated PKC-δ (Biosource) and serine 345 phosphorylated $p47^{phox}$, which was achieved using a polyclonal antibody (provided by Dr. El-Benna) directed against phospho-Ser345 by using as antigen the peptide QARPGPQ[pS]PGSPLEEER ($p47^{phox}$ amino acids 338-354), in the membrane fraction. The antibodies were diluted at concentrations of 1/1250 and 1/2500, respectively. Antibody against C-terminal sequence of human $p47^{phox}$ (also provided by Dr. El-Benna) was used at concentration of 1/1000 for detection of total $p47^{phox}$ levels. Hepatic content of IκBα, threonine 180 / tyrosine 182 phosphorylated p38 mitogen-activated protein kinase (MAPK), total p38 MAPK, threonine 183 / tyrosine 185 phosphorylated JNK, total JNK and serine phosphorylated IRS-1 and -2 were determined in the cytosolic fraction. Membranes were incubated overnight at a concentration of 1/1000 for IκBα antibody (Santa Cruz, CA), 1/500 for phospho-p38 MAPK antibody (Cell Signaling), 1/1000 for total p38 MAPK (Cell Signaling), 1/250 for phospho-JNK antibody (Santa Cruz, CA), and 1/500 for total JNK antibody (Santa Cruz, CA). Antibodies against IRS-1, (Serine 307) IRS-1, IRS-2 (Upstate) and (Serine 233) IRS-2 (Biosource) were added to the Western membranes at dilutions of 1/500, 1/1000, 1/500, and 1/500, respectively.

### 4.3.7 Protein Carbonyl Assay

Refer to the General Methods section for detailed protocol for measurement of protein carbonyl content.

### 4.3.8 Calculations

Refer to the General Methods section for calculations used in this study. Note that although specific activity was different in the basal vs clamp periods, this should not have affected the results because there was no change in specific activity in the same rat, which is the source of error. Moreover, a steady state was reached.

### 4.3.9 Statistical Analysis

One-way ANOVA for repeated measures was used to compare differences between treatments, using treatments (SAL vs. IH, IH+NAC, IH+RSV, NAC, and RSV, respectively) as independent variables in both the basal and clamp groups. Two-way ANOVA with interaction was used to compare differences between basal and clamp induced by treatment. Statistical calculations were performed using SAS software (Statistical Analysis System, Cary, NC). Significance was accepted at a $P<0.05$.

## 4.4 Results

The table shows plasma FFA, glucose, insulin and C-peptide levels in the basal fasting state and during the hyperinsulinemic-euglycemic clamp, as well as glucose infusion rate during the clamp. Plasma FFA levels increased by ~3-4 fold in the IH and IH plus NAC groups ($P<0.001$) and were lower during the hyperinsulinemic clamp than

during the basal fasting state, as expected, in all groups. NAC co-infusion did not affect plasma FFA levels obtained in the IH group. Plasma glucose levels were higher in the IH group ($P<0.01$) compared with the other groups in the basal experiments but were similar between all groups during the hyperinsulinemic clamp. Infusion of IH significantly increased plasma insulin and C-peptide levels in the basal experiments, suggesting that FFA increased endogenous insulin secretion. As expected, this was not reversed by IH+NAC. During the clamp, C-peptide levels decreased from the basal state in all the groups ($P<0.01$), illustrating that insulin secretion was almost completely suppressed by insulin. Since FFA decrease insulin clearance (58), insulin levels were matched by experimental design in the SAL and IH groups. This was obtained by insulin infusion at 2.5 mU/kg/min in the IH group. In the IH plus NAC and NAC alone groups, which received 5 mU/kg/min insulin as in the SAL group, the insulin levels were not different from the SAL group, indicating that insulin clearance was not affected. Glucose infusion rate during the clamp, which indicates whole body insulin sensitivity, was significantly lower in the IH group ($P<0.01$) compared to the other treatments.

In basal steady-state conditions, the rate of appearance of glucose (hepatic glucose production=endogenous glucose production, EGP) is equal to the rate of disappearance of glucose (glucose utilization, GU). IH was found to increase basal EGP=GU and to abolish the suppressive effect of insulin on EGP ($P<0.001$, Figure 1). Co-infusion of NAC with IH decreased the EGP both in basal state and during the clamp, and restored the suppressive effect of insulin on EGP. IH infusion also decreased GU (Figure 2) and co-infusion of NAC normalized GU during the clamp. NAC alone had no effect on EGP or GU. This finding is consistent with the finding that resveratrol, a

polyphenol antioxidant, also reversed IH-induced decrease in insulin-stimulated suppression of EGP (Figure 3).

The reversal of hepatic and peripheral insulin resistance induced by FFA elevation by NAC suggested a significant role of oxidative stress. To confirm that IH infusion induced oxidative stress specifically in the liver, hepatic protein carbonyl content was assessed. Protein carbonyls are increased by oxidative reactions and have been used as a marker of short-term oxidative stress (322;323). IH elevated hepatic protein carbonyl content 2-fold ($P<0.01$) and co-infusion of NAC completely inhibited this effect (Figure 4). NAC alone had no effect.

The source of ROS causing oxidative stress under various metabolic perturbations such as elevated glucose or FFA has been attributed mainly to either or both mitochondria and NADPH oxidase activity (211;212;324-326). To explore the role of NADPH oxidase activity, the phosphorylation of the $p47^{phox}$ subunit was examined. Phosphorylation of $p47^{phox}$ on serine 345, which as been correlated with NADPH oxidase activation, was markedly ($P<0.01$) increased by IH infusion. Of interest, co-infusion of NAC completely blocked $p47^{phox}$ phosphorylation (Figure 5), while NAC alone had no effect.

We previously found that PKC-δ activation was induced by IH infusion and that it is associated with insulin resistance in the liver (58). To examine the potential link between oxidative stress and PKC-δ activation in the mechanism of FFA-induced hepatic insulin resistance, we examined PKC-δ membrane translocation, an established marker of its activation. IH infusion induced hepatic PKC-δ translocation from the cytosol to the membrane (Figure 6). IH-induced PKC-δ membrane translocation, however, was not

prevented by NAC co-infusion, indicating that oxidative stress was likely not involved in this process. In addition, it has been demonstrated that PKC-δ activation in response to oxidative stress is associated with tyrosine phosphorylation of tyrosine 311 (327). We did not find any change in PKC-δ tyrosine 311 phosphorylation in response to IH or NAC infusion (Figure 7).

Several serine kinases have been implicated in insulin resistance. One such kinase is p38 MAPK, which can be activated by PKC-δ (82;328;329). In parallel with PKC-δ membrane translocation, phosphorylated p38 MAPK was increased by IH infusion (Figure 8) but NAC co-infusion did not reverse the IH-induced increase in phosphorylated p38 MAPK.

PKC-δ activation in the liver in FFA-induced insulin resistance has also been linked to IKKβ activation (54). To examine this association and the role of oxidative stress, the hepatic content of IκBα was determined. The infusion of IH caused a significant decrease in IκBα content, which reflects IKKβ activation (54). This was prevented by co-infusion of NAC (Figure 9). Another Ser/Thr kinase activated in insulin resistance states is JNK1, in particular in response to a high fat diet (207). IH infusion resulted in markedly increased JNK1 phosphorylation, measured with phospho-specific antibody which correlates with its activation. Similar to the results with IKKβ, NAC also prevented JNK1 activation by IH (Figure 10).

A consequence of IKKβ and/or JNK1 activation, which is thought to play a major role in the pathogenesis of insulin resistance, is serine phosphorylation of IRS-1 and potentially IRS-2 (118;119;122;207;244). To confirm that the activation of these serine/threonine kinases resulted in functional consequences, IRS-1 serine 307 (Figure

11) and IRS-2 serine 233 phosphorylation (Figure 12) were assessed with phospho-specific antibodies. IH increased both sites of phosphorylation of these key signaling molecules and both were prevented by co-infusion of NAC. It should be noted that in this model in the liver there was no change in total protein content of IRS-1 or IRS 2 (Figures 11 and 12). Furthermore, NAC co-infusion prevented IH-induced decrease in tyrosine phosphorylation of IRS-1 and IRS-2 (Figure 13), and in serine phosphorylation of Akt (Figure 14) in the liver.

## 4.5 Discussion

In the present study, we demonstrate that an antioxidant, NAC, prevented FFA-induced hepatic insulin resistance in conjunction with prevention of FFA-induced activation of NADPH oxidase and oxidative stress, IKKβ and JNK1 activation, and serine phosphorylation of IRS-1 and –2 without affecting PKC-δ activation in the liver. These results suggest that oxidative stress is a causal factor in the mechanism of FFA-induced hepatic insulin resistance and that a possible pathway for this defect encompasses in sequence PKC-δ activation, NADPH oxidase and oxidative stress, and IKKβ and JNK1 activation that eventually results in impaired hepatic insulin signaling.

Seven-hour infusion of IH elevated basal plasma FFA by approximately three-fold to levels commonly observed in uncontrolled diabetes. In the present study, IH infusion caused marked insulin resistance in both periphery and liver, as indicated by decreased peripheral glucose uptake and increased EGP during the clamp, respectively, in agreement with previous findings (58;330;331).

In the basal state, IH infusion increased EGP in spite of increased plasma insulin and glucose concentrations (Figure 1). Previous studies in humans and in 5-hour-fasted rats have revealed that IH infusion increases gluconeogenesis but does not affect EGP under basal conditions due to an autoregulatory decrease in glycogenolysis (74;332). However, this hepatic autoregulation was likely abolished in overnight-fasted rats used in the present study due to glycogen depletion, as previously reported (69). During the clamp, insulin infusion at 5 mU/kg/min suppressed EGP by approximately 60% in the SAL group while insulin-induced suppression of EGP was markedly diminished (~10%, $P<0.01$) in the IH group (Figure 1). Co-infusion of NAC, which prevented the IH-induced increase in hepatic protein carbonyl content, an oxidative stress marker, prevented the IH-induced defect in suppression of EGP, illustrating that oxidative stress is a major cause of FFA-induced hepatic insulin resistance.

Short-term lipid infusion has been shown to induce translocation of hepatic PKC-δ, a novel PKC isoform, from cytosol to the membrane (54;58), which is a marker of its activation. PKC may phosphorylate IRS-1 on serine/theronine residues thereby diminishing its tyrosine phosphorylation (317), although no PKC isoform has been directly linked to phosphorylation of IRS-1 on serine or threonine residues, such as serine 307, that are implicated in insulin resistance. Alternatively, PKC may activate other serine/threonine kinases which are downstream mediators of insulin resistance, such as IKKβ and JNK1 (120). In the present study, NAC completely abolished FFA-induced hepatic insulin resistance and serine phosphorylation of IRS-1 and -2 without affecting PKC-δ membrane translocation (Figure 6). This finding suggests that PKC-δ activation is either not causally related to or occurs upstream of oxidative stress in the pathway

leading to FFA-induced hepatic insulin resistance, which is consistent with results from cultured smooth muscle cells and endothelium where FFA-induced oxidative stress was found to be mediated by PKC (210). However, in adipocytes isolated from high-fat fed mice, oxidative stress increased PKC-δ activity (211;212). Therefore, we investigated the possibility that FFA-induced oxidative stress activates PKC-δ via tyrosine 311 phosphorylation, which is an alternative mechanism by which PKC-δ can be activated by ROS (327). However, IH infusion did not increase tyrosine 311 phosphorylation of liver PKC-δ. These findings indicates that FFA likely activate PKC-δ mainly via membrane translocation rather than via tyrosine phosphorylation and provides further evidence that PKC-δ activation occurs upstream of oxidative stress.

Other potential mediators of FFA-induced hepatic insulin resistance are pro-inflammatory and stress-activated serine kinases IKKβ and JNK1. In the present study, IH infusion decreased hepatic content of IκBα, which is evidence for IKKβ activation. IKKβ not only phosphorylates IκBα but also insulin signaling molecules such as IRS-1 on their serine residues (119) and plays a causal role in FFA-induced insulin resistance in skeletal muscle (187;233). Similarly, it was recently shown that liver IKKβ plays a causal role in hepatic insulin resistance caused by high-fat diet (231). Consistent with previous reports that ROS can activate IKKβ (216), NAC abolished the IH-induced decrease in hepatic IκBα content (Figure 6). Like IKKβ, JNK1 is also capable of phosphorylating IRS on serine residues (118;207;333) and increased JNK1 activity has been linked to FFA-induced hepatic insulin resistance *in vitro* (120;207;209;244) and to insulin resistance caused by high-fat feeding *in vivo* (207). In this study, IH infusion caused a marked increase in the levels of phosphorylated JNK1, which is the activated

form of the kinase, and this increase was reversed by NAC co-infusion. Together, these findings suggest that lipid infusion results in activation of both pro-inflammatory and stress-activated kinases via oxidative stress.

FFA decrease the ability of insulin to suppress EGP by impairing hepatic insulin signaling, as shown by decreased hepatic IRS-2-associated PI3-kinase activity in high-fat fed rats (131). Increased phosphorylation of insulin receptor and/or IRS on discrete serine or threonine sites decreases tyrosine phosphorylation and thus impairs insulin action (55;117;121;317). In particular, phosphorylation of IRS-1 on serine residue 307 has been shown to impair tyrosine phosphorylation (60;120;334). In the present study, NAC prevented the increase in serine phosphorylation of IRS-1 (serine 307) caused by IH infusion (Figure 11) in parallel with prevention of FFA-induced increases in markers of activation of serine kinases IKKβ and JNK1, but not PKC. Similarly, NAC prevented IH-induced increase in serine 233 phosphorylation of IRS-2 (Figure 12), which has been linked to insulin resistance (335), although it is unknown whether serine 233 phosphorylation interferes with tyrosine phosphorylation of IRS-2. Furthermore, NAC reversed IH-induced decrease in tyrosine phosphorylation of IRS-1 and 2 (Figure 13), and serine phosphorylation of Akt (Figure 14) in the liver. These findings that NAC is able to prevent the IH-induced impairment of hepatic insulin signaling implicates oxidative stress in the activation of serine kinases IKKβ and JNK1 (215;336).

NAC is a thiol antioxidant that increases cellular synthesis of reduced glutathione (337). Oxidative stress has been linked to multiple models of insulin resistance (321) and several studies have demonstrated that antioxidants improve insulin action. For instance, NAC improved insulin sensitivity in hyperinsulinemic patients with polycystic ovary

syndrome (320) and previous studies in our laboratory have shown that both NAC and taurine prevent insulin resistance caused by prolonged hyperglycemia in rats (193). Furthermore, glutathione treatment was shown to partially prevent IH-induced insulin resistance in humans, although tracer studies were not performed to localize its site of action (224). In the present study, NAC prevented IH-induced insulin resistance in both liver and peripheral tissues.

In the liver, NAC also reversed IH-induced increase in serine 345 phosphorylation of NADPH oxidase subunit $p47^{phox}$ measured in the membrane, a process which is required for NADPH oxidase activity prior to membrane translocation and assembly of its subunits (338). Previous studies have demonstrated that PKC activation can increase ROS via PKC-dependent activation of NADPH oxidase *in vitro* (210-212). Further support for a potential role of NADPH oxidase in hepatic insulin resistance comes from a study in which oxidative stress and NADPH oxidase activity were increased in the liver of Zucker fatty rats on high-fat diet (214). Recently, the direct inhibitory effect of NAC on expression and membrane translocation of NADPH oxidase subunits was demonstrated in the heart of streptozotocin-induced diabetic rats (339). These results suggest a role of ROS to augment their own production and a role of NAC to not only scavenge ROS, but also to decrease intracellular activity involved in their production.

A question that remains unanswered is whether, in the present study, PKC-δ directly mediated the activation of NADPH oxidase via serine phosphorylation of $p47^{phox}$. Several studies have reported that PKC can directly activate $p47^{phox}$ of the NADPH oxidase in both phagocytic and non-phagocytic cell types (213;335;340-343). In

particular, it was shown that PKC-δ can mediate both serine phosphorylation of $p47^{phox}$ and its membrane translocation in human neutrophiles (213); however, among numerous serine sites on $p47^{phox}$ which can be phosphorylated by PKC, PKC-δ was not capable of phosphorylating serine 345 (213). Instead, serine 345 of $p47^{phox}$ has been shown to be phosphorylated by MAP kinases, such as p38 MAPK and ERK 1/2 (338). Thus, it is possible that PKC-δ causes serine 345 phosphorylation of $p47^{phox}$ indirectly via p38 MAPK. Our finding that IH infusion increases PKC-δ membrane translocation and phosphorylation of p38 MAPK is consistent with this notion. However, both of these events were not affected by NAC co-infusion, suggesting that, while FFA-induced PKC-δ activation causes serine 345 phosphorylation of $p47^{phox}$ indirectly via p38 MAPK, NAC may exert a direct effect on the $p47^{phox}$ to reverse the IH-induced increase in serine 345 phosphorylation of the NADPH oxidase subunit. We cannot, however, exclude the possibility that FFA-induced ROS, independent of PKC, is sufficient to cause hepatic insulin resistance, although our data in Study 1 suggest that PKC-δ activation is causally related to FFA-induced hepatic insulin resistance.

NAC normalized basal glucose production, which may be partly due to amelioration of insulin resistance and partly due to a tendency toward higher basal insulin levels observed in the IH plus NAC group compared to the SAL or NAC alone groups. This is because the effect of IH infusion to increase basal insulin levels was not prevented by NAC, as expected, since FFA have a direct effect on β-cells to acutely increase insulin secretion, which is unrelated to oxidative stress. Unexpectedly, however, NAC also appeared to abolish FFA-induced decrease in insulin clearance, since elevation in insulin levels was not observed in the IH plus NAC group despite greater insulin

infusion rate than the IH group. Further studies are required to determine whether this normalizing effect on insulin clearance is due to normalization of IH-induced serine phosphorylation of insulin receptor or of adaptor molecules involved in insulin receptor internalization.

In conclusion, we have provided evidence that FFA-induced hepatic insulin resistance is associated with PKC-δ membrane translocation, phosphorylation of p38 MAPK, phosphorylation of NADPH oxidase subunit $p47^{phox}$ and oxidative stress, decreased IκBα and increased phosphorylation of JNK1, as well as impairment of insulin signaling in the liver. Furthermore, we have demonstrated that the antioxidant NAC prevents FFA-induced hepatic insulin resistance by 1) inhibiting NADPH oxidase and oxidative stress; 2) preventing activation of IKKβ and JNK1; 3) preventing increase in serine phosphorylation and decrease in tyrosine phosphorylation of IRS-1 and IRS-2; and 4) preventing decrease in serine phosphorylated Akt, without affecting membrane translocation of PKC-δ and increase in phosphorylated p38 MAPK. These data suggest that oxidative stress is a major causal factor and that PKC-δ activation may occur upstream of oxidative stress. Hence, we propose that a sequence of events leading to FFA-induced hepatic insulin resistance is FFA $\rightarrow$ PKC-δ $\rightarrow$ oxidative stress $\rightarrow$ IKKβ/JNK1 $\rightarrow$ IRS-1 and -2 serine phosphorylation $\rightarrow$ impaired hepatic insulin signaling.

| | Basal Period | | | | Clamp Period | | | |
|---|---|---|---|---|---|---|---|---|
| | SAL | IH | IH+NAC | NAC | SAL | IH [B] | IH +NAC | NAC |
| **FFA (mEq/l)** | 0.48±0.04 | 1.63±0.23[C] | 1.93±0.4[C] | 0.65±0.06 | 0.17±0.05 | 1.24 ±0.10[C] | 1.06±0.15[C] | 0.16±0.06 |
| **Glucose (mM/l)** | 6.7±0.3 | 7.3±0.2[D] | 6.7±0.4 | 6.8±0.3 | 6.2±0.1 | 6.3±0.2 | 6.9±0.1 | 7.2±0.1 |
| **Insulin (pM)** | 89±8 | 147±16[C] | 152±34 | 104±32 | 560±72 | 597±16 | 541±24 | 669±63 |
| **C-peptide (nM)** | 0.28±0.05 | 0.85±0.18[C] | 0.81±0.18[C] | 0.34±0.05 | 0.03±0.003 | 0.09±0.02[C] | 0.05±0.01 | 0.07±0.03 |
| **GIR (µmol/kg/min)** | — | — | — | — | 163±12 | 39.4±4.9[E] | 147±4.7 | 164±8.8 |

**Table.** Metabolic parameters and glucose infusion rate (GIR) in the basal state and during 2-hour hyperinsulinemic-euglycemic clamp[A] (n=6-9/group). [A] Data are means ± SEM and refer to the last 30 min of each experiment. [B] Insulin infusion rate = 2.5 mU/kg/min. [C] P<0.01 vs. SAL. [D] P<0.05 vs. SAL. [E] P<0.01 vs SAL, IH+NAC, and NAC

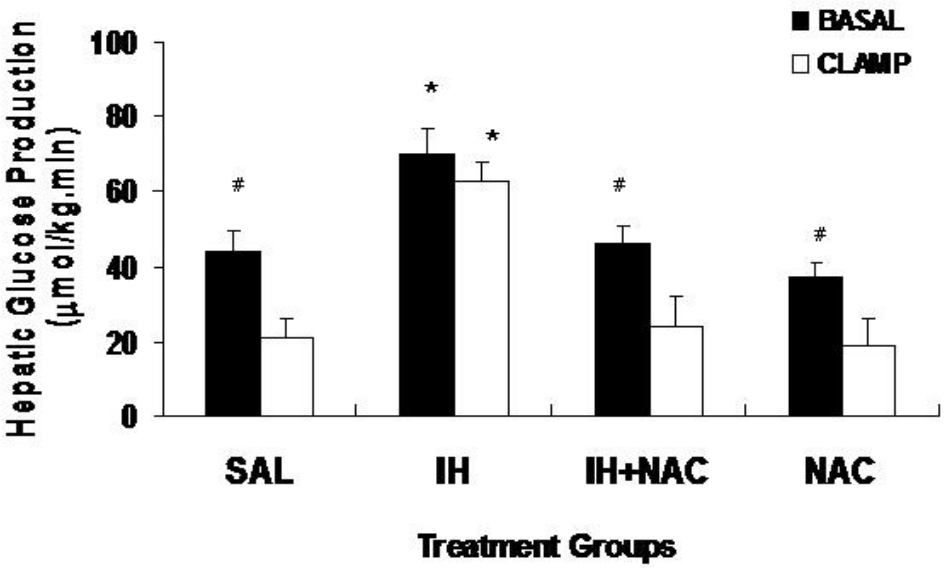



**Figure 1. The effect of NAC on IH-induced impairment of insulin-stimulated suppression of hepatic glucose production (HGP) (n=6-9/group).** Data are means ± SEM and refer to the last 30 min of each experiment. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. Basal: Basal fasting state. Clamp: 2h hyperinsulinemic-euglycemic clamp. *: P<0.01 IH vs. other treatment groups in the basal state and during the clamp. #: P<0.001, Clamp vs. Basal.

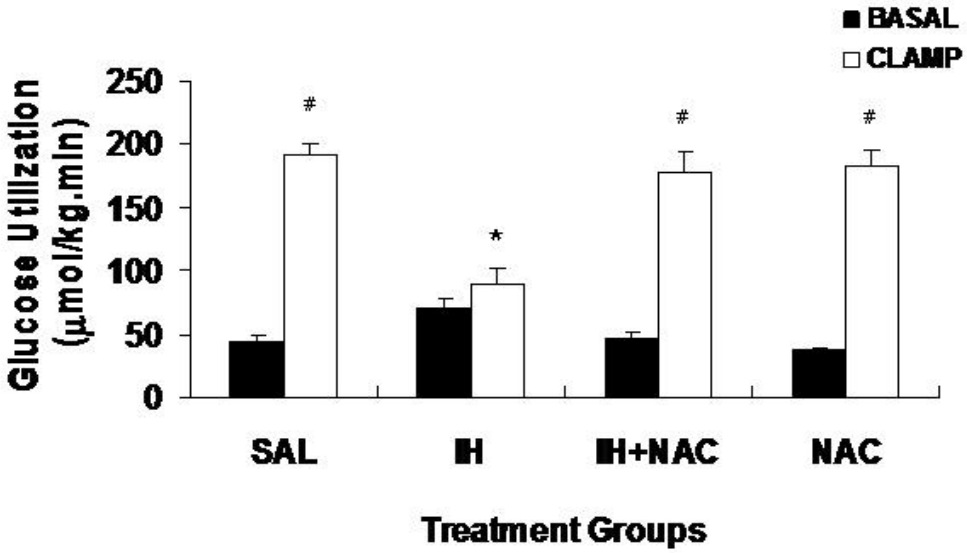
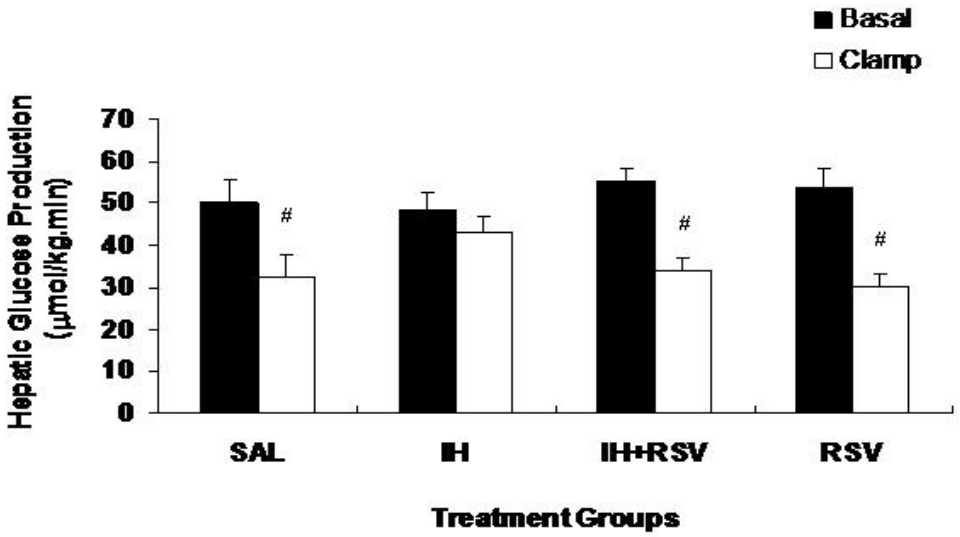



**Figure 2. The effect of NAC on IH-induced impairment of insulin-stimulated peripheral glucose utilization (n=6-9/group).** Data are means ± SEM and refer to the last 30 min of each experiment. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. Basal: Basal fasting state. Clamp: 2h hyperinsulinemic-euglycemic clamp. *: P<0.01 IH vs. other treatment groups during clamp. #: P<0.001, Clamp vs. Basal.

A

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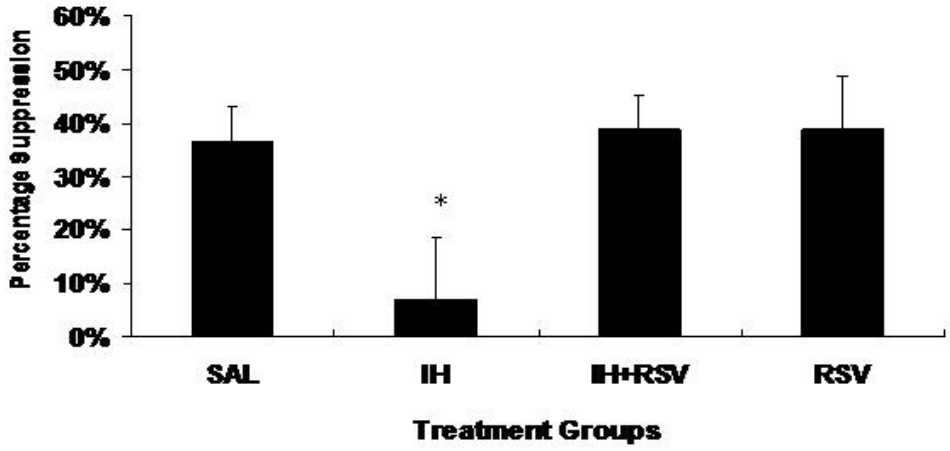



**Figure 3. Hepatic glucose production (HGP) in rats (n=6-9/group).** **A**: Effect of IH and resveratrol on HGP during the basal period and during the last 30 minutes of 2-hour hyperinsulinemic-euglycemic clamp. **B**: Effect of IH and resveratrol on insulin-induced percentage suppression of HGP from basal. Data are means ± SEM and refer to the last 30 min of each experiment. SAL: Saline. IH: Intralipid plus heparin. RSV: Resveratrol. Basal: Basal fasting state. Clamp: 2h hyperinsulinemic-euglycemic clamp. #: P<0.01, Clamp vs. Basal. *: P<0.05 IH vs. other treatment groups.

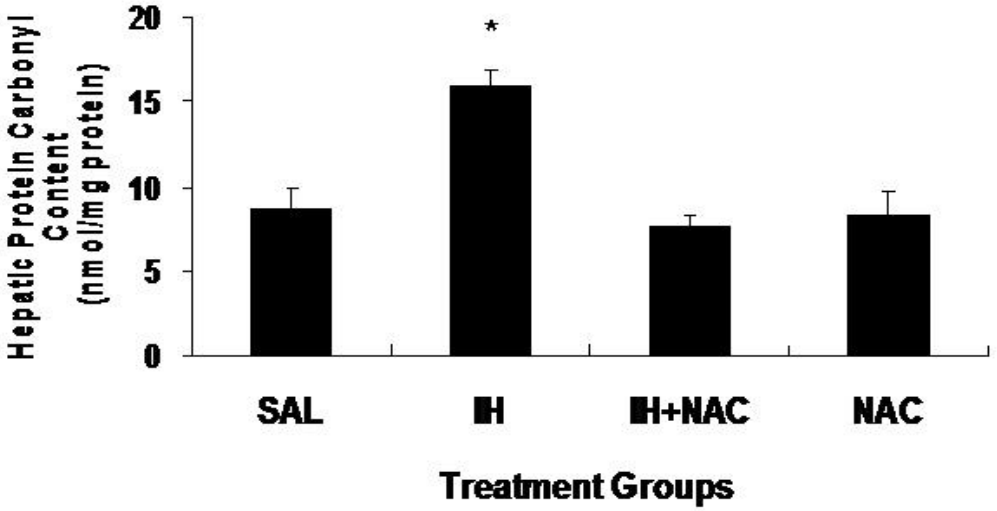



**Figure 4. Hepatic protein carbonyl content (n=6-9/group).** NAC alone did not have any effect. Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.01 vs. other treatment groups.

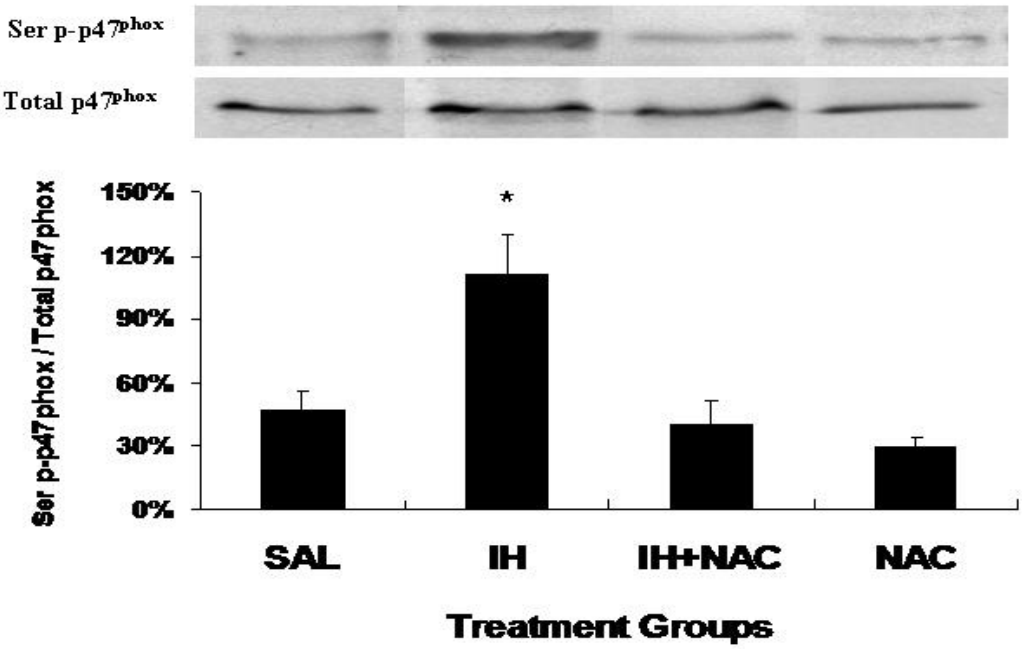



**Figure 5. Serine 345 phosphorylated $p47^{phox}$ subunit of NADPH oxidase in hepatic membrane fraction (n=6-9/group).** SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.01 vs. other treatment groups.

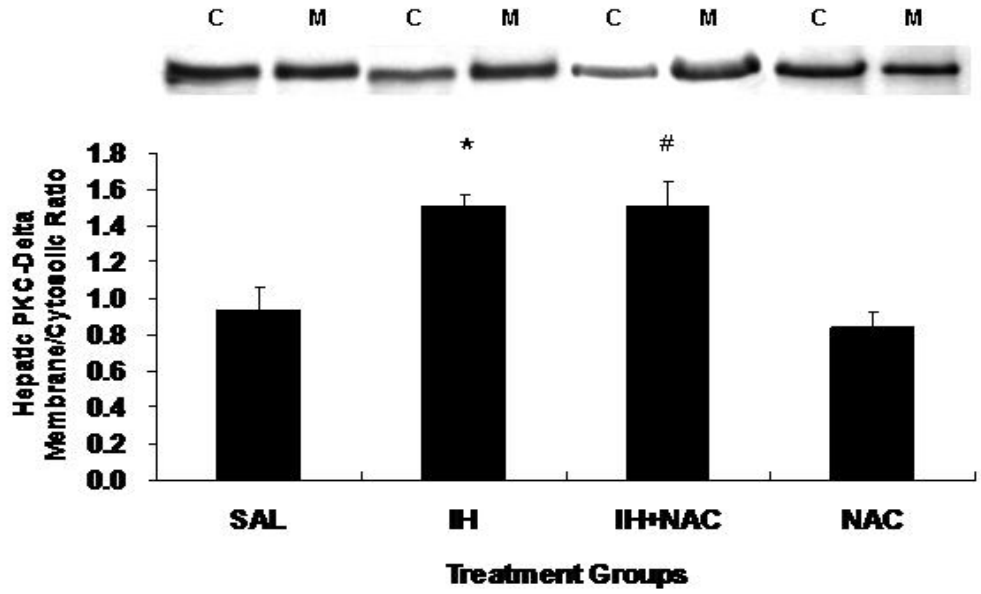



**Figure 6. The effect of IH infusion on hepatic PKC-δ membrane translocation (n=6-9/group).** Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.01 vs. SAL and NAC. #: P<0.05 vs. SAL and NAC. C: Cytosol M: Membrane

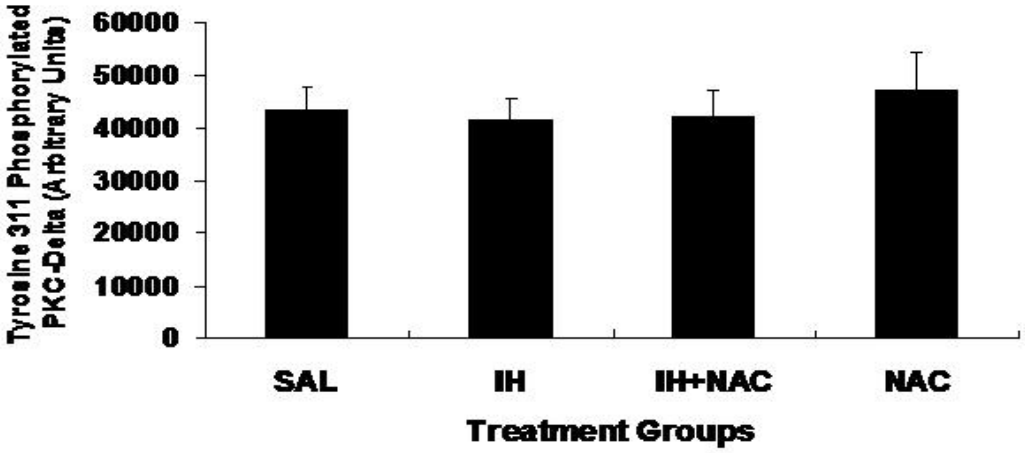



**Figure 7. The effect of IH infusion on tyrosine (311) phosphorylation of hepatic PKC-δ in the membrane fraction (n=6-9/group).** Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine.

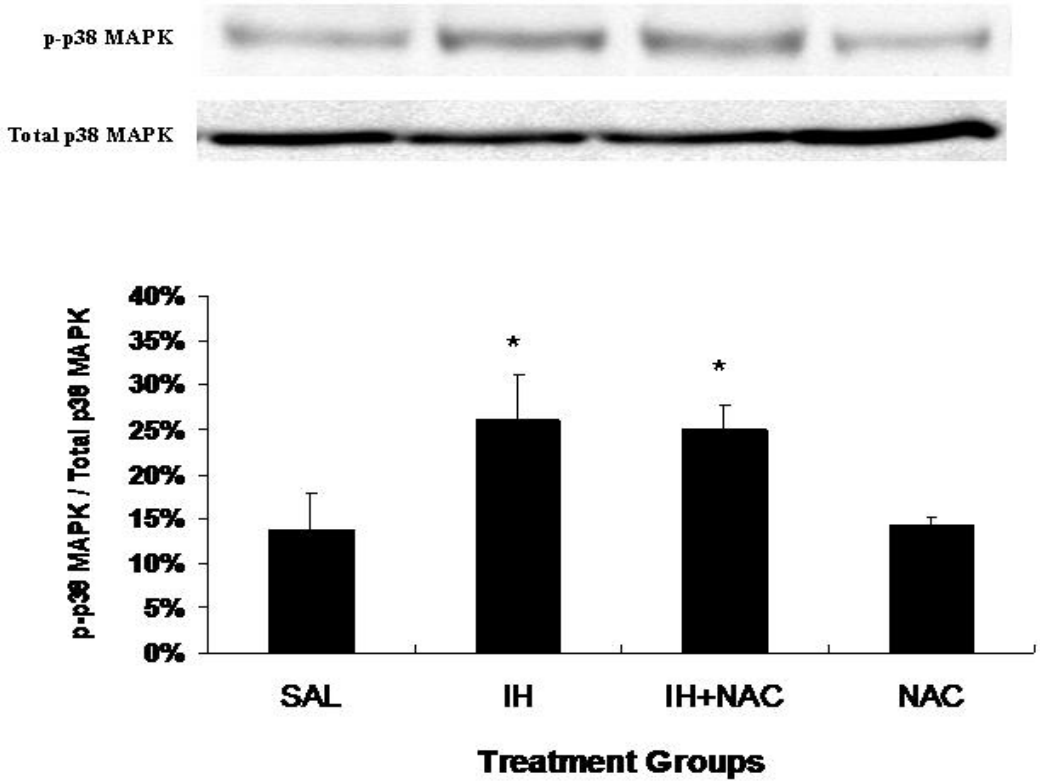



**Figure 8. Thr 180/Tyr 182 phosphorylated p38 mitogen-activated protein kinase (MAPK) levels in the liver tissue (n=6-9/group).** Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.05 vs. SAL.

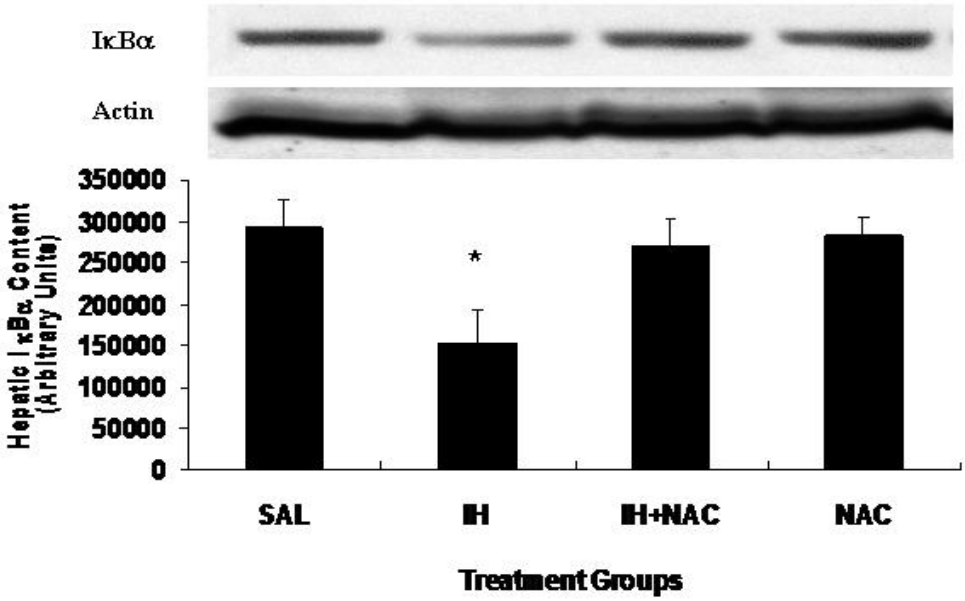



**Figure 9. Hepatic IκBα content (n=6-9/group).** Data are means ± SEM and refer to the last 30 min of each experiment. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.01 vs. other treatment groups.

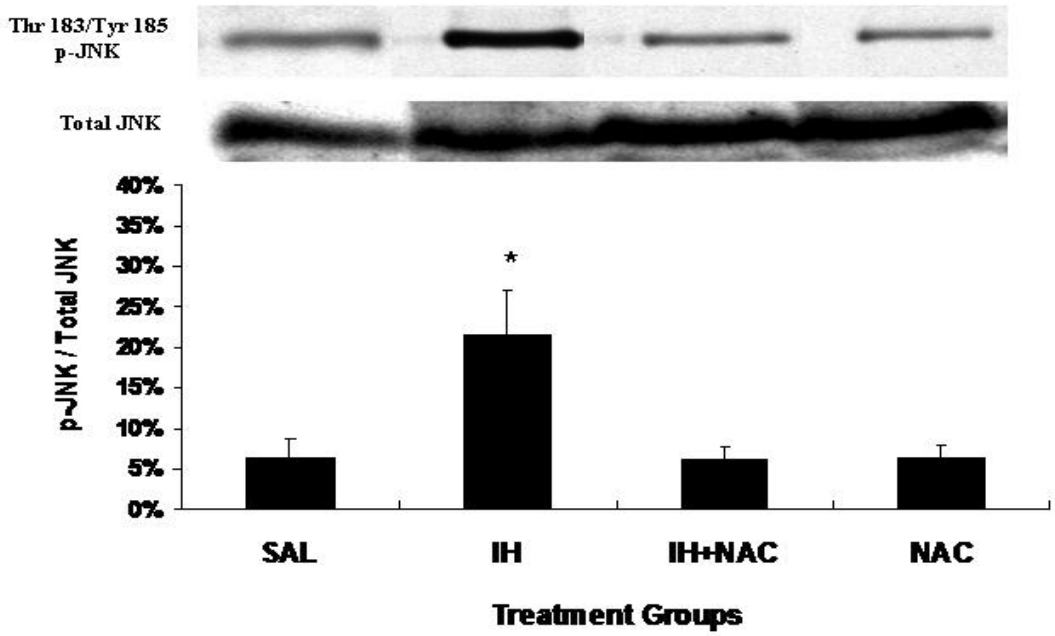



**Figure 10. Threonine (Thr) 183 / tyrosine (Tyr) 185 phosphorylated c-Jun $NH_2$-terminal kinase 1 (JNK1) levels in the liver tissue (n=6-9/group).** Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.01 vs. other treatment groups.

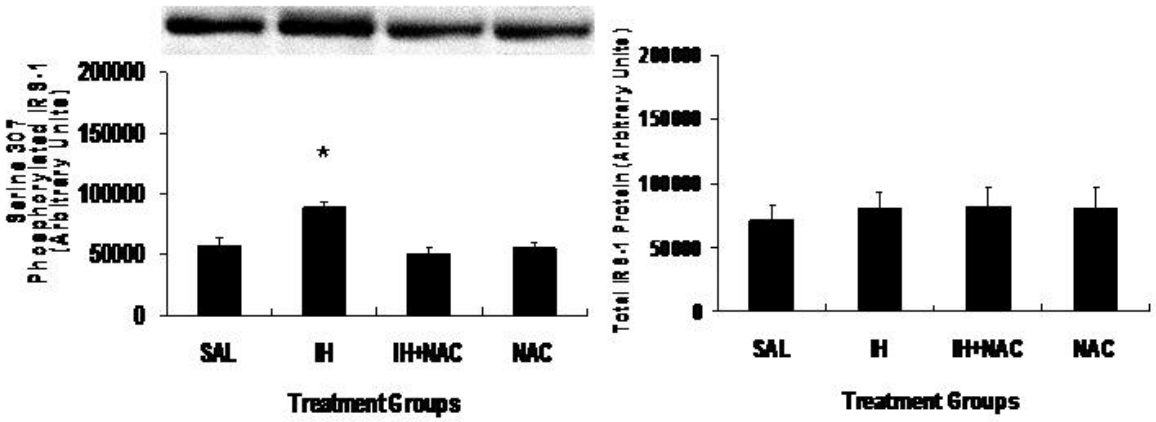



**Figure 11. Serine 307 phosphorylated and total IRS-1 levels in liver tissue (n=6-9/group).**
Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine.
*: P<0.01 vs. other treatment groups.

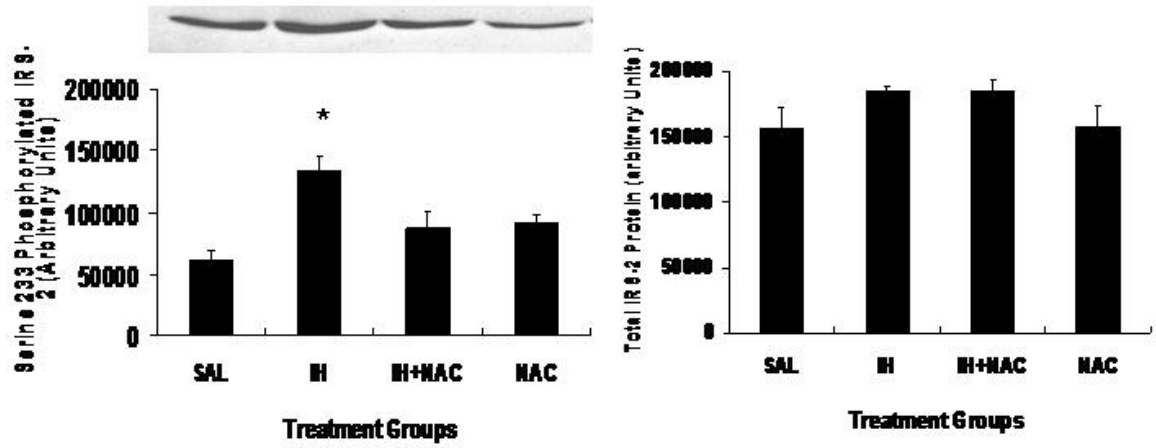



**Figure 12. Serine 233 phosphorylated and total IRS-1 levels in liver tissue (n=6-9/group).**
Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine.
*: P<0.01 vs. other treatment groups.

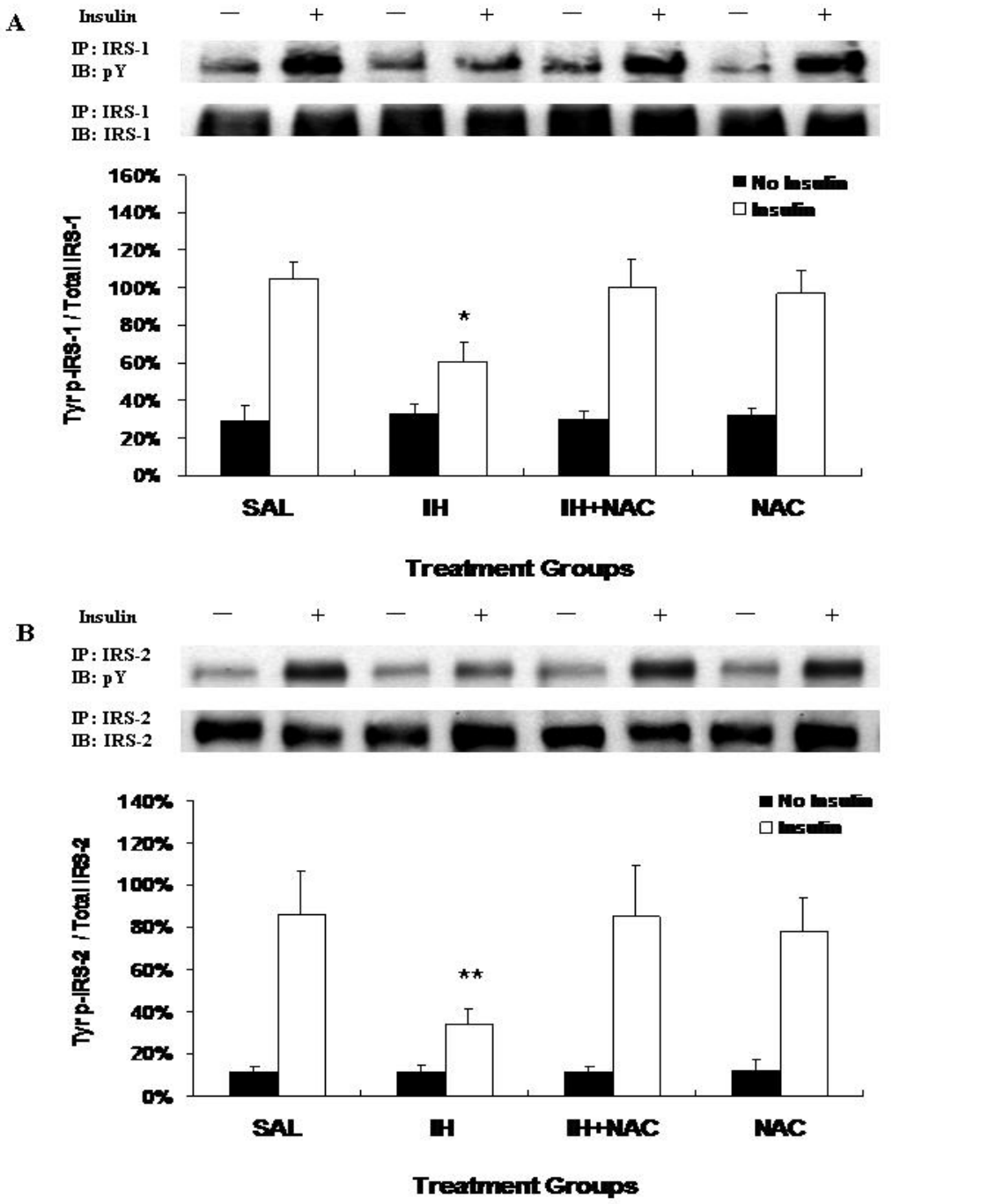



**Figure 13. Tyrosine phosphorylated IRS-1 (A) and IRS-2 (B) levels in liver tissue with or without insulin bolus (n=6-9/group).** Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.01 vs. other treatment groups **: P<0.05 vs other treatment groups.

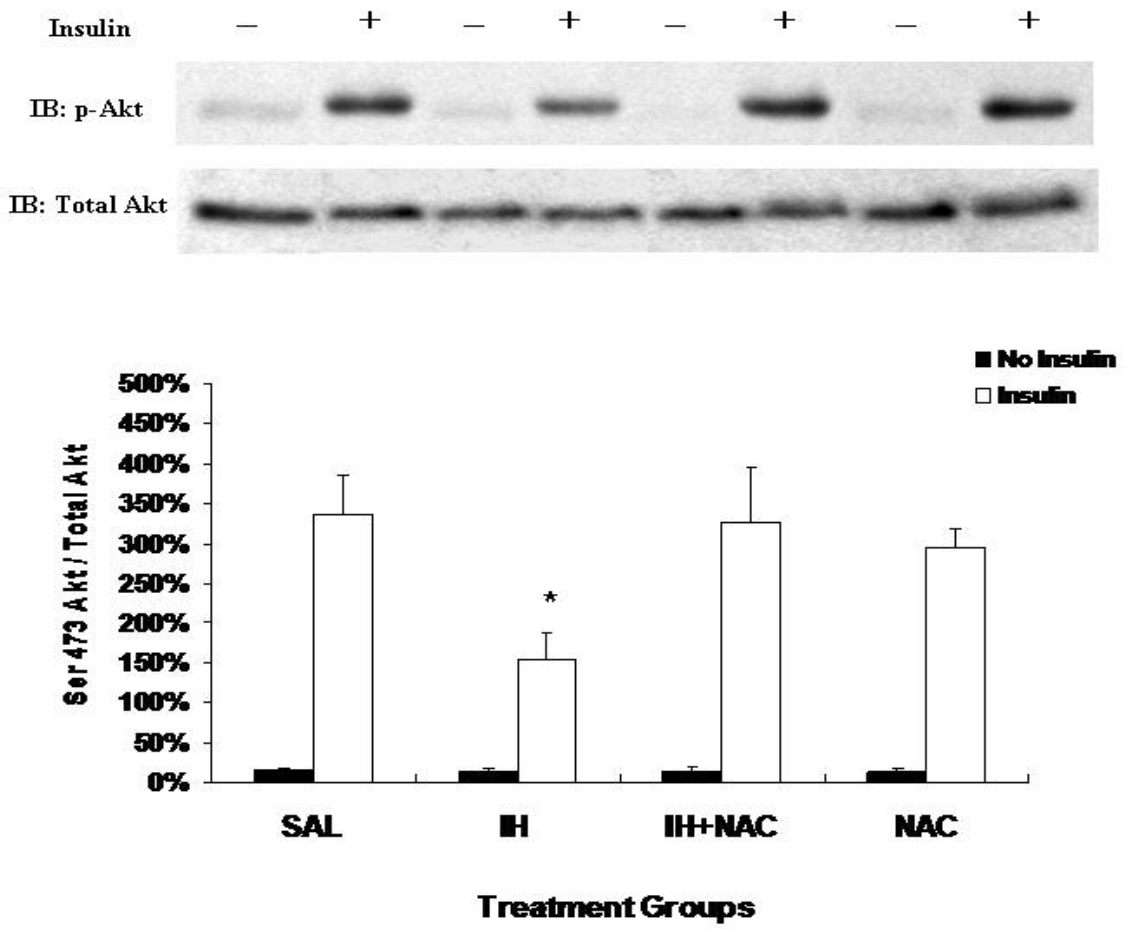



**Figure 14. Serine 437 phosphorylated Akt levels in liver tissue with or without insulin bolus (n=6-9/group).** Data are means ± SEM. SAL: Saline. IH: Intralipid plus heparin. NAC: N-acetyl-L-cysteine. *: P<0.05 vs. other treatment groups.

# 5

# Study 3

## *Salicylate Prevents Hepatic Insulin Resistance Caused by Short-Term Elevation of Free Fatty Acids In Vivo*

Park E, Wong V, Guan X, Oprescu AI, Giacca A. Salicylate Prevents Hepatic Insulin Resistance Caused by Short-Term Elevation of Free Fatty Acids In Vivo. *Journal of Endocrinology* 2007 Nov;195(2):323-31.

## 5.1 Abstract

Recent evidence indicates that inflammatory pathways are causally involved in insulin resistance. In particular, IκBα kinase β (IKKβ), which can impair insulin signaling directly via serine phosphorylation of insulin receptor substrates (IRS) and/or indirectly via induction of transcription of pro-inflammatory mediators, has been implicated in free fatty acid (FFA)-induced insulin resistance in skeletal muscle. However, it is unclear whether liver IKKβ activation plays a causal role in hepatic insulin resistance caused by acutely elevated FFA. In the present study, we wished to test the hypothesis that sodium salicylate, an inhibitor of IKKβ, prevents hepatic insulin resistance caused by a short-term lipid infusion. To do this, overnight-fasted Wistar rats were subject to a 7-hour intravenous infusion of either saline, Intralipid plus 20 U/ml heparin (IH; triglyceride emulsion that elevates FFA levels *in vivo*) with or without salicylate. Hyperinsulinemic-euglycemic clamp with tracer infusion was performed to assess insulin-induced stimulation of peripheral glucose utilization and suppression of endogenous glucose production (EGP). Infusion of IH markedly decreased insulin-induced stimulation of peripheral glucose utilization and suppression of EGP, which were completely prevented by salicylate co-infusion. Salicylate also prevented FFA-induced decrease in IκBα content, which indicates increased IKKβ activity, in the liver. Furthermore, salicylate reversed 1) an increase in serine phosphorylation of IRS-1 (ser 307) and IRS-2 (ser 233); 2) a decrease in tyrosine phosphorylation of IRS-1 and –2; and 3) a decrease in serine 473 phosphorylated Akt, which is a marker of Akt activation, in the liver. These results demonstrate that inhibition of IKKβ prevents FFA-induced

impairment of hepatic insulin signaling, thus implicating IKKβ as a causal mediator of hepatic insulin resistance caused by acutely elevated plasma FFA.

## 5.2 Introduction

Numerous studies have established a close relationship between obesity, insulin resistance, and type 2 diabetes. This link is attributed to a greater release of various adipocyte-derived products, such as cytokines, resistin, and free fatty acids (FFA), from the expanded adipose tissue in obesity. In particular, elevated circulating levels of FFA cause insulin resistance in both animals and humans (57;58;187;331;344). However, precise mechanisms by which FFA impair insulin action in the liver and peripheral tissues are incompletely understood.

Recent studies have demonstrated that FFA cause insulin resistance in skeletal muscle mainly via inhibition of tyrosine phosphorylation of insulin receptor substrate (IRS)-1 (57;60;233), which is a critical step in insulin signal transduction. This process is likely mediated by the phosphorylation of serine residues on IRS-1 by certain serine kinases, such as protein kinase C (PKC), inhibitor of IκBα kinase β (IKKβ), and c-Jun $NH_2$-terminal kinase (JNK) 1. Shulman (57;60;233) and others (54;56;187;345) have implicated accumulation of intramyocellular lipid metabolites (e.g. diacyl glycerol, long-chain fatty acyl CoA) and activation of serine kinases PKC (isoforms β and δ in humans, ε and θ in rodents) and IKKβ as potentially causal events in the pathway of FFA-induced insulin resistance in skeletal muscle. For instance, PKC-θ-null mice are protected from insulin resistance in the skeletal muscle caused by a short-term lipid infusion (57).

Furthermore, treatment with high dose sodium salicylate, an IKKβ inhibitor, or IKKβ-deficiency prevents fat-induced insulin resistance in rodent skeletal muscle (233).

In contrast, potentially causal roles of serine kinases PKC and IKKβ in FFA-induced hepatic insulin resistance have been less extensively investigated. Our laboratory has previously established an association between PKC-δ membrane translocation, a marker of its activation, and FFA-induced hepatic insulin resistance (58). This association has recently been confirmed by Boden et al. (54), who, in the same study, also linked hepatic IKKβ activation with FFA-induced hepatic insulin resistance. However, it has not been clearly shown whether IKKβ causes hepatic insulin resistance induced by a short-term elevation of circulating FFA. While Arkan et al. (231) have demonstrated that hepatocyte-specific IKKβ knockout mice are protected from hepatic insulin resistance caused by high-fat feeding or genetically-induced obesity, these models are associated with chronically elevated FFA levels, which may cause insulin resistance via a different mechanism than short-term FFA elevation. Thus, in the present study, we wished to test the hypothesis that IKKβ activation is causally involved in the mechanism of hepatic insulin resistance induced by acute FFA elevation. To do this, we used a rat model of short-term (7 hours) intravenous lipid infusion with or without co-infusion of sodium salicylate to examine whether inhibition of IKKβ results in restoration of hepatic insulin action via prevention of FFA-induced IRS-1 and IRS-2 serine phosphorylation.

## 5.3 Materials and Methods

### 5.3.1 Animal Models

Female Wistar rats (Charles River, Quebec, Canada) weighing 250-300g were used for experiments. The animals were maintained as described in the General Methods section except that they were fed a different rodent chow (Teklad Global 21% Protein Diet with 64% carbohydrate and 14% fat; Harland Teklad Global Diets, Madison, WI).

### 5.3.2 Surgery

After 3-5 days of adaptation to the facility, rats were anesthetized with isofluorane and indwelling catheters were inserted into the right internal jugular vein for infusion and the left carotid artery for blood sampling, as described in the General Methods section.

### 5.3.3 Experimental Design

After overnight fasting (10-12 hours), the rats (n=6-8/group) were subject to a 7-hour intravenous infusion of either saline (SAL), Intralipid plus heparin (IH; 20% Intralipid + 20 U/ml heparin at 5.5 μl/min), IH plus sodium salicylate (SS; 7 mg/kg bolus plus 0.117 mg/kg/min), or sodium salicylate alone. The dose of sodium salicylate (Sigma Aldrich) used in the study was derived from a previous *in vivo* study by Kim et al. in which SS treatment was shown to prevent FFA-induced insulin resistance in skeletal muscle (233). After 3 hours of infusion, [3-$^{3}$H] glucose was initiated (8 μCi bolus

followed by constant infusion at 0.15 μCi/min) to assess peripheral glucose utilization and endogenous glucose production. Hyperinsulinemic-euglycemic clamp was performed with tracer infusion during the last 2 hours of the 7-hour infusion period to assess hepatic and peripheral insulin sensitivity. During 30 minutes preceding the clamp ("basal period"), measurements were taken at 10-minute interval for plasma glucose, insulin, FFA, and [3-$^3$H] glucose specific activity. Refer to the General Methods section for detailed description of clamp procedure. All signaling results were obtained from the liver, soleus muscle, and fat samples taken at the end of the clamp.

### 5.3.4 Plasma Assays

Plasma glucose was measured with a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). Plasma radioactivity from [3-$^3$H] glucose was determined after deproteinization with $Ba(OH)_2$ and $ZnSO_4$, and subsequent evaporation to dryness. Aliquots of the [3-$^3$H] glucose and of the tritiated glucose infusate were assayed together with the plasma samples (58). Insulin levels in plasma were determined by radioimmunoassay (RIA) using a kit specific for rat insulin (but with 100% cross reactivity with porcine insulin used for infusion). Plasma FFA levels were measured using a colorimetric kit from Wako Industrials (Neuss, Germany). These procedures are described in detail in the General Methods section.

### 5.3.5 Immunoprecipitation and Western Blot Analysis

For determination of tyrosine phosphorylation, IRS-1 and IRS-2 were immunoprecipitated and then immunoblotted with phosphotyrosine antibody (Santa Cruz; 1:1000 dilution) and goat anti-mouse secondary antibody (Santa Cruz; 1:2000 dilution), as described in the General Methods section. Serine 473 phosphorylated Akt (Antibody purchased from Cell Signaling; 1:1000 dilution) was measured by Western blotting using the same liver lysates that were used for immunoprecipitation of IRS-1 and IRS-2.

Detailed procedures for Western blotting are described in the General Methods section. Polyclonal antibody specific for IκBα (Santa Cruz, CA) was used at a concentration of 1:2000. Antibodies against IRS-1, Serine 307 IRS-1, IRS-2 (Upstate) and Serine 233 IRS-2 (Biosource) were added to the Western membranes at dilutions of 1:500, 1:1000, 1:500, and 1:500, respectively.

### 5.3.6 Calculations

Glucose turnover (rate of appearance of glucose determined with [3-$^{3}$H] glucose) was calculated using Steele's non-steady-state equation (346), taking into account the extra tracer infused with the glucose infusate (306). EGP was calculated as described in the General Methods section.

### 5.3.7 Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to compare differences between treatment groups for the following parameters:

whole body insulin sensitivity, IκBα content, serine phosphorylated IRS-1 and –2, tyrosine phosphorylated IRS-1 and –2, total IRS-1 and IRS-2, and serine 473 phosphorlylated Akt. For plasma glucose, free fatty acids, and insulin as well as EGP and peripheral glucose utilization, we used two-way ANOVA with Tukey's post hoc test in order to compare results between treatment groups and, if necessary, to compare basal and clamp data within each group. Statistical calculations were performed using Statistica software (Statistical Analysis System, Cary, NC). Significance was accepted at a $P<0.05$.

## 5.4 Results

The table shows plasma glucose, FFA, and insulin levels during the basal period and hyperinsulinemic-euglycemic clamp. Plasma glucose levels were not different between treatments during the basal period or during the clamp. Plasma FFA levels which were measured during the basal period increased by ~100% in the IH and IH plus SS groups compared to the SAL group due to IH infusion ($P<0.01$). As expected, plasma FFA levels were lower during the hyperinsulinemic clamp than during the basal period in all treatment groups due to the anti-lipolytic and fat esterification effects of insulin. Plasma insulin levels were not different between groups during the basal period and were markedly elevated during the clamp compared to the basal period in all treatment groups due to insulin infusion.

Glucose infusion rate (GIR) during the last 30 minutes of the 2-hour hyperinsulinemic-euglycemic clamp is an indication of whole body insulin sensitivity. IH infusion significantly decreased GIR ($P<0.01$) compared to saline infusion (Figure 1).

Salicylate co-infusion with IH prevented the IH-induced decrease in GIR ($P<0.01$ vs IH). Salicylate alone did not have any effect.

During the basal period, EGP is equal to glucose utilization and there was no significant difference between treatment groups (Figure 2A and 3A). IH infusion decreased insulin-stimulated increase in peripheral glucose utilization during the clamp when compared with SAL infusion ($P<0.05$; Figure 2B). This IH-induced decrease was completely abolished by salicylate co-infusion ($P<0.05$ vs IH). The insulin-stimulated increase in peripheral glucose utilization in the SS alone group did not differ from the SAL group.

Hepatic insulin sensitivity is measured as the capacity of insulin to suppress the EGP from the basal state. In the SAL group, EGP decreased by 37% from basal during the last 30 minutes of hyperinsulinemic clamp. However, in IH group, suppression of EGP during clamp was only 7%, which is significantly less than suppression of EGP observed in the SAL group ($P<0.05$; Figure 3B). Co-infusion of salicylate prevented IH-induced decrease in suppression of EGP during the clamp ($P<0.05$ vs IH). Salicylate alone did not have any effect.

In order to assess the effect of salicylate on IKKβ activity in the liver, we measured hepatic IκBα content (Figure 4A), which is a marker of IKKβ activation as IκBα upon phosphorylation by IKKβ is targeted for degradation. IH infusion decreased hepatic IκBα levels ($P<0.05$), which were restored to control levels by salicylate co-infusion. Salicylate alone did not have any effect on hepatic IκBα levels. IH infusion also decreased IκBα content in the soleus muscle ($P<0.05$) and salicylate co-infusion reversed the IH-induced decrease in IκBα content ($P<0.05$ vs IH) (Figure 4B). Again,

salicylate alone did not have any effect on soleus muscle IκBα levels. In adipose tissue, however, IH infusion did not change IκBα content (Figure 4C).

IKKβ is known to phosphorylate IRS on serine residues (119), thereby decreasing the insulin-induced tyrosine phosphorylation. IH infusion markedly increased serine 307 phosphorylation of IRS-1 (Figure 5A; $P<0.05$) and serine 233 phosphorylation of IRS-2 (Figure 5B; $P<0.05$), Co-infusion of salicylate, however, completely reversed these effects of IH ($P<0.05$ vs IH). IH infusion, however, did not change total IRS-1 and IRS-2 levels (data not shown). Salicylate alone did not have any effect. Furthermore, IH infusion dramatically increased serine 307 phosphorylation of IRS-1 in the soleus muscle ($P<0.01$), which was completely reversed by salicylate co-infusion ($P<0.01$ vs IH) (Figure 5C). IH did not alter serine 307 phosphorylation of IRS-1 in the adipose tissue (Figure 5D).

Tyrosine phosphorylation of IRS by the insulin receptor is a critical step in insulin signaling. Shulman's laboratory has already shown that lipid infusion impairs tyrosine phosphorylation of IRS-1 in rat skeletal muscle (57;60;233). In liver, we found that IH infusion decreased tyrosine phosphorylation of IRS-1 (Figure 6A) and IRS–2 (Figure 6B; both $P<0.05$) and that salicylate co-infusion prevented this decrease (both $P<0.05$ vs IH)

When activated, Akt is phosphorylated at serine 473. IH infusion decreased serine 473 phosphorylated Akt ($P<0.05$) in the liver. This inhibitory effect of IH on Akt was prevented by salicylate co-infusion (Figure 7; $P<0.05$ vs IH).

## 5.5 Discussion

Recent studies have implicated inflammatory pathways, particularly IKKβ/nuclear factor κB (NFκB) system, in various animal models of insulin resistance (54;56;187;231-233). It is unclear, however, whether IKKβ activation is causally involved in the impairment of hepatic insulin action caused by short-term elevation of FFA. Therefore, the present study in rats was performed to investigate whether inhibition of IKKβ activity provides protection from FFA-induced hepatic insulin resistance. We demonstrate that treatment with high dose sodium salicylate completely prevents hepatic insulin resistance caused by short-term lipid infusion in association with prevention of IRS-1 and –2 serine phosphorylation and with improved hepatic insulin signaling.

As expected, intravenous infusion of IH markedly elevated plasma FFA levels. IH is a triglyceride emulsion containing heparin that is broken down to non-esterified fatty acids and glycerol *in vivo* by lipoprotein lipase. It is thus possible that glycerol derived from triglyerides by itself affects EGP measured in the present study; however, we have shown in a previous study (58) that glycerol infusion (5mg/kg/min) resulting in plasma glycerol levels similar to that achieved by 7 hours of IH infusion has no effect on EGP when compared with saline infusion either in the basal fasting state or during the hyperinsulinemic-euglycemic clamp.

The infusion rate of exogenous glucose during the clamp is an indication of whole body insulin sensitivity. Numerous studies (54;57;58;60;69;233;344;347) have shown that lipid infusion causes whole body insulin resistance and our results are consistent with these studies. The whole body insulin resistance caused by IH infusion was completely reversed with salicylate co-infusion, suggesting that the site of salicylate's

effect includes both liver and peripheral tissues. Using infusion of [3-$^3$H] tracer, which enabled us to separately assess hepatic and peripheral insulin sensitivity, we show that IH infusion causes both hepatic and peripheral insulin resistance, as indicated by decreases in insulin-induced suppression of EGP from basal and in insulin-stimulated peripheral glucose utilization, respectively. These results are in agreement with our previous findings (58). With salicylate co-infusion, IH-induced insulin resistance in both liver and periphery was completely prevented.

Consistent with the effect of salicylate to prevent IH-induced decrease in peripheral glucose utilization during the clamp, salicylate co-infusion prevented IH-induced decrease in IκBα content in parallel with normalization of IH-induced increase in serine 307 phosphorylation of IRS-1 in the skeletal muscle. In the adipose tissue, IH infusion did not alter IκBα content or serine 307 phosphorylation of IRS-1. Although FFA have been shown to decrease glucose uptake in association with the activation of IKKβ and increased serine 307 phosphorylation of IRS-1 in 3T3-L1 adipocytes (120), the differences in the experimental models used likely explains the discrepancies in the results. It is noteworthy that, in Kim's study (233), short-term lipid infusion did not alter insulin-stimulated glucose uptake in white adipose tissue, which is in keeping with our findings. These results indicate that liver and skeletal muscle were selectively targeted by salicylate and that prevention of FFA-induced peripheral insulin resistance by salicylate is associated with the reversal of serine phosphorylation of IRS-1 in skeletal muscle.

As far as we know, this study is the first to show that salicylate is effective in restoring hepatic insulin sensitivity in a model of acute FFA elevation. While recent studies (187;231) have demonstrated that IKKβ deficiency is protective against insulin

resistance caused by high-fat feeding or genetically-induced obesity in rodents, there is an important distinction in the experimental model of insulin resistance used between these studies and our present study. High-fat feeding and genetically-induced obesity usually result in chronically elevated FFA levels, which may induce insulin resistance via a different mechanism than acute FFA elevation. For instance, in high-fat-fed animals, inhibition of NFκB appears to be sufficient to restore insulin sensitivity without inhibition of IKKβ activity (232), suggesting that insulin resistance is secondary to transcriptional effects of NFκB, which may not be true after short-term lipid infusion. Furthermore, these models are associated with increased release of other adipocyte-derived factors than FFA that are known to induce insulin resistance, such as pro-inflammatory cytokines TNFα, IL-1β, and IL-6 (241;348-351). Accordingly, in these studies, insulin resistance cannot be attributed only to FFA. Instead, the model of insulin resistance we used, i.e. short-term intravenous lipid infusion, is similar to that used by Kim et al. (233) in their investigation of salicylate's effect on fat-induced insulin resistance in rat skeletal muscle. In this study, however, a high rate of insulin infusion (60 pmol//kg/min) used during the clamp completely suppressed EGP in all groups, potentially masking the effect of salicylate to prevent FFA-induced hepatic insulin resistance. By using much lower rate of insulin infusion (30 pmol/kg/min) and maintaining plasma glucose specific activity constant to avoid underestimation of glucose production during the clamp (352), we were able to reveal the ability of salicylate to prevent hepatic insulin resistance caused by FFA.

Numerous recent studies (54;231-233) have implicated activation of the IKKβ/ NFκB pathway in fat-induced insulin resistance, although it is not clear whether insulin

resistance is due to the direct inhibitory effect of IKKβ on insulin signaling or its indirect effect to promote NFκB-medicated production of pro-inflammatory cytokines. Since short-term fat infusion can activate IKKβ (54;233) and IKKβ can directly phosphorylate serine residues of IRS-1 (119), which prevents tyrosine phosphorylation of IRS and thus insulin signaling (353), it is likely that salicylate prevents fat-induced hepatic insulin resistance directly through prevention of IKKβ activity. In support of this notion, we found that salicylate completely prevents IH-induced decrease in hepatic IκBα content, which indicates increased IKKβ activity as IκBα upon phosphorylation by IKKβ is targeted for degradation. Interestingly, salicylate alone had no effect on hepatic IκBα content in the absence of elevated FFA, presumably because the dose of salicylate we used was not sufficiently high to suppress IKKβ activity beyond its baseline levels. Similarly, we have found in Study 2 that treatment with an antioxidant NAC, which prevented FFA-induced hepatic insulin resistance, did not, on its own, decrease oxidative stress in rat liver.

The preventive effect of salicylate on IH-induced IKKβ activation occurred in association with prevention of IH-induced increase in serine phosphorlyation and decrease in tyrosine phosphorylation of IRS-1 in the liver (Figures 5A and 6A, respectively). While there are numerous serine residues on IRS that are associated with impaired insulin signaling, serine 307 appears to be a critical site (334). For instance, TNFα, by triggering activation of JNK1, has been shown to increase serine phosphorylation of IRS-1 at 307 site, which decreases tyrosine phosphorylation of IRS-1 and thus impedes insulin signaling (117). It was recently shown that FFA cause insulin resistance through IKKβ- and JNK1-medicated serine (307) phosphorylation of IRS-1 in

3T3-L1 adipocytes (120). Furthermore, serine 307 phosphorylation of IRS-1 caused by short-term fat infusion was associated with decreased tyrosine phosphorylation of IRS-1 and impairment of insulin signaling in rat skeletal muscle, although the serine kinase responsible was not identified (60). Our results show that salicylate also prevents IH-induced increase in serine 233 phosphorylation and decrease in tyrosine phosphorylation of IRS-2 (Figures 5B and 6B, respectively), which has been linked to insulin resistance (335). Although it is still unknown whether serine 233 phosphorylation interferes with tyrosine phosphorylation of IRS-2, our finding of concomitant decrease in tyrosine phosphorylation of IRS-2 indicates that these processes may be linked.

In addition to defects in tyrosine phosphorylation of IRS, IH infusion was found to result in reduced levels of serine 473 phosphorylated Akt, which was prevented with salicylate co-infusion. Taken together, our results suggest that the effect of salicylate to restore hepatic insulin sensitivity is closely associated with its reversal of fat-induced impairment of hepatic insulin signaling.

Although our findings suggest that high dose salicylate inhibited IKKβ-mediated IRS-1 and –2 serine phosphorylation, thereby providing an explanation for hepatic insulin resistance, we cannot exclude the possibility that salicylate also prevented IKKβ-induced transcription of various pro-inflammatory cytokines, thereby inhibiting their autocrine effect to impair hepatic insulin signaling. However, relatively short exposure to elevated FFA may preclude IKKβ-induced transcription of pro-inflammatory genes. Furthermore, while salicylate is a relatively weak inhibitor of cyclooxygenase (COX) due to a lack of acetyl group that is required for deactivation of the enzyme, it is nonetheless possible that salicylate had some effect in inhibiting COX-mediated prostaglandin $E_2$

production, which may affect insulin resistance. However, given that neither a treatment with non-steroidal anti-inflammatory drugs, which are robust inhibitors of COX, nor COX deficiency prevented insulin resistance in Fao hepatoma cells and in obese mice (187), respectively, it is unlikely that potentially inhibitory effect of salicylate on prostaglandin played a role in the present study.

In summary, the present study demonstrates that 1) treatment with high-dose sodium salicylate prevents hepatic insulin resistance caused by short-term elevation of plasma FFA and 2) the effect of salicylate to restore hepatic insulin sensitivity occurs in association with prevention of FFA-induced hepatic IKKβ activation and corresponding impairment of hepatic insulin signaling. The findings of this study suggest that IKKβ is a causal mediator of hepatic insulin resistance induced by acutely elevated plasma FFA. Therefore, hepatic IKKβ represents a potential therapeutic target to prevent or treat fat-induced hepatic insulin resistance and associated diseases.

| | Basal Period | | | | Hyperinsulinemic-Euglycemic Clamp | | | |
|---|---|---|---|---|---|---|---|---|
| | SAL | IH | IH+SS | SS | SAL | IH | IH+SS | SS |
| **Glucose (mM/l)** | 6.77±0.36 | 7.07±0.29 | 6.79±0.34 | 7.23±0.80 | 6.86±0.31 | 6.74±0.39 | 6.87±0.29 | 7.04±0.90 |
| **FFA (mEq/l)** | 0.641 ±0.052 | 1.199* ±0.080 | 1.079* ±0.096 | 0.509 ±0.030 | 0.232# ±0.044 | 0.754*# ±0.091 | 0.674*# ±0.095 | 0.136 # ±0.012 |
| **Insulin (pM)** | 152.5 ±25 | 153.0 ±33 | 178.0 ±36 | 214.0 ±34 | 776.5† ±2.0 | 750.0† ±76 | 870† ±106 | 998.0 † ±65 |

**Table.** Plasma Glucose, Free Fatty Acids, and Insulin Levels During the Basal Period and Hyperinsulinemic-Euglycemic Clamp in Wistar Rats (n=6-8/group). *: P <0.01 vs SAL and SS groups. #: P<0.01 vs basal period. †: P<0.001 vs basal period.

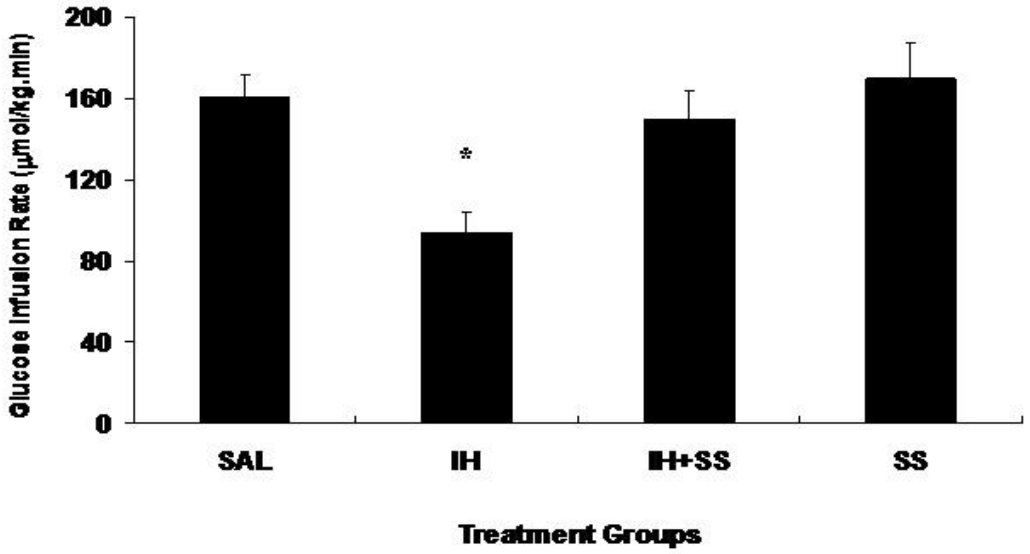



**Figure 1. Whole body insulin sensitivity during the last 30 minutes of 2-hour hyperinsulinemic-euglycemic clamp in Wistar rats (n=6-8/group).** Data are means ± SEM. SAL: Saline; IH: Intralipid plus heparin; IH+SS: Intralipid plus heparin co-infused with salicylate (0.117 mg/kg/min), SS: Salicylate alone. *: P<0.01 vs other treatment groups.

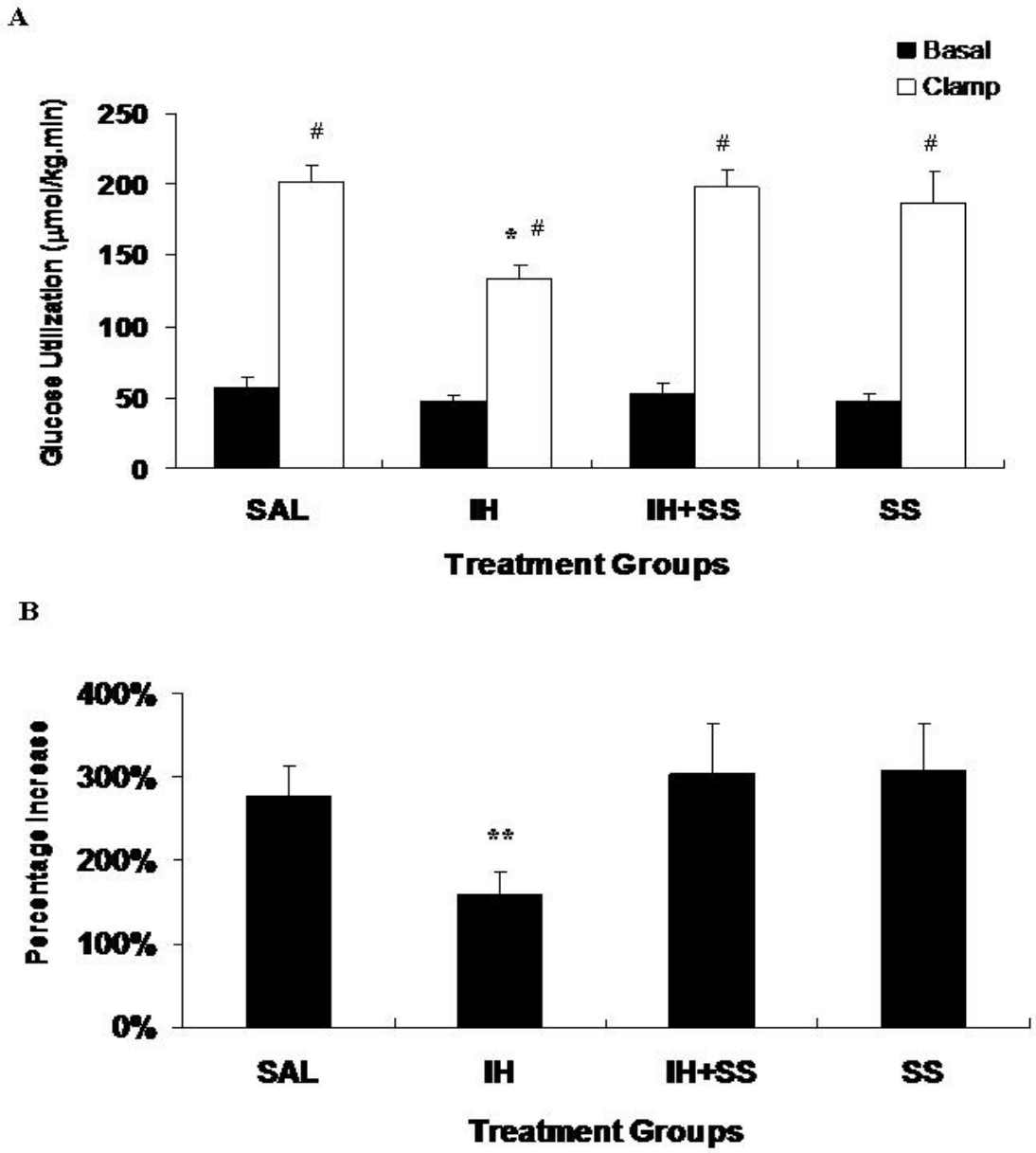



**Figure 2. Peripheral glucose utilization in rats (n=6-8/group). A:** Effect of IH and salicylate on peripheral glucose utilization during the basal period and during the last 30 minutes of 2-hour hyperinsulinemic-euglycemic clamp. **B:** Effect of IH and salicylate on insulin-induced percentageincrease in peripheral glucose utilization from basal. Data are means ± SEM. SAL: Saline; IH: Intralipid plus heparin; IH+SS: Intralipid plus heparin co-infused with salicylate (0.117 mg/kg/min), SS: Salicylate alone. *: P<0.01 vs other groups. **: P<0.05 vs other groups. #: P<0.001 vs basal period.

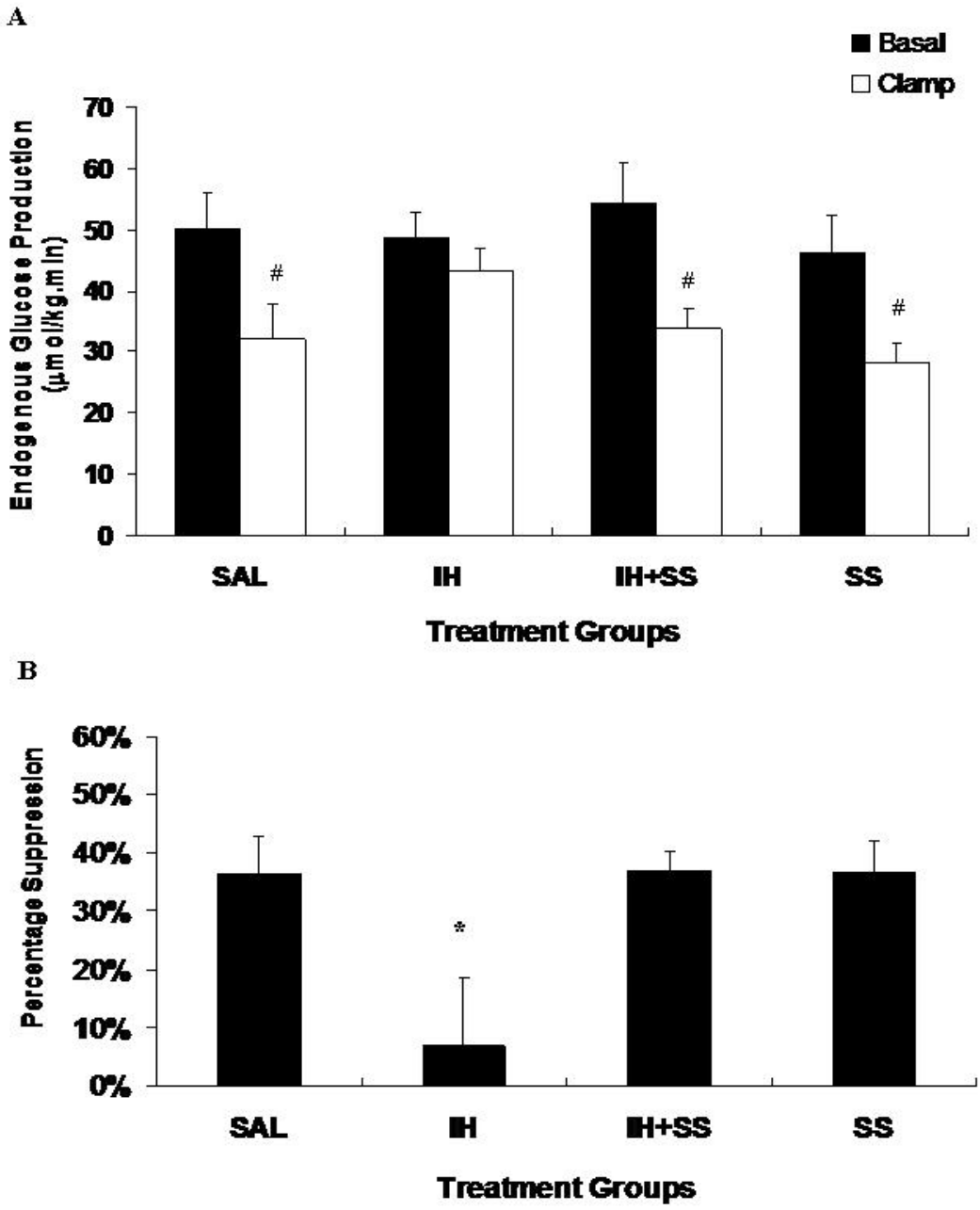
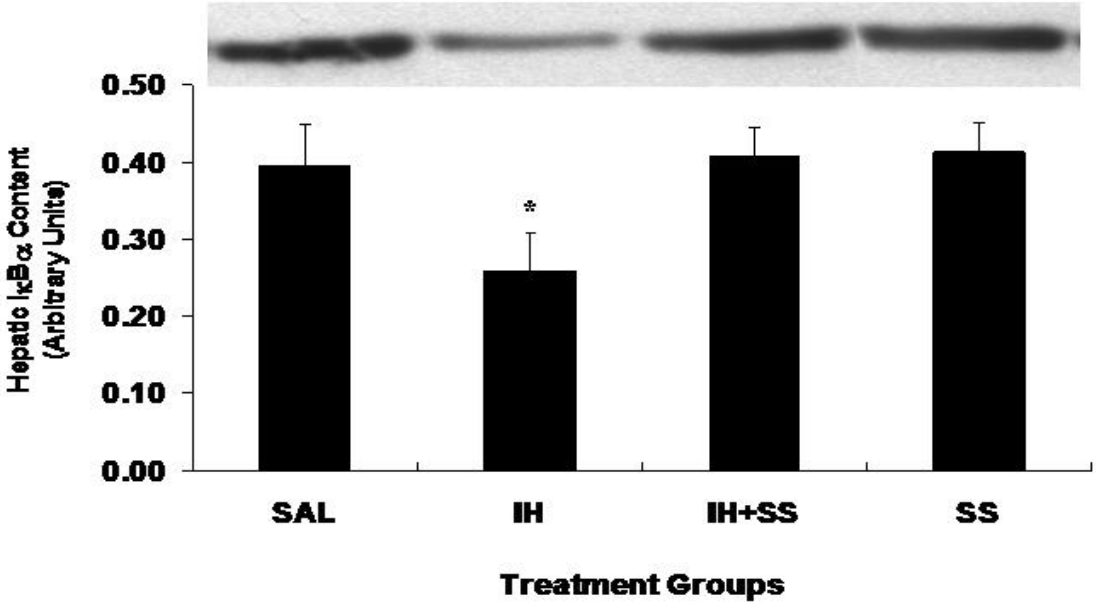
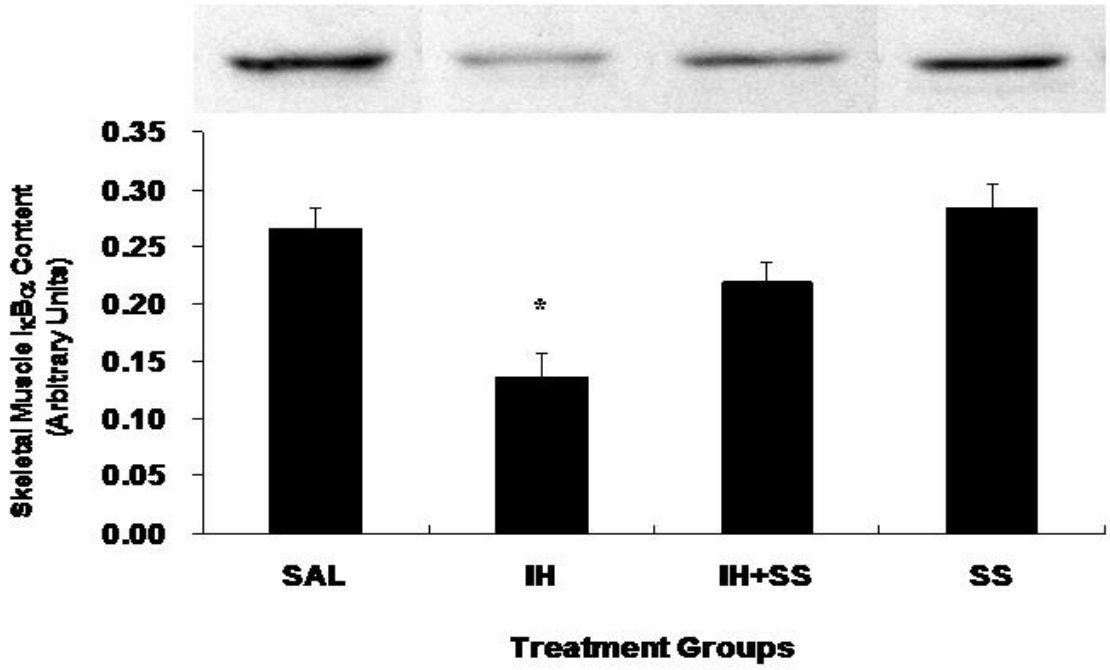



**Figure 3. Hepatic glucose production in rats (n=6-8/group).** **A**: Effect of IH and salicylate on hepatic glucose production (HGP) during the basal period and during the last 30 minutes of 2-hour hyperinsulinemic-euglycemic clamp. **B**: Effect of IH and salicylate on insulin-induced percentage suppression of hepatic glucose production from basal. Data are means ± SEM. SAL: Saline; IH: Intralipid plus heparin; IH+SS: Intralipid plus heparin co-infused with salicylate (0.117 mg/kg/min), SS: Salicylate alone. *: $P<0.05$ vs other groups. #: $P<0.01$ vs basal period.

A


0.50
0.40
0.30
0.20
0.10
0.00
Hepatic IκBα Content (Arbitrary Units)
*
SAL
IH
IH+SS
SS
Treatment Groups

B


0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Skeletal Muscle IκBα Content (Arbitrary Units)
*
SAL
IH
IH+SS
SS
Treatment Groups

C

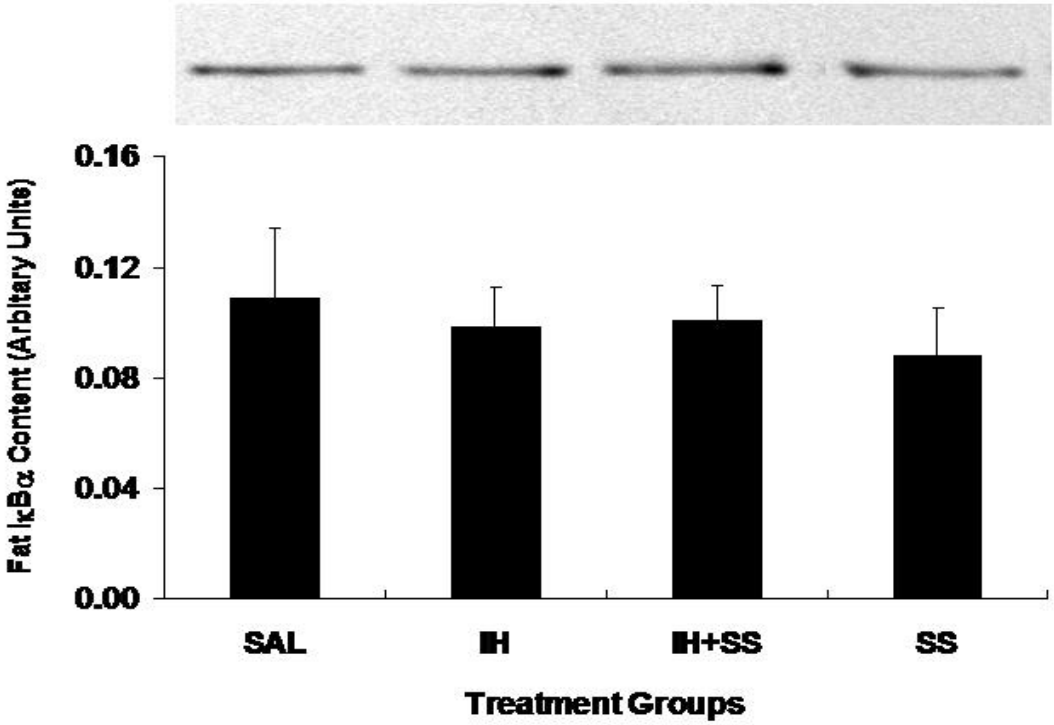
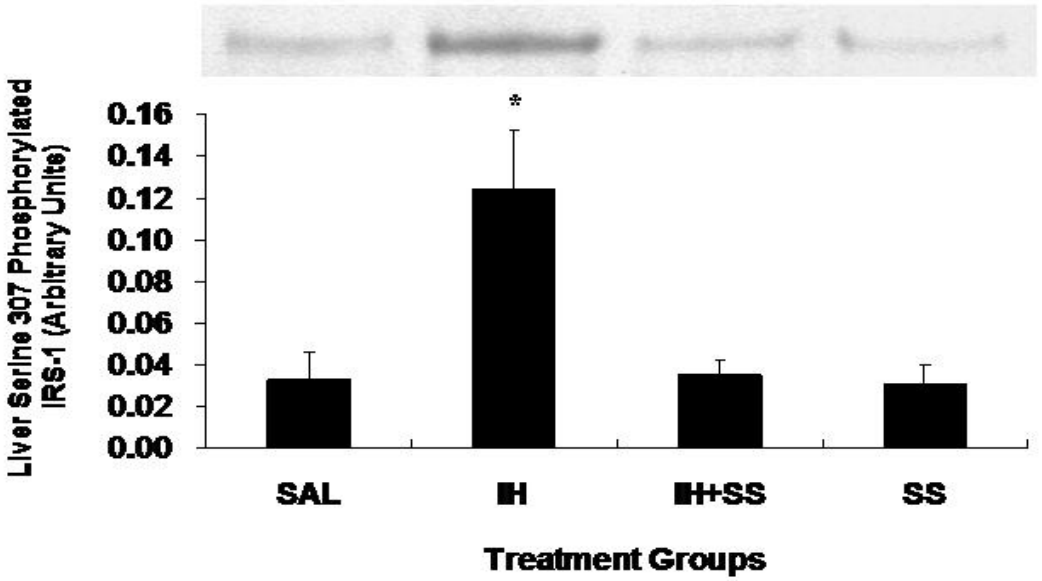
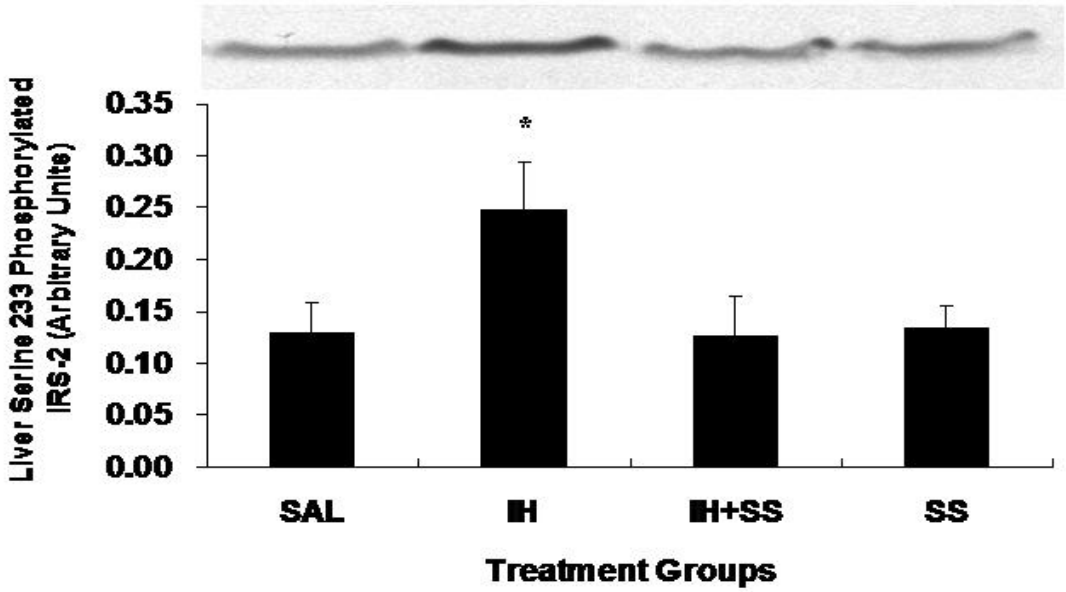



**Figure 4. IκBα content in the liver (A), skeletal muscle (B), and fat (C) following 2h hyperinsulinemic-euglycemic clamp in rats (n=6-8/group).** Data are means ± SEM. SAL: Saline; IH:Intralipid plus heparin; IH+SS: Intralipid plus heparin co-infused with salicylate (0.117 mg/kg/min), SS: Salicylate alone. *: P<0.05 vs other groups.

A


Liver Serine 307 Phosphorylated IRS-1 (Arbitrary Units)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
SAL
IH
IH+SS
SS
Treatment Groups

B


Liver Serine 233 Phosphorylated IRS-2 (Arbitrary Units)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
SAL
IH
IH+SS
SS
Treatment Groups

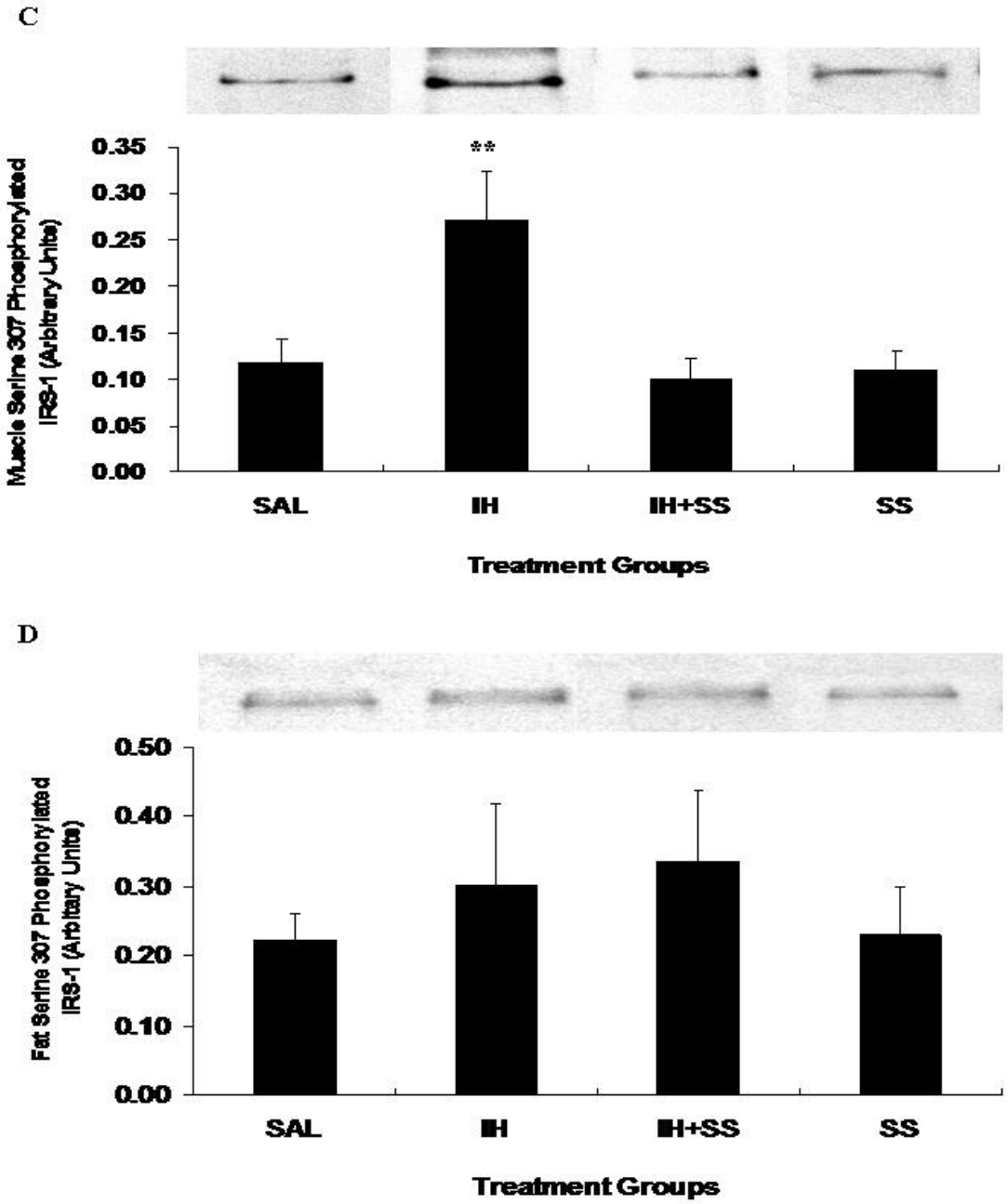



**Figure 5. Effect of IH and salicylate on serine phosphorylation of insulin receptor substrate (IRS) in the liver (A&B), skeletal muscle (C), and fat (D) following 2h hyperinsulinemic-euglycemic clamp (n=6-8/group).** A representative image of immunoblots is shown at the top of each graph. Data are means ± SEM. SAL: Saline; IH: Intralipid plus heparin; IH+SS: Intralipid plus heparin co-infused with salicylate (0.117 mg/kg/min), SS: Salicylate alone. *: P<0.05 vs other groups.

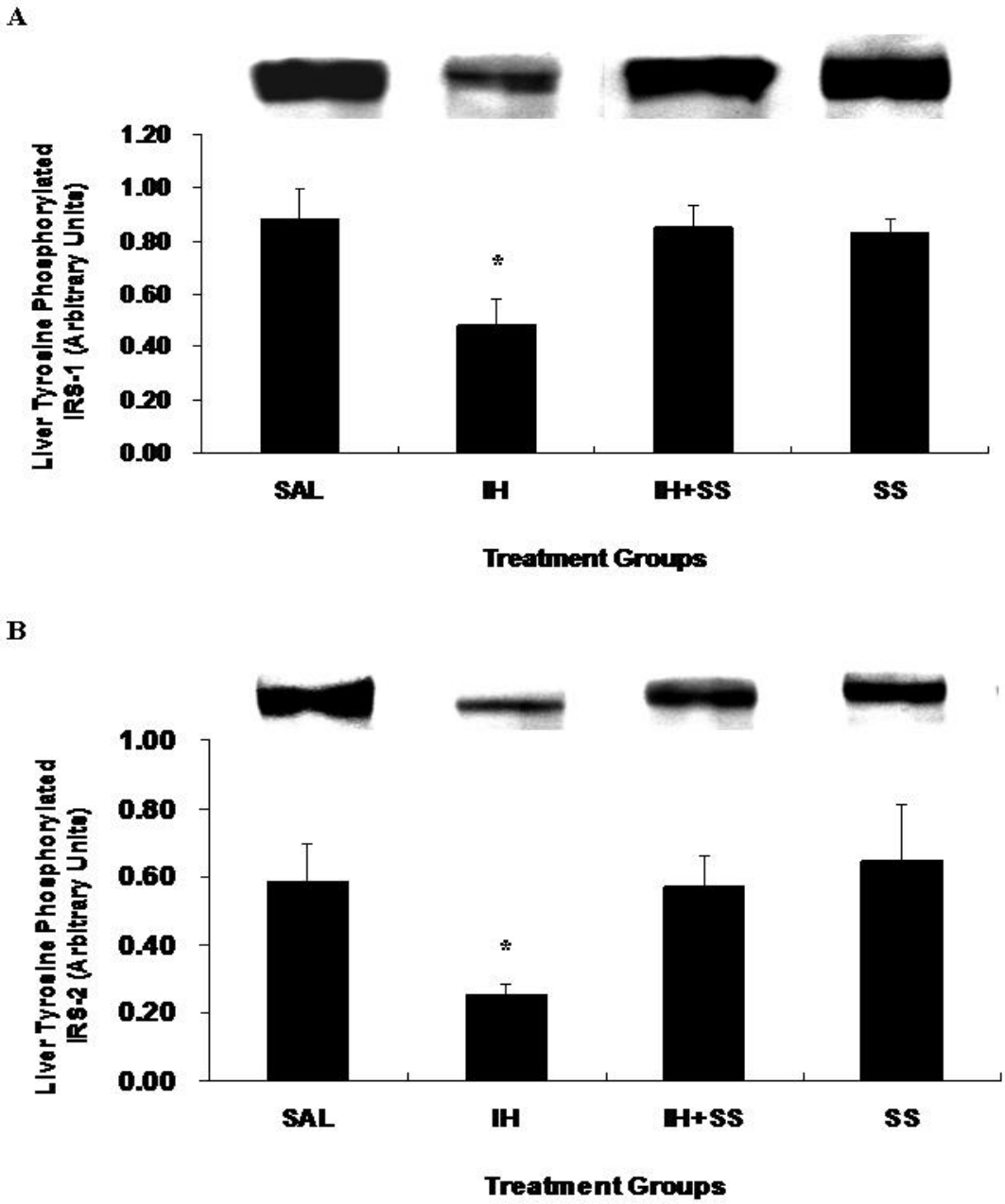



**Figure 6. Effect of IH and salicylate on tyrosine phosphorylation of insulin receptor substrate (IRS)-1 (A) and IRS-2 (B) following 2h hyperinsulinemic-euglycemic clamp in rat liver (n=6-8/group).** A representative image of immunoblots is shown at the top of each graph. Data are means ± SEM. SAL: Saline; IH: Intralipid plus heparin; IH+SS: Intralipid plus heparin co-infused with salicylate (0.117 mg/kg/min), SS: Salicylate alone. *: P<0.05 vs other groups.

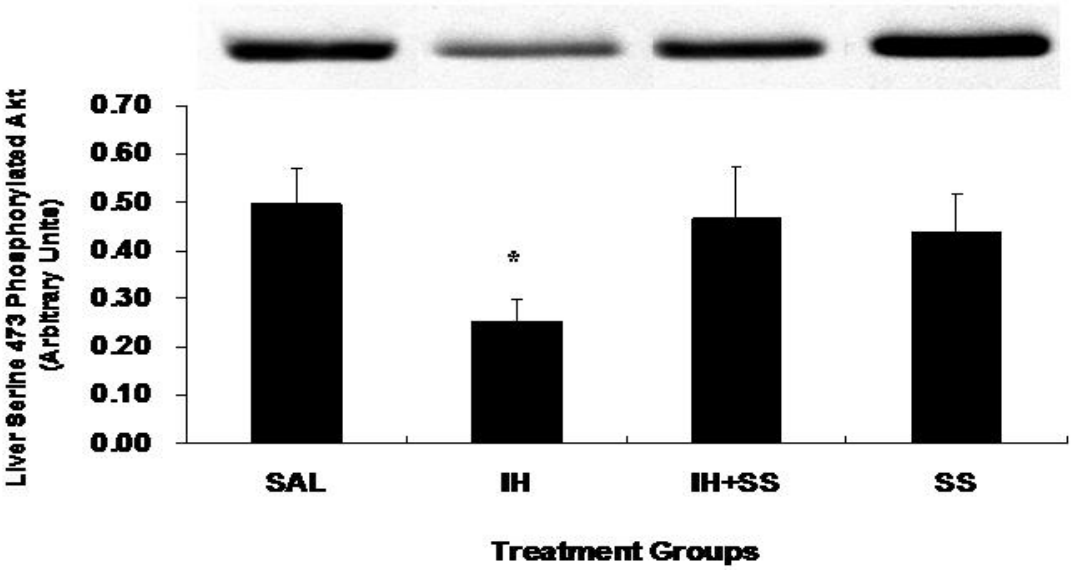



**Figure 7. Effect of IH and salicylate on serine 473 phosphorylated Akt following 2h hyperinsulinemic-euglycemic clamp in rat liver (n=6-8/group).** A representative image of immunoblots is shown at the top of each graph. Data are means ± SEM. SAL: Saline; IH: Intralipid plus heparin; IH+SS: Intralipid plus heparin co-infused with salicylate (0.117 mg/kg/min), SS: Salicylate alone. *: P<0.05 vs other groups.

# 6

# General Discussion

## *The Role of PKC-δ in the Mechanism of FFA-Induced Hepatic Insulin Resistance: a Causal Mediator or an Unrelated Marker?*

PKC has been implicated in insulin resistance both *in vitro* and *in vivo* (54;56-58;60;177;178;181). The serine/threonine kinase is capable of phosphorylating a number of specific serine/threonine residues on the insulin receptor and IRS *in vitro* (177;178). Furthermore, activation of conventional and novel PKC isoforms has been linked to FFA-induced insulin resistance in a species- and tissue-specific manner (βII and δ in human muscle; θ and ε in rodent muscle; δ and ε in rat liver) (54;56-58;60;181). For instance, the finding that PKC-θ-deficient mice are protected from muscle insulin resistance caused by a short-term lipid infusion implicated PKC-θ as a causal mediator of FFA-induced peripheral insulin resistance (57).

In the liver, PKC-δ may be a key player in FFA-induced insulin resistance. Previous studies in our laboratory (58), as well as those by others (54), have established an association between PKC-δ membrane translocation, a marker of its activation, and hepatic insulin resistance caused by a short-term lipid infusion. However, it is unknown whether PKC-δ activation is a necessary event, or merely an associated marker of, in the

mechanism of FFA-induced hepatic insulin resistance. The aim of Study 1 therefore was to examine whether an acute inhibition of liver PKC-δ protects against hepatic insulin resistance caused by short-term lipid infusion.

The results of the Study 1 show that a repeated i.p. injection of ASO against PKC-δ prevented FFA-induced hepatic insulin resistance in conjunction with a reduction of hepatic PKC-δ protein levels by approximately 50% and with prevention of FFA-induced membrane translocation of PKC-δ. The latter finding may be of particular significance as it indicates that the capacity of PKC-δ ASO to prevent FFA-induced hepatic insulin resistance was not only related to a reduction in hepatic PKC-δ protein but also to prevention of a key process involved in PKC-δ activation.

PKC-ε has been shown to mediate hepatic insulin resistance caused by a short-term high-fat diet (132;271). Not surprisingly, PKC-δ ASO did not inhibit PKC-ε expression in the liver, indicating that the ASO we used specifically targeted PKC-δ mRNA. Moreover, there was no evidence of PKC-ε membrane translocation in response to the lipid infusion. Given that lipid infusion was not associated with membrane translocation of any other PKC isoforms in the previous studies performed in our laboratory (182), PKC-δ activation appears to be specifically linked to hepatic insulin resistance caused by the short-term lipid infusion model. Different timing and duration of plasma and intracellular lipid accumulation in addition to different fat type may determine the activation of particular PKC isoform. Previous studies in our laboratory indicate that 7h lipid infusion results in modest intrahepatic accumulation of fat; however, it should be noted that fat may act directly through receptors, for instance TLR (354), to engage PKC-δ.

The finding that FFA-induced peripheral insulin resistance was not reversed by PKC-δ ASO treatment suggested that the inhibitory effect of ASO on PKC-δ protein expression was localized to the liver, although determination of PKC-δ expression in skeletal muscle and adipose tissue is required to confirm this. Based on the results of previous studies that utilized ASO *in vivo* (132;355), we expect that PKC-δ in skeletal muscle is not decreased appreciably in our study.

It is currently unknown whether PKC can directly impair insulin signaling *in vivo* via serine/threonine phosphorylation of insulin receptor and/or IRS. Several studies have demonstrated an association between PKC activation and increased serine phosphorylation or decreased tyrosine phosphorylation of IRS-1 in FFA-induced insulin resistance (57;60;132). However, one cannot conclude from the results of the studies that PKC was directly responsible for impaired insulin signaling; it is possible that PKC is an upstream mediator of intracellular signals, such as oxidative stress, that activate other serine/threonine kinases. In Study 1, PKC-δ ASO prevented IH-induced increase in serine phosphorylation of $p47^{phox}$, which is critical for activation of NADPH oxidase, suggesting that PKC-δ and NADPH oxidase-induced oxidative stress are mediators of FFA-induced hepatic insulin resistance. Further support for this notion comes from the results of the Study 2 in which the antioxidant NAC, which prevented FFA-induced hepatic insulin resistance, abolished FFA-induced oxidative stress in parallel with a reversal of FFA-induced decrease in hepatic IκBα content and phosphorylated JNK1, and impaired insulin signaling without affecting FFA-induced PKC-δ membrane translocation. These findings suggest that 1) PKC-δ activation may be an upstream event of oxidative stress and IKKβ/JNK1 in the pathway of FFA-induced hepatic insulin

resistance and 2) the serine kinase(s) responsible for increased serine phosphorylation of hepatic IRS molecules is IKKβ and/or JNK1 rather than PKC-δ. Further studies should assess whether the acute PKC-δ inhibition in the Study 1 is associated with concomitant decreases in oxidative stress, IKKB/JNK1 activity, and serine phosphorylation of IRS in the liver. Moreover, whether PKC-δ activates NADPH oxidase directly or, more likely, via another serine kinase such as p38 MAPK should be examined.

In summary, the results of the Study 1 suggest that PKC-δ plays a causal role in FFA-induced hepatic insulin resistance. However, whether PKC-δ is directly responsible for impairment of hepatic insulin signaling or it mediates activation of other downstream signals that impede insulin signaling remains to be elucidated. Future work to address this issue is warranted.

## *Is Oxidative Stress a Causal Mediator of FFA-Induced Hepatic Insulin Resistance?*

Oxidative stress is increased in insulin resistance and type 2 diabetes, and is a significant contributor to the pathogenesis of these diseases (324). Various antioxidants have been effective in improving insulin sensitivity in both humans and animals. In particular, previous studies in our laboratory have shown that antioxidants NAC and taurine prevent hepatic and peripheral insulin resistance caused by hyperglycemia (193). Although there is ample evidence for the involvement of oxidative stress in obesity-related insulin resistance, it is unknown whether oxidative stress is a causal mediator of FFA-induced hepatic insulin resistance; the objective of Study 2 was to address this issue.

In Study 2, NAC completely prevented FFA-induced hepatic insulin resistance, an effect that was replicated with another antioxidant, resveratrol. NAC also abolished FFA-induced increase in hepatic protein carbonyl content (a marker of protein oxidation thus oxidative stress) in association with complete reversal of FFA-induced increase in serine phosphorylation of IRS-1 and –2 in the liver. These findings demonstrate that oxidative stress is a causal mediator of hepatic insulin resistance caused by short-term lipid infusion and suggest that oxidative stress promotes activation of serine kinases that are capable of phosphorylating the IRS proteins. Indeed, the markers of activation of IKKβ and JNK1 were elevated following lipid infusion but were reversed with NAC co-infusion, providing evidence that these serine kinases lie downstream of oxidative stress in the pathway of FFA-induced hepatic insulin resistance.

Numerous studies have implicated IKKβ and JNK1 in insulin resistance (187;207;209;231-233). These serine kinases are capable of phosphorylating IRS on multiple sites, in particular serine 307 of IRS-1 (117;119). Accordingly, it is likely that IKKβ and JNK1 are directly responsible for serine phosphorylation of IRS-1 and-2 observed in Study 2. This notion is consistent with the results of Study 3, which showed that inhibition of IKKβ by salicylate is associated with prevention of FFA-induced decrease in tyrosine phosphorylation and increase in serine phosphorylation of IRS-1 and –2 in the rat liver. Although PKC is also a serine/threonine kinase capable of phosphorylating IRS (177;178), its direct involvement is not in keeping with our finding that serine phosphorylation of IRS-1 and –2 were reversed by NAC despite its inability to reverse FFA-induced membrane translocation of PKC-δ.

One of the potential ways by which FFA can increase oxidative stress is via PKC-dependent activation of NADPH oxidase (210-212). In Study 2, lipid infusion increased membrane content of serine 345 phosphorylated $p47^{phox}$ subunit of the NADPH oxidase and oxidative stress, as indicated by increased protein carbonyl levels, in the liver. NAC co-infusion not only abolished the FFA-induced increase in hepatic protein carbonyl levels but also prevented FFA-induced increase in the serine phosphorylation of $p47^{phox}$. Given that NAC did not prevent FFA-induced PKC-δ membrane translocation and increase in phosphorylated p38 MAPK, these findings raise a couple of important questions. First, is PKC-δ directly responsible for serine 345 phosphorylation of $p47^{phox}$, which is critical for activation of NADPH oxidase? PKC-δ is capable of phosphorylating multiple sites of $p47^{phox}$ (213); however, whether it can phosphorylate serine 345 of $p47^{phox}$ is unknown . It is more likely that PKC-δ mediates serine phosphorylation of $p47^{phox}$ indirectly, potentially via another serine kinase, p38 MAPK, which can be activated by PKC-δ (82;328;329). Our findings in Studies 1 and 2 that IH infusion causes PKC-δ membrane translocation, increased phosphorylation of p38 MAPK, and increased serine phosphorylation of $p47^{phox}$ while inhibition of PKC-δ using PKC-δ ASO prevents IH-induced serine phosphorylation of $p47^{phox}$ are consistent with this possibility. Second, given that NAC was not able to reverse IH-induced increase in phosphorylated p38 MAPK, how is NAC able to reverse FFA-induced increase in serine phosphorylation of $p47^{phox}$? One possibility is that antioxidants have a direct inhibitory effect, or oxidative stress has direct stimulatory effect, on $p47^{phox}$ serine phosphorylation, although this has not been clearly shown. However, a recent study showed that NAC decreases expression and membrane translocation of cytosolic subunits of NADPH oxidase in the cardiac

muscle of streptozotocin-induced diabetic rat (339). Similarly, α-lipoic acid reduced kidney expression of NADPH oxidase subunits $p22^{phox}$ and $p47^{phox}$ in streptozotocin-induced diabetic rats (356) while vitamin C and vitamin E decreased activation of vascular NADPH oxidase in spontaneously hypertensive rats (357). Further studies are required to elucidate the relationship between PKC-δ and NADPH oxidase in FFA-induced hepatic insulin resistance.

In summary, the results of Study 2 demonstrate that oxidative stress is causally involved in FFA-induced hepatic insulin resistance and suggest that oxidative stress mediates activation of downstream serine kinases IKKβ and JNK1 that impair hepatic insulin signaling. It is interesting to note that NAC did not prevent FFA-induced membrane translocation of PKC-δ and increase in phosphorylated p38 MAPK in the liver, indicating that PKC-δ and p38 MAPK may lie upstream of oxidative stress or are unrelated in the pathway of FFA-induced hepatic insulin resistance. The results of Study 1 support the former possibility, at least for PKC-δ, as they indicate that PKC-δ plays a causal role in the process. Future studies should examine the role of p38 MAPK.

## *Inflammatory and Stress-Activated Pathways in FFA-Induced Hepatic Insulin Resistance*

Obesity is now recognized as a state of chronic, low-grade inflammation. Many studies have causally linked the IKKβ/NFκB inflammatory pathway with obesity-related insulin resistance. Salicylate, an IKKβ inhibitor, is effective in reversing obesity and FFA-induced insulin resistance in rodent skeletal muscle (187;233). Moreover, mice with

hepatic over-expression of IKKβ develop systemic insulin resistance (232), which is reversed with salicylate treatment, while hepatocyte-specific IKKβ-null mice are protected from diet- and obesity-induced insulin resistance (231). These findings point to IKKβ as a key causal mediator of insulin resistance associated with obesity and reveal IKKβ as a potential target for treatment.

However, it is unknown whether IKKβ is a causal mediator of hepatic insulin resistance caused by elevated plasma FFA *per se*. Although Boden et al. demonstrated an association between reduced hepatic IκBα content (a marker of IKKβ activation as IκBα, upon phosphorylation by IKKβ, is targeted for degradation) and hepatic insulin resistance caused by acutely elevated FFA in rats (54), the relationship may be correlational rather than causal. Furthermore, the obesity models used in the aforementioned studies are generally associated with chronically elevated circulating FFA, which may cause hepatic insulin resistance via different mechanisms. Lastly, in the Kim's study (233), which utilized a model of acute lipid infusion, the high insulin dose used during the hyperinsulinemic-euglycemic clamp resulted in complete suppression of HGP in all groups, thereby masking potential effect of salicylate to reverse FFA-induced decrease in insulin-stimulated suppression of HGP. Therefore, the purpose of Study 3 was to investigate whether salicylate prevents hepatic insulin resistance caused by acutely elevated FFA *in vivo*.

In Study 3, we found that salicylate prevents FFA-induced decrease in insulin-stimulated suppression of HGP during the hyperinsulinemic-euglycemic clamp in association with reversal of FFA-induced decrease in hepatic IκBα content. This finding provides evidence that salicylate was effective in preventing hepatic IKKβ activation

caused by lipid infusion. Salicylate was also able to prevent FFA-induced impairment of a key insulin signaling step, namely tyrosine phosphorlyation of IRS-1 and –2 in the liver. It has been demonstrated that serine phosphorlyation of IRS on specific residues impairs the ability of insulin receptor to phosphorylate tyrosine sites of IRS and thus insulin signaling (118;358). Our results are consistent with this notion as lipid infusion was found to decrease tyrosine phosphorylation of IRS-1 and –2 in parallel with an increase in serine phosphorylation in the liver. This impairment of IRS-1 and -2 tyrosine phosphorylation by FFA was completely reversed by salicylate, indicating that IKKβ may be the serine kinase responsible for the defect, although the involvement of other serine/threonine kinases such as PKC and JNK cannot be ruled out. Moreover, whether PKC-δ activity and oxidative stress are affected by salicylate treatment should be examined. If these factors lie upstream of IKKβ in the pathway of FFA-induced hepatic insulin resistance, as we hypothesized, salicylate treatment would not be expected to alter their increase induced by FFA.

We also cannot exclude the possibility that the antagonistic effects of IKKβ activation on hepatic insulin signaling are indirect. When IKKβ is activated, it facilitates degradation of IκBα, thereby liberating NFκB to initiate transcription of various pro-inflammatory mediators, such as cytokines TNFα and IL-6, which may disrupt insulin signaling in an autocrine and feed-forward manner (232). Since IL-6 typically is not know to activate serine kinase, it is likely not a direct contributor to FFA-induced serine phosphorylation of IRS-1 and –2 in our study, although it can interfere with IRS tyrosine phosphorylation (45) and mediating IRS degradation (253) via SOCS proteins. TNFα, on the other hand, can activate IKKβ and JNK1(122;244;246), both of which are capable of

phosphorylating serine 307 of IRS-1 (117;119). In this regard, future studies should measure hepatic TNFα and IL-6 expression.

The results of Study 2 showed that lipid infusion causes hepatic insulin resistance in association with increased phosphorylated JNK1 in the liver, suggesting that JNK1 is a mediator of FFA-induced hepatic insulin resistance. Further support for this comes from a recent study, which showed that exposure of primary mouse hepatocytes to saturated fatty acids causes insulin resistance in parallel with activation of JNK1 (209). One potential strategy to examine whether JNK1 is causally involved in FFA-induced hepatic insulin resistance would be to utilize a short-term lipid infusion model with or without co-infusion of JNK1 inhibitor, for instance SP600125, in rats.

# 7

# Summary and Conclusions

## 7.1 Summary of Each Study in the Thesis

PKC-δ has been implicated in FFA-induced hepatic insulin resistance; however, it is unknown if its activation plays a causal role in the process. In Study 1, antisense oligonucleotide (ASO) against PKC-δ was administered intraperitoneally to rats in order to acutely inhibit liver PKC-δ protein expression. ASO treatment specifically decreased hepatic PKC-δ expression, as PKC-ε levels were unaltered. ASO-induced inhibition of PKC-δ expression resulted in prevention of hepatic insulin resistance caused by a short-term lipid infusion without affecting peripheral insulin resistance.

In Study 2, we wished to examine whether oxidative stress is a causal mediator of FFA-induced hepatic insulin resistance. Co-infusion of the antioxidant NAC with Intralipid plus heparin (IH) abolished IH-induced oxidative stress and reversed IH-induced increase in a marker of NADPH oxidase activity. NAC also prevented fat-induced hepatic insulin resistance in rats in parallel with the reversal of increased markers of IKKβ and JNK1activity and of increased serine phosphorylation of IRS-1 and –2 in the liver, without affecting hepatic PKC-δ membrane translocation and increase in phosphorylated p38 MAPK.

The role of IKKβ inflammatory pathway in hepatic insulin resistance caused by acutely elevated FFA is unclear. In Study 3, salicylate treatment prevented hepatic insulin resistance caused by a short-term IH infusion in rats. Salicylate also prevented FFA-induced decrease in hepatic IκBα content. This finding was associated with salicylate-mediated reversal of 1) increase in serine phosphorylation of IRS-1 and –2; 2) decrease in tyrosine phosphorylation; and 3) decrease in serine 473 Akt caused by acutely elevated FFA.

## 7.2 General Summary

By using in vivo lipid infusion model, the studies in this thesis have demonstrated that suppression of PKC-δ, abolishment of oxidative stress, or inhibition of IKKβ prevents hepatic insulin resistance caused by a short-term (7h) lipid infusion that results in acutely elevated circulating FFA.

## 7.3 General Conclusion

The results of the studies in this thesis together suggest that PKC-δ, oxidative stress, and IKKβ are causal mediators of FFA-induced hepatic insulin resistance. Furthermore, based on the results, it is proposed that FFA cause impairment of hepatic insulin action via these factors in a sequential manner (Figure 1 in the Introduction). Therefore, PKC-δ, oxidative stress, and IKKβ represent potential targets through nutrition (e.g. dietary antioxidants) and pharmacological agents (i.e. PKC inhibitors,

antioxidants, anti-inflammatory agents) to prevent or treat insulin resistance and associated diseases.

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# Limitations of the Studies

1. The short-term lipid infusion used in our studies is a convenient and useful model to acutely raise plasma FFA *in vivo*. Important distinctions exist between the short-term lipid infusion model and more chronic models, such as diet-induced obesity and high-fat feeding. Short-term lipid infusion allows examination of the effect of acutely elevated FFA *per se* on insulin action without potentially confounding influences of increased circulating factors found in rodent models of obesity. The duration of FFA elevation during short-term lipid infusion may be too short to cause substantial TG accumulation in the liver and skeletal muscle, which correlates highly with insulin resistance, although DAG was found to increase in the liver following a short-term lipid infusion (54). High-fat feeding is associated with increased lipid load via chylomicrons and hepatic TG accumulation, and may be associated with increased plasma FFA; thus high-fat feeding is a useful model to study chronic effects of fat overload. However, high-fat feeding can stimulate vagal afference, which could indirectly affect glucose production and can increase adipose tissue mass, which can alter the release of various adipocyte-derived substances that regulate insulin sensitivity. It should be clarified that, with neither lipid infusion nor high fat diet are FFA concentration in the target tissue known because of the action of tissue lipoprotein on plasma TG.

2. In all studies in this thesis, Intralipid plus heparin was infused intravenously to elevate plasma FFA levels in rats. Intralipid is composed mostly of polyunsaturated fatty acids whereas the most abundant fatty acids in circulation are oleate (monounsaturated fatty acid) and palmitate (saturated fatty acid); thus, the types of fat contained in Intralipid do not reflect the composition of circulating fatty acids in the body. However, it should be noted that preliminary studies with different types of fat show that at 7h all fats have the same effect on hepatic insulin sensitivity. Intralipid is a standardized and safe triglyceride emulsion that is commercially prepared and sterile. It is also commonly used in humans and animals, and therefore its use confers a number of technical advantages.

3. In Studies 1 and 3, new heparin used may have contributed to lower levels of plasma FFA achieved with Intralipid infusion than those of Study 2. This may explain the finding that, in Studies 1 and 3, IH infusion did not increase basal HGP or decrease insulin clearance (indicated by higher insulin levels than saline infusion during the clamp) as it did in Study 2, as rats used in Studies 1 and 3 may have been more insulin sensitive and more resistant to breakdown of hepatic glucose autoregulation compared to rats in Study 2. This observation raises an interesting possibility that hepatic glucose autoregulation and insulin clearance are not only related to the duration of fasting but also to the degree of insulin resistance.

# 9

# Future Directions

1. The studies in this thesis have shed light on the roles of oxidative stress and serine/threonine kinases in the mechanism of FFA-induced hepatic insulin resistance. The results of these studies suggest that PKC-δ activation is a causal event in the process; however, it is unknown whether PKC-δ directly impairs hepatic insulin signaling or is indirectly involved by activating other serine kinases via oxidative stress. In this regard, future work should examine whether the inhibition of PKC-δ using ASO abolishes oxidative stress and prevents activation of other serine kinases, such as IKKβ and JNK1. This would reveal whether oxidative stress and/or serine kinases participate in FFA-induced hepatic insulin resistance in a PKC-δ-dependent manner.

2. Similarly, we should determine whether, in the Study 3, salicylate alters FFA-induced PKC-δ activation and oxidative stress in order to test the hypothesis that the sequence of FFA-induced hepatic insulin resistance is PKC-δ → p38 MAPK → oxidative stress → IKKβ/JNK1 → impaired insulin signaling. If PKC-δ activation and oxidative stress indeed occur upstream of IKKβ, these parameters would be expected to remain unchanged by salicylate-induced inhibition of IKKβ. Moreover, since there is no

evidence for causal roles of p38 MAPK or NADPH oxidase, studies involving inhibitors of p38 MAPK (SB239063) and NADPH oxidase (apocynin) should be performed to further substantiate this pathway.

3. Evidence indicates that JNK1 plays an important role in insulin resistance (207-209;359). The results from Study 2 show that FFA cause hepatic insulin resistance in association with JNK1 activation in the liver. However, the nature of its contribution to FFA-induced hepatic insulin resistance is unclear. By using JNK1 inhibitor, SB239063, in our short-term lipid infusion model, future investigation should examine whether JNK1 is a causal mediator of hepatic insulin resistance caused by acutely elevated FFA.

4. The mechanism of hepatic insulin resistance caused by chronically elevated circulating FFA may be different than that caused by an acute FFA elevation. For instance, chronic elevation of FFA may activate a different PKC isoform than acute FFA elevation. The finding that high-fat feeding leads to activation of PKC-ε is consistent with this notion (131;132). Furthermore, chronic elevation of FFA may alter expression of gluconeogenic enzymes, such as PEPCK and G6Pase, and increase expression of gluconeogenic transcriptional regulators, FOXO-1 and PGC1α, via oxidative stress. Thus, future studies should investigate, by utilizing a prolonged (i.e.48 hours) lipid infusion with or without inhibitors, whether hepatic insulin resistance caused by chronically elevated FFA involve different players than those activated by acutely elevated FFA. In particular, it would be interesting to examine the potential role of p38 MAPK in the chronic model of FFA-induced hepatic insulin resistance (perhaps by using its specific

inhibitor, SB202190, *in vivo*), as prolonged exposure of primary hepatocytes to FFA causes insulin resistance via p38 MAPK (360) and p38 MAPK mediates the FFA-induced increase in the expression of key gluconeogenic enzymes and transcription regulators (361).

5. As mentioned in the previous section, we observed in Studies 1 and 3 that lower plasma FFA elevation than in Study 2 is associated with a lack of lipid-induced increase in basal HGP and of defect in insulin clearance we noted in Study 2. Thus, the potential relationship between elevated basal HGP and impaired insulin clearance, and hepatic insulin resistance is another potential topic for future investigation. In particular, it would be interesting to examine whether phosphorylation of insulin receptor and/or carcinoembryonic antigen-related cell adhesion molecule, which is critical for hepatic insulin clearance, is impaired in fat-induced hepatic insulin resistance.

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