LXR Acts as a Differentiator in the Regulation of FAS and G6PDH Gene Expression Under Insulin Resistant Conditions

Jaafar Hachem
Western Michigan University, jahachem@hotmail.com

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LXR ACTS AS A DIFFERENTIATOR IN THE REGULATION OF FAS AND G6PDH GENE EXPRESSION UNDER INSULIN RESISTANT CONDITIONS

by
Jaafar Hachem

A dissertation submitted to the Graduate College
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
Chemistry
Western Michigan University
December 2021

Doctoral Committee:
Susan R. Stapleton, Ph.D., Chair
David Huffman, Ph.D.
Ekk Sinn, Ph.D.
Pamela Hoppe, Ph.D.
LXR ACTS AS A DIFFERENTIATOR IN THE REGULATION OF FAS AND G6PDH GENE EXPRESSION UNDER INSULIN RESISTANT CONDITIONS

Jaafar Hachem, Ph.D.
Western Michigan University, 2021

Diabetes is a chronic disease that affects 10 percent of the world’s population and causes more than 1.5 million deaths a year and billions of dollars in associated health care cost. It can lead to very serious complications such as renal failure, liver cirrhosis, heart attack, and vision loss. The most common type of diabetes is type 2 diabetes. Type 2 diabetes arises when blood glucose levels remain chronically high due to insulin resistance. The reason for this elevation is due to the failure of insulin to allow tissues to uptake glucose causing problems in subsequent metabolic pathways. Over the years, it has been shown that insulin regulates key enzymes in both carbohydrate and fat metabolism. The insulin regulation of these enzymes occurs primarily via the phosphoinositol-3-kinase / protein kinase B (PI3K/Akt) pathway. Once glucose is transported into the cells, it is converted into glucose-6-phosphate and can enter metabolic pathways such as the pentose phosphate pathway, and fatty acid synthesis which are regulated by the key enzymes glucose-6-phosphate dehydrogenase (G6PDH), and fatty acid synthase (FAS), respectively. Previously, we established an insulin resistance model using glucosamine in primary rat hepatocytes and reported that under glucosamine induced insulin resistance, the insulin induction of G6PDH expression was suppressed, while there was no effect on the insulin induction of FAS expression. The mechanism of this differential regulation, however, is unknown. We also previously reported that the insulin induction of sterol regulatory element binding protein 1c (SREBP1c), a key transcription factor in the insulin induction of both G6PDH and FAS was suppressed under insulin resistant conditions suggesting that it was not involved in
the differential mechanism by insulin under insulin resistant conditions. In this study, we investigated the role of LXR, another important transcription factor in the regulation of metabolic genes by using its agonist TO901317. TO901317 had no impact on the insulin induction of G6PDH under normal or insulin resistant conditions. The effect of the LXR agonist on SREBP1c was muted in the presence of glucosamine however the LXR agonist’s effect on FAS expression was unaltered when glucosamine was present. LXR agonist did not have an effect on the insulin induced suppression of an inhibitor of SREPB1c processing, INSIG2 expression under normal or insulin resistant conditions. Taken together, these data strongly suggest that LXR acts as a differentiator in the regulation of FAS and G6PDH under glucosamine induced insulin resistant conditions.
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Jaafar Hachem
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<tr>
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<tr>
<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
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<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AMPL</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
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<td>ASP</td>
<td>Acylation Stimulating Protein</td>
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<td>Adenosine Triphosphate</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CCD</td>
<td>Charged Coupled Device</td>
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<td>CDC</td>
<td>Centers for Disease Control</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>Carbohydrate Response Element Binding Protein</td>
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<td>Coat Protein Complex II</td>
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<td>DMSO</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA-PK</td>
<td>DNA-Dependent Protein Kinase</td>
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<td>Ethylene Glycol-Bis (β-Aminoethyl Ether)-N,N,N′,N′-Tetraacetic Acid</td>
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<td>Endothelial Nitric Oxide Synthase</td>
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<td>Endoplasmic Reticulum</td>
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<td>Glucose Transporter 4</td>
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<tr>
<td>GSK</td>
<td>Glycogen Synthase Kinase</td>
</tr>
<tr>
<td>HBP</td>
<td>Hexosamine Biosynthetic Pathway</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-Piperazinethanesulfonic Acid</td>
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<td>3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A Reductase</td>
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<td>I</td>
<td>Insulin</td>
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<tr>
<td>INSIG</td>
<td>Insulin Induced Gene</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin Receptor</td>
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<td>Insulin Receptor Substrate-1</td>
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<tr>
<td>IRS-2</td>
<td>Insulin Receptor Substrate-2</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-Terminal Kinase</td>
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<td>Liver X Receptor</td>
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<td>LXRE</td>
<td>Liver X Receptor Element</td>
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<td>MAPK</td>
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<td>Mitogen-Activated Protein Kinase Kinase 4</td>
</tr>
<tr>
<td>MKK7</td>
<td>Mitogen-Activated Protein Kinase Kinase 7</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian Target of Rapamycin</td>
</tr>
<tr>
<td>mTORC</td>
<td>mTORM Complex</td>
</tr>
<tr>
<td>NA</td>
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<tr>
<td>NAFLD</td>
<td>Nonalcoholic Fatty Liver Disease</td>
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<td>NAPDH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
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<td>NF-Y</td>
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</tr>
<tr>
<td>NOX</td>
<td>NAPDH Oxidase</td>
</tr>
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<td>OGT</td>
<td>O-Linked N-Acetylglucosamine Transferase</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PDX-1</td>
<td>Insulin Promoter Factor</td>
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<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate Carboxykinase</td>
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<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIP2</td>
<td>Phosphatidylinositol 4, 5-Bisphosphate</td>
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<td>Phosphatidylinositol 3,4, 5-Trisphosphate</td>
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<td>PP1</td>
<td>Protein Phosphatase 1</td>
</tr>
<tr>
<td>PP2</td>
<td>Protein Phosphatase 2</td>
</tr>
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<td>PPP</td>
<td>Pentose Phosphate Pathway</td>
</tr>
<tr>
<td>PTP-1B</td>
<td>Protein Tyrosine Phosphatase 1B</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP Cleavage-Activating Protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
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<tr>
<td>Ser</td>
<td>Serine</td>
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<td>Sodium-Glucose Glutonporter-2</td>
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<td>Specificity Protein 1</td>
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<td>SRE</td>
<td>Sterol Regulatory Element</td>
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<td>Full Form</td>
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<td>-----------------------------------------------</td>
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<tr>
<td>SREBP1c</td>
<td>Sterol Regulatory Element Binding Protein 1c</td>
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<td>Streptozotocin</td>
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<td>T</td>
<td>TO9013137</td>
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<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
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<tr>
<td>TG</td>
<td>Triglyceride</td>
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<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous Sclerosis Complex</td>
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<tr>
<td>Tyr</td>
<td>Tyrosine</td>
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<tr>
<td>USF</td>
<td>Upstream Stimulatory Factor 1</td>
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Chapter 1

Introduction

1.1 Diabetes, Complications, and Impact

Diabetes is a disease that occurs when there is a chronically high amount of glucose in the blood, due to either a lack of insulin which leads to type 1 diabetes, or insulin resistance which leads to type 2 diabetes or a combination of both (1). Insulin is a hormone secreted by the beta cells of the pancreas that allow the cells to use excess glucose in the blood to maintain glucose homeostasis. Ninety percent of diagnosed diabetes cases are type 2 resulting from insulin resistance (2), highlighting the importance of investigating the mechanism by which insulin resistance evolves.

According to the Centers for Disease Control (CDC), in 2018 in the US, 34.2 million people have been diagnosed with diabetes and 88 million have pre-diabetes which equates to 11 and 34 percent of the US population, respectively. As of 2014, an estimated 422 million people or 8.5 percent of the world population were diagnosed with diabetes (3, 4). In the US, the number of people diagnosed with diabetes doubled in the last 20 years while a 4-fold increase was observed worldwide from 1980 to 2014 (3, 4). The number of deaths in the US in 2017 caused by diabetes was 270,702 and 1.5 million worldwide making it the 7th leading cause of death (3, 4). Diabetes if not controlled can lead to life threatening damage in the heart, blood vessels, eyes, kidneys, and nerves (3, 4). Medical care for the disease including doctor and hospital visits and medication have risen dramatically over the years (3). The cost of diabetes in the US including for the over 16 million people who visited the emergency room reached 327 billion dollars in 2017 according to the CDC and 825 billion dollars per year in the world.
according to the World Health Organization (3, 4). Risk factors for diabetes include obesity, not being physically active, smoking, and having a family member with diabetes (3).

Diabetes can be managed by changing diet, becoming more physical, taking insulin or other medicines. Treatment for individuals with type 1 diabetes involves insulin injections or the use of an insulin pump, frequent blood sugar checks, and carbohydrate counting. Treatment for individuals with type 2 diabetes primarily involves lifestyle changes, monitoring blood sugar levels, and taking diabetes medications that help with insulin sensitivity such as Metformin or insulin or both (5). However, even with those measures people can still have high amounts of blood glucose. Overdoing diabetic treatments can also lead to complications such as abnormally low amounts of blood glucose which can be deadly if not treated right away.

1.2 Glucose Homeostasis

Glucose homeostasis is a process that the body utilizes for the maintenance of blood glucose levels within a tight range of 4mM to 6mM to ensure normal body functions. This process is maintained by the balance of insulin and glucagon that are secreted by the pancreas (5). During fasting conditions, when blood glucose levels are low, the alpha cells of the pancreas secrete glucagon to drive the processes that elevate the blood glucose levels through gluconeogenesis (5). On the other hand, during feeding conditions, when the blood glucose levels are high, the beta cells of the pancreas release insulin to stimulate the processes that lower blood glucose levels through glycolysis, lipogenesis, glycogen synthesis, and protein synthesis (5–8).

Insulin acts on many tissues to main glucose homeostasis such as liver, muscle, and adipose tissues. Glucose uptake occurs when tissues such as muscle and adipose tissues take
glucose from the circulation for their energy needs for their cell processes. Glucose uptake occurs via moving glucose through glucose transporter 4 (GLUT4) in muscle and adipose cells. This process is regulated by insulin via the phosphoinositol-3-kinase PI3K/ protein kinase B Akt pathway (9). Muscle contraction also appear to enhance glucose uptake via GLUT4 on muscle cells due to enhanced calcium release from the cells. Acylation stimulating protein (ASP) 160 plays a key role in the insulin mediation of GLUT4 activity downstream of AKT. Insulin resistance inhibits the expression of GLUT4 leading to less glucose uptake which leads to higher blood glucose (10–13).

1.3 Insulin and the PI3K Pathway

Insulin which plays an important role in maintaining glucose homeostasis is an endocrine peptide hormone that is secreted by the beta cells of the pancreas in response to high glucose. It was discovered by Banting and Best in 1922. The structure of insulin consists of two polypeptide chains crosslinked by 3 disulfide bonds while the structure of the insulin receptor (IR) consists of 4 subunits that are joined by disulfide bonds (14, 15). The regulatory action of insulin in response to high glucose is exerted on the target cells such as the liver and skeletal muscle by binding to the insulin receptor located on the plasma membrane of those cells (16). When insulin binds to the alpha subunit of the IR it activates the tyrosine kinase in the beta subunit via auto phosphorylation of itself (Figure 1). This causes a series of activations and inactivations of proteins inside the cells through a pathway known as the insulin signaling cascade. The tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1) by the IR activates the insulin signaling cascade to regulate enzymes that control growth and metabolism (17).
The portion of the insulin signaling cascade that plays a large role in the regulation of metabolic enzymes is called the (PI3K)/Akt pathway. Upon binding of insulin to its receptor, the tyrosine phosphorylation of IRS-1 would activate the PI3K protein by binding the p85 and p110 subunits of the PI3K protein which induces the phosphorylation of phosphatidylinositol 4, 5-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (18). PIP3 binds Akt which allows it to translocate from the cytoplasm and bind the cell membrane. Then phosphoinositide-dependent protein kinase-1 (PDK-1) binds Akt and allows for the phosphorylation of Akt at Thr308 (19). The phosphorylation of Akt at Thr308 allows for the activation of the mammalian target of rapamycin (mTOR pathway) and DNA-PK. MTORC2, which is part of the mTOR pathway, and DNA-PK phosphorylate Akt at Ser473. Those two phosphorylation events on Akt in response to insulin lead to the full activation of Akt. The full activation of Akt stimulates and inhibits key effectors that play a role in the regulation of metabolic pathways and processes such as glucose homeostasis. For example, Akt phosphorylates glycogen synthase kinase (GSK3β) at Ser9 and Ser21 in response to insulin which activates glycogen synthase (GS), an important enzyme in the conversion of excess glucose to glycogen (19). Akt also activates the (mTOR) pathway by activating the mTOR complex 1 (mTORC1) protein and Ras homolog enriched in brain (RHEB) protein by inhibiting both tuberous sclerosis complex 1 (TSC1) and tuberous sclerosis complex 2 (TSC2). The RHEB protein which is a Ras dependent protein also assists in the activation of mTORC1 complex (20). The activation of the mTOR pathway leads to the activation of sterol regulatory element binding protein 1c (SREBP1c), an important transcription factor in the regulation of lipogenesis (21, 22). Akt has been shown to also phosphorylate forkhead box protein O 1 (FOXO1), an important transcription factor in the activation of gluconeogenesis,
which leads to its translocation from the nucleus to the cytoplasm to be degraded by the 14-3-3 protein degradation process (23).

1.4 Metabolic Pathways Regulated by Insulin
1.4.1 Fatty Acid Synthesis

Fatty acid synthesis is an important metabolic pathway for energy and glucose homeostasis (24). It is a pathway that allows for the conversion of excess glucose from high carbohydrate diets into fatty acids. Fatty acids are precursors needed to synthesize molecules for the purpose of energy storage for future need. Similar to many other metabolic pathways, fatty acid synthesis is tightly regulated by hormonal and nutritional status such as high insulin / low glucagon in response to feeding and fasting (25). Fatty acid synthesis occurs in the cytosol and the endoplasmic reticulum (ER) of cells of the liver and adipose tissues. During the process of fatty acid synthesis, a sequential extension of an alkanoic chain two carbons at a time occurs where acetyl units derived from either glucose or acetate are added to an initial starting molecule such as acetyl coenzyme A (CoA). This is followed by a series of reductive biosynthesis reactions to form the 16-carbon palmitate. The following equation summarizes the production of palmitate from acetyl CoA.

\[
8 \text{Acetyl CoA} + 7\text{ATP} + 14\text{NADPH} + 14\text{H}^+ \rightarrow \text{Palmitate} + 7\text{ADP} + 7\text{P}_i + 8\text{CoA} + 14\text{NADP}^+ + 6\text{H}_2\text{O}
\]
The first committed step in the synthesis of fatty acids is catalyzed by the enzyme acetyl CoA carboxylase (ACC) which converts acetyl CoA to malonyl CoA. The process of fatty acid synthesis from malonyl CoA, the second committed step in fatty acid synthesis, is catalyzed by a single multifunctional enzyme called fatty acid synthase (FAS) (26). This process starts with condensation of the acetyl and malonyl moieties coupled with an energetically favored decarboxylation to form an acetoacyl, a beta-ketoacyl. The acetoacyl is then reduced to form the fatty acyl-CoA that condenses again with another malonyl moiety to form an elongated beta-ketoacyl (24, 25). Seven cycles of chain extension and beta carbon processing according to the equation above form the 16-carbon palmitate. Palmitate is then converted to triglycerides for storage (27).

1.4.2 Pentose Phosphate Pathway

The Pentose Phosphate Pathway (PPP) is another pathway that is important to glucose homeostasis. Its role in glucose homeostasis is to shunt glucose away from glycolysis to provide needed molecules for other cellular functions. The goal of this pathway is to provide precursors necessary for nucleotide and amino acid biosynthesis and fatty acid synthesis. It also provides the reducing agents needed for anabolism and to combat oxidative stress. Discovered in the 1930s by Warburg, the pathway converts glucose-6-phosphate (G6P) into carbon dioxide (CO₂), and produces nicotinamide adenine dinucleotide phosphate (NADPH) and ribulose 5- phosphate in the oxidative branch. The non-oxidative branch then starts with ribulose 5 phosphate being isomerized to form ribose-5-phosphate and xylulose-5-phosphate, molecules needed for nucleic acid synthesis. If there is no need for nucleic acid synthesis, then the pathway would supply the
metabolites that are in need for glycolysis (28). The committed step of PPP is the enzyme glucose-6-phosphate dehydrogenase (G6PDH). Similar to fatty acid synthesis, PPP is tightly regulated by hormonal and nutritional status, including high insulin due to feeding, and high glucagon due to fasting (29).

1.5 Importance of the Regulation of Gene Expression and Posttranslational Modification in the Control of Metabolic Pathways

Flux through the metabolic pathways is controlled by their regulatory enzymes. Enzyme activity can be regulated by the controlling the expression of its corresponding gene. Gene expression according to the National Human Genome Research Institute is a process by which the cell uses the information encoded in the DNA to direct the synthesis of a functional protein of the cell. During this process, the cell transcribes a messenger RNA from the DNA in a process called transcription. This messenger RNA then undergoes a process called translation where the translation RNA reads the sequence of the messenger RNA by groups of 3 bases which correspond to 1 of each 20 amino acids to build a protein sequence in the ribosome. Subsequently to the synthesis of the protein, phosphorylation and glycosylation can change the function via changing the conformation of a protein. These posttranslational modifications play an important role in activating and deactivating signaling pathways that are important for cellular functions. Insulin regulates the rate of protein synthesis for the regulatory enzymes in the metabolic pathways by altering the expression of the gene that codes for the subsequent protein or altering the translation of the mRNA into protein sequences. Insulin also regulates the posttranslational modifications of many proteins in it signaling pathway such as its inhibitory effect on FOXO1 via phosphorylation. Insulin regulates the expression of over 100 genes via its
signaling pathway, with most of metabolic processes it regulates occurring via the PI3K pathway (30). In gluconeogenesis for example, FOXO1, a transcription factor, binds the iron response element (IRE) unit of the promoter regions of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6pase) to activate the expression of those genes for enzyme synthesis (31). That binding is inhibited when insulin phosphorylates FOXO1 which translocates it from the nucleus to the cytoplasm to initiate degradation.

1.6 Genes Involved in the Regulation of Fat Synthesis

1.6.1 Fatty Acid Synthase (FAS)

FAS is a complex homodimeric (552 kDa) enzyme that regulates de novo lipogenesis by catalyzing the synthesis of the free fatty acid (FFA) palmitate, a 16-carbon chain fatty acid, from acetyl CoA and malonyl CoA in a NAPDH dependent manner (32, 33). FAS is also responsible for the synthesis of other fatty acids such as myristate, laureate, and shorter chain fatty acids (34).

Like many enzymes that regulate metabolic pathways, changes in the nutritional status alters the amount of FAS present. For example, FAS is upregulated in response to diets with high carbohydrate (35). Polyunsaturated fats reduce the expression of FAS by inhibiting SREBP1c, a key transcription factor in the regulation of FAS expression (36, 37). Under normal fasting conditions, glucagon via cAMP inhibits the expression of FAS in rat liver, while under feeding conditions, insulin stimulates the expression of FAS. Under diabetic conditions however, refeeding did not stimulate the increase in FAS expression compared to normal conditions, an indication in type 1 diabetes that the insulin induction of FAS is severely reduced (38). In obese mice, insulin resistance reduces glucagon inhibition of fatty acid synthesis, an indication that insulin resistance
leads to higher fatty acid synthesis (39). As shown in Figure 1, FAS is induced by insulin via the PI3K/Akt pathway that activates a series of transcription factors that are involved in the expression of FAS (40). One transcription factor, SREBP1c, which binds to the SRE element of the promoter region of FAS is regulated by signal proteins such as mTOR which is downstream of Akt (41). Another transcription factor that is involved in the regulation of FAS expression in response to insulin is LXR, which binds to the LXRE element of the promoter region of FAS (42). USF, downstream of PP1, is an effector of Akt that binds to the EBOX of the promoter of FAS and
regulates the expression of FAS by recruiting SREBP1c to the promoter. USF has been shown to be an absolute requirement for insulin induction of FAS expression (43–45).

Figure 1. Schematic Diagram Showing the Insulin Induction of FAS Expression Via the PI3K/Akt Pathway.
1.7 Genes Involved in the Regulation of the PPP

1.7.1 Glucose 6 Phosphate Dehydrogenase (G6PDH)

G6PDH is a housekeeping enzyme that catalyzes the rate determining step of the oxidative phase of the PPP (46). It was first discovered in 1931 in red blood cells and yeast and is found in most cell types in the body (47). It controls the flux of oxidative PPP which determines how much of the G6P gets shunted into the PPP. G6PDH is present in both monomeric and dimeric forms which are inactive and active, respectively (48). G6PDH which is a highly specific enzyme for NADP+, a precursor for NADPH, makes it an important source of NADPH which is needed for fatty acid synthesis and maintaining redox balance (49). G6PDH is typically present in tetramer form that cannot be inhibited by NADPH. However high pH and ionic strength can cause the dissociation of the tetramer to a dimer which allows NADPH to inhibit G6PDH activity by dissociating the dimer form of G6PDH to the inactive monomer form of G6PDH (50). Similar to many regulatory enzymes in other metabolic pathways, G6PDH is regulated by hormonal and nutritional status such as high insulin/low glucagon in response to feeding and fasting (50, 51). Carbohydrate increases the expression and activity of G6PDH while polyunsaturated fatty acids inhibit the expression of G6PDH in rats and mice (52, 53). AMPK is a key mediator in the inhibition of G6PDH by polyunsaturated fatty acids in rat hepatocytes (54). Similar to FAS, insulin induces the expression of G6PDH via the mTOR pathway downstream of Akt (figure 2) which leads to the activation of SREBP1c that binds to the SRE element of the promoter of G6PDH (55). SREBP1c acting as a key regulator of gene expression of G6PDH was shown by the overexpression of SREBP1 in HEK-293 cells, suggesting a role for SREBP1c
in the insulin regulation of G6PDH (56). Unlike FAS, the G6PDH promoter region does not contain a site for the liver x receptor suggesting that the liver x receptor does not regulate G6PDH (57).

Figure 2. Schematic Diagram Showing the Insulin Induction of G6PDH Expression Via the PI3K/Akt Pathway.
1.8 Transcription Factors Involved in the Regulation of FAS and/or G6PDH

1.8.1 Sterol Regulatory Element Binding Protein 1c (SREBP1c)

SREBPs are a family of proteins around 1150 amino acids long and are primarily bound to the endoplasmic reticulum (ER). They are regulated in various ways which include transcription, proteolytic cleavage of the precursor for release off the ER, and posttranslational modifications (50). The three forms of SREBP are SREBP1a, SREBP1c, and SREBP2. SREBP1c is involved in fatty acid synthesis and storage, SREBP1a is involved in global lipid synthesis and cell growth, and SREBP2 is involved in cholesterol biosynthesis. SREBP1a is only predominant in certain tissues such as intestinal, epithelial, heart, macrophage, and bone marrow dendritic cells. SREBP1c however, is the predominant isoform in most other tissues (56). In the liver, where fat synthesis occurs, SREBP1c is expressed 10 times more than SREBP1a making it the major isoform in the liver (60). SREBPs are synthesized as a precursor (pSREBP) bound to the SREBP cleavage-activating protein (SCAP)/INSIG complex. This complex is bound to the ER to keep SREBPs in the inactive form. In response to signaling molecules such as insulin, INSIG expression is inhibited. This inhibition then allows the dissociation of SREBPs from the ER. SREBPs are then translocated to the golgi apparatus via binding to the Sec23/24 proteins of the coat protein complex II (COPII) vesicles. Once in the golgi, they are cleaved by site 1 protease (S1P) and site 2 protease (S2P) to become the mature active form of SREBPs (nSREBP) (61, 62).

Insulin regulates exclusively SREBP1c transcription and proteolytic processing in the liver via the mTOR pathway (63, 64). The role of the mTOR pathway in the insulin induction of
SREBP1c expression was confirmed when rapamycin, an inhibitor of the pathway was added in mouse liver cells and in diabetic mice (65).

For insulin to fully activate the transcription of SREBP1c, the binding of several transcription factors is required including SREBP1c itself, liver x receptor (LXR), nuclear factor Y (NF-Y), and specificity protein 1 (SP-1) (66). SP-1 and NF-Y play a role in activating the SRE region of the promoter to help insulin induce the transcription of SREBP1c (66). LXR plays a role in the insulin induction of SREBP1c via binding two LXRE sites in the promoter of SREBP1c which along with the serum response element (SRE) site plays a cooperative role in insulin induction of SREBP1c via LXR (67). FOXO1, a transcription factor involved in the activation of gluconeogenesis has been shown to be an inhibitor of SREBP1c via two mechanisms. The first mechanism is inhibiting the transcriptional activity of LXR by inhibiting LXR binding to the LXRE in mouse liver and HEPG2 cells (68, 69). The second mechanism is inhibiting SP1 and SREBP1c transcriptional activity on the SRE unit of the promoter of SREBP1c in mouse livers and primary rat hepatocytes (70, 71).

In addition to the insulin induction of SREBP1c expression, insulin also induces the proteolytic processing of SREBP1c, a requirement for its activity on its target genes. Insulin, via P70 S6 kinase, which is downstream of the mTOR protein, phosphorylates the pSREBP1c which increases the affinity of the SREBP1c-SCAP complex to the Sec23, 24 of the COPII (72, 73). Insulin also induces SREBP1c processing by suppressing INSIG2 in an mTOR independent manner which allows for the dissociation of pSREBP1c from the ER, a requirement for the initiation of processing of pSREBP1c (74, 75).

On the other hand, glycogen synthase kinase 3β (GSK3β) an inhibitor of glycogen synthesis, has been shown to inhibit SREBP1c processing by phosphorylating it at Ser73 which
initiates its degradation (76, 77). AMPK also inhibits SREBP1c processing by phosphorylating it at Ser372 (78).

1.8.2 Liver X Receptor (LXR)

Liver X Receptors are nuclear receptors that are very important for regulating lipid metabolism. They exist in two isoforms, LXRα and LXRβ. LXRα was cloned in 1995 by Patricia Willy and shown to form a heterodimer with the RXR to bind to the LXRE site in the promoter regions of the target genes (79). LXRβ which is also known as OR-1 was cloned by Teboul in 1995 (80). LXRα is highly expressed in liver, kidney, intestine and adipose tissues, whereas LXRβ is expressed in nearly all tissues (81). LXRβ is mainly involved in cholesterol synthesis (82) while LXRα has been shown to be an important transcription factor in the regulation of lipogenic genes via binding to the LXRE element in the promoter region of its target genes such as FAS and SREBP1c (83, 84). The binding of LXR to the LXRE elements of SREBP1c and FAS was confirmed in studies done by Liang and Joseph using expression plasmids of LXR and nSREBPs, and by using LXR agonist TO901317 in culture and whole animal hepatocytes (83, 84). These studies also confirmed LXR as a key transcription factor in the induction of SREBP1c and FAS using knockout and overexpression studies in cultured hepatocytes and in fed mice (83, 85). LXR can also induce the expression of FAS independent of SREBP1c. This independency was confirmed by overexpression and inhibition of SREBP1c studies in mice liver (42, 83). LXR has been shown to be a key transcription factor in the insulin induction of FAS expression via SREBP1c. This induction requires the LXR binding to C/EBPβ, another key transcription involved in the transcription of SREBP1c in rat hepatocytes and livers from whole mice (86, 87) LXR can be activated independent of insulin via O-glycosylation in response to high glucose and glucosamine leading to higher induction of its target genes in culture and diabetic mice (85, 88,
AMPK, which is inhibited by insulin has been shown to inhibit LXRα activity by phosphorylating it at an unknown threonine residue and via the inhibition of S6K1 in the livers of whole mouse fed with high fat diet and HEPG2 and rat hepatoma cell lines (90, 91).

1.8.3 Insulin Induced Gene (INSIG)

INSIGs are recently discovered genes that are involved in the regulation of lipogenesis and cholesterol synthesis. The INSIG family consists of 2 isoforms, INSIG1, also known as CL-6, and INSIG2. INSIGs regulate lipid metabolism and cholesterol synthesis via binding to SCAP proteins and 3-hydroxy-3-methyl-glutaryl (HMG) reductase, an enzyme involved in cholesterol synthesis (92). INSIG1 was first discovered in liver and fibroblast cells in the 1990s (93). INSIG2 was discovered in 2002 in hamster ovary cells. INSIG1 and INSIG2 are 59 percent identical. INSIGs play an important role in the inhibition of SREBP processing. In the presence of sterols, INSIG2 blocks the processing of SREBP (74). With INSIGs being an inhibitor of SREBPs including SREBP1c, INSIGs play a large role in the antilipogenic effect on the liver. INSIGs being a modulator of lipogenesis have been confirmed by overexpression studies of INSIGs performed in livers of obese mice (94). Insulin regulates SREBP1c induced lipogenesis by reducing the expression of INSIG2 causing the dissociation of the SCAP-SREBP1c complex from INSIG2 protein allowing the complex to be released from the ER to begin SREBP1c processing (95). During long term insulin treatment, insulin would induce expression of INSIG1 due to increased nSREBP1c since INSIG1 is a target of nSREBP1c. This might limit SREBP1c processing since INSIG1 is also an inhibitor of SREBP1c (75). This was confirmed by a study that showed that overexpression of INSIG1 limited the processing of insulin induced SREBP1c processing via inhibiting INSIG2 (96). It has also been shown that the insulin induced processing
of SREBP1c via suppressing INSIG2 occurs downstream of Akt but independent of the mTOR pathway (20).

1.9 Types of Insulin Resistance

Insulin resistance occurs when the target cells, such as muscle, liver, or fat cells, do not respond well to insulin. Genetic defects of the genes that code for certain proteins in the insulin signaling cascade starting with insulin receptor and IRS-1 can cause insulin resistance (97–99). Also, lipid and fatty acid accumulation, oxidative stress, O-glycosylation, and ER stress can cause insulin resistance (15). When insulin resistance leads to chronically higher than normal levels of blood glucose, the person will be classified as a patient with type 2 diabetes. For more than a half century, insulin resistance and type two diabetes have been linked to each other, therefore insulin resistance is so far the most powerful predictor of type 2 diabetes (96).

1.9.1 Hexosamine Biosynthetic Pathway and Insulin Resistance

The hexosamine biosynthetic pathway (HBP) is a metabolic pathway that branches off glycolysis at fructose-6-phosphate (F6P) to produce UDP-N-acetylglucosamine from glucose (UDP-GlcNAc), acetyl CoA, glutamine, and UTP. UDP-GlcNAc is a precursor needed for the activation of O-glycosyl transferase (OGT) which is responsible for the O-glycosylation of proteins and lipids (101). HBP was first linked to insulin resistance by Marshall in 1991. The role of glutamine fructose-6-phosphate amidotransferase (GFAT) and OGT in decreasing the insulin response and decreasing glucose tolerance were confirmed by overexpression of OGT in transgenic mice and inhibiting GFAT (102, 103). Glucosamine, a carbohydrate that bypasses GFAT and drives the HBP forward without any control, was able to decrease insulin sensitivity and glucose tolerance by decreasing IRS-1 phosphorylation which inhibited its association to the
p85 subunit of the PI3K protein (104–106). Downstream of PI3K, the insulin induction of mTOR was also shown to be weakened in response to glucosamine (107). This indicates that glucosamine induces insulin resistance by affecting IRS-1 activity which leads to the weakening of downstream signaling pathways such as the PI3/Akt pathway.

1.9.2 Oxidative Stress Induced Insulin Resistance

Oxidative stress occurs when excess endogenous reactive oxygen species (ROS) damage the cells and manipulate signaling pathways (108). Examples of ROS include superoxide and hydrogen peroxide. ROS are mostly generated in the electron transport chain of the mitochondria when the proton motive force is high and the coenzyme Q pool is reduced leading to less ATP made (109). ROS are also generated via activating enzymes such NAPDH oxidase (NOX) and endothelial nitric oxide synthase (eNOS) outside the mitochondria (110). While a minimal amount of ROS is needed to maintain important cellular functions, an excess of ROS can lead to cellular damage resulting from modification of the thiol groups on cystine residues of proteins when can alter their function (111). Hyperglycemia induces oxidative stress via to the activation of PKC in endothelial cells which drives glucose flux through the polyol pathway. The polyol pathway is a pathway that allows the conversion of glucose to sorbitol via aldose reductase and then to fructose via aldose reductase dehydrogenase. In this process, NAPDH gets oxidized to NADP+ (112). The less NADPH means less reduced glutathione available to combat oxidative stress. This results in the generation of ROS such as superoxide (113–115). The production of superoxide leads to the inhibition of glyceraldehyde-3-phosphate which leads to more glucose being shuttled through the HBP pathway (116). These studies indicate that hyperglycemia leads to oxidative stress by enhancing the production of ROS which can lead to the increased shuttling of glucose via HBP.
Hyperglycemia induced oxidative stress decreases the mitogen activated protein kinase phosphatase 1 (MKP-1) in rat vascular smooth muscle cells that leads to excessive p38 mitogen activated protein kinase (MAPK) pathway activation (117). The c-Jun N-terminal kinase (JNK) pathway is a pathway that is stimulated by stress and inflammation via the activation of mitogen activated protein kinase kinase 4 (MKK4) and mitogen activated protein kinase kinase 7 (MKK7) of the MAPK family as a result of stress and inflammation. The activation of the JNK pathway involves the phosphorylation of JNK by MKK4 and MKK7 in response to stress indicators at Tyr184 and Thr183 respectively. These phosphorylation events allow the translocation of JNK to the nucleus which phosphorylates the c-Jun protein at Ser63 and Ser73 leading to the activation of many proteins involved in cell proliferation and apoptosis (118–122). The JNK pathway has been shown to phosphorylate IRS-1 at Ser307 leading to the inhibition of IRS-1 activity which can lead to insulin resistance (123). Oxidative has been shown to induce insulin resistance via decreasing IRS-1 and IRS-2 protein levels leading to a decreased Akt activity (124). It has also been shown that oxidative stress mediated by FOXO1 translocation to the nucleus is due to active JNK pathway also induces beta cell dysfunction triggering type 1 diabetes. FOXO1 induced beta cell function by the induction of the JNK pathway was confirmed by the inhibition of the insulin promoter factor-1 (PDX-1), an important protein in the maturation of β-cells to secrete insulin (125, 126). This is an indication that oxidative stress is important inducer of insulin resistance through reducing the insulin signaling via IRS-1, and reducing insulin secretion from the beta cells, triggering both type 1 and type 2 diabetes.
1.9.3 Palmitate Induced Insulin Resistance

Palmitate has been linked to insulin resistance through oxidative stress inhibiting the PI3K pathway. Protein tyrosine phosphatase 1B (PTP-1B), a negative regulator of the tyrosine phosphorylation of IR and IRS-1, protein levels were elevated in c2c12 skeletal cells under palmitate induced insulin resistance leading to the reduction of tyrosine phosphorylation of IRS-1 leading to a reduction in the PI3K/Akt pathway activity. The role of PTP-B1 as a key factor in palmitate induced insulin resistance was confirmed by knocking out PTP-B1 in skeletal cells (127). Another way that palmitate induces insulin resistance is via oxidative stress. An increase in ROS species produced leading increase in the phosphorylation of p38 MAPK and JNK. The increase in the activity of the p38 MAPK and JNK proteins leads to a reduced tyrosine phosphorylation of IRS-1 which leads to a reduced insulin inhibition of gluconeogenic genes via the PI3K pathway thereby increasing gluconeogenesis. The increase in gluconeogenesis leads to elevated glucose levels, a consequence of insulin resistance, causing diabetes (128–130). The role of JNK in palmitate induced insulin resistance effect on the PI3K/Akt was confirmed by inhibiting the JNK pathway in H4IIE cells leading to the restoring of PI3K/Akt pathway activity (130).

1.10 Insulin Resistance and Fatty Acid Synthesis and PPP

Interestingly, while palmitate induced insulin resistance reduces the insulin inhibitory effect on gluconeogenesis, the insulin induction of fatty acid synthesis is not affected as evident by the increase in the expression of lipogenic genes and the steady amount of FFA shown in culture and whole animals (131, 132). The buildup of fatty acids in response to insulin resistance
leads to excess lipid storage in the liver which develops the condition called Non-Alcoholic Fatty Liver Disease (NAFLD) (133). NAFLD can range from excess fatty liver, to hepatic steatosis to liver cirrhosis due to excess fat from lipolysis in the adipose tissues, to excess lipogenesis, and decreased fatty acid oxidation in the liver (134, 135). There is a strong link between obesity induced insulin resistance and NAFLD as more than 90 percent of patients with type 2 diabetes have NAFLD (136). NAFLD can further enhance insulin resistance via elevated levels of SREBPs. In obese mice and rat hepatocytes, elevated SREBP1c activity leads to a reduction in IRS-2 protein levels. The reduction of IRS-2 activity leads to lower Ser473 Akt phosphorylation which leads to higher gluconeogenic gene expression resulting in lower glucose tolerance. The role of SREBPs as an important mediator in inducing insulin resistance via decreasing IRS-2 mediated insulin signaling was confirmed using overexpression and knockout of SREBP1c in mice livers (137–139).

There has been conflicting evidence on the role or regulation of G6PDH in insulin resistance and hence PPP. For example, it has been shown that high glucose activates the PKA pathway in endothelial cells which inhibits the expression and activity of G6PDH which contributes to increased oxidative stress These findings were also shown in STZ treated rat endothelial cells (140, 141). It has also been documented that G6PDH activity and expression is reduced under type 1 diabetes as shown in STZ treated mice livers (142). This indicates that high glucose in STZ treated mice via the PKA pathway might result in decreased G6PDH expression and activity. Others have shown that G6PDH contributes to insulin resistance by increasing oxidative stress and increasing lipogenesis via increasing NADPH available for fatty acid synthesis (143) On the other hand, G6PDH over expression leads to larger β-pancreatic cells which leads to improved insulin secretion under hyperglycemic conditions (144).
1.11 LXR and Insulin Resistance

LXR has been shown to have antidiabetic effects in studies using the LXR agonist. LXR agonist is a synthetic ligand that binds to LXR to cause its activation. There are many agonists for LXRs, one of them is TO9013137 which has been shown to induce a 3.5-fold activation increase in LXRα compared to a 1.5-fold activation of LXRβ (145). Overactivation of LXR via its agonist has been shown to improve glucose tolerance, due to inhibition of oxidative stress which leads to the deactivating of the JNK pathway. The deactivation of the JNK pathway leads to increased insulin sensitivity by restoring Akt activity. The restoration in Akt activity leads to the reduction of gluconeogenic gene expression which leads to decreased glucose output in culture and diabetic obese mice (132, 146, 147). The decrease in glucose output is correlated with increased glucose uptake due to an elevation in glucose transporter 4 (GLUT4) expression and activity in adipocytes of (147, 148). Interestingly, mice treated with LXR agonist for 3 weeks via intraperitoneal injection resulted in the induction of insulin resistance due to increased lipogenesis as evident by an increase in free fatty acids, hyperinsulinemia, and decreased glucose tolerance (149). These results indicate that despite LXR having antidiabetic effects, prolonged treatment with LXR might induce insulin resistance due to the induction of fatty acid synthesis, suggesting more studies are needed to clarify.

1.12 Significance and Hypothesis

Treatment of insulin resistance, a hallmark of type 2 diabetes, has been a goal of scientists and health care professionals for years. Therefore, it is important to fully understand the mechanism of insulin resistance to help find the treatment. Understanding the mechanism helps identify potential proteins to target with synthetic drugs. It is also important to find out whether
that target protein regulates other pathways not involved in insulin signaling due to the side
effects that may arise from inhibiting or overactivating that target protein. It is also important to
understand the different mechanisms of insulin resistance and their effects on the metabolic
pathways since insulin resistance might have different effects on the different metabolic
pathway. For example, as discussed before fat induced insulin resistance suppresses the insulin
inhibitory effect on gluconeogenesis but leaves the insulin induction of fatty acid synthesis
unabated. How this happens is still not well understand. Understanding the effect of insulin
resistance on metabolic pathways may lead to inventing drugs that directly address the
dysregulation of the metabolic pathways. These drugs will lead to a significant improvement in
treatment of diabetes since current diabetic medicines only address insulin sensitivity and
glucose uptake and urine excretion. These medicines do not address the core of the problem
which is the dysregulation of metabolic pathways due to insulin resistance.

Many models of insulin resistance have been established in animals and cells in culture.
One of those models uses glucosamine to stimulate the HBP and has been adapted in our lab.
Evidence shows that palmitate induced insulin resistance leads to the activation of the HBP
(150). Initial evidence that we have in our lab shows that insulin regulates G6PDH and FAS, the
key enzymes in PPP and fatty acid synthesis, respectively differently under glucosamine induced
insulin resistance. However, the reason for this regulation is not well understood. Therefore, the
goal of this study is to find the differentiator that is responsible for the differential regulation of
the gene expression of FAS and G6PDH under glucosamine induced insulin resistance.
Chapter 2

Materials and Methods

2.1 Hepatocyte Isolation and Treatment

Male Sprague Dawley Rats SAS SD strain code 400 were purchased from Charles Rivers Laboratories Kingston NY. Rats were housed in the Institutional Animal Care and Use Committee approved facility at Western Michigan University and maintained on Rodent Diet 5001 rat chow with drinking water ad libitum under 12-hour light and dark cycles until they weighed over 240g. Animals were food deprived 48 hours prior to hepatocyte isolation and anesthetized with 50mg/kg of Pentobarbital via IP injection prior to hepatocyte isolation. Hepatocytes were isolated using the collagenase hyaluronidase perfusion and digestion method (151). Briefly, the liver was first perfused via the portal vein with a perfusion solution containing 0.102M sodium chloride, 0.0087M sucrose, 0.0151M HEPES, 0.0005M EGTA and > 5400 units heparin sodium salt from porcine intestinal mucosa (H3149, Sigma Aldrich) at a rate of 50ml/min for a minimum of 2 minutes prior to switching to the digestion solution. Then the digestion solution containing 0.102 M sodium chloride, 0.0087M sucrose, 0.0151M HEPES, 0.002M potassium chloride, 0.001M calcium chloride dihydrate, 30mg/l trypsin inhibitor from glyceine max (T6522, Sigma Aldrich) and 0.05g of 300 unit/mg collagenase type 4 (LS004188, Worthington) was run through the liver at a rate of 50ml/min for a maximum of 2 minutes. Cells were isolated through a sterile double layered cheese cloth using a solution containing 0.102M sodium chloride, 0.0087M sucrose, 0.0151M HEPES, 0.002M potassium chloride, 0.001M calcium chloride dihydrate, 30mg/l trypsin inhibitor from glyceine max (T6522, Sigma Aldrich) and 0.5 percent Bovine Serum Albumin (BSA) (A9418, Sigma Aldrich).
The hepatocytes were washed twice with Waymouth media MB-752/1 (W1625, Sigma Aldrich) with 0.5 percent BSA (A9418, Sigma Aldrich) by centrifugation at around 560 rpm for 3 minutes. Cell count and viability was estimated using a hematocytometer and the trypan blue dye exclusion method. The cells were then plated at 90 % confluency on sterilized Falcon 60x15mm tissue culture dishes coated with collagen type 1 from rat tail (CB-40236, Corning) and incubated at 37 degrees Celsius for 4 hours. Cells were then washed and incubated at 37 degrees Celsius in Waymouth media MB-752/1 overnight under a humidified atmosphere of 5 % CO2 and 95 % air at 37 °C. The cells were next washed and incubated in DMEM low glucose media with no phenol red (11054020, Gibco) and supplemented with 0.29g/l of glutamine, 0.1g/l of streptomycin salt, and 0.15g/l penicillin G. Treatment with bovine insulin (I-1882 Sigma Aldrich), glucosamine (G1514, Sigma Aldrich), TO901317 (T2320, Sigma Aldrich), DMSO, or combination of those treatments were added to the cell culture media containing the hepatocytes. The treatments are summarized in Table 2.

Table 2. Treatment Schedule for Primary Rat Hepatocytes in Low Glucose Media in Culture.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Glucosamine (G) (1mM)</th>
<th>Insulin (I) (44nM)</th>
<th>TO901317 (T) 1uM</th>
<th>DMSO</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PDH</td>
<td>18 hrs.</td>
<td>18 hrs.</td>
<td>18 hrs.</td>
<td>18 hrs.</td>
</tr>
<tr>
<td>SREBP1c</td>
<td>18 hrs.</td>
<td>18 hrs.</td>
<td>18 hrs.</td>
<td>18 hrs.</td>
</tr>
</tbody>
</table>
2.2 RNA Isolation

Media was aspirated from the cells, and 200μl to 300μl of Trizol Reagent (15596018, Thermo Fisher Scientific) were added to the plate. The cells were scraped and then were transferred into a microcentrifuge tube. The samples were incubated for 5 minutes at room temperature and then 0.2ml of chloroform isoamyl alcohol 24:1 (v: v) (C0549, Sigma Aldrich) per 1 ml of Trizol was added to the samples. The samples were mixed by pipetting up and down several times and incubated for 3 minutes at room temperature. Then the samples were centrifuged for 15 minutes at 12000xg at 4 degrees Celsius. The top clear layer was transferred to a new tube, and 0.5ml of 2-propanol was added per 1 ml of Trizol used. The samples were mixed by pipetting up and down several times and incubated for 10 minutes at room temperature, and then centrifuged for 10 minutes at 12000xg at 4 degrees Celsius. The supernatant was removed, and the RNA pellet was resuspended and washed twice with ice cold 75 percent ethanol. The RNA pellet was resuspended and dissolved in nuclease free water and the concentration and the purity of the RNA was measured using a Thermo Scientific nano drop spectrophotometer.

2.3 RT-PCR

4μg of cDNA were synthesized using the High-Capacity cDNA Reverse Transcription kit with RNase inhibitor (4374966, Thermo Fisher Scientific). Briefly, 10μl of isolated RNA solution containing 4μg of RNA was added to 10μl of master mix containing the reverse transcription
buffer, reverse transcriptase, RNAase inhibitor, and random primers. The mixture was then incubated at 37 degrees Celsius for 2 hours in a thermocycler. Then qRT-PCR was performed using the 96 well optical microplate. Each well contained 5µl of cDNA solution (100ng of cDNA), 1µl of primer solution, 4µl of nuclease free water, and 10µl of TaqMan Fast Advanced Master Mix solution (4444556, Thermofisher Scientific). The primers used for the various reactions are summarized in table 2-2. The qRT-PCR was performed in a Step One Plus Real Time PCR machine (4376600, Thermofisher Scientific) in 40 cycles where each cycle was 95 degrees Celsius for 1 second and 60 degrees Celsius for 20 seconds, after initial incubation at 50 degrees Celsius for 2 minutes and 95 degrees Celsius for 2 minutes. The results were obtained by measuring mRNA fold change compared to no addition which was set at one. β-actin (ACTB) was used as an endogenous control.

Table 3. Primers Used for RT-PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Catalog</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD</td>
<td>Rn01529640_g1</td>
</tr>
<tr>
<td>SREBF1</td>
<td>Rn01495769_m1</td>
</tr>
<tr>
<td>FASN</td>
<td>Rn00569117_m1</td>
</tr>
<tr>
<td>ACTB</td>
<td>Rn00667869_m1</td>
</tr>
<tr>
<td>INSIG2</td>
<td>Rn00710111_m1</td>
</tr>
</tbody>
</table>

2.4 Protein Assay

Media was aspirated from cells after treatment. Cells were washed one time with ice cold phosphate buffered saline (PBS). Then 100µl of radioimmunoprecipitation assay (RIPA) buffer
(R-0278, Sigma Aldrich) containing 1:100µl of protease cocktail inhibitor (P-8340, Sigma Aldrich) was added to each plate. Cells were scraped and the lysate was transferred to a microcentrifuge tube and incubated for 5 minutes on ice. The lysate was then centrifuged at 8000xg for 10 minutes and the supernatant was transferred to a new cold microcentrifuge tube. The sample protein concentration was then assessed using Pierce BCA protein assay kit (Thermo Scientific) according to the protocol supplied by the vendor. Briefly, samples were first diluted by 10-fold. Then 10µl of protein sample was mixed with 200µl of a 50:1 (v: v) dilution of solution A and B and incubated at 37 degrees Celsius for 30 minutes. The absorbance of the BCA/copper/protein complex was measured at 562nm wavelength, and the concentration was determined by plotting against a standard curve utilizing various concentrations of BSA.

2.5 Western Blots

For the western blot analysis, 30µg of total protein was mixed with 3.33 ul of 6x Lamenli sample buffer (J61337, Alfa Aesar) and diluted with water for a total volume of 20µl. The mixture was heated to 70 degrees Celsius for 10 minutes and then loaded and separated on a 10 percent Tris-SDS polyacrylamide gel at 150V. The proteins in the gel were transferred onto a PVDF membrane using the semi dry method at 20V for 8 minutes (IB401001, Thermofisher Scientific). The membranes were incubated in blocking buffer (5 percent skim milk in TBS containing 0.1 percent Tween 20 and then washed 3x for 5 minutes each with TBS containing 0.1 percent Tween 20. The membranes were incubated in primary antibody (table 2-3) in 5 percent fat free BSA in TBS containing 0.1 percent Tween 20 with the recommended dilutions by the manufacturer overnight at 4 degrees Celsius. The membranes were washed 3x for 5 minutes each to remove the primary antibody with TBS containing 0.1 percent Tween 20. The membranes were then incubated in the secondary antibody (table 2-4) in a solution of 5 percent
fat free BSA in TBS containing 0.1 percent Tween 20 for 1 hour at room temperature. The membranes were washed to remove the secondary antibody 3x for 5 minutes each with TBS containing 0.1 percent Tween 20. To obtain results, the membrane was incubated with Super Signal™ West Pico PLUS Chemiluminescent Substrate (34579, Thermofisher Scientific) for 5 minutes at room temperature. The blots were visualized under a CCD camera.

Table 4. Primary Antibodies Used for Western Blot.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Catalog Number/ Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-SREBP-1 Mouse Antibody (2A4)</td>
<td>sc-13551/ Santa Cruz</td>
</tr>
<tr>
<td>Anti β-Actin Rabbit Antibody (13E5)</td>
<td>4970/ Cell Signaling Technology</td>
</tr>
</tbody>
</table>

Table 5. Secondary Antibody Used for Western Blot.

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Catalog Number/Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse IgG, HRP-linked Antibody</td>
<td>7076S/ Cell Signaling Technology</td>
</tr>
<tr>
<td>Anti-rabbit IgG, HRP-linked Antibody</td>
<td>7074S/ Cell Signaling Technology</td>
</tr>
</tbody>
</table>
Chapter 3

Results

3.1 Insulin Mediated Expression of G6PDH

As the rate limiting enzyme in the pentose phosphate pathway, G6PDH is an important enzyme in both carbohydrate and oxidation-reduction metabolism. Early studies on the expression of the gene for G6PDH demonstrated regulation of its expression by diet and hormones such as insulin (50, 51). Therefore, it is important to confirm that insulin induces the expression of G6PDH in our model. Therefore, the effect of insulin on G6PDH expression was evaluated by measuring G6PDH mRNA. We confirmed that insulin significantly induced the expression of G6PDH compared to a no addition control as shown in Figure 3.
Figure 3. Insulin Mediated Expression of G6PDH.

The change in expression of G6PDH was measured using the primer G6PD, Assay ID Rn01529640_g1. Primary rat hepatocytes were incubated in low glucose media. 44nM insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. The fold change was expressed as the mean +/- S.E.(N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.

**** Denotes significance of p<0.0001.

3.2 Insulin Mediated Expression of FAS
FAS is the 2\textsuperscript{nd} committed step in fat synthesis and catalyzes the synthesis of fatty acids from carbohydrates. Previous studies have shown that it is regulated primarily via changes in nutritional status which can induce hormonal changes such as releasing insulin and glucagon (41, 44, 152, 153). Therefore, we wanted to confirm that insulin also induces FAS expression in our system. The effect of insulin on FAS expression was evaluated by measuring FAS mRNA. We confirmed that insulin significantly induced the expression of FAS compared to a no addition control as shown in Figure 4.

![FASN Gene Expression](image)

Figure 4. Insulin Mediated Expression of FAS.

The change in expression of FAS was measured using the primer FASN, Assay ID Rn00569117\_m1. Primary rat hepatocytes were incubated in low glucose media. 44nM insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA
compared to non-treated cells. The fold changed was expressed as the mean +/- S.E.(N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.
**Denotes significance of p<0.01.

3.3 Insulin Mediated Expression of SREBP1c

As previously discussed, the insulin induction of G6PDH and FAS expression via the PI3K/Akt signal pathway is mediated through transcriptions factors. In evaluating those transcription factors, we find that SREBP1c is the common transcription factor that is involved in the insulin induction of both G6PDH and FAS. FAS and G6PDH both contain an SRE element where SREBP1c would bind to allow the expression of the genes (40, 57). Therefore, it is important to confirm that insulin induces the expression of SREBP1c in our system. The effect of insulin on SREBP1c expression was evaluated by measuring SREBP1c mRNA. The results shown in Figure 5 confirm that insulin significantly induced the expression of SREBP1c compared to the no addition control.
Figure 5. Insulin Mediated Expression of SREBP1c.

The change in expression of SREBP1c was measured using the primer SREBF1, Assay ID Rn01495769_m1. Primary rat hepatocytes were incubated in low glucose media. 44nM insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. The fold changed was expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism. ** Denotes significance of p<0.01.

3.4 Insulin Mediated Suppression of INSIG2 Expression

INSIG2 is a very important protein that helps control lipogenesis via its ability to inhibit SREBP1c processing (154). SREBP1c processing, is activated by insulin through 2 mechanisms; one uses the mTOR pathway, and the other inhibits INSIG2 downstream of Akt independent of the mTOR pathway (72). Insulin suppresses the expression of INSIG2 via the Akt pathway.
independent of the mTOR pathway which allows for the processing of SREBP1c. The processed mature form of SREBP1c is then able to bind to promoter regions of target genes such as FAS (20, 75). Therefore, it is important to confirm that insulin suppresses the expression of INSIG2 in our system. We evaluated the insulin effect on INSIG2 expression and found that indeed insulin significantly suppresses the expression of INSIG2 as measured by a decrease in its mRNA compared to no addition control as shown in Figure 6.

**Figure 6. Insulin Mediated Suppression of INSIG2 Expression.**

The change in expression of INSIG2 was measured using the primer INSIG2, Assay ID Rn00710111_m1. Primary hepatocytes were incubated in low glucose media. 44nM insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. The fold change was expressed as the mean +/- S.E.(N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism. **** Denotes significance of p<0.0001.
3.5 Effect of Glucosamine on the Insulin Induction of G6PDH Expression

The expression of G6PDH and its activity is suppressed in metabolic diseases such as Type I diabetes (155, 156). However, there are conflicting reports regarding its expression under insulin resistant conditions. This may be in part due to the role of G6PDH in regulating oxidative stress due to the production of NADPH, a reducing agent needed for the reduction of ROS (140, 157–162). Oxidative stress has been documented as a potential cause of insulin resistance (108, 129, 130, 163). Excessive production of NADPH can also lead to increased oxidative stress therefore contributing to insulin resistance (157).

Previously, our laboratory has shown that insulin normalizes the expression of G6PDH in Streptozotocin induced diabetic animals (142), and induces its expression via the mTOR pathway in primary rat hepatocytes (55). Others have shown that this induction of G6PDH by insulin is partially mediated through SREBP1c (56, 164, 165). Using a model of insulin resistance induced by glucosamine, others in our lab have shown that the insulin induction of G6PDH expression is suppressed (166). These results have been confirmed as shown in Figure 7.
Figure 7. Insulin Mediated Expression of G6PDH in the Presence of Glucosamine.

Primary rat hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. The fold changed were expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.

**** Denotes significance of p<0.0001.
*** Denotes significance of p<0.001.
* Denotes significance of p<0.05.

3.6 Effect of Glucosamine on the Insulin Induction of FAS.

Compounds other than glucosamine have been used to induce insulin resistance and their effect on metabolism has been measured. For example, under palmitate induced insulin resistance the insulin induction of fatty acid synthesis continues unabated (132, 137–139, 167). Studies have shown that palmitate induces insulin resistance through two different mechanisms, one through increasing flow in the HBP and the other by increasing oxidative stress (132, 168).
Interestingly, oxidative stress also activates the HBP (116). Given that glucosamine induces insulin resistance through bypassing the regulatory enzyme in HBP, it seems logical to conclude that similar to palmitate induced insulin resistance, the induction of FAS expression by insulin would continue unabated with glucosamine. Indeed, others in our lab evaluated this possibility and showed that glucosamine does not affect the insulin induction of FAS expression (166) as shown in Figure 8.

This indicates that under glucosamine induced insulin resistant conditions, there is one mechanism that allows glucosamine to suppress the insulin induction of G6PDH expression while another mechanism is allows the insulin induction of FAS to continue unabated.

**FASN Gene Expression**

![FASN Gene Expression](image)

Figure 8. Insulin Mediated Expression of FAS in the Presence of Glucosamine.

The change in expression of FAS was measured using the primer FASN, Assay ID Rn00569117_m1. Primary rat hepatocytes were incubated in low glucose media with or without
glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. The fold changed were expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.

**Denotes significance of p<0.01.
*Denotes significance of p<0.05.
ns Denotes no significance.

3.7 Glucosamine Effect on the Insulin Induced Expression of SREBP1c.

SREBP1c is a common transcription factor that mediates the insulin induction of both FAS and G6PDH (29, 40). Others in our lab have shown that the insulin induction of SREBP1c expression is suppressed under glucosamine induced insulin resistance (Ruff, 2015) and these results are confirmed as shown in Figure 9. Since the insulin induced expression of SREBP1c is suppressed with glucosamine similar to G6PDH, it suggests that a different mechanism that does not involve SREBP1c is at work for FAS.
Figure 9. Insulin Mediated Expression of SREBP1c in the Presence of Glucosamine.

Primary rat hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. The fold changes were expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.

**Denotes significance of p<0.01.
*Denotes significance of p<0.05.
ns Denotes no significance.

3.8 Glucosamine Effect on the Insulin Induced Suppression of INSIG2 Expression.

As discussed earlier, INSIG2 is an inhibitor of SREBP1c processing (154). The expression of INSIG2 is suppressed by insulin in an Akt dependent manner (20). Others have shown in our lab that glucosamine suppresses the insulin induced phosphorylation of Akt. With INSIG2, downstream of Akt, it is safe to conclude that glucosamine suppresses the insulin effect on INSIG2 expression. Therefore, the effect of glucosamine on the insulin induced suppression
of INSIG2 expression was evaluated and as shown in Figure 10, surprisingly, glucosamine did not have a significant effect on the insulin induced suppression of INSIG2 expression. With these results similar to the FAS results, it would suggest that the mechanism that glucosamine uses to allow the insulin effect of FAS and INSIG2 expression to continue is the same. These results would also suggest that under glucosamine induced insulin resistance, any available SREBP1c will be processed.

Figure 10. Insulin Mediated Suppression of INSIG2 Expression in the Presence of Glucosamine.

The change in expression of INSIG2 was measured using the primer INSIG2, Assay ID Rn00710111_m1. Primary hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44n insulin (I) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. The fold changed were
expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.

**** Denotes significance of p<0.0001.
** Denotes significance of p <0.01.
ns Denotes no significance.

3.9 Effect of LXRα Agonist on the Insulin Induction of G6PDH Expression with or without Glucosamine

Given that glucosamine differentially effected the insulin induction of G6PDH and FAS expression and that the effect of glucosamine on the insulin induction of SREBP1c expression mirrored the effect of G6PDH, suggests that other factor(s) beyond SREBP1c is involved in maintaining the insulin induction of FAS expression with glucosamine. Thus, the promoter regions for both FAS and G6PDH were evaluated for a possible clue to explain this difference. We found that besides both genes containing a binding site for SREBP1c, they both contain a site for USF while only the FAS promoter contains a binding site for LXR (40, 57).

LXR is a transcription factor that is involved in the regulation of lipogenesis and cholesterol synthesis. LXRα is highly expressed in the liver and has been shown to induce the expression of lipogenic genes such as FAS and SREBP1c (40).

LXRα agonist effect on insulin resistance by other compounds was evaluated to offer a clue about the possibility of LXR being the differentiator in the regulation of gene expression of G6PDH and FAS under glucosamine induced insulin resistance. We found that LXRα through its agonist is able to overcome the insulin resistant effect of fat by inhibiting oxidative stress (132). Interestingly HBP is also activated by oxidative stress. With glucosamine bypassing the committed step in HBP to induce insulin resistance, it makes sense to conclude that LXRα would also overcome the glucosamine effect on the insulin induction of G6PDH. Therefore, we
evaluated whether LXRα through its agonist is able to overcome the glucosamine effect on the insulin induction of G6PDH. We found that LXRα agonist did not affect the basal expression of G6PDH compared to the no addition control, nor did it effect the insulin induction of G6PDH. LXRα agonist also did not affect the glucosamine effect on the insulin induction of G6PDH expression or the basal expression with glucosamine as shown in Figure 11.

This indicates that unlike with palmitate, LXRα agonist was not able to overcome the glucosamine effect on the insulin induction of G6PDH. With G6PDH not containing an LXRE to allow LXR to bind to its promoter, it might be possible, that the presence of an LXRE on the promoter of the target gene is a key for LXRα through its agonist to overcome the insulin resistant effect on the target gene.
Figure 11. Insulin Mediated Expression of G6PDH in the Presence of Glucosamine and LXR Agonist TO901317.

The expression of G6PDH was measured using the primer G6PD, Assay ID Rn01529640_g1. Primary rat hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. 1uM LXR agonist TO901317 (T) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. DMSO was used as a negative control. The fold changed were expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.
3.10 Effect of LXRα Agonist on the Insulin Induction of FAS Expression with or without Glucosamine

FAS as discussed above contains of LXRE element to allow LXRα to bind to its promoter region. Others have shown that LXRα through its agonist induces the expression of FAS independent of SREBP1c processing. Glucosamine did not have an effect on the insulin induction of FAS expression. Glucosamine also activates LXRα via glycosylation (85, 89). Therefore, it becomes possible that LXRα is the mediator in the mechanism that allows glucosamine to work to allow the insulin induction of FAS to continue unabated.

Therefore, we evaluated the LXRα agonist effect on the insulin induction of FAS expression. We found as indicated in Figure 12 that LXRα agonist indeed significantly induced the expression of FAS compared to no addition control. There was also an insulin additive effect on the LXRα induction of FAS. Glucosamine did not affect the LXRα agonist induction of FAS expression alone and with insulin. This indicates that LXRα might be the mediator in the mechanism that glucosamine uses to allow the insulin induction of FAS to continue.
Figure 12. Insulin Mediated Expression of FAS in the Presence of Glucosamine and LXR Agonist TO901317.

The change in expression of FAS was measured using the primer FASN, Assay ID Rn00569117_m1. Primary hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. 1μM LXR agonist TO901317 (T) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. DMSO was used as a negative control. The fold changed were expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.
3.11 LXRα Agonist Effect on the Insulin Induction of SREBP1c Expression with or without Glucosamine

SREBP1c contains an LXRE on its promoter region for LXR binding (69). Under fat induced insulin resistance, SREBP1c expression was elevated with LXRα agonist (132). As discussed above, there is also evidence that palmitate induced insulin resistance induces the HBP. Glucosamine induces insulin resistance via bypassing the committed step in the HBP. Therefore, it is logical to say that LXRα agonist overcomes the glucosamine induced insulin resistance on SREBP1c. Therefore, we evaluated the LXRα agonist effect on the glucosamine effect on the insulin induction of SREBP1c expression.

As shown in Figure 13, we confirmed that LXRα agonist significantly induced the expression of SREBP1c compared to no addition control. There also is an insulin additive effect on the LXRα induction of SREBP1c expression. Glucosamine however, significantly suppressed the LXRα induction of SREBP1c expression alone and with insulin. These results further solidify the possibility that the mechanism that glucosamine uses to work on FAS is not through SREBP1c. This also indicates that in contrast with FAS, and similar to G6PDH, the other mechanism that glucosamine uses to suppress the insulin induction of SREBP1c is not affected by LXRα agonist. This further solidifies the possibility of glucosamine working on FAS through LXR to allow the insulin induction of FAS expression to continue.
Figure 13. Insulin Mediated Expression of SREBP1c in the Presence of Glucosamine and LXR Agonist TO901317.

The change in expression of Srebp1c was measured using the primer Srebf1, Assay ID Rn01495769_m1. Primary rat hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. 1uM LXR agonist TO901317 (T) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. DMSO was used as a negative control. The fold changes were expressed as the mean +/- S.E. (N=7). Significance was evaluated using a One-way Anova test using Graphpad Prism.

*** Denotes significance of p<0.001.
** Denotes significance of p<0.01.
* Denotes significance of p<0.05.
ns Denotes no significance.
3.12 Effect of LXRα Agonist on the Insulin Induced Suppression of INSIG2 with or without Glucosamine

According to our results in Figure 13, we found that compared to no addition control, LXRα agonist still significantly induced the expression of SREBP1c. We also found according to our results in Figure 11, glucosamine did not affect the insulin induced suppression of INSIG2, an inhibitor of SREBP1c processing. In evaluating the LXRα agonist on SREBP1c processing, some have shown that LXRα agonist inhibits SREBP1c processing which is correlated with an increase in INSIG2 mRNA and protein in mice livers and rat hepatocytes (42, 87). In culture, it has also been shown that LXRα through its agonist does not have an effect on the insulin induced suppression of INSIG2 (87). Others have shown that low concentrations of LXRα agonist elevated SREBP1c processing in mice livers of high fat fed mice (169). With the glucosamine effect on the insulin induced suppression of INSIG2 expression similar to FAS, it becomes possible that LXRα agonist might not affect the insulin effect on INSIG2. Therefore, we evaluated the LXRα agonist effect on the insulin effect on INSIG2. We found that according to Figure 14, that LXRα agonist did not affect the basal expression of INSIG2 compared to no addition control, nor did it effect the insulin induced suppression of INSIG2 expression. LXRα agonist did not alter the basal expression of INSIG2 expression with glucosamine, nor did it effect the insulin induced suppression of INSIG2 with glucosamine. This indicates that LXRα does not have an effect on the insulin induced suppression of INSIG2 under normal or glucosamine induced insulin resistance.
Figure 14. Insulin Mediated Expression of INSIG2 in the Presence of Glucosamine and LXR Agonist TO901317.

The change in expression of INSIG2 was measured using the primer INSIG2, Assay ID Rn00710111_m1. Primary rat hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. 1uM LXR agonist TO901317 (T) was added where indicated and incubated for 18 hrs. Values indicate fold increase in mRNA compared to non-treated cells. DMSO was used as a negative control. The fold changed were expressed as the mean +/- S.E. (N=7) Significance was evaluated using a One-way Anova test using Graphpad Prism.

**** Denotes significance of p<0.0001.
*** Denotes significance of p <0.001.
** Denotes significance of p<0.01
ns Denotes no significance.
3.13 Effect of LXRα Agonist on SREBP1c Processing with or without Glucosamine

Our results in Figure 13 show that LXRα though its agonist still significantly induces the expression of SREBP1c compared to no addition control. Our results in Figure 14 also show that LXRα did not affect INSIG2 expression, nor did it effect the insulin effect on INSIG2 expression with or without glucosamine. Therefore, it becomes logical to say that LXRα through its agonist might not affect SREBP1c processing. Therefore, we evaluated the LXRα agonist effect on SREBP1c processing, and found as show in Figure 15 that similar to SREBP1c expression, LXRα significantly increases the amount of precursor SREBP1c protein compared to no addition control, and that glucosamine significantly reduces it. There was also a significantly increased amount of mature SREBP1c as a result of LXRα agonist inducing high amounts of precursor SREBP1c, however glucosamine did not have an effect on the amount of mature SREBP1c protein induced by LXRα. This indicates that LXRα might not have an effect on SREBP1c processing. These results along with the results from the LXRα agonist effect on INSIG2 expression would solidify that whatever pSREBP1c is available to be processed will be processed.
Figure 15. Effect of LXRα Agonist on SREBP1c Processing.

Western blots were used to measure the amount of Srebp1c protein, the antibody used to detect SREBP1c was anti-SREBP1c mouse 2A-4 (sc-13551, Santa Cruz) and the antibody for beta-actin was anti β-actin rabbit (13E5) (4970, Cell Signaling Technology). Primary rat hepatocytes were incubated in low glucose media with or without glucosamine (G) for 18 hrs. 44nM insulin (I) was added where indicated and incubated for 18 hrs. 1μM LXR agonist TO901317 (T) was added where indicated and incubated for 18 hrs. DMSO was used as a negative control.
Lipogenesis is an important complex metabolic process that allows for the conversion of excess carbohydrates to triglycerides (TG) that are stored mainly in the liver and adipose tissues. The process starts with converting acetyl CoA, an intermediate metabolite from glycolysis and other metabolic pathways to the 16-carbon fatty acid chain, palmitate. Palmitate then undergoes desaturation and esterification to become triglycerides. The amount of fat accumulation is determined by the balance between fatty acid synthesis and fatty acid oxidation (170). Therefore, any dysregulation due to obesity, type 2 diabetes or oxidative stress can lead to excessive fat accumulation which results in non-alcoholic fatty liver disease (NAFLD). NALFD can range from excess fatty liver, to hepatic steatosis to liver cirrhosis due to excess fat from lipolysis in the adipose tissues, to excess lipogenesis, and decreased fatty acid oxidation (134, 135). The complications from NALFD including obesity and cardiovascular issues have heightened the importance of understanding how lipogenesis is regulated and what happens when dysregulation occurs.

The rate of lipogenesis is determined by two key lipogenic enzymes that catalyze the committed steps of this metabolic pathway, ACC which catalyzes the 1st committed step and FAS which catalyzes the 2nd committed step of fatty acid synthesis. ACC is a complex molecule that catalyzes the formation of malonyl CoA from acetyl CoA. The catalytic site of the complex molecule contains 2 catalytic sites, the first one is a biotin carboxylase site at which biotin, a required cofactor, is carboxylated, the second one is a carboxylase site that transfers the carboxylate group to acetyl CoA to form malonyl CoA (171, 172). FAS is a multicomplex
homodimeric enzyme that catalyzes the second committed step of fatty acid synthesis. The FAS monomer contains six catalytic sites which are beta-ketoacyl synthase (KS), acetyl/malonyl transacylase (AT/MT), beta-hydroxyacyl dehydratase (DH), enoyl reductase (ER), beta-ketoacyl reductase (KR), acyl carrier protein (ACP), and thioesterase (TE) that sequentially aid in the synthesis of the 16-carbon chain palmitate from acetyl CoA and malonyl CoA (173, 174).

Like other metabolic pathways, lipogenesis is regulated by the nutritional and hormonal status. During fasting conditions, circulating glucose is low and glucagon levels are high which lead to inhibition of lipogenesis and the stimulation of fatty acid oxidation. Under feeding conditions however, circulating glucose and insulin levels are high which leads to the induction of lipogenesis and the inhibition of fatty acid oxidation. Insulin activation is stimulated through specific signaling pathways such as the PI3K pathway and downstream signal molecules such as Akt, the atypical protein kinase C (aPKC), and the mTOR pathway, as well protein phosphatases such as PP1 and PP2 (41, 175–180).

Glucose and insulin both regulate the lipogenic enzymes through changes in gene expression and posttranslational modifications such as phosphorylation and dephosphorylation of the enzymes themselves. For example, insulin and glucose activate ACC by dephosphorylation by PP1 in many cell types such as beta cells, hepatocytes, and adipocytes (24, 181–185). Insulin and glucose also regulate the gene expression of FAS and ACC via the mTOR pathway and PP1 which control the expression and activity of the transcription factors on the promoter regions of both genes (40, 158, 166–169).

PPP is another important metabolic pathway that is regulated by insulin, diet, and oxidative stress. The role of this pathway is to produce NAPDH, a cofactor needed for the synthesis of fatty acids and to combat oxidative stress. It also has a role to produce the ribose-5-phosphate needed
for nucleic acid synthesis. The rate of glucose flux into the PPP is controlled by G6PDH, the enzyme that catalyzes the 1st committed step. G6PDH, first discovered in 1931, is a protein that is active in dimer or tetramer form due to G6P and NADP binding to it (29). Like other metabolic pathways, the regulation of the PPP is tightly controlled and flux through the pathway can vary due to the nutritional and hormonal status, and oxidative stress (29).

Earlier studies in whole animals and cells in culture have shown that under fasting conditions or low glucose and insulin conditions the activity and expression levels of G6PDH was reduced. While under feeding conditions, in response to insulin and glucose, the activity and expression levels of G6PDH were elevated (55, 142, 190, 191). Insulin induces the expression of G6PDH in hepatocytes via the mTOR pathway downstream of the PI3K/Akt pathway (55).

The transcriptional regulation of lipogenic genes has been a focus of study for many years (20, 40, 45, 65, 70, 84, 192–194). It has been shown that the insulin regulation of lipogenic genes involves the changes in gene expression and posttranslational modifications of the transcription factors ChREBP, SREBP1c, LXR, and USF (40, 195). SREBPs are bHLH-LZ transcription factors that bind as dimers to the sterol regulatory elements (SRE) of the promoter regions of target genes involved in metabolic pathways. There are 3 isoforms of SREBPs which are SREBP1a, SREBP1c, and SREBP2, with SREBP1c being most predominately expressed in the liver and responsible for the regulation of the expression of lipogenic genes (192). Overexpression studies of SREBP1c in fasted mice and knockout studies of SREBP1c in refed mice demonstrated that SREBP1c is a key transcription factor for the induction of lipogenic genes (196). Cell culture studies have shown a role of SREBP1c in the insulin induction of FAS and G6PDH, the 2 metabolic genes included in this study (29, 180, 197).
The activity of SREBP1c on its target genes requires the proteolytic processing of the enzyme. The protein is synthesized in an inactive form which is located at the ER as a larger complex with SCAP and INSIG proteins (72). SCAP proteins are ER sterol sensor proteins that escalate SREBPs from the ER to golgi by binding with the COPII vesicles of the golgi. INSIG are proteins when highly expressed are bound to the SCAP to prevent its binding to the COPII vesicles of the golgi. Once SREBP1c is cleaved, the mature N-terminal SREBP1c is translocated to the nucleus where it can now bind to the SRE elements of its target genes. Insulin induces the proteolytic processing of SREBP1c via 2 different pathways downstream of Akt to induce FAS expression (74, 75, 95). One pathway involves the insulin suppression of INSIG2 gene expression independent of the mTOR pathway. The other pathway involves the P70 S6 kinase downstream of the mTOR pathway. P70 S6 kinase is a serine threonine kinase that is part of the mTOR pathway that is downstream of PI3K. It is involved in many processes such as metabolism, cell growth and survival (198).

Over the years it has been established that glucosamine, a molecule that bypasses GFAT and activates HBP, induces insulin resistance in various cell types and whole animals. Glucosamine induces insulin resistance by lowering insulin sensitivity by reducing IRS-1 tyrosine phosphorylation. This decrease in active IRS-1 leads to the reduction of the PI3K/Akt signaling pathway along with the reduction of the insulin induced activation of the mTOR pathway downstream of Akt, which then leads to decreased glucose tolerance (102, 104, 107, 199). OGT, the key enzyme in O-glycosylation of proteins, was shown to play a key role in inducing insulin resistance through its overexpression in vivo in mice (103). Glucosamine was also able to elevate FAS and ACC gene expression in adipocytes (200). However, others in our lab have seen little effect of glucosamine on the expression of FAS in rat hepatocytes (166).
Our laboratory utilized glucosamine to establish an insulin resistant model in primary rat hepatocytes in culture to study G6PDH, the key enzyme in regulating the glucose shunt through PPP, and FAS, the key enzyme in the regulation of fatty acid synthesis from excess glucose. Others in our lab have evaluated the effect of glucosamine on the insulin induction of G6PDH and FAS. They found as expected that glucosamine would suppress the insulin induction of G6PDH expression. Surprisingly though, they found that glucosamine did not affect the insulin induction of FAS even though insulin regulation of both FAS and G6PDH is mediated through the PI3K/Akt pathway. Others in our lab have shown in hepatocytes that glucosamine suppresses the insulin induction of Akt phosphorylation at Ser473. These results suggest a possible mechanism to disrupt the insulin signal pathway since for insulin to fully activate the PI3K/Akt pathway, Akt must be phosphorylated by PDK-1, which allows the mTORC2 to phosphorylate Akt at Ser473. Others have shown in adipocytes that the insulin induction of Akt phosphorylation at Thr308 was suppressed by glucosamine (201). Although our laboratory had not investigated the phosphorylation status of Thr308 on the Akt protein with glucosamine, it is anticipated that results similar to those found in adipocytes could also be found. Since our results support the findings that glucosamine affects the PI3K/Akt pathway it suggests that there must be a different mechanism not involving this pathway to keep the insulin induction of FAS expression unabated with glucosamine.

To evaluate which mechanism glucosamine utilizes to manifest the effects on FAS expression, the transcription factors involved in the insulin induction of G6PDH, and FAS expression must be evaluated. From the literature, it is known that insulin induces the expression of G6PDH via SREBP1c binding to the SRE of the promoter of G6PDH. G6PDH contains a binding site for USF similar to FAS but the role of this site has yet to be determined. The binding
site for USF in FAS is required for SREBP1c to be recruited to the SRE element during the insulin induction of FAS expression (202). The insulin induction of SREBP1c requires the cooperation of LXR, SREBP1c, NF-Y, and SP1 to fully induce the expression of SREBP1c downstream of the mTOR pathway (66). G6PDH also contains several SP1 binding sites, but none have been shown to be involved in the insulin induction of G6PDH to date (57). Under refeeding conditions, LXRα agonist can induce the expression of FAS independent SREBP1c (42), an indication that LXR can induce the expression of FAS independent of SREBP1c.

Since SREBP1c is part of the mechanism by which both G6PDH and FAS are induced by insulin suggests that it might not be part of the mechanism through which glucosamine exerts its effect in hepatocytes. This assumption is substantiated by our results that show that similar to G6PDH glucosamine did suppress the insulin induction SREBP1c expression. Some other mechanism must be in play for FAS since induction of its expression by insulin was not suppressed by glucosamine.

In addition to insulin inducing the expression of SREBP1c, insulin also induces the processing of SREBP1c in an Akt dependent manner (20, 72). SREBP1c processing is required for the SREBP1c to bind to the SRE element of its target gene. INSIG2 has been shown to inhibit SREBP1c processing by keeping it bound to the ER in a complex with SCAP. When insulin suppresses the expression of INSIG2, the amount of INSIG2 protein also is reduced leading to the dissociation of the pSREBP1c from the ER to initiate the processing of SREBP1c. The insulin suppression of INSIG2 happens in an Akt dependent but mTOR independent mechanism. As discussed above, glucosamine suppresses the phosphorylation of Akt induced by insulin. Therefore, one would expect that glucosamine would minimize the insulin induced suppression of INSIG2 expression. Surprisingly however, we found that glucosamine did not have an effect on
the insulin suppression of INSIG2. Therefore, similar to FAS expression, glucosamine utilizes a different mechanism to allow the insulin suppression on INSIG2 suppression to continue. Since glucosamine reduces the insulin induced phosphorylation and activation of Akt, it seems plausible that the Akt pathway must be important in the glucosamine suppression of the insulin induction of G6PDH. Also, since glucosamine does not suppress the insulin induction of FAS, another mechanism or pathway must be involved. In reviewing the literature and evaluating other possible mechanisms, it was found that insulin induced suppression of INSIG2 down stream of Akt is mediated by CREBZF in liver cells of mice (203). CREB/ATF bZIP transcription factor (CREBZF) is a co repressor that inhibits the cAMP mediated transcription of cAMP responsive genes such as INSIG2 (203). This study also showed that under fat induced insulin resistance, the expression of CREBZF is elevated in livers of high carbohydrate/ high fat mice (203). Although no studies to date have linked CREBZF to glycosylation or glucosamine, it is interesting to speculate that glucosamine induced insulin resistance might influence the expression of CREBZF.

With SREBP1c possibly not involved in the mechanism that allows the insulin induction of FAS to continue in the presence of glucosamine, our attention turned to another transcription factor that is involved in the regulation of FAS and SREBP1c which is LXR. LXR is a transcription factor that is involved in the insulin induction of FAS (204). It is a transcription factor that belongs to the nuclear hormone receptor superfamily of the sterol regulated transcription factors that are activated by ligands binding to them such as oxysterols (40). It has been shown before that LXR is a key transcription factor downstream of Akt that is involved in the insulin induction of FAS and SREBP1c (53). LXR is downstream of S6K1 of the mTOR pathway and has been shown to be activated under palmitate induced insulin resistance (167). In the presence of palmitate, the protein expression levels of mTOR, S6K1, LXRα, SREBP-1c, FAS, ACC1 and phosphorylated
mTOR, S6K1, and mRNA expression levels of lipogenesis-related markers were significantly upregulated in a time-dependent manner demonstrating that palmitate-induced lipid accumulation is associated with the increase in both mTOR/S6K1 and LXRα levels via the upregulation of downstream lipogenic genes.

LXRα through its agonist has been shown to overcome fat induced insulin resistance and its effect on gluconeogenesis without effecting fatty acid synthesis, as indicated by constant TG synthesis, (132) by preventing oxidative stress which inhibits the JNK pathway, a pathway that inhibits the insulin induced activation of the PI3K/Akt pathway in obese mice (132). This inhibition leads to the restoration of the insulin induction of Akt phosphorylation at Ser473 (132). In the same study, the insulin induction of SREBP1c is not affected in obese mice possibly due to the activation of the mTOR pathway independent of Akt activation in these animals. This conclusion is supported by a study that shows that in culture, palmitate, a molecule that mimics fat induced insulin resistance, induces SREBP1c expression and subsequently FAS expression (167).

With oxidative stress activating the HBP and LXRα agonist inhibiting oxidative stress to restore Akt phosphorylation at Ser473, one might question whether LXRα overcomes the glucosamine effect on the insulin induction of G6PDH and SREBP1c. Interestingly, our results show that LXRα did not overcome the glucosamine effect on the insulin induction of both G6PDH and SREBP1c. Moreover, glucosamine also suppressed the LXRα induction of SREBP1c expression. This is an indication that oxidative stress might not be involved in the glucosamine effect on the induction of SREBP1c expression by both LXR and insulin and on the insulin induction of G6PDH expression. Others in our lab have shown using the JNK inhibitor SP600125, that inhibiting that the JNK pathway, a pathway activated by oxidative stress, did not ameliorate the glucosamine effect on G6PDH and SREBP1c expression nor did it change the expression of
FAS expression when glucosamine was added, supporting the conclusion that oxidative stress is not a mediator in these findings. In evaluating the mechanisms that regulate LXRα in the literature, it was shown that AMPK which is activated by glucosamine suppresses the LXRα agonist induction of SREBP1c in rat hepatoma cells (205, 206). So, it is possible that glucosamine could activate the AMPK protein thereby reducing the LXRα induction of SREBP1c expression.

Since inhibiting the JNK pathway did not change the insulin induction of FAS when glucosamine was added, other mechanism(s) must be involved. We showed in this study that glucosamine did not affect the LXRα agonist induction of FAS alone or with insulin, which is a different outcome than what happened with SREBP1c. With both promoter regions containing an LXRE, this would indicate that other factors beyond LXR binding to the target gene are involved in the glucosamine effect on the metabolic genes if glucosamine acts on FAS via LXRα. In examining those factors, one of proteins involved in the regulation of a FAS and G6PDH is INSIG2. INSIG2 as discussed above, is an inhibitor of SREBP1c processing.

Our results show that glucosamine does not affect the insulin induced suppression of INSIG2 similar to the no effect of glucosamine on the insulin induction of FAS. Therefore, it becomes important to understand whether LXRα agonist has a similar effect on INSIG2. Others have shown that LXRα agonist elevated the expression of INSIG2 correlated with a decrease in SREBP1c, however when insulin was present the agonist did not overcome the suppression of INSIG2 expression. Therefore, LXRα does not affect the insulin action on INSIG2 (42, 87). Another study showed that the under high fat diet, the LXRα agonist still elevated SREBP1c processing (169). In our study, the LXRα agonist did not affect the expression of INSIG2 alone or the insulin effect on INSIG2 with or without glucosamine.
In examining the regulation of INSIG2 by other factors, it has been shown that glucagon activates INSIG2 to inhibit lipogenesis by shutting down SREBP1c processing (194, 207). AMPK, which is inhibited by insulin also activates INSIG2 expression and protein activity (208). AMPK increases LXRα expression and protein levels in macrophages while other studies have shown AMPK decreases the LXR induced expression of SREBP1c correlated with decreased nSREBP1c (91, 205). With glucosamine activating AMPK pathway (206, 209), we would expect an increase in INSIG2 expression with glucosamine, but there was no effect on the basal expression of INSIG2 in our study.

With LXRα agonist not affecting INSIG2 expression, we would expect similar outcome with SREBP1c processing. We found that the protein amounts of nSREBP1c induced by LXRα agonist was not affected by glucosamine despite a reduction in the amount of pSREBP1c induced by LXRα agonist. These results correlate with the SREBP1c expression being reduced by glucosamine, and the INSIG2 expression not effected by glucosamine. So, processing of available SREBP1c might is not impacted by glucosamine. Therefore, whatever SREBP1c is available will be processed. These results further support the possibility that SREBP1c is not involved in the mechanism that allows glucosamine to work on FAS expression.
Chapter 5

Conclusion and Significance

The goal of this study was to examine the insulin resistant effect on the insulin regulation of two important metabolic genes, FAS and G6PDH. We utilized glucosamine as an insulin resistant model due to many studies that show that excess glucose causes higher flux through the HBP which causes insulin resistance. In this study, we focused on the regulation of the gene expression of G6PDH and FAS under glucosamine induced insulin resistant conditions. These enzymes that are important in the regulation of PPP and fatty acid synthesis respectively, two important pathways in maintaining glucose homeostasis. We concluded that glucosamine suppressed the insulin induction of G6PDH expression, while it did not affect the insulin induction of FAS expression. SREBP1c, a common transcription factor that is involved in the insulin induction of both G6PDH and FAS expression, was also suppressed by glucosamine. The insulin induced suppression of INSIG2 expression, a protein that inhibits SREBP1c processing, was not affected by glucosamine. With LXRα being a transcription factor involved in the regulation of SREBP1c and FAS expression, we evaluated its effects on our target molecules using a LXRα agonist, TO901317. We concluded that the LXRα agonist did not have an effect on the basal expression of G6PDH, nor the insulin induction of G6PDH expression. LXRα agonist also did not have an effect on either the basal expression or the insulin induction of G6PDH expression in the presence of glucosamine. Interestingly, the LXRα agonist alone increased the basal expression of FAS and this increase appears to be additive with insulin. Glucosamine did not influence the LXRα agonist induction of FAS expression alone or with
insulin, an indication that glucosamine might utilize LXRα to allow the insulin induction of FAS to continue. Similar to FAS, LXRα agonist induces the basal expression of SREBP1c, and insulin has an additive effect on this induction. Although glucosamine significantly suppressed the LXRα agonist effect on SREBP1c expression alone and with insulin, the level of SREBP1c expression was still higher than baseline indicating a significant amount of SREBP1c was available for processing. These results tend to suggest that SREBP1c might not be the differentiator in the regulation of G6PDH and FAS under glucosamine induced insulin resistance.

To uncover whether SREBP1c was processed to its mature form under these experimental conditions, we evaluated the expression of INSIG2 which is an inhibitor of SREBP1c processing. LXRα agonist did not have an effect on the basal expression of INSIG2, nor the insulin induced suppression of INSIG2 expression, and these expression levels were unaltered in the presence of glucosamine. The results suggest that under our experimental conditions with LXRα agonist any available SREBP1c could be processed to its mature form. Western blot analysis confirmed that the precursor form of SREBP1c induced by LXRα agonist continued to be processed to the mature form. These results support the conclusion that glucosamine regulates the insulin induced expression G6PDH and FAS via two different mechanisms. The first mechanism involves glucosamine reducing the insulin induction of G6PDH expression by reducing the expression of SREBP1c. The second mechanism involves glucosamine acting via LXRα to allow the insulin induction of FAS expression to continue despite lower pSREBP1c protein levels.

Our study is important to help understand the mechanism of insulin resistance which can lead to finding additional possible treatments for diabetes. In other models of insulin resistance
such as palmitate induced insulin resistance, it is well documented that the insulin induction of fatty acid synthesis, based on continuation of normal TG values, continues unabated. This model mimics fat induced insulin resistance. While fatty acid synthesis under these conditions continues unabated, other pathways such as the insulin induced inhibition of gluconeogenesis is suppressed leading to an imbalance in glucose homeostasis that leads to higher blood sugar.

The higher blood sugar which is a characteristic of diabetes results in many complications such as eye, nerve, and renal damage. Therefore, it is very important to understand all aspects of this disease. Our study focuses on the glucosamine effect on the insulin regulation of FAS and G6PDH, key enzymes in carbohydrate metabolism and fat synthesis to help better understand mechanisms of diabetes that may lead to additional treatments for the disease. In our study, we found that glucosamine suppresses the insulin induction of SREBP1c, a common transcription factor involved in the insulin induction of both G6PDH and FAS expression. This is a significant variance from other models of insulin resistance that show that the insulin induction of SREBP1c is not affected by insulin resistance. The suppression of the insulin induction of SREBP1c by glucosamine would mean that the insulin induction of FAS and G6PDH expression should be suppressed by glucosamine. However, in our model, the insulin induction of FAS expression is not affected by glucosamine, unlike the insulin induction of G6PDH expression which is suppressed by glucosamine similar to SREBP1c. So, we addressed how would glucosamine allow the insulin induction of FAS to continue even though SREBP1c expression is reduced. Could it be possible that glucosamine modulates other transcription factors? Other common factors include USF which is required for the insulin induction of FAS by recruiting SREBP1c, and ChREBP which has been shown to regulate FAS expression in response to glucose (40, 57). ChREBP and USF has not been linked to the regulation of G6PDH expression.
yet. Therefore, USF and ChREBP regulation of G6PDH can be evaluated in future studies.

LXRα is an important transcription factor in the regulation of the insulin induction of FAS and SREBP1c expression but not for G6PDH, so our efforts focused on this protein. Although, we found that glucosamine suppresses the LXRα induction of SREBP1c alone and with insulin, there was still a significant amount of SREBP1c being expressed under these conditions. Glucosamine, however, had no effect on the LXRα agonist induction on FAS expression alone or with insulin. This is a significant outcome because other models of insulin resistance show that the induction of SREBP1c expression is correlated to the induction of fatty acid synthesis.

Results from others show that the insulin induction of FAS requires USF to recruit SREBP1c to the promoter, therefore USF is necessary to stimulate the insulin induction of FAS via SREBP1c (193, 202, 210). To confirm that in our system, we would need to evaluate whether insulin induces the binding of SREBP1c and USF to the promoter regions of FAS by first showing that insulin induces the binding of SREBP1c to the promoter region of FAS. Since USF binding to the promoter region of FAS does not change in response to insulin, then we will need to evaluate the SREBP1c-USF binding in response to insulin. Since insulin phosphorylates USF via DNA-PK, then the insulin induced phosphorylation of USF would need to be evaluated.

LXRα agonist induction of SREBP1c expression is still significant under glucosamine induced insulin resistance. Western blot analysis demonstrated that in cells treated with the LXRα agonist, SREBP1c processing is not affected with or without glucosamine. The insulin induced suppression of INSIG2 expression was also not affected under the same conditions. Also, in our study, glucosamine suppresses the insulin induction of SREBP1c to near basal levels suggesting that another mechanism is in play that can bypass SREBP1c and allow the insulin
induced expression of FAS to continue. This maybe through the LXRα because LXRα is required for the insulin induction of FAS (204).

With SREBP1c still significantly expressed by LXRα despite the suppressive effects of glucosamine, and the processing of SREBP1c not impacted under these conditions, the question arises as to whether the insulin induction of USF/SREBP1c binding also suppressed by glucosamine? If that is the case, then that further supports that glucosamine uses a mechanism that bypasses SREBP1c to allow the insulin induction of FAS to continue unabated in our conditions. This mechanism could also be through LXRα, since our results show no glucosamine effect on the insulin and LXRα induction of FAS expression.

Our study provides very important information about how insulin resistance effects the signaling pathways that directly regulate the metabolic pathways. Current diabetic medicines focus on many processes that do not target the enzymes that directly regulate the metabolic processes. For example, sodium-glucose cotransporter-2 (SGLT-2) inhibitors such as canagliflozin help release the sugars into the kidney for excretion in the urine. Alpha glucoside inhibitors suppress the breakdown of complex of carbohydrates into sugars to prevent glucose from quickly entering the circulation. Other medications such as amylin and meglitinides help insulin secretion. Metformin, one of the more popular drugs, helps increase insulin sensitivity to stimulate glucose uptake and inhibit glucose production. However, none of the current diabetic medicines directly address the metabolic pathways that regulate glucose homeostasis. Our study provides important information about the regulation of G6PDH and FAS, two important enzymes in the regulation of PPP and fatty acid synthesis respectively to help maintain glucose homeostasis. Perhaps from our study and future studies that will evolve from it, new diabetic medicines that address the metabolic processes that maintain glucose homeostasis will replace
the current medicines that only help insulin sensitivity, glucose uptake, and inhibition of glucagon release. These medicines do not currently address the core problem of the disease which is the dysregulation of the metabolic pathways by insulin resistance. The information from our study and future studies that evolve from it could pave the way to finding new diabetic medicines that address the expression key metabolic pathway enzymes such as G6PDH and FAS under insulin resistant conditions to be able to maintain glucose homeostasis in a more efficient and better way under insulin resistant conditions, a hallmark of type 2 diabetes.
Appendix
IACUC Approval Form for Use of Primary Rat Hepatocytes

WESTERN MICHIGAN UNIVERSITY
Institutional Animal Care and Use Committee
ANNUAL REVIEW OF VERTEBRATE ANIMAL USE

PROJECT OR COURSE TITLE: Regulation Of Gene Expression In Hepatocytes
IACUC Protocol Number: 10-05-03
Date of Review Request: 02/12/21
Date of Last Approval: 5-20-20
Purpose of project (select one): ☐ Teaching ☒ Research ☐ Other (specify):

PRINCIPAL INVESTIGATOR OR ADVISOR
Name: John Splibergen
Department: Biological Sciences
Title: Professor
Electronic Mail Address: john.splibergen@wmich.edu

CO-PRINCIPAL OR STUDENT INVESTIGATOR
Name: Jooah Hachem
Department: Chemistry
Electronic Mail Address: jooh.hachem@wmich.edu
Title: Graduate Student

1. The research, as approved by the IACUC, is completed:
☐ Yes (Continue with items 4-5 below.) ☒ No (Continue with items 2-5 below.)

If the answer to any of the following questions (items 2-4) is “Yes,” please provide a detailed explanation on an attached sheet of paper. Include details of any modifications made to the protocol based on new findings or publications, adverse events or mortalities.

2. Have there been any changes in Principal or Co-Principal Investigators? ☐ Yes ☒ No

3. Have there been any new findings or publications relative to this research that require you to alter your study? ☐ Yes ☒ No

Describe the sources used to determine the availability of new findings or publications:
☐ No search conducted (Please provide a justification on an attached sheet.)
☐ Animal Welfare Information Center (AWIC)
☒ Search of literature databases (select all applicable)
☐ AGRICOLA ☐ Current Research Information Service (CRIS)
☐ Biological Abstracts ☒ Medline
☐ Other (please specify):
Date of search: 03/02/21
Years covered by the search: 2016-2021
Key words: rodent models and primary cell isolation, insulin resistance, regulation of FAS and G6PDH gene expression, transcription factors- SREBP-1c, FOXO, PPARα, liver X receptor and INSR.
☐ Additional search strategy narrative:

4. Are there any adverse events, in terms of animal well-being, or mortalities to report as a result of this research? ☐ Yes ☒ No

Cumulative number of mortalities:

5. Animal usage: Number of animals used during this quarter (3 months): 0
Cumulative number of animals used to date: 20

Signed 04/20/2021

Principal/Investigator/Faculty Advisor Signature Date

IACUC REVIEW AND APPROVAL
Upon review of the relevant information regarding this protocol, the IACUC approval for this project has been extended for one year from the date of this signature.

Signed 05/03/2021

IACUC Chair Signature Date

Revised 05/2019 WMU IACUC All other copies obsolete.
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