

The Effects of Low-Dose Endotoxin on Whole Body Glucose Metabolism

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ABSTRACT

Obese individuals present with an increased inflammatory tone as compared to healthy, normal-weight individuals, which is associated with insulin resistance. One factor hypothesized to contribute to increased inflammation in obese and diabetic states is elevated blood endotoxin levels, also known as metabolic endotoxemia. In healthy rodents (non-obese and insulin sensitive), there is evidence that blood endotoxin levels fluctuate over the course of the day with elevations in the post-prandial state that return to baseline levels in the post-absorptive state. High-fat feeding in these animals altered these fluctuations causing endotoxin levels to remain high throughout the day. The effects of alterations in endotoxin levels on glucose metabolism are not understood. The goal of this study was to determine the effects of short-term and long-term increases in endotoxin of a low magnitude on insulin signaling in a human primary cell line as well as the effects of short-term endotoxin treatments on glucose homeostasis in a C57/B16 mouse model. First, we tested the hypothesis in cell culture that short-term low-dose endotoxin treatments would enhance insulin-signaling and glycogen synthesis while long-term treatments would have inhibitory effects. Under our second hypothesis, we examined whether short-term low-dose treatments of endotoxin would contribute to improvements in glucose tolerance in a mouse model. In contrast to our first hypothesis, short-term endotoxin treatments did not improve insulin signaling or glycogen synthesis although long-term treatments did contribute to decreases in glycogen synthesis.

Interestingly, short-term endotoxin treatments resulted in significant improvements in glucose clearance in the mouse model; this is believed to be partly attributed to LPS inhibiting gluconeogenesis. Future studies are necessary to understand the mechanisms responsible for altered glucose metabolism in response to low magnitude changes in LPS levels.

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Chapter 1: Introduction

Inflammation is a key mediator linking obesity and Type 2 Diabetes^{1,2}. Normal-weight individuals exhibit an immune response to various instigators such as sickness, injury, that is resolved following removal of the instigator³. These same individuals also show an immune response following ingestion of high-fat meals. Obese individuals show a chronically heightened immune response that can negatively affect insulin signaling processes and this can cause insulin resistance^{4,5}.

A mechanism thought to contribute to this chronically elevated inflammatory response is known as metabolic endotoxemia^{4,6,7}. Metabolic endotoxemia was first coined by Cani, et al.⁴ to describe the chronic low grade inflammation resulting from elevated circulating endotoxin concentrations. Endotoxin has been shown to activate certain members of the toll-like family of pattern recognition receptors and this can cause an inflammatory signaling response. Research from both mouse and human models has attributed chronic increases of endotoxin to the development of metabolic diseases including obesity and insulin resistance^{4,7,8}. In normal individuals (insulin sensitive and normal weight) endotoxin levels behave in a cyclical manner, reaching a peak during the postprandial period following a meal⁴. The above mentioned studies performed by Cani et al., in which rodents were fed a high-fat diet for 4 weeks, showed increased levels of endotoxin that did not fluctuate as they did in rodents fed a normal diet, but remained at high levels throughout the day. This response represents a vital connection between the diet and the consequent changes in endotoxin levels.

The objective of this study was to determine the effects of low levels of endotoxin (lipopolysaccharide or LPS) on insulin-stimulated glucose clearance through the use of acute and chronic treatments in cell culture and in a mouse model using acute treatments. This

approach is unique because it uses doses of endotoxin that are both physiologically relevant to humans and low enough as to avoid a systemic inflammatory response, which could confound the results. Using this approach, our hypotheses are that transient increases in endotoxin levels will improve glucose tolerance in an animal model, whilst using cell culture studies, prolonged treatments with endotoxin will harm insulin signaling and therefore impair normal glucose homeostasis. This study will lend insight into the changes in glucose metabolism that can occur as a result of normal fluctuations in endotoxin levels.

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Chapter 2: REVIEW OF LITERATURE

1. Background.

Obesity, insulin resistance, and heart disease have long been associated with one another as being primary metabolic diseases and are often grouped together under the term, metabolic syndrome¹⁻⁴. Epidemiological data suggest that approximately 24% of the American population (47 million people) is affected by the metabolic syndrome^{5,6}. Of these individuals, 10% of women and 15% of men present with normal glucose tolerance; 42% and 64% with impaired fasting glucose; and 78% and 84% with type 2 diabetes⁷. Most patients (> 80%) with type 2 diabetes have metabolic syndrome, but the converse is not necessarily true⁷. These data stress the importance of understanding the complex relationship between the three diseases and, in particular, the mechanisms that underlie altered glucose metabolism. It is understood that the relationship between the three diseases is a complex one that cannot be simplified to a linear relationship of causation. However, the most supported model suggests that an obese state and the metabolic environment associated with it can cause insulin resistance and heart disease abnormalities^{8,9}. This model suggests an insulin resistant state can contribute directly to the development of coronary artery disease and further exacerbate the obese state^{10,11}.

It is important to determine the mechanisms that allow for an altered metabolic environment to contribute to the development of the insulin resistant state. One of the key mediators to its development and its contribution to the pathogenesis of dyslipidemia and heart disease is the inflammatory environment¹²⁻¹⁴. Normal-weight individuals exhibit increases in the presence of inflammatory molecules with injury or sickness that returns to normal levels once the instigator

is removed. In the case of obese individuals, there is a persistent low-grade inflammation that can directly affect insulin signaling processes causing insulin resistance that can contribute to the development of heart disease^{15,16}.

One of the possible contributing factors to the altered inflammatory environment that contributes to these diseases is metabolic endotoxemia¹⁷⁻¹⁹. Cani et al. first coined the term metabolic endotoxemia in order to describe the chronic increases in blood endotoxin levels that are seen with prolonged (4 weeks) high-fat feeding in rodents. Endotoxin is the major glycolipid component of the outer membrane of gram-negative bacteria that comprise approximately 70% of the total bacteria in the gut¹⁹. There is a limited understanding of how the diet can alter levels of endotoxin. One of the possible mechanisms explaining changes in endotoxin levels in these disease states are the concomitant changes seen within the gut microbiome of afflicted individuals²⁰⁻²². Gut microbiome refers to the bacteria that colonize the length of the intestinal tract. Obese and diabetic individuals have significantly different bacterial profiles in their gut and these differences correlate with changes in endotoxin levels as well as insulin sensitivity^{19,23}. Another possible mechanism is through alterations in proteins that control gut barrier function²⁴.

Much work remains to determine the role of endotoxin in mediating the inflammatory environment and the dynamics of endotoxin involvement in glucose metabolism. It is known that endotoxin can activate certain of the toll-like family of pattern recognition receptors, specifically the toll-like receptor 4 (TLR4) and this can initiate an inflammatory response^{25,26}. Activation of TLR4 can contribute to the defects in insulin signaling that are seen in an insulin-

resistant state²⁷. Various models exist in which inflammatory molecules of the TLR4 pathway have been deleted or inhibited and there is protection against insulin resistance²⁸. Many of the deleterious effects of endotoxin on glucose metabolism and insulin signaling exist in chronic or long-term situations in which endotoxin levels remain elevated for many hours. What remains to be fully understood is how acute or short-term changes in endotoxin levels can elicit effects on the insulin signaling machinery and whole-body glucose metabolism. More importantly there is little research to determine what constitutes a significant enough change in endotoxin levels to effect glucose metabolism. A significant proportion of research in this area uses levels of endotoxin that elicit an inflammatory response. This includes work showing that acute treatments with extremely high dosages (sepsis) of endotoxin in animals can cause increases in glucose uptake²⁹. These levels of endotoxin do not represent fluctuation such as those seen following meals.

This review will seek to explain how the diet-influenced changes in the gut environment can partially explain the relationship between an obese state and the associated insulin resistant state. Importantly, we will determine the involvement of the gut in mediating changes in endotoxin, and how these changes can both negatively and positively affect glucose metabolism.

2. The Metabolic Syndrome

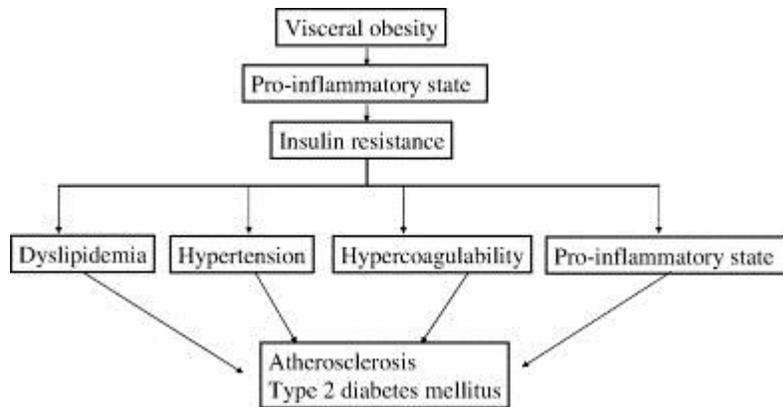


Figure 2.1 Pathogenesis of The Metabolic Syndrome

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The term metabolic syndrome refers to a group of metabolic disturbances that all contribute to cardiovascular disease³⁰.

Figure 2.1 represents the current view of the pathogenesis of the metabolic syndrome. The disturbances involved include abnormalities in glucose tolerance such as

hyperinsulinemia and insulin resistance as well as central obesity, dyslipidemia, and hypertension³⁰⁻³². The concept that these abnormalities are associated with and causative of one another has been suggested for nearly 90 years; however, with a sharp increase in the prevalence of these disorders over the last 20 years, there has been much attention given to the mechanisms underlying the syndrome. The relationship between these disorders is complex and it is possible to present with either one or any combination of the disorders, it becomes important to determine a criteria for diagnosing the metabolic syndrome. There are multiple definitions of these criteria given by various sources with fairly good agreement that glucose intolerance, hyperlipidemia, and obesity are central to its development and diagnosis. There remains a clear association between these diseases and developing an understanding of relationships will be essential to the treatment of the metabolic syndrome. This relationship

has been difficult to discern, it is likely driven by various factors including those that are genetic, epigenetic, and environmental in nature. Insulin resistance and obesity are often thought to be the essential co-morbidities to the development of the other disease outcomes^{33,34}. Literature suggests that obesity often precedes and directly contributes to the defects in insulin sensitivity and lipid handling^{33,35,36}. However, it is important to recognize that insulin resistance can cause alterations in the handling of FFA that can contribute to the development of obesity^{37,38}. Thus, it is most likely that these abnormalities are directly contributing to the progression of metabolic syndrome. Increases in fat stores as well as decreases in fatty acid oxidation lead to various changes including increases in circulating free fatty acids that can significantly alter normal metabolic activities including the handling of glucose and fatty acids within insulin sensitive tissues^{38,39}. Lean, normal weight individuals typically rely on a particular substrate (glucose, fatty acids or proteins) for energy production depending on feeding status and energy need. The ability to switch between these various substrates is known as the Randle Cycle and this becomes dys-regulated in overweight individuals^{40,41}. Obese individuals show a preference for oxidizing glucose and storing fatty acids in comparison to lean individuals⁴².

The inability to properly regulate substrate preference and subsequent increases in circulating free fatty acids can greatly contribute to individuals becoming more insulin resistant and obese as well as developing cardiovascular issues⁴³. Mechanisms controlling insulin resistance include circulating fatty acids interfering with signaling processes involved in the insulin-dependent uptake of substrates into tissues^{43,44}. Fatty acids can impair activation of protein kinase C- λ and protein kinase C- ζ as well as the derivatives of fatty acids such as acyl-CoA

and ceramide being able to directly inhibit AKT, an important signaling intermediate in insulin sensitive tissues⁴⁵. Increases in free fatty acids and their intermediates in the circulation can also cause disruptions in mitochondrial oxidative phosphorylation that can add to the excess storage of fatty acids in various tissues⁴⁶. There also appears to be defects within the endoplasmic reticulum that leads to a cellular stress response that can cause serine-phosphorylation of the insulin receptor, rendering it un-responsive⁴⁷.

This model in which obesity and altered lipid handling often precede and cause the defects in insulin signaling, also suggests that altered insulin responsiveness works to worsen the defects in lipid handling. To this point, disruptions in insulin signaling and glucose tolerance often are used in describing the pathophysiology of the metabolic syndrome. These findings suggests that glucose control and the inflammatory environment that contribute to altered glucose handling could be an important area to target for treatments.

3. Glucose Metabolism

Important to understanding the mechanisms involved in the development of insulin resistance and type 2 diabetes is an understanding of glucose metabolism in healthy individuals. Carbohydrates from the diet are the main energy source for humans and they primarily take the form of starches⁴⁸. A typical human diet will consist of approximately 250 grams per day of carbohydrates⁴⁸. Overconsumption of carbohydrates can contribute to the development of obesity and insulin resistance^{49,50}. The digestion of carbohydrates begins in the mouth with α -amylase hydrolyzing the glycosidic bonds in carbohydrates⁵¹. This produces short chain polysaccharides and maltases that are more easily digested later⁵¹. Digestion of other

carbohydrates such as disaccharides occurs in the brush border of the small intestine⁵². The brush border contains enzymes that are specific to the breakdown of these disaccharides. Once carbohydrates are sufficiently broken down by the enzymes of the mouth and small intestine they are available for uptake⁵². This uptake occurs first across the apical membrane of the small intestine by a variety of transporters and the specific transporter is determined by the structure of the carbohydrate⁵². Hexoses such as glucose and fructose are transported by the sodium/glucose co-transporter1 (SGLT 1) which is dependent on ATP. Fructose is transported by GLUT5 which functions through facilitated diffusion. Once across the apical membrane carbohydrates must cross the basolateral membrane to reach the blood. There are 3 destinations for glucose and other carbohydrates once inside the enterocyte.⁵² Approximately 15% will leak back into the intestine while the other 85% will enter circulation. Of this 85%, approximately 60% of entry will occur with the help of Glut2 and the other 25% through diffusion⁵². The monosaccharide's fructose and galactose are not strictly regulated by hormonal control of insulin, this review will focus on glucose as its uptake is strictly regulated by insulin and it is nutritionally the most important monosaccharide.

The primary defects involved in insulin resistance are the inability of insulin sensitive tissues to recognize and respond to increases in blood glucose, as well as the inability of the body to appropriately initiate a hormonal response to the increases in glucose⁸. This review will focus on the inability to properly respond to increases in glucose and do so as the mechanisms relate to inflammation in both a hormonal and a tissue specific manner.

4. Glucose Homeostasis During Normal and Insulin Resistant Conditions

Hormonal Influence.

The release of metabolic hormones is primary to controlling glucose homeostasis⁵³. Glucose levels must be tightly regulated to ensure that tissues are able to meet energy needs. Despite the fact that an individual can be under various stages of feeding or fasting, glucose levels in the blood are always maintained at a level between 4 and 7mM in healthy individuals⁵⁴. This is due to the regulation of glucose levels by absorption in the gut, hepatic glucose production and glucose uptake in tissues. Hepatic glucose production and glucose uptake in tissues are both tightly regulated by hormones⁵⁵.

Under the fed state, achieved following ingestion of a meal, the primary hormone involved in glucose homeostasis is insulin. Insulin is released from the pancreatic beta cells in response to an increase in blood glucose levels but also to a lesser degree in response to increases in the levels of fatty acids and protein. Once insulin is released from the pancreas it circulates in the blood where it can then bind to tissues expressing the insulin receptor. These tissues are wide ranging but the primary tissues expressing these receptors are skeletal muscle, adipose tissue, and liver⁵⁶. Once bound by insulin these receptors initiate a signaling cascade that leads to the uptake of primarily glucose but also fatty acids are metabolized for energy production. Insulin controls glucose uptake through increases in the transcriptional and translational expression of the glucose transporter Glut4⁵⁷. Insulin also controls the expression and activity of enzymes involved in the synthesis of glycogen, lipids, and proteins as well the oxidation of glucose through increases in enzymes controlling glycolysis in various tissues. To maintain glucose

homeostasis, insulin must inhibit the processes controlling endogenous glucose production (gluconeogenesis) as well as other catabolic processes including glycogenolysis, lipolysis, and protein breakdown⁵⁸.

In individuals who are insulin-resistant, tissues are unable to respond to changes in blood insulin levels. The latter contributes to abnormally high blood glucose levels following a meal. The pancreas responds to the elevated blood glucose by increasing insulin production and secretion (hyperinsulinemia) to counteract the abnormal glucose levels⁵⁹. However, this mechanism of overproduction that is seen in the pre-diabetic state is only able to function for a certain period of time after which the beta cells begin to malfunction and further disease progression occurs. This overproduction of insulin greatly contributes to the development of Type 2 diabetes and cardiovascular disease through the increases in fat storage as well as through further degradation of insulin sensitivity. Evidence suggests that prolonged insulin treatment is sufficient for preventing the acute action of insulin on Foxo1 phosphorylation and Glut4 cellular membrane trafficking^{60,61}. Insulin can also inhibit *Irs2* gene transcription in the liver and promote IRS2 ubiquitination, both leading to disruption of normal insulin sensitivity⁶².

Under fasted conditions the primary hormone regulating glucose homeostasis is glucagon. Glucagon is released from the alpha cells of the pancreas in response to mixed nutrient meals, oral or intravenous (IV) amino acids, activation of the autonomic nervous system (ANS) and hypoglycemia⁶³. The primary functions of glucagon are to stimulate the breakdown of glycogen in the liver and skeletal muscle and to up-regulate gluconeogenesis in the liver. These

result in glucose being released into the blood that can be accessed for energy purposes by tissues. Gluconeogenesis is the process by which glucose is synthesized from glucose precursors derived from fatty acids and amino acids. This happens through up-regulation of lipolytic enzymes in adipose tissue, freeing substrate for glucose production. Glucagon also down-regulates most of the before mentioned processes that insulin up-regulates⁶³. This switch or adaptation that occurs with these metabolic hormones between the different feeding statuses is known as the Randle cycle. When tissues become insulin-resistant there is an inability to make this switch in substrate preference and glucose becomes the sole substrate used by the tissues for energy production⁶⁴.

Glucagon also plays a role in the development and exacerbation of insulin resistance into Type 2 diabetes⁶⁴. One of the central features of Type 2 diabetes is hyperglycemia. This results not only from tissues being insensitive to insulin and unable to uptake glucose from the blood, but also from dysregulation of hepatic glucose production⁶⁴. Normally, when insulin is present in the blood, enzymes regulating hepatic glucose production are down-regulated; however, in an insulin resistant state this regulation is faulty and hepatic glucose production continues, further contributing to the damaging state of hyperglycemia. Along with this there is a defect in the alpha-cells that secrete glucagon resulting in chronically increased levels of glucagon being present in the blood⁶⁵. Hepatic insulin resistance results in an inability to properly respond to the changing levels of glucose in the blood, further exacerbating the hyperglycemic state⁶⁵.

Role of Skeletal Muscle in Maintaining Glucose Homeostasis.

Skeletal muscle comprises 40-50% of total body mass in humans and therefore plays a pivotal role in the maintenance of whole-body glucose homeostasis⁶⁶. Skeletal muscle is the primary insulin-responsive tissue in the post-prandial period and because of this the mechanisms underlying insulin resistance are deeply rooted in the skeletal muscle and contribute greatly to the whole body defects in glucose homeostasis⁶⁷. Typically, when blood glucose levels increase following a meal, there is release of insulin from the pancreas into the blood stream. Insulin then binds to the insulin receptor and this initiates a signaling cascade that results in glucose being transported across the plasma membrane and into the cytosol. Once within the cytosol glucose is phosphorylated and subsequently directed towards its most useful fate, determined by the energy need of the cell at that time. When the cell is in a state of energy demand, glucose will be converted to pyruvate by glycolysis and then directed toward the mitochondria where the TCA cycle and oxidative phosphorylation will convert glucose into ATP to meet energy demands. When energy needs are successfully met, glucose can be stored within the cell in the form of glycogen, to be accessed during periods of energy demand. During situations of energy surplus, pyruvate can also be directed towards fatty acid synthesis. The series of events that lead to glucose being transported into the cell are complex and defects within this signaling cascade are often seen with insulin resistance¹².

Immediately upon binding, the insulin-receptor complexes internalize by receptor-mediated endocytosis to initiate several intracellular sequences of events, one of which is to facilitate glucose transport into skeletal and cardiac muscle. Insulin is able to exert its effects on nutrient uptake through two primary pathways⁶¹. The first pathway is the Insulin Receptor Substrate-1

(IRS-1) dependent pathway and the second, an IRS-1 independent mechanism. The IRS-1 dependent pathway has two pathways of action. One is the PI3K-AKT and the other the Ras-MAPK pathway⁶⁸. The PI3k pathway is the one primarily involved in the metabolic action of insulin including glucose uptake. The Ras-MAPK pathway is involved in gene expression but also interacts with the PI3K pathway in controlling cell growth and differentiation⁶⁹. Typically, phosphorylation of the insulin receptor leads to tyrosine phosphorylation of IRS-1. This renders IRS-1 active and causes signal transduction. IRS-1 can be phosphorylated at various tyrosine residues, all of which will cause this activation⁷⁰. Serine activation acts in the opposite manner and prevents transmission of the signal downstream⁷¹. This becomes an important concept in understanding many of the perturbations that occur with insulin resistance being caused by inflammatory processes.

There are other substrates involved in mediating insulin action and subsequently glucose uptake that are independent of IRS-1 signaling. Two of these are able to act on more classical insulin signaling pathways while the third acts independently. Both the Shc/Grb2 protein complex and the Gαq/11 heterotrimeric G protein act on either the PI3K or Ras/MAPK pathways^{68,72,73}. The proto-oncogene product Cbl can interact with the Cbl binding protein at the Insulin receptor and stimulate Glut4 translocation that is independent of any of the classical pathways⁷⁴.

Uptake of glucose into muscle cells is controlled by a family of glucose transporters known as GLUT⁵⁷. There are various isoforms within the GLUT family, however, GLUT4 and GLUT1 are those primarily expressed in skeletal muscle⁷⁵. GLUT4 is involved in the insulin-dependent

uptake of glucose following a meal. GLUT4 resides within intracellular vesicles under non-insulin stimulated conditions and is transported to the cell surface in the presence of insulin where it docks and fuses with the plasma membrane to allow for engulfment of glucose molecules⁷⁶. GLUT4 also mediates contraction-stimulated glucose uptake into the cell⁷⁷. The pathway that controls contraction mediated glucose uptake during exercise appears to be completely independent of the insulin stimulated glucose uptake pathway and largely appears to be controlled by calcium release from the sarcoplasm⁷⁷. GLUT1 is ubiquitously expressed in the plasma membrane and primarily is involved in the basal or non-insulin stimulated uptake of glucose into the cell⁷⁸.

In skeletal muscle of insulin-resistant individuals, defects can arise at various points within the insulin-signaling pathway and each can contribute negatively to whole-body glucose homeostasis. Alterations in the expression, binding, phosphorylation state, and/or kinase activity of the insulin receptor can account for the defects in glucose uptake^{79,80}. The insulin receptor can undergo serine phosphorylation and this can down-regulate its function. Serine phosphorylation can be controlled through both chronically high insulin levels as well as increases in the presence of inflammatory molecules⁸¹. It has been shown that the insulin receptor gene can be negatively affected through inheritance patterns as well as through diet modulation⁷⁹. Homozygous mice void of the insulin receptor gene die in the neonatal stage whereas, heterozygous mice are healthy⁸². Mice with knockout of the IRS1 gene present as mildly insulin resistant, however; when the two mutations are combined in mice they become severely insulin resistant and diabetic⁸². The IRS1 protein itself has also been shown to contribute to the defect. Diabetic tissues have been shown with reduced levels of IRS1 protein

as well decreased tyrosine phosphorylation of IRS1⁸³. This suggests, while defects in the insulin receptor may contribute to the pathology of insulin resistance, it most likely does not stand alone.

Events downstream of the insulin receptor/ IRS1 complex can also contribute greatly to the defect. The molecule that has been shown to be completely necessary for insulin-stimulated Glut4 translocation is PI3K⁵⁷. PI3K is activated through its phosphorylation by IRS1 and it appears that increased levels of FFA related to obese and high fat conditions may play a role in down-regulating the activation of PI3K⁸⁴. Insulin-resistant individuals have a decrease in the association between IRS1 and PI3K, however, this does not down-regulate the expression of AKT which is the downstream effector of PI3K⁷⁹. This suggests that maximal activation of PI3K is not necessary to induce glucose uptake and that PI3K itself cannot completely control the defects in insulin signaling⁷⁹.

There may also be defects present in the Glut4 vesicle machinery^{85,86}. In an insulin resistant state, there are decreases in both the number and functionality of these vesicles and this is related to an increase in inflammatory markers⁸⁷.

Role of the Liver in Maintaining Glucose Homeostasis.

While insulin resistant skeletal muscle can contribute to whole body defects in glucose homeostasis, alterations in how the liver senses nutrients and manages glucose output can also add to these issues⁸⁸. As mentioned previously, output of glucose from the liver is tightly controlled by the hormone glucagon. In states of fasting hepatic glucose production as well as glycogenolysis are up-regulated to maintain glucose levels within a normal range and this is

mediated by glucagon. Once glucose levels begin to rise following a meal, insulin release inhibits the production of glucagon resulting in decreased hepatic glucose production and glycogen breakdown. The liver plays a vital role in the uptake and distribution of nutrients to various peripheral tissues and requires tight control of the liver enzymes (Figure 4.1) involved. Therefore, dysregulation within the liver as seen with insulin resistance can have dire consequences on whole body metabolism.

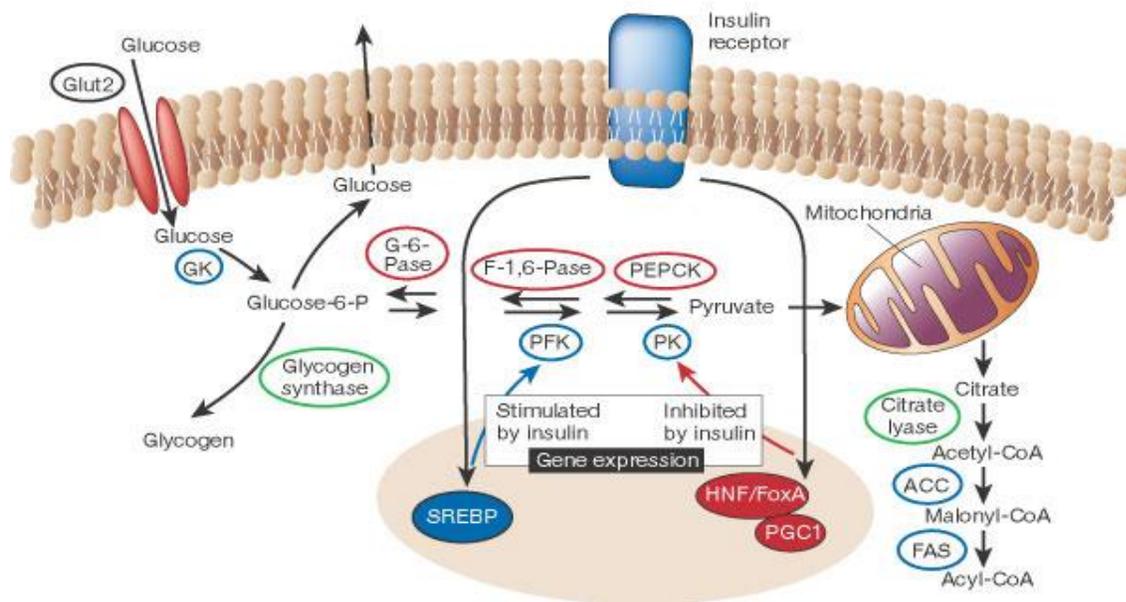


Figure 4.1 Glucose Metabolism in the Liver
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The role of the liver in maintaining blood glucose levels begins and ends with the glucose transporter GLUT2⁸⁹. GLUT2 works to uptake glucose from the blood into the liver following a meal as well as in transporting glucose out of the liver during periods of low blood glucose⁸⁹. Typically, insulin is able to exert its effects on the liver through the phosphorylation and de-

phosphorylation as well as through transcriptional regulation of the key enzymes involved in glycolysis, glycogen synthesis, and gluconeogenic pathways. Insulin inhibits gluconeogenesis at several key points. It inhibits the transcription of the gene encoding phosphoenolpyruvate carboxylase (PEPCK), the rate-limiting step in gluconeogenesis⁹⁰. It also causes decreases in the transcription of the genes encoding fructose-1,6-bisphosphatase and glucose-6-phosphatase, and increases in the transcription of glycolytic enzymes such as glucokinase and pyruvate kinase⁹¹. Insulin also up-regulates the expression and activity of glycogen synthase. Covalent modification of these enzymes through phosphorylation can also control which pathway is favored. Phosphorylation and de-phosphorylation are tightly controlled through a set of kinases and phosphatases specific to each enzyme.

With insulin resistant states there is an inability within the liver to properly regulate the switch between states of glycolysis and glycogen synthesis and states of gluconeogenesis⁶³. This serves to worsen the whole body effects of glucose intolerance by further increasing blood glucose levels, already at high levels from the inability of peripheral tissues to clear glucose.

5. Inflammation Mediated by TLR4 leads to Disruptions in Glucose

Homeostasis.

As mentioned previously, the central defect of insulin resistance is the inability of tissues to recognize and bind insulin and to properly initiate a signaling cascade intracellularly leading to translocation of glucose transporters to the cell surface⁸⁵. A chronically elevated immune response, primarily in the form the production of pro-inflammatory molecules, has long been associated with the changes in glucose metabolism that are seen with insulin resistant and

diabetic states^{8,13,21,68,92}. The production and release of harmful pro-inflammatory molecules can occur following ingestion of diets high in fats, both in short term and long term situations, and the production of inflammatory molecules following this is a central cause of the defects in insulin signaling as observed in insulin resistant individuals⁹³. A key mediator of the inflammatory response is toll-like receptor 4 (TLR4). TLR4 is an activator of pro-inflammatory pathways, specifically those pathways involved with inhibiting insulin signaling^{27,68}. Obese and diabetic subjects have significantly elevated TLR4 gene expression and protein content in muscle and TLR4 muscle protein content correlates with the severity of insulin resistance⁴². This review will focus on the role of TLR4 mediated inflammation on influencing disease specific control of glucose metabolism.

Role of TLR4 in Inflammation and Insulin Resistance

TLRs are a family of pattern-recognition receptors that play a critical role in the innate immune system by initiating a pro-inflammatory signaling response to microbial pathogens^{94,95}. TLR4, the best-characterized TLR, binds primarily to lipopolysaccharide (LPS) of gram-negative bacterial cell walls as well as other ligands. Upon binding of ligands to TLR4 and its co-receptors CD14 and MD-2, the adaptor protein myeloid differentiation factor 88 (MyD88) is recruited to the Toll/IL-1 receptor (TIR) domain of the receptor^{96,97}. Interaction of the TIR domain of TLR4 and MyD88 triggers a downstream signaling cascade, leading to activation of the NF- κ B pathway, which then activates the transcription of many pro-inflammatory genes encoding pro-inflammatory molecules including cytokines, chemokines, and other effectors of the innate immune response⁹⁸. It has been shown that TLR4 expression as well as the inflammatory molecules regulated by TLR4 each have a connection to defects in insulin

signaling²⁷. When the TLR is knocked out of mice, these mice are protected from diet induced obesity and insulin resistance^{99,100}. The transcription factor primarily activated by TLR4 is NfKB^{95,99,101}. NfKB forms a regulatory complex with IkkB. When IkkB is phosphorylated there is subsequent activation of NfKB^{102,103}. One hundred years ago, Salicylates were used to treat high blood glucose and were shown to be an effective tool. It was later discovered that they were acting through the inhibition of IkkB. It has since been discovered that this effectiveness is only in high doses and these high doses can cause severe side-effects in patients⁶⁸. More recent studies have confirmed this effect of Salicylates and shown that these effects are similar when IkkB is knocked down through transgenic alteration¹⁰². IkkB can also have direct effects on IRS1 through its ability to serine phosphorylate and consequently down-regulate the molecule⁷¹.

The inflammatory molecule production through binding by NFκB, and as shown in Figure 4.1

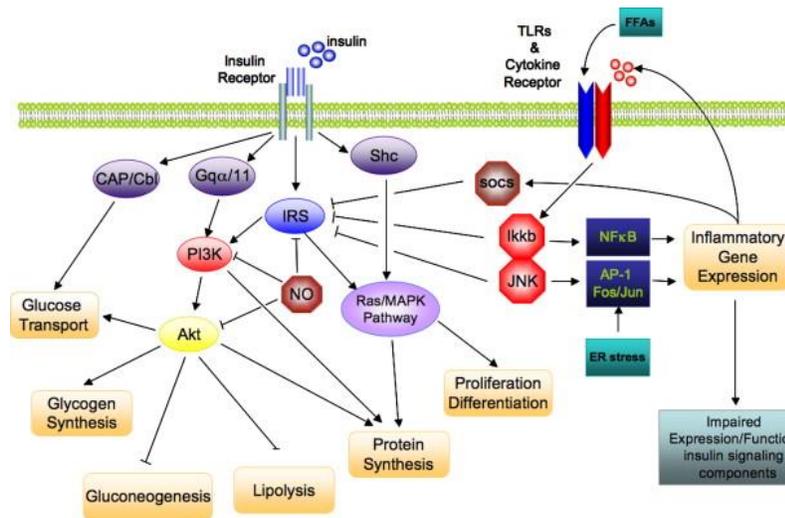


Figure 5.1 Inflammation and Insulin Resistance

**Reproduced from (2008). "Inflammation and insulin resistance." *FEBS Letters*, 582(1): 97-105. DOI: 10.1016/j.febslet.2007.11.057 with permission of Elsevier Publishing.

is wide-ranging¹⁰⁴. There are numerous molecules that directly interact with the insulin signaling machinery, causing defects. The most prominent of these molecules are Tnf- α , Il-6, and Il-1 α /Il-1 β ^{104,105}. Tnf- α has been shown to directly inhibit insulin signaling through

serine phosphorylation of IRS-1^{106,107}. Mice that lack Tnf show improvements in insulin sensitivity when compared to WT mice, when fed a high-fat diet¹⁰⁸⁻¹¹⁰. Obese humans show improvements in Tnf levels and insulin sensitivity when they undergo a weight-loss regimen^{111,112}. Il-1 α /Il1- β expression levels both show direct correlation to insulin sensitivity and transcriptional regulation of insulin signaling machinery¹¹³. Il-1 α /Il1- β KO mice have lower fasting glucose and insulin levels and have greater insulin sensitivity than do control mice¹¹⁴. Il1-a/b has been shown to directly target the insulin receptor by way of serine phosphorylation and also has roles in limiting the transcription of IRS1¹¹⁵. Il-6 can also contribute to defects. Research shows that insulin sensitivity increases in mice treated with anti-IL6 antibodies and concentrations of IL-6 decrease in humans undergoing weight loss regimens^{116,117}. In addition, IL-6 is increased in obese subjects compared to lean and this correlates with defects in insulin sensitivity¹¹⁸. In addition to the IkkB /NF κ B signaling complex, TLR4 activation can also work to stimulate inflammatory molecule production through the JNK/AP-1 pathway and this has similar deleterious effects on insulin signaling⁶⁸. Furthermore, there are numerous other inflammatory processes that can also be affected directly through this mechanism, each being able to contribute negatively to insulin signaling and glucose tolerance. Nitric oxide (NO) is an endogenous signaling molecule produced by nitric oxide synthase¹¹⁹. Normally, NO acts as a signal transduction molecule involved in vasodilation, but also is involved in mediating insulin resistance. Activation of TLR4 signaling can contribute directly to up-regulation of nitric oxide synthase and this can contribute to defects in insulin signaling^{120,121}. In the insulin signaling pathway, NO can interfere with AKT signaling and result in the degradation of IRS1 in skeletal muscle¹²². When NOS is inhibited in animal models they are protected from insulin resistance¹²². It appears that the mechanism

for this involves nitrosylation of serine residues on the insulin signaling machinery¹²³. Another inflammatory mechanism having harmful effects on insulin signaling is endoplasmic reticulum stress. TLR4 KO mice are protected against HFD-induced ER stress and this results in protection against diet induced glucose intolerance¹²⁴. While TLR4 signaling is essential for the initiation of the ER stress response, it remains to be fully understood how TLR4 signaling activates this response.

TLR4 mediated Beta-Cell destruction

Another mechanism by which TLR4 signaling can contribute to Type 2 Diabetes is through destruction of the beta-cells of the pancreas^{125,126}. Type 2 diabetes is characterized as not only a defect in the ability of tissues to recognize and bind insulin, but is also contributed to a defect in the ability of the pancreas to function normally in producing insulin. Pre-diabetics, or those individuals who have become insulin resistant while still maintaining normal glucose homeostasis, do so because the pancreas over-produces insulin, thus allowing the tissues to uptake enough glucose to maintain normal blood glucose levels, a process termed beta cell compensation¹²⁷. However, this mechanism eventually fails and this is largely contributed to the failure of the beta-cells themselves¹²⁷. There remains no shortage of hypotheses related to how this is being controlled and it remains a distinct possibility that a wide range of factors are involved. The failure of beta cells is contributed to increases in beta cell apoptosis as well as decreases in beta cell proliferation. The role of TLR4 in the stimulation of beta cell apoptosis has been well documented¹²⁸. Similarly to its role in inhibiting insulin signaling in peripheral tissues, TLR4 activation in the beta cell leads to the recruitment of various cytokines as well as the activation of the ER stress response and the creation of reactive oxygen species, all of

which can contribute to cell death. The interferon γ -inducible protein 10 (CXCL10) is shown to be up-regulated in beta cells of diabetic subjects and acts as a ligand for TLR4 signaling¹²⁸. A TLR4 (-/-) cell line (HEK 293), demonstrate protection against the diabetogenic effects of treatment with CXCL10. Additionally, it has been shown that CXCL10 treatment results in a sustained up-regulation of the AKT signaling pathway in beta-cells. This pathway is important for beta cell proliferation, but sustained activation also can have roles in apoptosis, and it has been hypothesized that this is a primary mechanism contributing to beta cell death. Another important inflammatory molecule mediating defects in beta cell function is IL-1 β ¹²⁹. IL-1 β has been shown to be regulated by TLR4 as well as by other stimuli including certain macronutrients such as glucose and FA's¹²⁹. Chronically elevated blood glucose levels as well as prolonged increases in FFA levels can activate IL-1 β and this can contribute directly to beta cell death¹²⁹.

6. Mechanisms Contributing to TLR4 Activation

Ligands for TLR4

It is clearly understood that TLR4 activation and signaling lead to increases in inflammatory molecules that contribute to the dysregulation of glucose metabolism. An important and yet to be definitively determined question related to TLR4 inflammatory signaling, are the mechanisms that cause the activation of TLR4. The mechanisms by which TLR4 inflammatory signaling becomes activated represent an important point of control in the process that could possibly be manipulated by treatments. For TLR4 signaling to initiate, the receptor must be bound by its ligand. There have been as many as 23 hypothesized ligands for TLR4¹³⁰. However, many of these ligands have been subsequently disproven as activators of TLR4

signaling as they were merely contaminated by lipopolysaccharide (LPS) like substances. LPS is the primary ligand for TLR4^{131,132}. A point of debate relating to what can activate TLR4 is whether activators are of microbial origin or are certain endogenous molecules capable of activating. Certain of these ligands, including fatty acids (FA) have been shown to act as ligands but this remains controversial¹³³. Microbial components such as LPS from the gut remain are a more likely source directly altered by the diet and contributing directly to diet-induced inflammation^{18,24,28}.

Fatty Acids as a TLR4 Ligand.

Obese individuals often present with increased levels of circulating fatty acids in their blood compared to lean individuals^{43,134}. These same individuals also have increased levels of cytokines and other inflammatory molecules that track with the increases in FFA¹⁵. Research has suggested a role for the FFA in activating the inflammatory response that produces the molecules capable of interfering with glucose metabolism¹³⁵. Previous work has suggested a role for FA's in activating TNF α and JNK signaling which can interfere with insulin signaling machinery, but an exact mechanism by which this occurred was unknown. Shi et al. showed that in both macrophages and adipose tissue, FA's could initiate inflammatory signaling through a TLR4 dependent mechanism¹³³. The use of TLR4 KO models both in-vitro and in-vivo showed a role for TLR4 activation by FA's in mediating defects in insulin signaling¹³³. Additional research in this area has shown FA's activating TLR4 in skeletal muscle cells^{99,136}. Saturated fatty acids (SFA) but not unsaturated fatty acids (UFA) can initiate an inflammatory response through TLR4 activation¹³⁷. UFA such as those of the ω -3 variety tend to exhibit anti-inflammatory properties¹³⁸. The mechanism by which FA's activate TLR4 remains to be

understood but two possibilities remain likely, one being that SFA induced ceramide biosynthesis is dependent on TLR4 signaling and this contributes to defects in insulin signaling^{139,140}. A more likely hypothesis explaining this is that increased LPS levels activate the TLR4/CD14 signaling machinery and this leads to up-regulation of the TLR2 which is a known receptor for FA's¹³⁷.

Metabolic endotoxemia

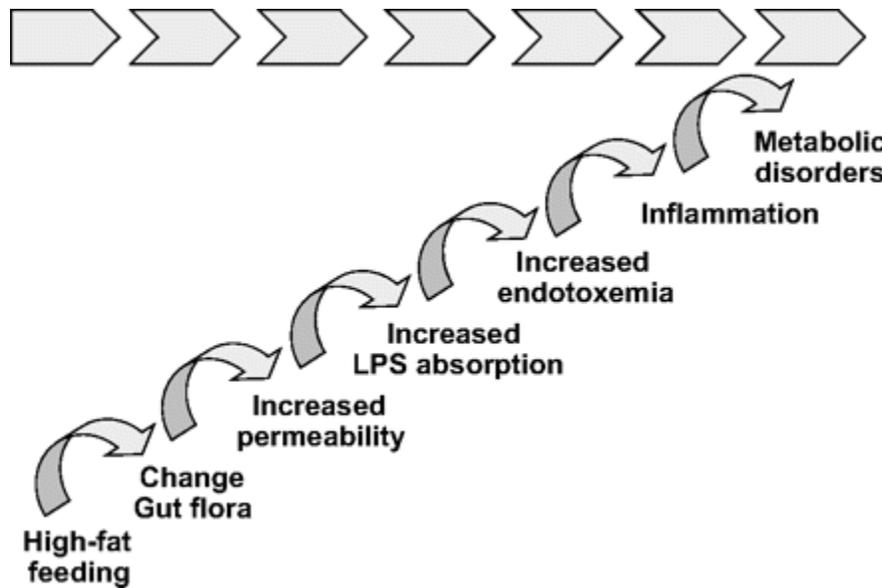
Microbial components like LPS act as the primary ligand for TLR4 and are able to initiate an inflammatory response^{131,132}. Treatment with LPS in both cell and animal models causes a wide range of negative effects on glucose metabolism including insulin resistance^{19,141,142}. Because LPS can influence the immune response and cause changes in metabolism it becomes important to understand LPS as a possible link between the diet and systemic inflammation. A prevailing theory about how LPS controls inflammation and metabolism, states that changes in the composition of the gut microbiome and in the integrity of the gut cell wall caused by the diet can allow the leakage of LPS into circulation, where it then binds TLR4 in the tissues. This increase in LPS levels thought to control systemic inflammation and negatively influence metabolism is known as metabolic endotoxemia¹⁹. It is thought that dietary fat facilitates the development of metabolic endotoxemia (e.g., increased plasma LPS levels)^{143,144}. This process can be initiated by physiological mechanisms, such as the transport of LPS from the gut lumen toward target tissues by newly synthesized chylomicrons from epithelial intestinal cells in response to fat feeding¹⁴⁵. This mechanism could contribute to the higher plasma LPS levels and low-grade inflammation observed with a high-fat diet. Under this theory, gram-negative bacteria would be favorable for the production of LPS and concomitant decreases in the

viability of the tight junction proteins in the gut would lead to leakage of LPS into circulation and this would be a mechanism directly contributing to the development of metabolic disease¹⁸.

The gut microbiome is a microscopic ecosystem composed of trillions of bacteria that thrive on the intestinal contents of the host to produce both waste products and energy¹⁴⁶. These bacteria work to convert fibrous material in the form of indigestible plant polysaccharides to energy usable by the host and this can largely influence the metabolic health of the host¹⁴⁷. Gut bacteria can also play roles in regulating gut hormones and the composition of gut bacteria can influence the host ability to harvest energy from macronutrients¹⁴⁷. Crucial to this story is how the species composition of the gut bacteria can influence metabolic outcomes. Mammalian gut microbiota belongs predominantly to four bacterial phyla: the Gram-negative *Bacteroidetes* and *Proteobacteria* and the Gram-positive *Actinobacteria* and *Firmicutes*²³. A great deal of research has been done examining the correlations between which of these prevailing species of bacteria occupy the gut, and the metabolic phenotype of the host-organism, and these findings remain somewhat controversial. It has been shown that high-fat diets alter the gut microbiota by supporting increases in those bacteria in the *Firmicutes* phylum and decreasing those within *Bacteroidetes*¹⁴⁸, and this finding was shown to be conserved across animals and humans¹⁴⁸. However, other studies have shown that gram-negative bacteria such as *Bacteroidetes* are in fact increased in obese and diabetic individuals. This outcome points to the likelihood that the mechanism contributing to metabolic alterations is a leaky gut and that alterations in levels of various gut microbiota are secondary to this. Obese individuals also contain greater numbers of bacteria that are more efficient at extracting energy from the diet than other strains of bacteria^{149,150}. Other work suggesting the gut microbiome as an important mediator of metabolism was conducted in germ-free mice, or mice lacking an intestinal

microbiota^{20,151}. Backhed et al. showed that germ-free animals fed a high-fat diet were protected from obesity and insulin resistance¹⁵². Similarly, when these animals were colonized with the gut bacteria from conventional mice, there was a 60% increase in the body fat of these animals that tracked with decreases in insulin sensitivity¹⁵².

As it relates to metabolic endotoxemia, the cell walls of the Gram-negative bacteria contain LPS¹⁵³. These LPS-producing bacteria have been shown to be higher in both obese individuals and those with Type II diabetes and this tracks with reductions in the beneficial strains of bacteria¹⁵⁴. In a study in which mice were fed a high-fat diet (Figure 6.1), and there was evidence of pronounced endotoxemia, there was an increased ratio of gram-negative to gram-



positive bacteria¹⁹. In this study, these changes coincided with negative changes in glucose metabolism. In situations where the TLR4/CD14 signaling machinery has been knocked out of animals given a high-fat diet, there was protection

Figure 6.1 Hypothesis for bacteria-induced metabolic disease.
 **Reproduced from Diabetes June 2008 vol. 57 no. 6 1470-1481 doi: 10.2337/db07-1403 with permission from Diabetes Journal.

against the development of obesity and subsequent inflammation and insulin resistance that was not seen in control animals¹⁹. This supports the hypothesis that high-fat diets alter the gut microbial environment leading to up-regulation of the immune response that can be harmful to

metabolism. Taken together there appears to be a crucial role for the innate immune system, through the LPS/TLR4 signaling cascade in mediating the interaction between high-fat diets and the negative metabolic outcomes.

7. The Role of LPS in Glucose Metabolism.

Changes in LPS levels can have profound effects on glucose metabolism. As mentioned above, LPS acts a ligand for TLR4 and its activation contributes to defects in glucose homeostasis. More specifically, the effects of LPS on glucose homeostasis can vary greatly depending on both the duration and intensity of the increase in LPS. LPS concentrations can transiently rise after a meal and LPS levels have been shown to increase significantly in response to high-fat meals¹⁵⁵ which is a proposed risk factor for the chronic inflammation in obesity. Short-term increases in LPS levels, whether they be small or large, can be either beneficial or harmful to glucose metabolism depending on dose. Chronically elevated LPS levels are harmful to insulin signaling and glucose homeostasis across a wide range of doses. A question related to this is how long must LPS levels remain elevated for harmful effects to take place? Following a 2hr treatment with LPS in humans there were increases in insulin sensitivity. In the same study, a 24hr treatment resulted in insulin resistance^{156,157}. It is known that prolonged activation of inflammatory pathways, as seen with infections and certain diseases, is harmful to glucose metabolism. Another important factor concerning LPS and glucose metabolism is how different dosages can affect glucose metabolism. A great deal of research has been done looking at how septic (very high) levels of LPS result in hyperglycemia in the short-term and hypoglycemia in the long-term^{158,159}. The hypoglycemia is a result of impaired hormone-stimulated hepatic glucose production and increased insulin-independent glucose uptake in

multiple tissues^{160,161}. The insulin resistance is related to impaired insulin action in the liver or muscle through both direct and indirect effects of LPS. However, such high levels give little insight into how LPS effects glucose metabolism in situations such as metabolic endotoxemia. More recent work has begun to elucidate the effects of lower doses of LPS on glucose homeostasis and remains largely inconclusive. Certain studies using acute low-dose LPS have resulted in improvements in glucose clearance^{157,162,163}, however; other work has suggested that low-doses are sufficient to initiate an inflammatory response that becomes harmful to glucose homeostasis. It is known that the increases in glucose clearance following acute challenges with LPS involve the interaction between the gut and the pancreas that results in increases in insulin production through GLP-1 and it appears these changes are partially causative of the changes observed in peripheral tissues such as the liver and skeletal muscle. Taken together, literature indicates that the relationship between endotoxemia and glucose homeostasis is rather complex, and it appears that the effects of LPS on glucose homeostasis might be dependent on the metabolic context and the experimental conditions used including in particular the magnitude and duration of the endotoxemic challenge. Whilst it is better understood how long-term activation of inflammatory pathways by LPS can result in decreased insulin sensitivity and hyperglycemia, much less is known regarding short-term variations in LPS levels.

Mechanisms Contributing to Improvements in Glucose Clearance.

The exact mechanisms that contribute to the low-dose LPS-mediated acute improvements in glucose metabolism are poorly understood. Much of the work appears to be in conflict with one another and this may be largely due to differences in experimental design, particularly in

regards to the dosages and timing of LPS treatments in these experiments. One possible mechanism leading to improvements in glucose clearance is LPS exerting an insulin-like action on tissues to stimulate glucose disposal¹⁶⁴. Another more likely mechanism, shown across much of the literature, associates LPS treatment with changes in pancreatic function. A well-known characteristic of LPS treatment in both animals and humans models is a sharp increase in the production and release of insulin from the pancreas¹⁶⁴⁻¹⁶⁶. There remain various possibilities responsible for this. One is that LPS can stimulate pancreatic insulin secretion^{167,168}. Pancreas removed from animals treated with LPS show increases in both basal insulin secretion and GSIS¹⁶⁹. In disagreement with this neither perfusion of excised pancreas with LPS, nor LPS treatment of cultured pancreatic b-cells were able to directly increase insulin secretion which suggests the involvement of mediators¹⁶⁸.

Another possible mediator of the effect of LPS on insulin secretion is Glucagon-like Peptide 1 (GLP-1). GLP-1 is a known inducer of insulin-secretion and more recently has been shown to be significantly up-regulated in the blood following treatment with LPS and this occurs during both fasted and insulin-stimulated conditions¹⁶². In a recent study increases in GLP-1 were positively correlated with increases in glucose-stimulated insulin secretion. When a GLP-1 receptor antagonist or a GLP-1 receptor KO animal model was used the effects of the increased GLP-1 in blood was negated. Similar findings have been made when GLP-1 is infused in to humans where there are increases in β -cell function and insulin sensitivity in peripheral tissues¹⁷⁰. This data suggests GLP-1 as an important downstream mediator of the effects of LPS on glucose metabolism.

There also remains the possibility that inflammatory mediators are involved directly in stimulating the increased secretion of insulin from the pancreas seen with acute LPS treatment. IL6 has been shown to stimulate insulin secretion¹⁷¹ through a GLP-1 dependent mechanism. As with its contrasting roles in inflammation (pro and anti-inflammatory actions), IL-6 has similar roles in mediating insulin action and glucose disposal. It can augment and inhibit insulin action in-vivo¹⁷² depending on amount and context. IL-6 has also demonstrated involvement in the enteroendocrine pathway through which its action on insulin secretion can be explained. IL6 increases GLP1 production through increased pro-glucagon transcription and PC1/3 expression¹⁷¹. LPS also contributes to sensitization of adenylyl cyclase activity and increases the production of cAMP, which leads to an up-regulation of pro-glucagon gene expression¹⁷³. Neural pathways may also explain the up-regulation of GLP-1 as sensory nerves, known to be involved in the glucose response to endotoxin, increase the expression of the neuropeptide calcitonin gene-related protein (CGRP) an inducer of GLP-1¹⁷⁴. Finally, a direct effect of the LPS challenge on TLR4 signaling might make a significant contribution to the LPS-induced production of GLP1. Indeed, it has recently been shown that enteroendocrine cells expressed functional TLR4, and hypoglycemia as well as GSIS were reported to be blunted in TLR4-deficient mice¹⁷⁵.

6. Conclusions and Unanswered Questions.

There has long been a clear association between heightened inflammatory responses and defects in glucose homeostasis such as that seen in situations such as Type 2 Diabetes. The immune-mediated mechanisms controlling the defects in glucose control have also been well defined and it is understood that a westernized (high-carbohydrate/high-fat) diet can directly

contribute to the activation of these immune responses. Little is known, however, regarding the mechanisms connecting the westernized diet and a chronically activated immune response. A hypothesized mechanism that has become prevalent in this area of study is bacterially derived endotoxin from the gut contributes to systemic inflammation and defect in glucose metabolism. It is thought that westernized diets can alter the microbial composition of the gut, favoring those bacteria capable of producing endotoxin in addition to altering the function of the gut so that endotoxin is able to enter circulation. There are many questions to be answered within this hypothesis including how diets can alter the microbial composition of the gut, as well what these changes mean for whole-body metabolism. There are many questions regarding the mechanism controlling the ability of endotoxin to escape from the gut and remain functional. Also, it is yet to be determined how changes in endotoxin contribute to metabolism. It is well understood that large amounts of endotoxin are harmful, but it remains to be understood how small changes in endotoxin levels can affect metabolism. Answering these questions will provide an opportunity to possibly alter these mechanisms and render treatments to the diseases caused by these mechanisms.

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Chapter 3: SPECIFIC AIMS

Obesity has long been associated with the development of insulin resistance and heart disease¹⁻³. The chronic low-grade inflammation caused by an obese state has been shown to directly inhibit insulin signaling processes and contribute directly to the development of coronary heart disease^{4,5}. Obese individuals present with modestly elevated levels of blood endotoxin^{6,7}. Endotoxin, the toll-like receptor 4 ligand, elicits an inflammatory response and elevated levels have been associated with derangements in metabolic processes^{8,9}. Unpublished work from our lab has shown that in healthy, non-obese, insulin-sensitive humans, endotoxin levels increase in the post-prandial state and return to baseline in the post-absorptive state. To date, little evidence exists to show the role of these fluctuations in glucose metabolism.

We propose to use acute and chronic treatments in cell culture and acute treatments in a mouse model to study the association between endotoxin levels and resultant changes in glucose metabolism. We will use a method in which human primary skeletal muscle cells are treated with low levels of endotoxin for an acute (2hrs) or chronic (24hrs) time frame followed by treatment with insulin in order to determine effects on the insulin signaling machinery. We also propose using a C57/B16 mouse model in which mice are given a single, low dose injection of endotoxin in order to mimic an acute change. Endotoxin will be delivered by way of an IP injection. This approach is unique because it uses doses of endotoxin that are both physiologically relevant to humans and low enough to avoid a systemic inflammatory response, which could confound the results. Using this approach will give insight into the following specific aims:

THE SPECIFIC AIM OF THIS DISSERTATION PROJECT IS:

SPECIFIC AIM: To determine the effects of low levels (50pg/ml or 0.01ug/kg) of endotoxin (lipopolysaccharide or LPS) on insulin stimulated glucose clearance in a human primary skeletal muscle cell culture model as well as in a C57/B16 mouse model.

Hypothesis: Transient increases in endotoxin levels improve insulin-stimulated glucose uptake, whilst chronically elevated blood endotoxin levels have negative effects on insulin signaling and therefore impair normal glucose homeostasis.

Objective 1: To test the hypothesis that low doses of endotoxin are beneficial to the insulin signaling machinery during acute (2hr) and harmful over chronic (24hr) incubations in a human primary muscle cell line.

Objective 2: To test the hypothesis that an acute injection of endotoxin in a C57/B16 mouse line will improve glucose tolerance through an insulin dependent mechanism.

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Chapter 4: EXPERIMENTAL DESIGN

SPECIFIC AIM: To determine the effects of low levels of endotoxin (lipopolysaccharide or LPS) on insulin stimulated glucose clearance in a human primary skeletal muscle cell culture model as well as in a C57/Bl6 mouse model.

Hypothesis: *Transient increases in endotoxin levels are beneficial and serve to enhance insulin stimulated glucose uptake, whilst chronically elevated blood endotoxin levels have deleterious effects on insulin signaling and therefore impair normal glucose homeostasis.*

Objective 1: To test the hypothesis that low doses of endotoxin are beneficial to the insulin signaling machinery during acute (2hr) and harmful over chronic (24hr) incubations in a human primary muscle cell line.

Research Model:

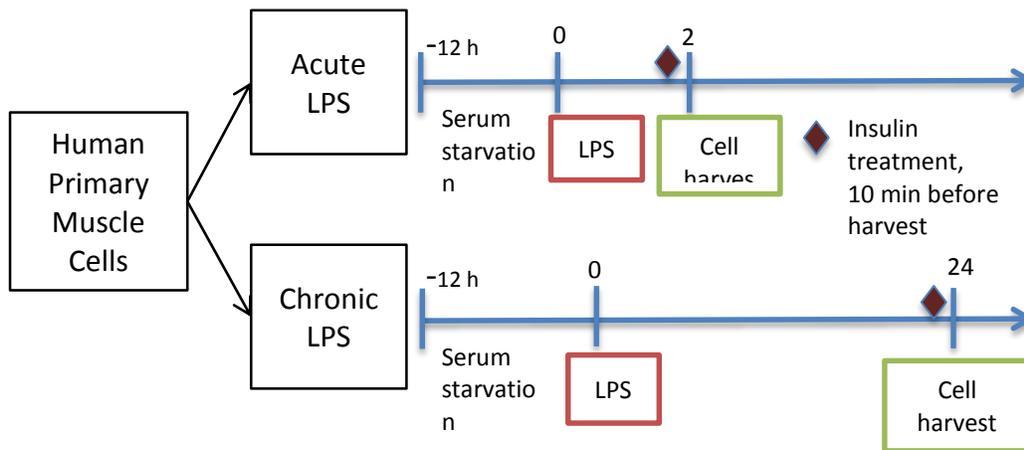


Figure 4.1 Experimental design for cell culture studies

Cell Culture: A human primary muscle cell culture model will be used as this gives the most significance as it pertains to the mechanisms at play in humans. Because approximately 80% of insulin-stimulated glucose uptake occurs in muscle tissue, this model will allow us to draw conclusions regarding the effect of LPS on a whole-body level. Our laboratory has used this cell line extensively and preliminary data demonstrate the feasibility of this cell line for the proposed studies. These cells were obtained from biopsies done on the vastus-lateralis muscle of human subjects.

Experimental Strategy: As shown in the above figure 4.1, fully differentiated cell cultures will be serum deprived for 12h and then divided into one of 8 groups, (2hr LPS w/ insulin, 2hr LPS w/o insulin, 2hr saline w/insulin, 2hr saline w/o insulin) or (24hr LPS w/ insulin, 24hr LPS w/o insulin, 24hr saline w/insulin, 24hr saline w/o insulin). 2hr treatments will be used to mimic acute situations and 24hr treatments will be used for chronic situations. Cells will be treated with a low-dose of endotoxin (20EU/ml) or saline for these time periods. These dosages of endotoxin differ significantly from what has been used in similar experiments in other laboratories in that they are much lower. Following each of the treatments, the cells will be treated with insulin (100nm) for 10mins and then harvested in protein lysis buffer for further examination. Endpoint measures for this study are shown in table 1.

Table 1: Metabolic Endpoint Measurements for Objective 1 Cell Culture Studies

• Glycogen Incorporation*
• Gene expression of proteins involved in glucose metabolism and immune response*
• Protein levels of AKT and p-AKT*

- p-AKT is the phosphorylated serine/threonine-specific protein kinase, which when activated through phosphorylation by binding of insulin to the insulin receptor, and activates intracellular signaling cascades that leads to uptake of glucose into the cell.
- * Further described in extended methods section.

Objective 2: To test the hypothesis that an acute injection of endotoxin in a C57/Bl6 mouse line will improve glucose tolerance through an insulin dependent mechanism.

Animal Model: The second arm of the proposed study will use an in-vivo model in which two cohorts of C57Bl/6 mice will be given an acute (4hours) injection of LPS or saline and whole-body glucose homeostasis mechanisms will be assessed.

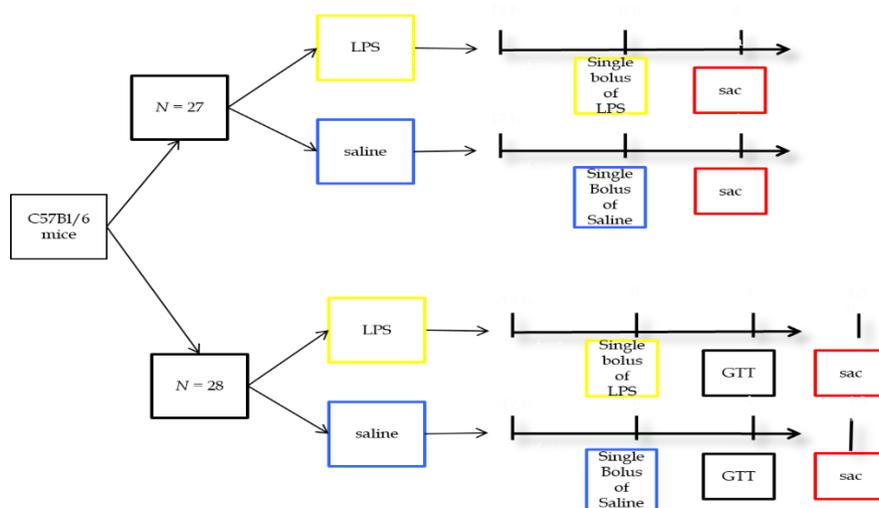


Figure 4.2 Experimental Design for Animal Studies

Experimental Strategy: As shown in figure 4.2 mice will be randomly divided into two cohorts. Both groups of mice will be fasted for 12 hours prior to the experimentation. Following the fast mice will be given either a one-time low-dose (0.01ug/kg BW) bolus of LPS or a one-time bolus of saline through an IP injection. Following treatment with LPS or saline,

one group of animals will be sacrificed, while the other will be given an IP injection of glucose (1g/kg BW). Thirty minutes after injection of the glucose in the second cohort, these mice will be sacrificed and harvesting of red and white skeletal muscle, liver, pancreas, and white adipose tissue will be harvested. From prior experiments using similar methods, we expect insulin release to peak at this time.

In addition, we propose to perform a glucose disposal assay in which a separate cohort of animals is given a low-dose of LPS followed 4hrs later by an injection of 10 µCi of 2-deoxy-D-[1,2-³H] glucose intraperitoneally. This is followed by a glucose tolerance test. At the 120 minute time-point, animals will be sacrificed and red and white skeletal muscle, liver, pancreas, and white adipose tissues will be harvested. This will allow for determination of the contribution of the various tissues to the expected whole-body glucose disposal affect.

Table 2: Metabolic Endpoint Measurements for Objective 2 Animal Studies

<ul style="list-style-type: none"> • Gene expression of proteins involved in glucose metabolism and immune response*
<ul style="list-style-type: none"> • Protein expression of Insulin signaling and glucose uptake proteins*
<ul style="list-style-type: none"> • Whole-body glucose homeostasis via Glucose tolerance test
<ul style="list-style-type: none"> • Plasma Insulin levels*
<ul style="list-style-type: none"> • Glucose disposal in peripheral tissues via In-vivo glucose uptake assay*

- *Further described in extended methods section.

Extended Methods

Serum Insulin measures. Measured using commercially available kit according to the manufacturer's instructions (ALPCO Diagnostics, Salem, NH).

Glucose disposal in adipose and skeletal muscle tissues and glucose incorporation into glycogen in liver in vivo. Mice will be fasted for 6 h (6 AM-12 PM) and then injected intraperitoneally with 2-deoxy-D-[1,2-³H]glucose (10 μCi) in either saline or glucose (2 g/kg body wt). Blood will be collected at 0, 15, 30, 60, and 120 min after the injections for the determination of blood glucose and 2-deoxy-D-[1,2-³H]glucose, and tissues will be dissected and processed at 120 min. Insoluble material will be pelleted from the tissue homogenates in water without perchloric acid treatment

RNA extraction and qRT-PCR. RNA will be extracted using an RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR will be performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression in rodent skeletal muscle and cell culture will be normalized to β-actin RNA levels. Target gene expression in human skeletal muscle primary cell culture will be normalized to cyclophilin B RNA levels. Primers and 5' FAM-labeled TaqMan probes were purchased as prevalidated assays (ABI). Relative quantification of target genes will be calculated using the $2^{-\Delta C_T}$ method. Derivation of the $2^{-\Delta C_T}$ equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859).

Western blotting. Western analysis will be performed using cell lysates harvested in Mammalian Cell Lysis Buffer (Sigma Aldrich). Proteins (30 μg) will be separated using a 10% Criterion-Tris·HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blots will be probed with primary antibodies

against β -actin (1:1,000; Cell Signaling, Danvers, MA), AKT1/2 (1:1,000; Cell Signaling, Danvers, MA) and IRS1 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-rabbit, mouse, or goat secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Proteins were visualized using Super-Signal Chemiluminescent Substrate (Pierce, Rockville, IL) and a ChemiDoc XRS Imaging System (Bio-Rad).

Western Blotting of membrane bound Glut4. Muscle tissue will be homogenized in homogenization buffer consisting of 50mM Hepes, 10mM EDTA, 100mM NaF, 50 mM Na pyrophosphate, and 10mM Na orthovanadate. Protease inhibitor cocktail will be added prior to homogenization at 10ul/ml final concentration. Homogenization will be done in 2ml glass homogenizer tube until slurry appears homogenous. Homogenate will be centrifuged at 150k x g for 1hr at 4°C in a Beckman Ultracentrifuge. Following spin supernatant will be removed and collected as cytosolic fraction for later processing. Pellet will be re-homogenized in homogenization buffer and 10% triton x-100 for solubilization of membrane-bound proteins. Following a 30-min incubation period, homogenates will be spun in micro-centrifuge at 16k x g for 15 mins and supernatant will be harvested as membrane protein fraction. Protein fractions will be separated on a 10% Criterion-Tris·HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blots will be probed with primary antibodies against Glut4 (1:1,000; Cell Signaling, Danvers, MA) followed by anti-mouse secondary antibody (1:10,000 Jackson ImmunoResearch Laboratories, West Grove, PA).

**Chapter 5: Acute Low-Dose Endotoxin Treatments Result in Improved Whole-Body
Glucose Homeostasis in Mice.**

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Abstract

Obese individuals present with an increased inflammatory tone as compared to healthy, normal-weight individuals, which is associated with insulin resistance. One factor hypothesized to contribute to increased inflammation in obese and diabetic states is elevated blood endotoxin levels, a condition known as metabolic endotoxemia. In non-obese and insulin sensitive individuals circulating endotoxin concentrations fluctuate over the course of the day with elevations in the post-prandial state that return to baseline levels in the post-absorptive state. Evidence suggests that high-fat feeding alters these fluctuations causing endotoxin levels to remain high throughout the day. The effects of alterations in endotoxin levels on glucose metabolism remain to be understood. The goal of this study was to determine the effects of both short-term and long-term increases in endotoxin of a low magnitude on the insulin signaling machinery in a human primary cell line as well as the effects of short-term endotoxin treatments on glucose homeostasis in a C57/Bl6 mouse model. First, we tested the hypothesis that short-term low-dose endotoxin treatments would augment insulin-signaling and glycogen synthesis while long-term treatments would have inhibitory results in the cell culture model. Second, we examined if these short-term low dose treatments of endotoxin would contribute to similar improvements in whole-body glucose homeostasis in a mouse model. In contrast to our first prediction, short-term endotoxin treatments did not improve insulin signaling or glycogen synthesis, while long-term treatments did contribute to decreases in glycogen synthesis. Interestingly, short-term endotoxin treatments resulted in significant improvements in glucose homeostasis in the mouse model; this is believed to be at least partly attributed to an inhibitory action of LPS on liver glucose production. Additional studies are necessary to

understand the mechanisms responsible for altered glucose metabolism in response to low magnitude changes in LPS levels.

Introduction

A key mediator of the relationship between obesity and Type 2 diabetes mellitus is inflammation^{1,2}. Normal-weight individuals exhibit an immune response to various instigators such as sickness, injury, or even high-fat meals that is resolved following removal of the instigator³. Obese individuals show a chronically heightened immune response that can negatively affect insulin signaling processes and this can cause insulin resistance^{4,5}.

A mechanism thought to contribute to this chronically elevated inflammatory response is known as metabolic endotoxemia^{4,6,7}. Metabolic endotoxemia was first coined by Cani et al.⁴ to describe the chronic increases in blood endotoxin levels that are seen with prolonged high-fat feeding in rodents. Endotoxin has been shown to activate the toll-like receptor 4 (TLR4) that has been well-classified as able to initiate an inflammatory signaling response. Research from both mouse models and human has attributed chronic increases of endotoxin to the development of metabolic diseases including obesity and insulin resistance^{4,7,8}. Studies have shown that endotoxin levels are typically 0.2 ng/ml in the fasted state and can increase to 2 ng/ml depending on the feeding status of an individual. Endotoxin levels behave in a cyclical manner, at the lowest levels during fasting periods and reaching a peak during the postprandial period following a meal⁴. Studies performed by Cani et al. in which rodents were fed a high-fat diet for 4 weeks, exhibited sustained increases in circulating endotoxin levels in comparison to animals fed a control diet. The latter represents a vital connection between the diet and the consequent changes in endotoxin levels.

These chronic increases in endotoxin are thought to contribute to activation of inflammatory processes that have long been associated with detrimental roles in glucose metabolism primarily through inhibitory actions on insulin signaling^{9,10}. Much less is known about whether short-term changes in LPS levels following meals are capable of causing an inflammatory response. Previous studies aimed at determining the effects of short term LPS treatments on glucose metabolism have shown severe hypoglycemia, due in large part to increased glucose uptake along with inhibition of hepatic glucose production¹¹⁻¹³. These studies used extremely high doses of LPS, many at septicemic levels. Doses of this magnitude lend little relevance to situations such as those seen with metabolic endotoxemia. Previous work from our laboratory has identified a unique role for these short-term increases in LPS of a low magnitude, such as those seen with feeding, being able to cause significant changes in glucose metabolism in skeletal muscle cells¹⁴. This work showed that short-term treatments with low doses of LPS were capable of causing significant increases in glucose uptake and oxidation as well as glycogen synthesis and lactate production.

The objective of these studies was to determine the effects of these low-magnitude short-term changes in LPS on glucose metabolism through the use of acute and chronic treatments in both cell culture and a mouse model. This approach is unique in that it uses doses of endotoxin that are both physiologically relevant to humans and yet much lower than dosages used in other studies of this nature. Using this approach, our hypotheses were that short-term low-magnitude increases in endotoxin levels would be beneficial and serve to enhance insulin stimulated glucose uptake both in cells and animals, whereas long-term treatments in cell culture would result in a decrease in insulin signaling.

Taken together, our findings suggest that short-term increases in LPS are in fact beneficial to glucose metabolism in an animal model, and this occurs in part due to inhibitory actions on gluconeogenesis in the liver. Also, it appears long-term treatments of LPS in a skeletal muscle cell line prove to negatively influence glycogen synthesis, thereby indicating a negative influence of LPS on glucose metabolism over the long-term.

Methods and Materials

Cell Culture. Studies were conducted using human primary myotubes taken from vastus lateralis muscles of human subjects. Muscle cells were isolated, cultured, and grown to ~80% confluence in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were then differentiated into myotubes in DMEM containing 2% horse serum, 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen). All experiments were performed on Day 7 of differentiation.

INSI Cell Culture Studies. Cells were grown in RPMI-1640 until 70% confluency then pretreated with glucose free KRB buffer for 1 hr. Cells were then washed with KRB buffer then incubated in KRB buffer containing 2mM glucose with and without 50pg/ml LPS for 2 hrs. Insulin secreted in the supernatant was measured by ELISA kit. Cells were lysed and protein concentration was measured. Insulin was normalized to the total protein

LPS treatment in cell culture experiments. Cell culture experiments were performed with 50 pg/ml of LPS (*E. coli* 0111:B4, catalog no. L2630; Sigma-Aldrich). All LPS preparations were made using endotoxin-free water. In all of the studies, treatment time was either 2 hours or 24 hours, and insulin was added to the plates 10 minutes before harvesting. Cell culture plates

were washed three times with PBS before initiating collection for any other assays described below.

Glycogen Synthesis. Following treatment with LPS as described above cells were pre-incubated in Krebs's Ringer buffer for 5 hours, then washed with PBS and glycogen synthesis was determined by the addition of 1.25 $\mu\text{Ci/mL}$ of $[\text{U-}^{14}\text{C}]$ -glucose in low glucose DMEM. Following 3 hours of incubation, the plates were placed on ice and washed 3 times with ice-cold PBS. Cells were harvested in 30% KOH and placed in tubes containing 35 μL of 60 mg/mL glycogen. Cells were vortexed and incubated for 20 minutes at 80°C . Cells were then placed on ice and 0.5 ml of 100% EtOH was added to each tube to precipitate glycogen. Tubes were centrifuged and the supernatant discarded. 70% EtOH was then added to the tubes, vortexed, and centrifuged again. Supernatant was removed and tubes were allowed to air-dry. Pellets were re-suspended in 0.5 ml dH_2O and vortexed until glycogen was completely dissolved. Glycogen synthesis was calculated based upon specific activity and expressed relative to protein content.

Animals. Animal studies were performed under an approved protocol by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State University. Multiple studies were conducted using 12-week old male C57/B16 mice from the Jackson Laboratory (Bar Harbor, ME). The first experiments assessed glucose clearance abilities via a glucose tolerance test from overnight-fasted, saline ($n = 12$) and LPS-injected ($n = 10$) mice. These mice were fasted overnight (12 hours) then given an intraperitoneal injection of either saline ($n=12$) or low-dose (0.1 $\mu\text{g/kg BW}$) of LPS ($n=14$) followed 4 hours later by an intraperitoneal injection of glucose (2 g/kg BW). Blood glucose measurements were taken every 30 minutes until 120 minutes. The second experiments occurred 2 weeks later and sought

to assess non-insulin dependent tissue specific effects of LPS on glucose clearance. In this experiment, the same animals used above were again fasted overnight and given an injection of saline or low-dose LPS and 4 hours post-injection were sacrificed and tissues were harvested for measurement of gene and protein expression of inflammatory (TNF α , IL6, and TLR4) and glucose metabolism (PCX, PFK, Glut2, PEPCK) markers. LPS from *Escherichia coli* 0111:B4 was used for all studies (catalog no. L2630; Sigma-Aldrich, St. Louis, MO). Mice for all studies were maintained on a normal chow diet and a 12:12-h light-dark cycle. A second cohort of 12-week old C57/Bl6 mice was used to assess the insulin-dependent effects of LPS on glucose metabolism. These animals were fasted overnight for 12 hours and given an injection of saline or LPS as described above. Four hours following treatment, the animals were given an injection of glucose similar to the glucose tolerance test described above. However, in this case animals were sacrificed at the 30 minute time point of this study and tissues and blood were harvested to assess insulin signaling markers (Total-AKT, P-AKT, Glut4) in tissues as well as insulin levels (ALPCO Diagnostics, Salem, NH) in the blood. *Glucose disposal in adipose, skeletal muscle tissues, and glucose incorporation into glycogen in liver.* Mice were fasted for 12 hours and then injected with either saline or low-dose (0.1 ug/kg BW) LPS intraperitoneally. After 4 hours, mice were injected intraperitoneally with 2-deoxy-D-[1,2-³H]glucose (10 μ Ci) in either saline or glucose (2 g/kg BW) and blood was collected at 0, 15, 30, 60, and 120 minutes after the injections as previously described¹⁵. Blood was used for the determination of blood glucose and 2-deoxy-D-[1,2-³H]glucose levels, and tissues were dissected and processed at 120 minutes for determination of tissue uptake. Insoluble material was pelleted from the tissue homogenates in water with perchloric acid treatment.

Pyruvate Challenge to Assess Gluconeogenesis. Mice were fasted for 12 hours and then injected with either saline or low-dose (0.1 ug/kg BW) LPS intraperitoneally. After 4 hours, mice were injected intraperitoneally with sodium pyruvate at 2 g/kg BW (Sigma). Blood glucose readings were taken in 30 minute increments out to 2 hours as previously described^{16,17}.

RNA extraction and qRT-PCR. RNA was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using an ABI PRISM 7900 Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression in rodent skeletal muscle was normalized to β -actin RNA levels. Human skeletal muscle primary cell culture was normalized to cyclophilin B RNA levels. Primers and 5' FAM-labeled TaqMan probes were purchased as prevalidated assays (Applied Biosystems, Foster City, CA). Relative quantification of target genes was calculated using the $2^{-\Delta C_T}$ method. Derivation of the $2^{-\Delta C_T}$ equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859).

Western Blotting. Western analysis was performed using cell lysates harvested in Mammalian Cell Lysis Buffer (Sigma Aldrich). Proteins (30 μ g) were separated using a 10% Criterion-Tris-HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Blots were probed with primary antibodies against β -actin (1:1,000; Cell Signaling, Danvers, MA). Total AKT1/2 (1:1,000; Cell Signaling, Danvers, MA) and P-AKT1/2 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) followed by anti-rabbit secondary antibodies (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Proteins were visualized using Super-Signal

Chemiluminescent Substrate (Pierce, Rockville, IL) and a ChemiDoc XRS Imaging System (Bio-Rad, Hercules, CA).

Western Blotting of membrane bound Glut4. Muscle tissue was homogenized in homogenization buffer consisting of 50 mM Hepes, 10 mM EDTA, 100 mM NaF, 50 mM Na pyrophosphate, and 10 mM Na orthovanadate. Protease inhibitor cocktail was added prior to homogenization at 10 μ l/ml final concentration. Homogenization was done in 2 ml glass homogenizer tube until slurry appears homogenous. Homogenate was centrifuged at 150k x g for 1hr at 4°C in a Beckman Ultracentrifuge. Following spin, supernatant was removed and collected as cytosolic fraction for later processing. The pellet was re-homogenized in homogenization buffer and 10% Triton x-100 for solubilization of membrane-bound proteins. Following a 30-min incubation period, homogenates were spun in micro-centrifuge at 16k x g for 15 minutes and supernatant was harvested as membrane protein fraction. Protein fractions were separated on a 10% Criterion-Tris·HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Blots were probed with primary antibodies against Glut4 (1:1,000; Cell Signaling, Danvers, MA) followed by anti-mouse secondary antibody (1:10,000 Jackson ImmunoResearch Laboratories, West Grove, PA).

Statistics. Data are presented as mean \pm standard error of the mean (SEM). To compare saline versus LPS treatments and insulin versus non-insulin treatments in cell culture, protein data were analyzed using two-way ANOVA while mRNA data were analyzed by one-way ANOVA. To compare saline versus LPS groups in mouse studies, data were analyzed using an unpaired *t-test*. Results were considered significant at $p < 0.05$.

Results

Acute LPS treatments contribute to increased insulin secretion in an INS1 cell line.

Previous work from our lab has shown significant increases in measures of glucose flux following acute (2 hour) treatments with low-dose (50 pg/ml) LPS and based on this finding we sought to determine if LPS could be involved in mediating other processes relating to glucose metabolism. Other labs have shown a role for LPS in stimulating insulin secretion from the pancreas; however, this work was done using very high doses of LPS and did not provide for our low-dose studies. Using an INS1 (cultured pancreatic beta-cell) line we investigated whether low-dose LPS (20 EU/ml) could affect insulin secretion. We observed significant increases in insulin secretion (Figure 1A) in the LPS treated group ($p < 0.05$) compared to control. Interestingly, this differed when cells were treated with a higher concentration (11 mM) (Figure 1B) of glucose. In this group, the effect was blunted and no significant increase in insulin secretion was seen. This suggested a role for low-dose LPS in stimulating insulin secretion and identified a possible mechanistic target for our animal studies.

Chronic LPS treatments contribute to decreased glycogen synthesis independent of changes in insulin signaling in a Human Primary skeletal muscle cell line. Having previously identified a role for low-doses of LPS in contributing to significant improvements in glucose flux in mouse and human muscle cells, we sought to determine if these changes were occurring through an influence on insulin signaling as well as on glycogen synthesis. Using acute (2 hour) and chronic (24 hour) treatments of LPS followed by a 10 minute insulin treatment, we found that neither time frame resulted in significant changes in expression of genes related to metabolic or inflammatory functions (Figure 2). Unexpectedly, neither time frame of LPS

treatment resulted in significant alterations in either P-AKT or P-IRS protein expression, two main components of insulin signaling (Figure 3A-D). Because no changes were shown with insulin signaling between the two groups we expected that our endpoint measure, glycogen synthesis would also show no changes. However, as demonstrated in Figure 4B, the 24 hour LPS treatment resulted in a significant ($p<0.05$) decrease in glycogen synthesis compared to the control group while the 2 hour treatment showed no changes. This data suggests that long-term LPS treatments can play a harmful role in glucose handling as we hypothesized; however, these data suggest that mechanisms other than those that are insulin dependent are controlling these changes.

Acute low-dose LPS contributes to significant improvements in whole-body glucose tolerance in animals. Following cell culture experiments that showed no changes in glucose metabolism after acute treatments with LPS, we wanted to determine if the same was true in an animal model. We injected animals with low dose (0.1 $\mu\text{g}/\text{kg}$) LPS and 4 hours later performed a glucose tolerance test. As shown in Figure 5A, LPS injected animals show a significantly enhanced glucose tolerance compared to their saline injected counterparts. As shown in Figure 5B, LPS treatment resulted in a significantly lower Area Under the Curve (AUC) ($p<0.001$) compared to the saline group. To determine if the improvement in glucose clearance was due to an increase in insulin secretion, we measured blood serum for insulin levels. Figure 6A shows no significant difference in these levels. When protein expression of insulin signaling markers P-AKT and total-AKT were examined in red skeletal muscle, there were significant ($p<0.05$) increases in both (Figure 6B-C). Increases in an insulin signaling component suggested a possible increase in its downstream effector, Glut4. We examined

whether there were significant changes in Glut4 protein expression at the plasma membrane (Figure 6D), as this would indicate an increase in glucose uptake into the muscle. However, protein expression of Glut4 remained unchanged between the treatment groups suggesting that the AKT expression data was an anomaly. In agreement with this data that suggests skeletal muscle was not a major player in the improved glucose clearance effect seen with LPS treatment, gene expression data from red and white skeletal muscle under basal (non-insulin stimulated) (Figure 7) or insulin-stimulated (Figure 8) conditions showed no major changes.

Acute low-dose LPS treatment causes significant improvements in whole-body glucose tolerance independent of changes in uptake of glucose into tissues. Because LPS treated animal's exhibit improved whole body glucose tolerance not caused by increased insulin secretion or Glut4 dependent skeletal muscle uptake, we sought to identify the tissues primarily responsible for the effect. Within the glucose uptake assay employed, LPS treated animals ($n=5$) again showed improved glucose clearance abilities compared to saline treated control animals ($n=5$) (Figure 9A). AUC analysis shows heightened response to glucose occurred primarily at the 15 min (Figure 9B) ($p<0.05$) and 30 min (Figure 9C) ($p<0.05$) time points. Figure 10 represents the uptake of 2-DOG into skeletal muscle (Figure 10A-B), adipose tissues (Figure 10C-D), and glycogen incorporation in the liver (Figure 10E). None of the tissues examined showed significant differences between the saline and LPS treated animals suggesting that tissue uptake was not the primary mechanism responsible for improvements in glucose clearance shown with low dose LPS treatment.

Animals given acute low-dose LPS treatments exhibit decreased gluconeogenic capabilities as well as altered gene expression of enzymes involved in liver glucose handling.

Because low-dose LPS contributes to a significant enhancement of glucose clearance independent of insulin stimulated glucose uptake into the primary insulin responsive tissues, we next wanted to identify if the effect on glucose clearance was at least partially a byproduct of decreased gluconeogenesis (GNG). Figure 11 shows fasting blood glucose levels in animals 4 hours after LPS injection. Blood glucose levels are significantly ($p < 0.05$) lower in the LPS treated group compared to control, suggesting LPS may have an inhibitory effect on endogenous glucose production. To test this theory of altered GNG, we used a pyruvate challenge assay. Animals were fasted overnight and then given an injection of low-dose LPS followed 4 hours later by an injection of sodium pyruvate (2 g/kg BW). Blood glucose measures were taken at baseline (time 0) and every 30 minutes out to 2 hours. Figure 12A shows significantly lower blood glucose levels in the LPS treated versus saline treated group. Figure 12B represents the AUC for this study. These results suggest that LPS is in fact contributing to a decrease in GNG capabilities, and this at least partially explaining the significant improvement in glucose clearance in LPS treated animals.

In order to determine potential mechanisms underlying the effect of LPS on GNG as well as whole-body glucose control, we examined the gene expression levels of various liver enzymes involved in these processes as well as the expression levels of genes that contribute to inflammation. We measured these under both basal and insulin-stimulated conditions in order to differentiate any effects specific to one or the other. Figure 13 shows that under basal conditions there were significant alterations in gene expression within the liver. Interestingly, gene expression of phosphoenolpyruvate carboxy kinase (PEPCK) (Figure 13A), the key

enzyme catalyzing gluconeogenesis, was unaltered by LPS treatment. However, other markers of glucose flux in the liver were significantly decreased, including phospho-fructokinase (PFK) (Figure 13C) ($p < 0.01$) a key enzyme involved in glycolysis and Glut2 ($p < 0.001$) the primary transporter controlling the movement of glucose into and out of the liver. Decreases in the gene expression of these markers point towards a decreased glucose flux in the liver, and in combination with the pyruvate challenge data suggest an effect for LPS on inhibiting both the production and oxidation of glucose. These changes were in concert with increased gene expression of (Figure 13 E) TNF α ($p < 0.05$), signifying an increased inflammatory response; however, IL-6 mRNA expression showed a significant ($p < 0.01$) decrease in the liver. These data are interesting because no other tissue showed a heightened inflammatory response in these studies.

Under insulin stimulated conditions, there were similar alterations in gene expression (Figure 14). In this case, pyruvate carboxylase kinase (PCX) (Figure 14A), a crucial control point for glycolysis, showed a decreased gene expression ($p < 0.05$) that was not supported by similar changes in PFK (Figure 14B). Glut2 gene expression (Figure 14D) was significantly decreased ($p < 0.05$) in these animals. PEPCK gene expression was unaltered with LPS treatment (Figures 14C). As with non-insulin stimulated conditions TNF α expression (Figure 14F) was again significantly ($p < 0.05$) increased with LPS treatment while IL6 was unchanged (Figure 14E).

Discussion

Changes in LPS levels in the blood can have profound effects on glucose metabolism^{8,14,18}. LPS is a ligand for TLR4 and its activation contributes greatly to defects in

glucose homeostasis such as seen with the metabolic syndrome^{14,19}. The effects of LPS on glucose homeostasis can vary greatly depending on both the duration and intensity of the increase in LPS. LPS concentrations can transiently rise after a normal meal and LPS levels have been shown to increase significantly in response to high-fat meals²⁰ which is a proposed risk factor for the chronic inflammation in obesity. Previous work by others fails to determine whether short-term increases in LPS levels, whether they be small or large, are beneficial or harmful to glucose metabolism. Chronically elevated LPS levels have been shown to have harmful effects on insulin signaling and glucose homeostasis. Another poorly understood factor concerning LPS and glucose metabolism is how different dosages can affect glucose metabolism. A great deal of research has been done looking at how septic (very high) levels of LPS result in hyperglycemia in the short-term and hypoglycemia in the long-term^{21,22}. The hypoglycemia is a result of impaired hormone-stimulated hepatic glucose production and increased insulin-independent glucose uptake in multiple tissues^{23,24}. The insulin resistance is related to impaired insulin action in the liver or muscle through both direct and indirect effects of LPS. However, such high levels give little insight into how LPS affects glucose metabolism in situations such as metabolic endotoxemia. Certain studies using acute low-dose LPS have resulted in improvements in glucose clearance^{13,25,26}; however, other work has suggested that low-doses are sufficient to initiate an inflammatory response that becomes harmful to glucose homeostasis. The increases in glucose clearance following acute challenges with LPS involve the interaction between the gut and the pancreas and results in increases in insulin production through GLP-1²⁵. These changes are partially causative of the changes observed in peripheral tissues such as the liver and skeletal muscle.

These findings suggest that the relationship between endotoxemia and glucose homeostasis is rather complex and that the effects of LPS on glucose homeostasis might be dependent on the metabolic context and the experimental conditions used including in particular the magnitude and duration of the endotoxemic challenge. The goal of the present studies was to better understand this relationship between LPS and glucose metabolism in the context of magnitude and duration of LPS challenge.

Current studies conducted in an INS1 beta cell line show increased insulin secretion (Figure 1A) when treated with low-doses of LPS. This confirms previous reports in animal models that show the pancreas as a target tissue for LPS^{13,27}. Using a human primary skeletal muscle cell line, we show that long-term challenges can result in decreased utilization of glucose (Figure 3C), thus confirming our hypothesis that long-term LPS would have deleterious effects on insulin responsiveness.

The present studies show a prominent role for low magnitude short-term changes in LPS in contributing to significant alterations in glucose metabolism in a C57/Bl6 mouse model. This includes data showing that a low-dose LPS challenge contributes to a significant improvement in glucose tolerance (Figure 5A) and that these improvements occur independent of changes in insulin secretion (Figure 6A). These improvements also occur independent of changes in insulin signaling (Figure 6B and 6C) and glucose uptake into the primary insulin-responsive tissues (Figure 10). A low-dose LPS challenge in animals also resulted in a significant decrease in gluconeogenesis shown via a pyruvate challenge (Figure 12), and this occurred without concomitant changes in expression levels of PEPCK, the primary control point for gluconeogenesis in the liver. LPS challenge greatly affected gene expression of enzymes crucial to glucose handling in the liver. Decreases in PFK, Glut2, and PCX gene

expression (Figure 13 and 15) in the liver were shown with LPS treatment. These data all suggest a decrease in glucose oxidation. This data is in disagreement with others in that decreased GNG in the liver following short-term LPS treatment is typically shown in concert with increased glycolysis with a primary mechanism being the up-regulation of PFK¹² and Fructose 2,6 Bisphosphate¹¹. However, this mechanism is related to using LPS of a much higher dose than the present study. Along with this, there was a significant increase in the gene expression of TNF α and IL-6 in the liver (Figures 13 and 15). Taken with the fact that other tissues involved in the handling of glucose failed to show changes in inflammatory markers, this suggests that inflammatory markers may be playing a role in regulating hepatic glucose production/oxidation.

While the significant decrease in GNG shown with low-dose LPS treatment could possibly explain the significantly lower AUC for the glucose tolerance curve of these same animals, there remains various other possibilities explaining this effect. Primary to this is the role of the immune cell. Low-doses of LPS very similar to those used in the present studies can activate immune cells²⁸, particularly macrophages, causing significant up-regulation of the activity of these cells. This increased activity of immune cells means their energy requirements are increased²⁹. Previous studies have shown increased glucose uptake into macrophages following treatment with LPS³⁰. Activation of macrophages and other immune cells was not measured in the current study and represents an important limitation of the study as this could possibly explain the increase in glucose clearance with these animals. Gene expression of inflammatory markers in the liver presented herein would suggest a heightened inflammatory response in the liver and could be evidence in support of a heightened activation of immune cells contributing to the effects on glucose metabolism.

In addition to the above mentioned limitations of these studies, a few critical limitations remain. These include the failure to show the effects of LPS over a chronic time frame in our animals model as well as limitations in what can be taken from our functional data in animals, both of which would have certainly strengthened the findings a great deal. In regards to not having a chronic timeframe component to the animal study, an inability to use methodology similar to that used in the acute study prevented us from including this part of the study. In the acute study, mice were given a single IP injection of LPS or saline but, in the case of a chronic study using the same methods, animals would need to be given repeated injections over a prolonged period and this can have dire consequences on outcomes through the induction of a systemic inflammatory response. Ideally, these studies could have been done using implanted catheters in which LPS or saline could be delivered through the carotid artery and blood could be sampled from the jugular vein. This would have allowed for a better understanding of the dynamics of endotoxin levels in the blood following infusion, which this study did not and could be seen as another drawback. In terms of limitations to our functional data, it could be said that a glucose tolerance test is not a sufficient assessment of glucose homeostasis being that measuring plasma glucose alone is not representative of situations where glucose production and its utilization increase to the same extent without any change in glucose concentration³⁰. The use of tracers and clamp methods would have given us a better understanding of glucose dynamics following LPS treatment.

Conclusions

In summary, research regarding the role of low magnitude, short-term changes in LPS on mediating changes in glucose metabolism is largely non-existent. Most work giving insight

into the involvement of LPS in glucose metabolism has been done using very-high doses, characteristic of conditions such as septicemia. Our lab has taken interest in how low-magnitude changes in LPS can elicit changes in glucose handling, particularly in how these changes vary over time because they are characteristic of the metabolic syndrome. Our research indicates that short-term low-magnitude LPS injections in animals can cause drastic improvements in the ability of animals to clear glucose. This improved glucose clearing appears independent of any changes in insulin levels or in uptake in to insulin sensitive tissues. Animals also show decreased gluconeogenic capabilities that appear independent of gene or protein expression of PEPCK. These animals show decreases in enzymes controlling glucose flux through the liver as well increased gene expression of inflammatory markers in the liver. Taken together, these data indicate that low-magnitude, short term changes in LPS can have significant effects on whole body glucose metabolism and this likely occurs through its direct effects on the liver.

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Figures

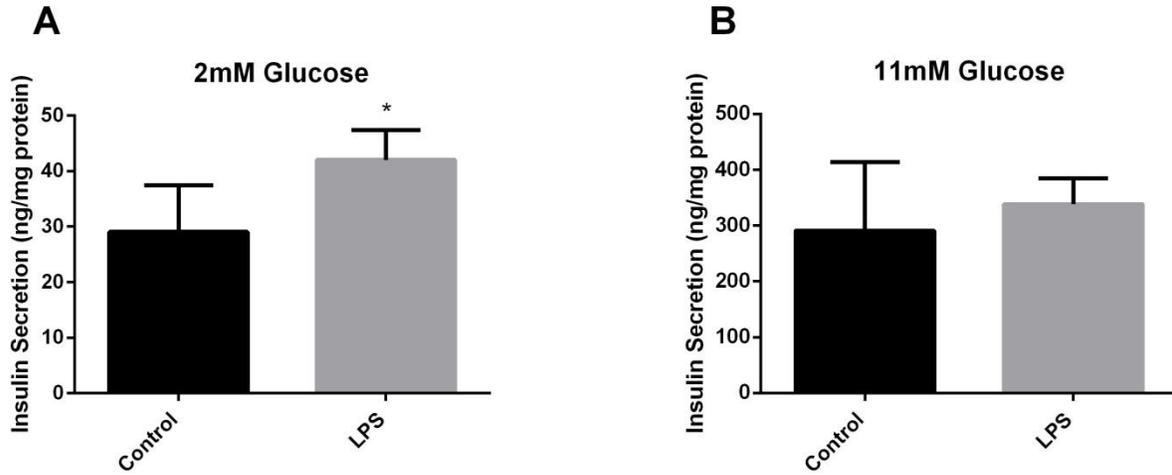


Figure 1. Acute low-dose LPS treatments in INS1 cells increase insulin secretion. INS1 cells were treated with LPS for 2 hours then, following addition of glucose at either 2 mM (A) or 11 mM (B) concentrations, insulin secretion was measured. Data are presented as mean±SEM. Different from control. *, $p < 0.05$.

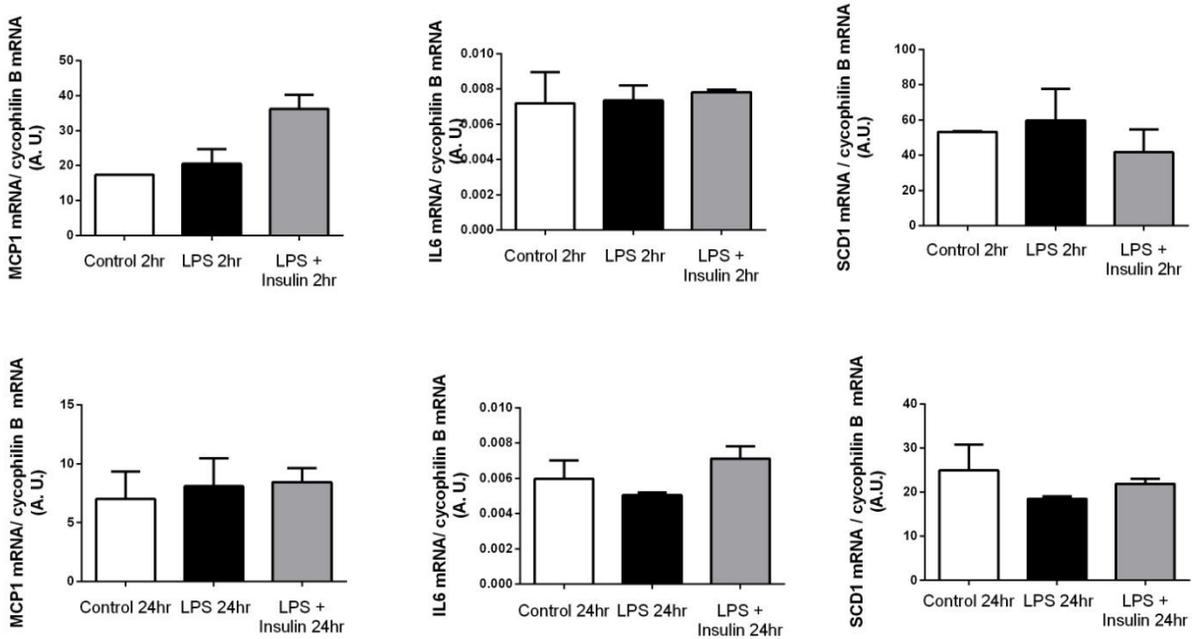


Figure 2. Gene-expression of metabolic and inflammatory genes is unaffected by neither acute nor chronic LPS treatments. Cells were treated with serum-free media (control), LPS media, or LPS media+insulin for 2 hours or 24 hours followed by 10 minute insulin

treatment. Cells were harvested and mRNA was extracted to measure gene expression of (A) MCP1, (B) IL-6, and (C) SCD-1 with 2hr LPS treatment or 24hr LPS treatment (D-F). Data are presented as mean±SEM. *, $p < 0.05$

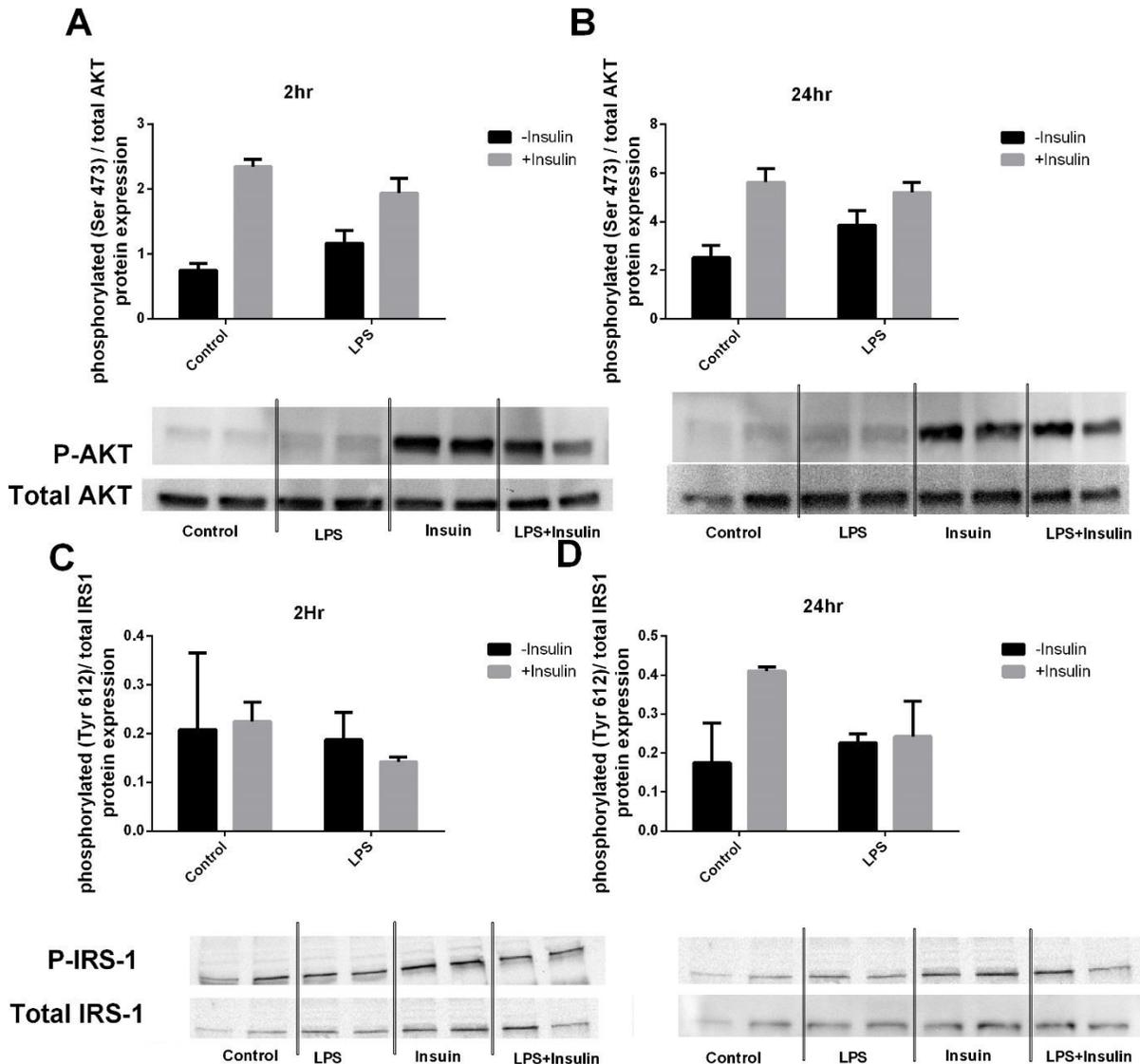


Figure 3. Protein content of insulin-signaling markers is unaffected by *neither acute nor chronic* LPS treatments in a Human Primary cell line. Serum-starved cells were treated for either 2 hours (A and C) or 24 hours (B and D) with low-dose LPS followed by a 10-minute insulin treatment. Cells were then harvested and P-AKT and P-IRS1 protein expression were measured via western blotting during both 2 hour (A and C) and 24 hour (B and D). Data are presented as mean±SEM. *, $p < 0.05$.

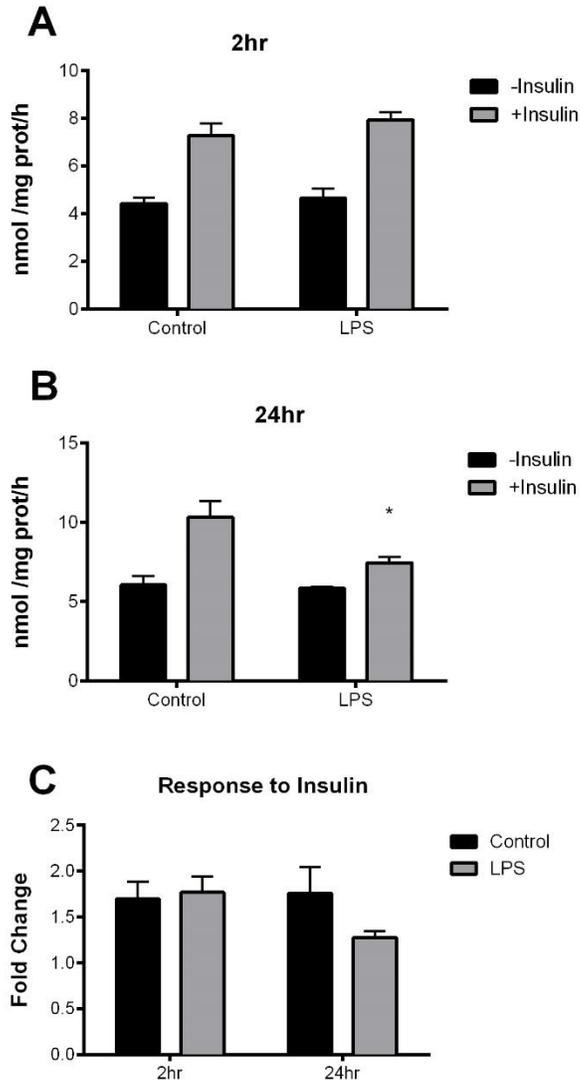


Figure 4. Chronic LPS treatments contribute to decreases in insulin-stimulated glycogen synthesis in a Human Primary cell line. Serum-starved cells were treated with LPS for either 2 or 24 hours then incubated for 3 hours with [U-¹⁴C]-glucose. Cells were then washed and harvested and glycogen synthesis (A and B) was measured. (C) Represents fold change in response to insulin between control and LPS treatments in the 2 hour and 24 hour groups. Data are presented as mean±SEM. Different from control. *, $p < .05$.

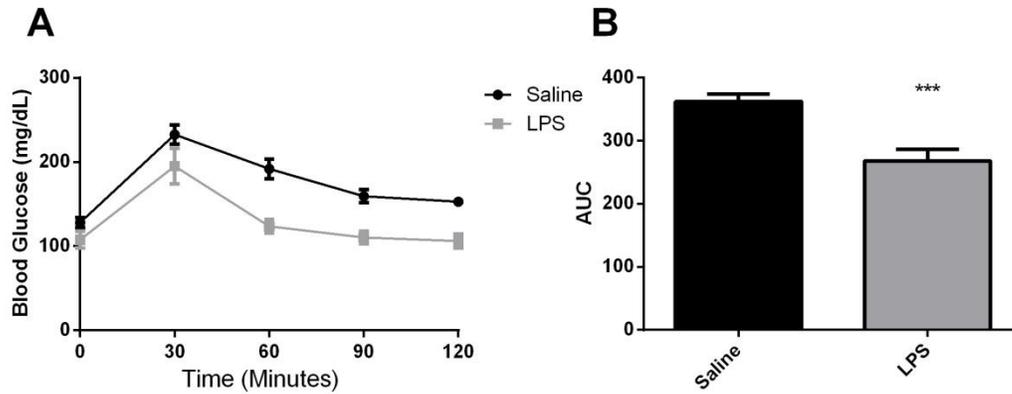


Figure 5. Mice injected with low-dose LPS exhibit improved glucose tolerance compared with WT littermates. C57/B16J mice were injected with low dose (0.1 ug/kg) LPS ($n=10$) or saline ($n=12$) followed 4 hours later by GTT (A). (B) Represents area under curve for GTT graph. Data are presented as mean \pm SEM. Different from control. ***, $p<0.01$.

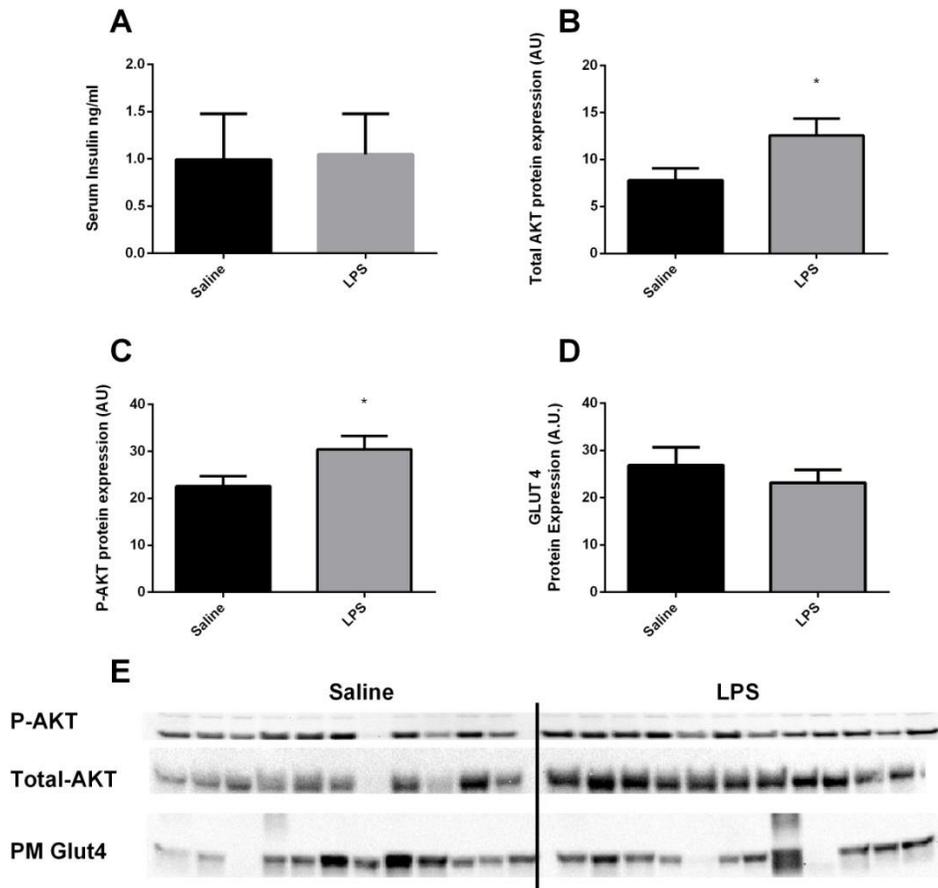


Figure 6. Serum insulin levels and plasma membrane Glut4 protein are unchanged while phosphorylated and total-AKT protein expression levels are up-regulated following LPS treatment. (A) Serum insulin levels were measured in blood from LPS ($n=14$) or saline ($n=13$) injected animals. (B-D) Protein expression levels of P-AKT, Total-AKT, and plasma

membrane bound Glut4. (E) Corresponding western blots of these proteins. Data are presented as mean±SEM. Different from control. *, $p<0.05$.

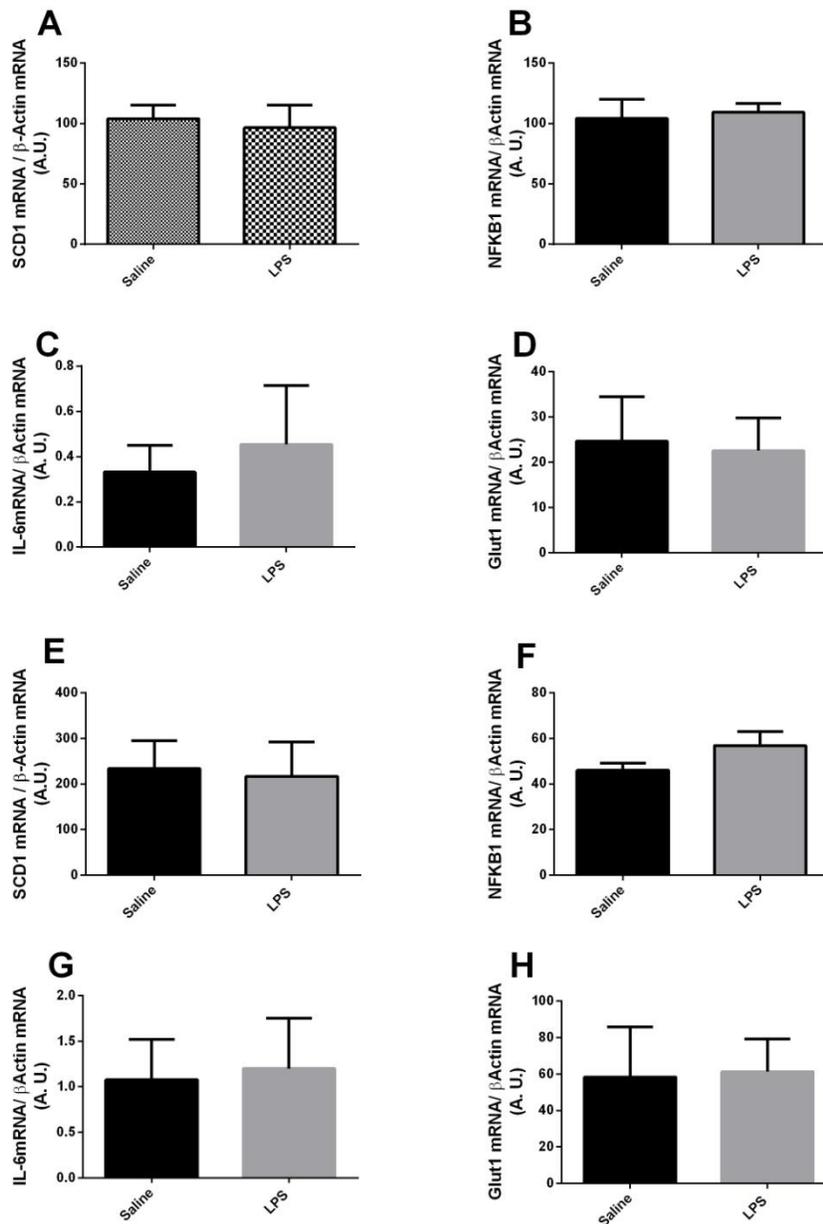


Figure 7. Gene expression of metabolic and inflammatory genes are unchanged under basal conditions in red and white skeletal muscle following acute low-dose LPS treatments. Gene expression data from red (A-D) and white (E-H) skeletal muscle of LPS ($n=14$) or saline ($n=13$) injected animals. Data are presented as mean±SEM. *, $p<0.05$.

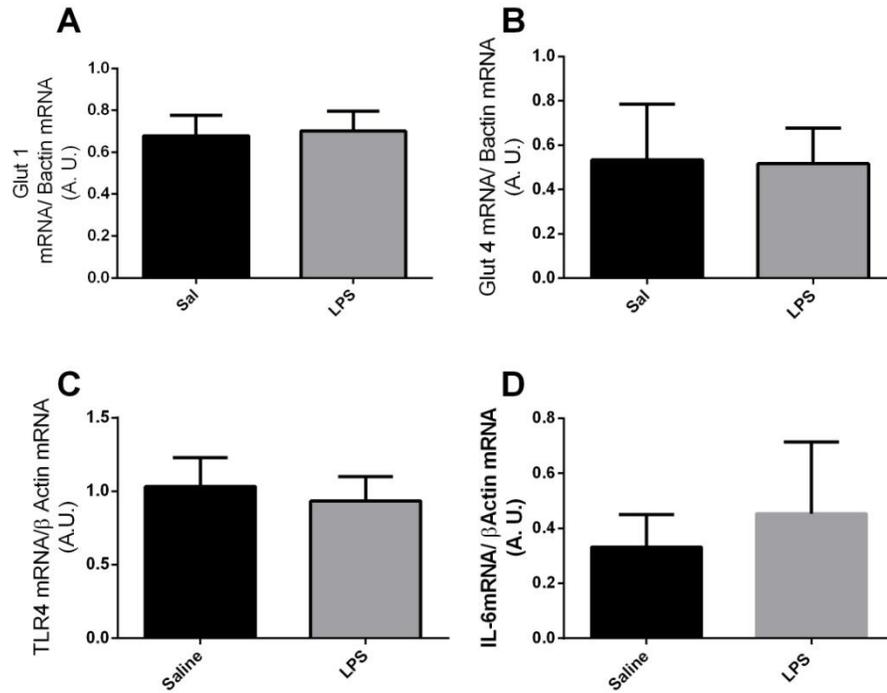


Figure 8. Gene expression of metabolic and inflammatory genes are unchanged under insulin-stimulated conditions in skeletal muscle following acute low-dose LPS treatments. Gene expression data from red (A-D) skeletal muscle of saline (n=13) or LPS (n=14) injected animals. Data are presented as mean±SEM. *, $p < 0.05$.

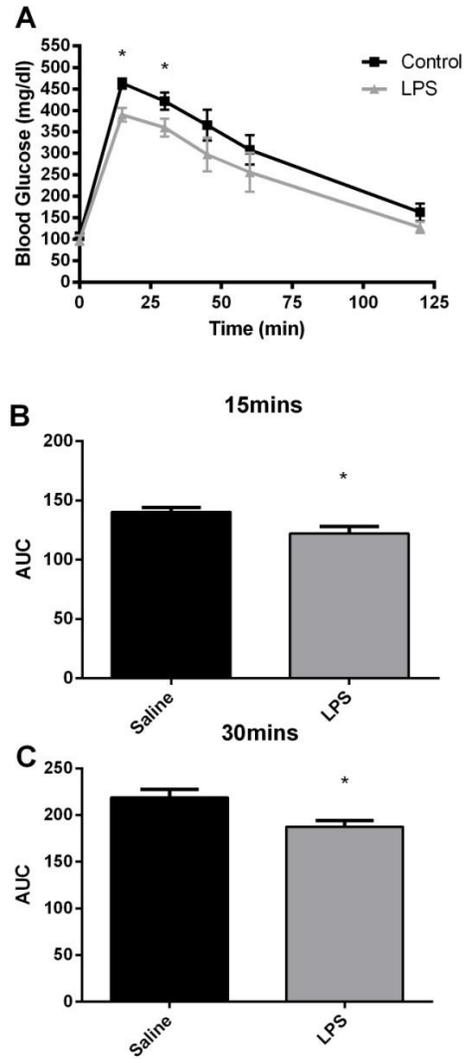


Figure 9. *LPS-injected animal's exhibit enhanced glucose tolerance during glucose uptake assay compared to WT littermates.* (A) Glucose tolerance curve of saline (n=5) or LPS (n=5) injected animals, 4hours post-injection. (B) Blood glucose levels of animals 15 (B) and 30 (C) minutes after glucose injection. Data are presented as mean±SEM. *P ≤.05.

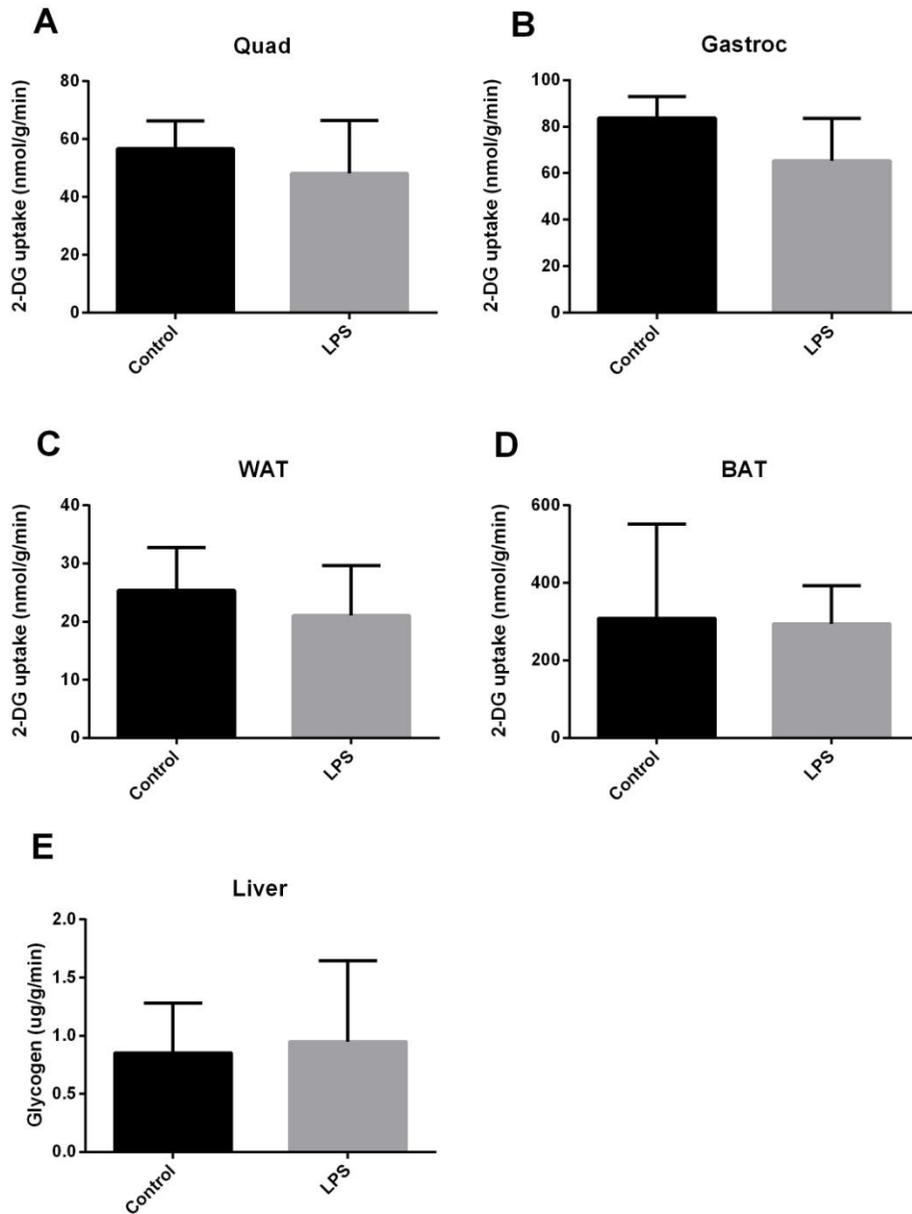


Figure 10. Uptake of 2-Deoxyglucose into muscle and adipose tissues and incorporation into liver glycogen is unaffected by LPS treatment. Uptake of 2-DOG 4hours following LPS or saline injection in (A) quadriceps, (B) gastrocnemius, (C) white adipose tissue, (D) brown adipose tissue, and (E) 2-DOG incorporation into liver glycogen. Data are presented as mean±SEM. *, $p < 0.05$.

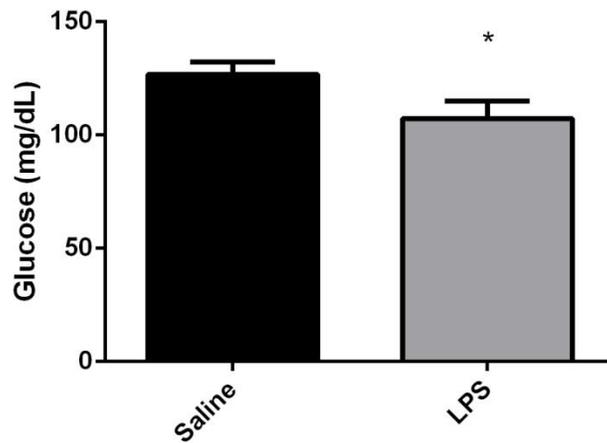


Figure 11. Fasting blood glucose levels are significantly lower in LPS versus saline treated animals. Fasting blood glucose levels from LPS ($n=10$) or saline ($n=12$) treated animals 4 hours after injection. Data are presented as mean \pm SEM. *, $p < .05$.

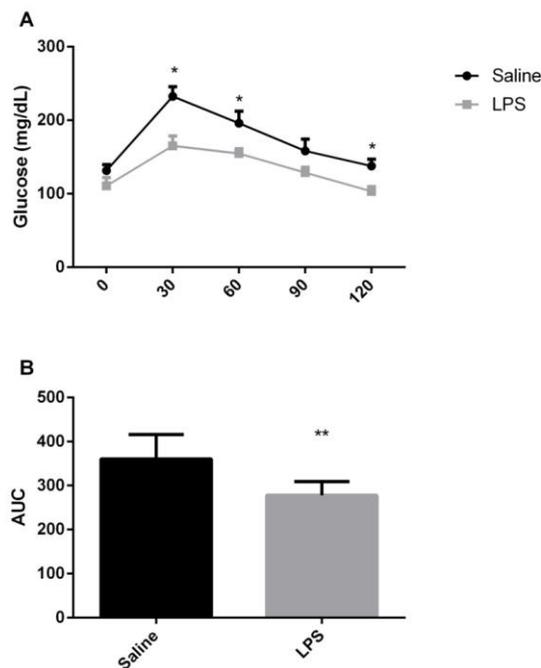


Figure 12. LPS treated animals exhibit decreased gluconeogenesis following pyruvate challenge. Overnight fasted animals were injected with LPS ($n=6$) or saline ($n=6$) then 4 hours later injected with sodium pyruvate to assess their abilities to perform gluconeogenesis. Data are presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

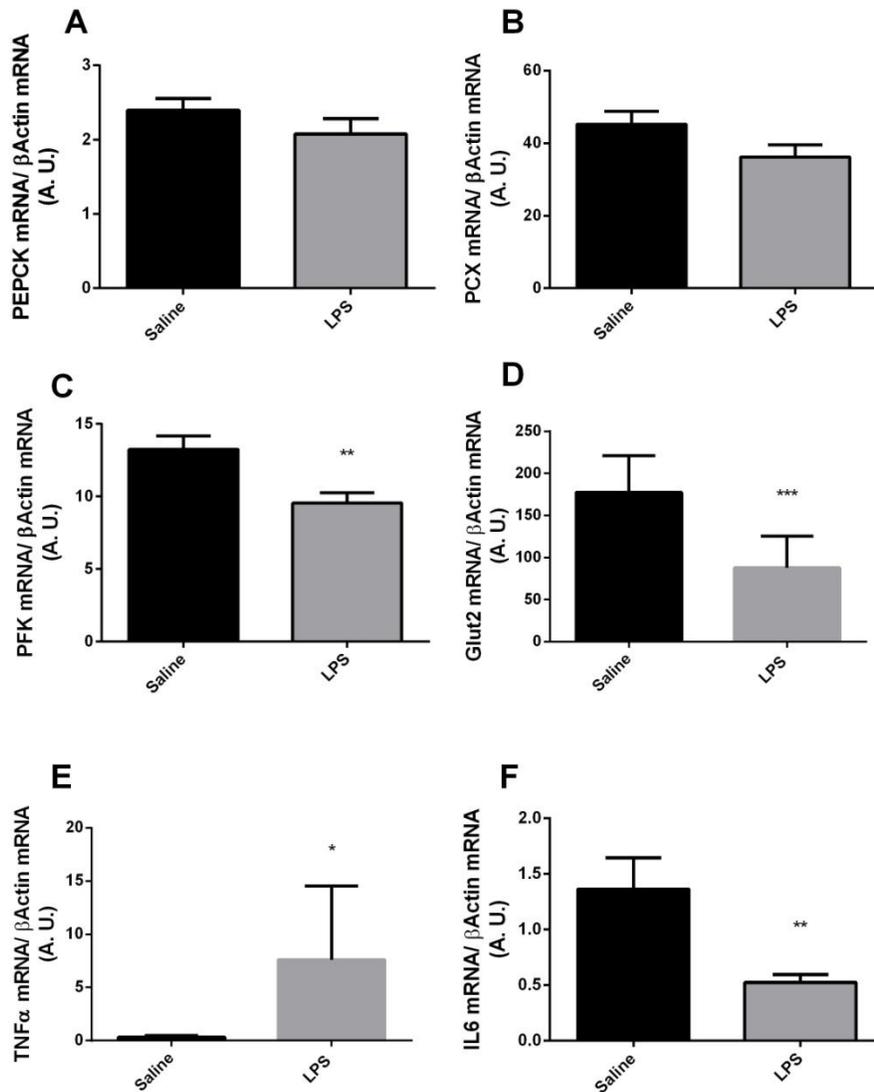


Figure 13. LPS treatment causes significant changes in the expression of genes involved in liver glucose flux and inflammation under basal conditions. Expression of genes involved in glycolysis (B and C), gluconeogenesis (A), and inflammation (E and F) from liver of animals harvested 4hours after injection with LPS ($n=10$) or saline ($n=12$). Data presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

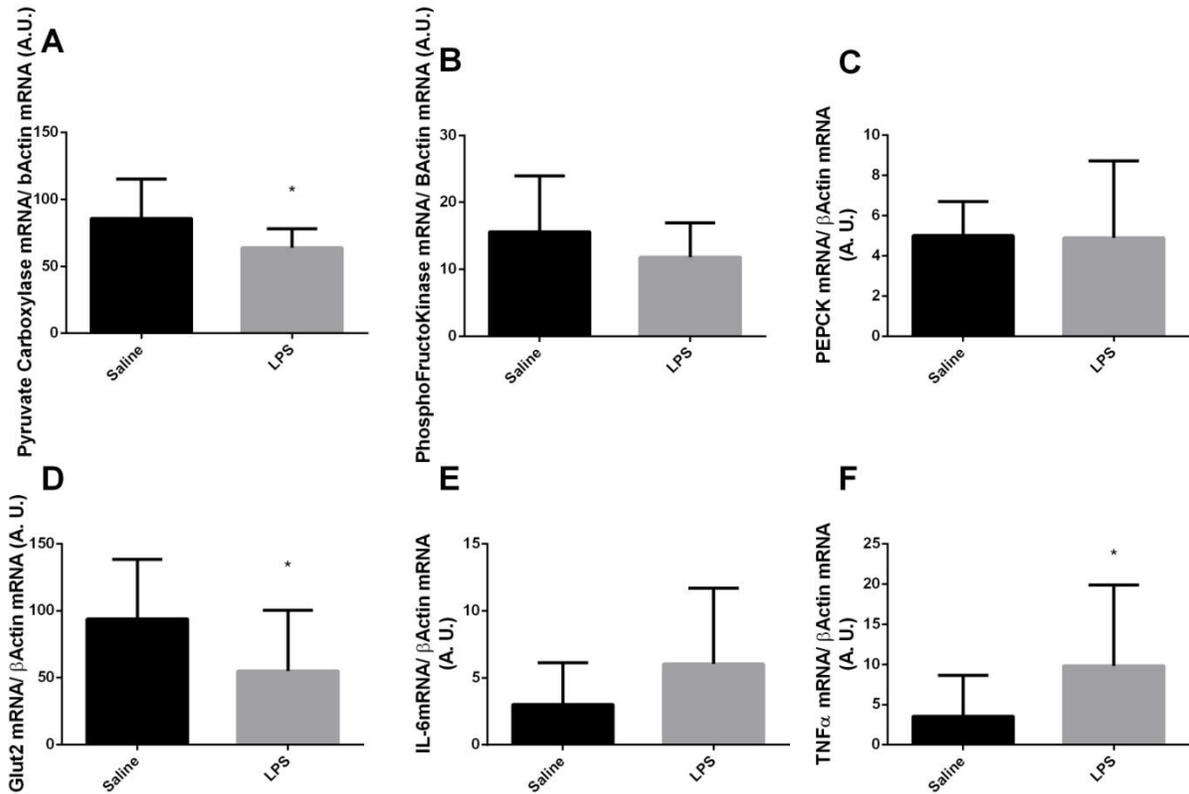


Figure 14. LPS treatment causes significant changes in the expression of genes involved in liver glucose flux and inflammation under insulin-stimulated conditions. Expression of genes involved in glycolysis (A and B), gluconeogenesis (C), and inflammation (E and F) from liver of animals harvested 4 hours after injection with LPS ($n=10$) or saline ($n=12$) and 30 minutes following injection of glucose. Data presented as mean \pm SEM. * $p < 0.05$.

Chapter 6: Conclusions and Future Directions.

Changes in LPS levels in the blood can have profound effects on glucose metabolism¹⁻
³. LPS is a ligand for TLR4 and its activation contributes greatly to defects in glucose homeostasis such as that seen with the metabolic syndrome^{1,4}. It is known that the effects of LPS on glucose homeostasis can vary greatly depending on both the duration and intensity of the increase in LPS. LPS concentrations can transiently rise after a normal meal and LPS levels have been shown to increase significantly in response to high-fat meals⁵ which is a proposed risk factor for the chronic inflammation in obesity. Research disagrees if these short-term increases in LPS levels, whether they be small or large, are beneficial or harmful to glucose metabolism. Chronically elevated LPS levels have been shown to have harmful effects on insulin signaling and glucose homeostasis. Another poorly understood factor concerning LPS and glucose metabolism is how different dosages can affect glucose metabolism. Most studies have used very high levels and this is difficult to translate into how LPS effects glucose metabolism in situations such as metabolic endotoxemia. Certain studies using acute low-dose LPS have resulted in improvements in glucose clearance⁶⁻⁸, however; other work has suggested that low-doses are sufficient to initiate an inflammatory response that becomes harmful to glucose homeostasis. The increases in glucose clearance following acute challenges with LPS possibly involve the interaction between the gut and the pancreas and results in increases in insulin production through GLP-1⁶. These changes are partially causative of the changes observed in peripheral tissues such as the liver and skeletal muscle.

These findings suggest that the relationship between LPS levels in the blood and glucose homeostasis is rather complex, and that the effects of LPS on glucose homeostasis might be dependent on the metabolic context and the experimental conditions used including

in particular the magnitude and duration of the endotoxemic challenge. The goal of our studies was to better understand this relationship between LPS and glucose metabolism in the context of magnitude and duration of LPS challenge. These studies show that in an INS1 beta cell line low-dose LPS treatment results in increased insulin secretion as well as trends towards increased glucose uptake. This outcome confirms previous reports using animal models suggesting the pancreas as a target tissue for LPS^{8,9}. Using a human primary skeletal muscle cell line, we show that long-term challenges can result in decreased utilization of glucose (Figure 3C), thus confirming our hypothesis that long-term LPS would have deleterious effects on insulin responsiveness.

Our studies show that a low-dose LPS challenge contributes to a significant increase in glucose clearance in an animal model and that this increase in glucose uptake occurs independent of changes in insulin secretion as well as without increased insulin signaling and glucose uptake into the primary insulin-responsive tissues. A low-dose LPS challenge in animals also resulted in a significant decrease in gluconeogenesis shown via a pyruvate challenge, and this occurred without concomitant changes in expression levels of PEPCK, the primary control point for gluconeogenesis in the liver. LPS challenge greatly affected gene expression of enzymes crucial to glucose handling in the liver. Decreases in PFK, Glut2, and PCX gene expression in the liver were shown with LPS treatment. These data all suggest a decrease in glucose oxidation. This data taken along with the pyruvate challenge data present an interesting paradigm in which decreases in enzymes controlling glycolysis fail to contribute to increases in the expression of PEPCK or in subsequent increases in gluconeogenesis as would be expected. These data are in disagreement with others in that decreased GNG in the liver following short-term LPS treatment is typically shown in concert with increased

glycolysis with a primary mechanism being the up-regulation of PFK¹⁰ and Fructose 2,6 Bisphosphate¹¹. However, this mechanism is related to using LPS of a much higher dose than the present study. Glut2 protein expression data from these studies was in disagreement with gene expression data in that LPS caused significant increases. This could possibly be explained as a compensatory mechanism elicited in response to the decrease in gene expression and could suggest transcriptional regulation as a primary mechanism for LPS involvement in the liver. Along with this there was a significant increase in the gene expression of TNF α and IL-6 in the liver, this taken with the fact that other tissues involved in the handling of glucose failed to show changes in inflammatory markers, suggests that inflammatory markers may be playing a role in regulating hepatic glucose production/oxidation.

Future directions of this research would include an investigation of how chronic treatments with low-dose LPS in animals affects glucose metabolism. It could be hypothesized that a chronic treatment would result in a heightened systemic inflammation that would be detrimental to glucose clearance and result in hyperglycemia. Ideally, these studies could have been done using implanted catheters in which LPS or saline could be delivered through the carotid artery and blood could be sampled from the jugular vein. This would allow for a better understanding of the dynamics of endotoxin levels in the blood following infusion, which this study did not and would aid in the understanding of this research area. In addition, studies aimed at better understanding the dynamics in glucose handling following LPS treatment are warranted. Glucose tolerance tests are not representative of situations where glucose production and its utilization increase to the same extent without any change in glucose concentration¹² and the use of tracers and clamp methods would give a better understanding of glucose dynamics following LPS treatment.

There remains other possibilities explaining the effect of LPS on glucose metabolism. Primary to this is the role of the immune cell. Low-doses of LPS very similar to those used in the present studies have been shown to activate immune cells¹³, particularly macrophages, causing significant up-regulation of the activity of these cells. This increased activity of immune cells means their energy requirements are increased¹⁴. Previous studies have shown increased glucose uptake into macrophages following treatment with LPS¹². Activation of macrophages and other immune cells was not measured in the current study and could possibly explain the increase in glucose clearance with these animals. Future studies using isolated macrophages from tissues involved in glucose handling would give great insight into their ability to alter glucose homeostasis following LPS treatment. Gene expression of inflammatory markers in the liver in the studies mentioned above would suggest a heightened inflammatory response in the liver and could be evidence in support of a heightened activation of immune cells contributing to the effects on glucose metabolism.

In summary, research regarding the role of low magnitude, short-term changes in LPS on mediating changes in glucose metabolism is largely, non-existent. There is little question that increases in LPS levels are capable of causing a wide-range of effects on metabolism. Our lab has taken interest in how low-magnitude changes in LPS can elicit changes in glucose handling, particularly in how these changes vary over time. These changes are characteristic of those seen with the metabolic syndrome. Our research indicates that short-term low-magnitude LPS injections in animals can cause drastic improvements in the ability of animals to clear glucose. This improved glucose clearing appears independent of any changes in insulin levels or in uptake in to insulin sensitive tissues. Animals also show decreased gluconeogenic capabilities that appear independent of gene or protein expression of PEPCK. These animals

show decreases in enzymes controlling glucose flux through the liver as well increased gene expression of inflammatory markers in the liver. Taken together, these data indicate in the mouse that low-magnitude, short term changes in LPS can have significant effects on whole body glucose metabolism in the mouse and this likely occurs through its direct effects on the liver.

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