The Role of Fatty Acids on Toll-like Receptor 4 Regulation of Substrate Metabolism with Obesity

Ryan Peyton McMillan

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The Department of Human Nutrition, Foods and Exercise

Matthew W. Hulver, Ph.D., Chair
Kevin P. Davy, Ph.D.
Deborah J. Good, Ph.D.
Robert W. Grange, Ph.D.
Liwu Li, Ph.D.

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Abstract

Growing evidence suggests that obesity and associated metabolic dysregulation occurs in concert with chronic low-grade inflammation. Toll-like receptors (TLR) are transmembrane receptors that play an important role in innate immunity and the induction of inflammatory responses. Our laboratory has observed that TLR4 expression is elevated in the skeletal muscle of obese humans and is associated with reduced fatty acid (FA) oxidation and increased lipid synthesis. Additionally, activation of this pathway by lipopolysaccharide (LPS), ex vivo, results in a shift in substrate metabolism favoring glucose as an energy substrate and preferential storage of FA in intracellular lipid depots. The purpose of this study was to examine the effects of saturated vs. monounsaturated FA on TLR4 transcription and signaling using ex vivo and in vivo models. C2C12 myotubes were incubated in FA-enriched growth medium with varying ratios of palmitate and oleate for 12 hours. Following FA treatment, cells were either collected for measures of mRNA and protein levels of TLR4 or challenged with LPS (500 ng/mL) for 2 hours to assess TLR4 mediated changes in interleukin-6 (IL-6) and glucose and fatty acid metabolism. TLR4 mRNA and protein content were increased in stepwise fashion with higher palmitate concentration ($p<0.05$). This was associated with an exacerbated LPS effect on IL-6 mRNA and protein levels, and glucose and fatty acid metabolism. To determine if these effects are translated to an in-vivo model, C57BL/6 mice were fed high saturated fat (HSF), high monounsaturated fat (HMF), and control diets for 10 weeks. Following the dietary intervention, animals were challenged with I.P. injections of either saline or LPS (~25µg/mouse), sacrificed 4 hours post-injection, and red and white gastrocnemius muscle were harvested for measures of expression and protein levels of TLR4 and IL-6, and glucose and fatty acid metabolism. TLR4 mRNA and protein levels were not altered with either the HSF or HMF diets.

However, there was a heightened LPS response with regards to increases in IL-6 and TNF-α, and enhanced shifts in substrate metabolism following the HSF diet ($p<0.05$). These effects were not observed in response to the HMF diet. In conclusion, these data demonstrate that a milieu of high saturated fatty acids results in elevated sensitization of the TLR4 pathway in skeletal muscle. These results provide insight into how a westernized diet, one enriched in saturated fat, may link chronic inflammation with obesity-associated metabolic abnormalities.

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Chapter 1: Introduction

An emerging pattern of increased food availability and over-nutrition combined with reduced physical activity has resulted in a dramatic increase in weight gain in the United States (1). Obesity is tightly associated with a variety of metabolic disorders such as Type 2 diabetes (T2D), hyperlipidemia, and cardiovascular disease (2-5). Skeletal muscle comprises approximately 40% of total body mass and is the predominant site for substrate disposal, and therefore; is primarily responsible for much of the metabolic dysregulation that accompanies obesity (6). The skeletal muscle of obese humans is highly glycolytic, and characterized by a reduced proportion of type 1 muscle fibers and a reduced capacity to oxidize fat (7-9). Hyperlipidemia, a condition associated with obesity, is known to induce a myriad of complications to skeletal muscle physiology. Lipid over-exposure has been shown to be detrimental to skeletal muscle glucose homeostasis and promotes the progression of insulin resistance to T2D (10-14).

Growing evidence suggests that obesity and metabolic disorders, including insulin resistance and T2D, occur in concert with chronic low-grade inflammation (15, 16). Toll-like receptor 4 (TLR4), a transmembrane receptor and integral to the innate immune response, is particularly involved in the recognition of lipopolysaccharide (LPS), a product of the outer membrane of gram-negative bacteria (17, 18). TLR4 plays an important role in promoting pro-inflammatory responses through activation of transcription factors such as nuclear factor kappa B (NFkB) (19). The receptor is expressed ubiquitously; located not only on immune cells such as macrophages, but is also present in several peripheral tissues including skeletal muscle (19). This suggests a potential role for the TLR4 pathway in cellular functions apart from innate immunity and a potential link between immunity, chronic inflammation and obesity.

There is evidence to suggest that TLR4 signaling plays a critical role in mediating vascular responses to diet-induced obesity. Kim et al. (20) report that vascular inflammation in response to prolonged fatty acid exposure is dependent on TLR4 function. In addition, TLR4 has been implicated in the development of fatty acid induced

insulin resistance (21). Shi et al demonstrated that free fatty acids could activate the receptor in both macrophages and adipose tissue, which resulted in impaired insulin signaling.

TLR4 is present in skeletal muscle and when activated, via LPS, can induce an inflammatory response (22). Whether TLR4 plays a role in other cellular processes in skeletal muscle, in particular those associated with obesity, has yet to be fully elucidated. There is evidence reporting increased TLR4 expression and protein content in the skeletal muscle of obese humans (23). Additionally, activation of this pathway has been shown to be causal to a metabolic phenotype consistent to what is routinely observed with obesity: increased glycolytic activity, and a partitioning of fatty acids toward storage as triglyceride and away from oxidative pathways. However, the relationship between the obesogenic environment, particularly lipid-oversupply to skeletal muscle, and TLR4 regulation is not well understood. Therefore, the central objective of the present study is to investigate the role of fatty acid surplus on the regulation of skeletal muscle TLR4 and associated TLR4-dependent metabolic defects.

Chapter 2: Review of the Literature

The Obese Metabolic Phenotype

Obesity is invading Westernized societies at epidemic rates and currently affects more than 300 million people worldwide. Currently in the United States, 65% of adults are overweight, 31% are obese, and the annual cost of overweight and obesity in the U.S. is approaching $125 billion per year (1). The association between obesity and chronic disease has been well established. The combination of overconsumption of calorie-rich foods and reduced physical activity lead to peripheral organ and tissue dysfunction and has promoted a dramatic increase in the incidence of cardiovascular disease, type 2 diabetes (T2D), hypertension, and other obesity-driven metabolic defects (2-5).

Skeletal muscle is the predominant location for substrate disposal and subsequent metabolism. Skeletal muscle comprises greater than 40% of total body mass and is responsible for at least 80% of postprandial glucose clearance (6). Therefore, it is not surprising that defects in this tissue are primarily responsible for much of the metabolic dysregulation that accompanies obesity. Tanner et al. (9) has shown that there appears to be a relationship between obese skeletal muscle and fiber type. Obese individuals typically possess fewer type I (oxidative) and more type IIB (glycolytic) muscle fibers than their lean counterparts. This presents metabolically as a preference for glucose as a substrate while partitioning fatty acids toward synthesis as triglyceride and away from oxidative pathways.

One of the hallmark characteristics of obesity is highlighted by what many experts have coined “metabolic inflexibility”; a condition defined as the inability of peripheral tissues to transition from lipid to carbohydrate metabolism in the presence of insulin. During postabsorptive conditions, such as after an overnight fast, the predominant fuel source for skeletal muscle is lipid. In these conditions, despite no differences in fatty acid uptake, obese individuals exhibit lower rates of fatty acid oxidation compared to lean

subjects (24). Likewise, in obesity, despite elevated levels of glucose uptake in the basal state, there is a well-characterized defect in the ability to respond to insulin (25-27).

In line with this paradigm, results from a number of investigations have shown a reduction in whole-body fatty acid oxidation with obesity (28, 29). Whether this impairment in fatty acid oxidation is due to a reduction in mitochondrial content or mitochondrial function has been an issue of debate. Studies measuring the ratio of electron transport chain capacity to mitochondrial DNA suggest an imbalance and thus mitochondrial dysfunction (30, 31). Also it has been suggested that the proportion of fatty acids that are completely oxidized to CO2 is diminished with obesity and this incomplete oxidation may lead to a build-up of metabolic intermediates, which promote oxidative damage in the mitochondria compromising its function (32). The results of other studies, which have directly measured oxygen consumption from mitochondria, isolated from lean and obese humans found no differences in function (33, 34). Although there were significant reductions in whole-body fatty acid oxidation in these studies, once corrected for citrate synthase activity, the effect was abolished suggesting instead, that reductions in mitochondrial content are the primary cause for decreased fatty acid oxidation with obesity. Holloway et al. (35) have shown that indeed muscle mitochondrial content was markedly reduced in a group of age-matched lean and obese humans. Despite these inconsistencies in regards to the mechanism, there is wide consensus that whole-body fatty acid oxidation is reduced in the obese state.

Hyperlipidemia, a condition characterized by excessive lipids in the bloodstream, is associated with obesity and known to induce a myriad of complications to skeletal muscle physiology. Excess lipid delivery to peripheral tissues, particularly skeletal muscle, in conjunction with a reduced capacity to oxidize fat, presents the tissue with the dilemma of matching supply with uptake and oxidation. This results in an excessive accumulation of intramuscular triglycerides (IMTG) a phenomenon that occurs in concert with reduced insulin stimulated glucose transport and impaired insulin signal transduction (10-14). Interestingly, measurements of intramuscular lipids correlate most

closely with insulin resistance than any other commonly measured indices including body mass index, total body fat and waist-hip ratios (36). Now commonly agreed upon is the notion that IMTG is not a direct cause of lipid-induced insulin resistance but instead is simply a marker for elevated cytosolic lipid accumulation. Instead, it is triglyceride intermediates such as fatty acyl CoA's and diacylglycerols that accumulate within the skeletal muscle fibers of this population and evidence has shown that these moieties directly interact and enhance the activity of protein kinase C (PKC) (37). Activation of PKC leads to serine phosphorylation of insulin receptor substrate-1 (IRS-1). Serine phosphorylation impairs insulin receptor-mediated tyrosine phophorylation of IRS-1 and thus inhibits IRS-1 from interacting with phosphatidylinositol-3-kinase (PI3-kinase). This in turn, inhibits further downstream signaling to protein kinase B (PKB) and eventually inactivation of glycogen synthase kinase. These processes prevent insulin-stimulated GLUT-4 translocation and ultimately impair glucose uptake and glycogen synthesis.

Evidence for the important role of triglyceride intermediates and insulin signaling is supported by work from Liu and colleagues (38). Demonstrated in these studies was that upregulation of the triglyceride synthesizing enzyme DGAT in mice resulted in an increase in IMTG but improved insulin sensitivity. This suggests that the partitioning of bioactive, harmful lipid intermediates into triglyceride protects from activation of the PKC pathway and subsequent fatty acid-induced insulin resistance.

In addition to the chronic effects associated with lipid over-supply and subsequent ectopic accumulation, acute lipid loads are also shown to be quite toxic to insulin signaling. Yu et al. (39) show that following a 5hr lipid infusion in rodents, intracellular fatty acyl-CoA's levels increased approximately 6-fold and this was associated with increased diacyglycerol levels and PKC activation. This in turn, resulted in a significant impairment to insulin-stimulated glucose transport.

In addition to the PKC/PI3-Kinase mechanism by which lipids induce insulin resistance, other mechanisms have been demonstrated including one centered on the "glucose-

fatty acid cycle" or Randle cycle. It is proposed that fatty acids induce insulin resistance by inhibiting pyruvate dehydrogenase and increasing cellular citrate concentrations. Citrate is a potent allosteric inhibitor to phosphofructokinase (PFK), a rate limiting enzyme of glycolysis. Inhibition of PFK leads to a backload of its substrate, glucose-6-phosphate, which in turn inhibits hexokinase activity and thus reduces glucose uptake and oxidation. Furthermore, GLUT 4, the primary protein responsible for insulin-regulated glucose uptake in skeletal muscle, has been shown to be significantly reduced in response to chronically elevated free fatty acid (FFA) levels (40-41).

Obesity and Inflammation

In recent years, evidence has accumulated to corroborate the notion that obesity, and its associated comorbidities, are tightly associated with systemic, chronic low-grade inflammation (15-16). This concept was first conceived in 1993 when Hotamisligil and colleagues (42) demonstrated that adipocytes, once thought to be inert storage sites for triglyceride, constitutively express and secrete the proinflammatory cytokine tumor necrosis factor alpha (TNF-α). Furthermore, it was established that TNF-α expression levels are significantly elevated adipocytes from obese animals (ob/ob mouse, db/db mouse, and fa/fa zucker rat). Neutralization of TNF-α by a soluble TNF-α receptor in these animals leads to improvements in metabolic function and decreased insulin resistance. These data were the first to establish a link between expression levels of a proinflammatory marker and metabolic disease. Subsequent to these findings, it was later discovered that weight loss resulted in a significant decline in both adipose tissue and plasma TNF-α (43-45). Since then, it has been confirmed by multiple groups that obese humans present with higher circulating levels of the proinflammatory proteins C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), and interleukin-6 (IL-6) (46-48).

One possible mechanism linking obesity and inflammation involves the role of the adipokine, adiponectin. Adiponectin is released from adipose tissue and serves as a key mediator in obesity-induced insulin resistance and peripheral tissue inflammation.

Acting as a negative regulator to inflammation, adiponectin is shown to increase mitochondrial biogenesis and reduces inflammation by opposing the effects of TNF-α (49). Counterintuitively, obese individuals with greater adiposity are linked to less production and secretion of this insulin-sensitizing, anti-inflammatory agent (50). Thus, it is conceived that reduced circulating levels adiponectin, characteristic of the obese state, may be a prime contributing factor to inflammation associated with obesity. Indeed, treatment with adiponectin in obese mice resulted in an attenuation of inflammatory markers, reduced serum hyperglycemia, and improved insulin sensitivity (49).

Another possible mechanism linking obesity to systemic inflammation involves regulation by leptin. Leptin is an adipokine produced and secreted by adipocytes, which serves as a master regulator of appetite and energy balance (51). Despite the satiety-inducing and appetite-reducing effects of leptin, obese individuals generally present as having higher circulating levels than non-obese subjects (51). These individuals are typically resistant to leptin's effect in much the same way T2D patients are resistant to insulin (52). Leptin has been shown to induce oxidative stress and activate nuclear factor-kappa B (NFkB), a proinflammatory transcription factor. This, in turn, leads to the increased secretion of monocyte chemoattractant protein (MCP-1), TNF-α, IL-6, and CRP, all of which act toward promoting a pro-inflammatory environment (53, 54). However, ob/ob mice, which lack leptin, and db/db mice, which lack the leptin receptor, do present with an inflammatory phenotype suggesting that other mechanisms, in addition to leptin, must play a role in obesity-associated inflammation.

Fatty acids, particularly those of saturated nature, have been shown to elicit both local and systemic inflammatory responses in a variety of peripheral tissues, most notably adipose and skeletal muscle. In white adipose tissue (WAT), chronic lipid oversupply results in hypertrophied adipocytes, which secrete proinflammatory agents and promote systemic inflammation. One such secreted chemokine is MCP-1, which acts to recruit macrophages to expanding adipose tissue and further exacerbate the inflammatory processes (55). Fatty acids promote inflammatory responses not only by expanding

WAT, but also by inducing apoptosis through oxidative and endoplasmic reticulum stress, accumulation of diacylgycerols and ceramides, and activation of the PKC signaling (56, 57); a proinflammatory mechanism operating in much the same way that fatty acids induce insulin resistance in this tissue.

It is important to note that the production and secretion of proinflammatory cytokines and chemokines is not only limited to adipose tissue, and those cells associated with immunity, but also is very pronounced in skeletal muscle. Several reports have confirmed that myocytes produce a significant source of proinflammatory mediators IL-6, interleukin-1β (IL-1B), TNF-α, among others; and secrete these into local interstitial fluid in response to fatty acid exposure (58).

Interestingly, the contribution of fatty acids to insulin resistance and inflammation appears to be mediated by their chemical nature and composition. Whereas saturated fatty acids have routinely been shown to elicit a strong lipotoxic, inflammatory response in skeletal muscle, monounsaturated fatty acids do not seem to induce the same effect. Palmitate, a saturated fatty acid, is known to regulate the expression of TNF-α and activate NFkB, which has been linked to fatty-acid induced inflammation/insulin resistance (56, 57). Weigert et al. (56) demonstrate that incubation of human myotubes in the presence of physiological levels of palmitate induces both rapid (30min and 1hr) as well as chronic (24 and 48hr) increases in NFkB nuclear translocation and DNA binding. This was associated with concomitant increases of 6-fold and 3-fold in IL-6 mRNA and protein, respectively. Incubation with the monounsaturated fatty acid oleate, under identical conditions, did not generate the same inflammatory response. Coll and colleagues have shown that oleate reverses the palmitate-induced inflammatory response (59). Incubation with palmitate alone was shown to phosphorylate IRS-1 on its serine residues and have detrimental effects on insulin signaling in C2C12 myotubes, while these changes were not evident with oleate incubation. Interestingly, co-incubation of palmitate and oleate reversed this effect in a concentration-dependent manner (59).

The mechanism(s) by which oleate prevents the deleterious effects of saturated fatty acids on skeletal muscle are not fully elucidated but a few theories have emerged. Oleate, but not palmitate, has been shown to increase expression levels of carnitine palmitoyl transferase-1 (CPT-1) (59). CPT-1 facilitates the entry of fatty acids into the mitochondrial matrix and thus regulates fatty acid oxidation. Increased levels of CPT-1 would expect to be beneficial towards reducing cytosolic lipotoxicity by funneling harmful bioactive lipid intermediates, such as fatty acyl-CoA's, into the mitochondria for β-oxidation. This, in turn, prevents their cytosolic accumulation, their synthesis into diacylglycerols, and subsequent activation of the PKC-NFkB pathway and resulting insulin desensitization.

Another observation that may explain the mechanism by which monounsaturated fatty acids are protective to saturated fat-induced inflammation involves regulation by the triglyceride synthesizing enzyme diacylglycerol transferase 2 (Dgat2). Palmitate exposure has been shown to decrease levels of Dgat2 and is predominately incorporated into diacylglycerol as opposed to triglyceride (59). Oleate is more preferentially incorporated into triglyceride and co-incubating oleate with palmitate prevents this fall in Dgat2 (59). It is important to re-emphasize that intracellular lipid incorporation into triglyceride is protective due to a reduction in the formation of more toxic lipid species. Thus, in summary, it appears that oleate exerts it protective effects intracellularly by 1) increasing β-oxidation via increasing fatty acid entry into the mitochondria, and 2) decreasing formation of harmful lipid intermediates by preventing the decrease in Dgat2 and partitioning lipid moieties into triglyceride form.

In humans, whether inflammation is an early characteristic pre-disposing one to obesity, or if it is simply an effect of an obesogenic environment has not been clarified. Above normal concentrations of inflammation-sensitive proteins have been shown to predict future weight gain (60), however long-term studies in humans are lacking and additional studies should be designed to further investigate the causal relationship between obesity and inflammation.

Toll-Like Receptors, Innate Immunity, and Inflammation

The innate immune system provides for the first-line of defense against invading pathogens and is found throughout animal life. The primary objective of the innate immune system is recognition of distinct pathogen-associated molecular patterns (PAMPs) presented by bacteria, viruses and other pathogens and initiating a rapid response via intracellular signaling pathways that culminate in the production of proinflammatory products designed to combat infection. Host cells contain a variety of cell-surface receptors designed to recognize these molecular patterns.

One such family of receptors includes the toll-like receptors (TLR's). TLR's were first described in the drosophila and were recognized to be vital to the establishment of dorso-ventral integrity in the developing embryo (19). The Toll signaling pathway in the drosophila is very similar to the mammalian IL-1 pathway, which culminates in the activation of NFkB and transcription of genes responsible for immune and inflammatory processes. Soon after the discovery of the Toll receptor in drosophila, the mammalian homologue was identified (61). Since then, at least 13 mammalian TLR's have been recognized and are universally characterized by a leucine-rich repeat domain extracellularly and a TIR domain intracellularly. They are classified into distinct groups based upon the type of PAMPs they recognize and respond to.

Toll-like receptor 4 (TLR4) was the first mammalian TLR identified and was shown to be involved in the recognition of lipopolysaccharide (LPS), a component on the outer walls of gram-negative bacteria. The structure of LPS consists of a core polysaccharide with polysaccharide side chains and an imbedded lipid A portion; the lipid A moiety of which has been shown to be critical component of LPS to TLR4 sensing and exerting its toxic effects. Recognition of LPS by TLR4 requires the cooperation of additional proteins. LPS binds to LPS-binding protein in serum and this complex is recognized on the cell surface by CD14 (62). CD14 lacks an intracellular domain and consequently recruits

MD-2, an adaptor protein to complex with the intracellular domain of TLR4. This TLR4/MD-2 complex is then primed to bind the lipid A portion of the LPS molecule and initiate downstream cytosolic signaling.

Intracellular signaling through TLR4 requires the combined cooperation of several signaling molecules commencing with NFkB activation and gene transcription. Specifically, once TLR4 is stimulated by ligands, such as LPS, the adapter proteins, MyD88 and TIR domain-containing adaptor-inducing IFN-β (TRIF), are recruited to the receptor complex (63). This initiates a downstream signaling cascade involving the phosphorylation and activation of many complexes culminating in the activation of the transcription factor, NFkB. Upon activation, NFkB translocates to the nucleus and induces the expression of many inflammatory cytokines including TNF-α and IL-6 (19).

LPS-stimulation of TLR4 in MyD88-defiencient macrophages results in NFkB activation and transcription of proinflammatory genes, thus suggesting a MyD88-independent pathway by which LPS may signal through TLR4 (64). Later work has confirmed that indeed there exists a MyD88-independent signaling route, which involves the adaptor proteins TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM). The LPS–induced MyD88 independent pathway appears to regulate a different subset of genes including IP-10, GARG16, and IRG-1 (65).

In addition to LPS, TLR4 appears to recognize endogenous ligands. For example, heat shock proteins have been shown to bind TLR4 in macrophages and dendritic cells and represent a mechanism by which the cell may respond to stress (19). For example, heat shock protein 60 has been shown to be implicated in the inflammatory response to atherosclerosis (66). Consistent with this, mice defective in TLR4 show an impaired ability to respond to heat shock proteins with an appropriate cytokine-based inflammatory response (67-69).

Since their discovery about a decade ago, much progress has been made in characterizing the TLR's and identifying their role in regulating inflammatory responses

to invading pathogens. The role of TLR's, and specifically TLR4, in modulating physiological pathways independent of immune function, including those associated with chronic disease, are only now beginning to emerge.

Linking TLR4 and Obesity

The link between inflammation, obesity and diabetes is well established. TLR4, a mediator of inflammation is proposed to play an integral role in this relationship. Given that the receptor is expressed ubiquitously; located not only on immune cells such as macrophages, but also present in several peripheral tissues including adipose tissue and skeletal muscle suggests that the receptor may be an integral player in obesity-related inflammatory processes (70-72).

Adipocytes from obese subject's characteristically manifest inflammatory-like properties including the production and release of proinflammatory cytokines, and in this way resemble macrophages in their ability to elicit and inflammatory response (73, 74). Based on this evidence, it has been hypothesized that TLR4 mediates lipid-induced insulin resistance and inflammation in peripheral tissues and that this phenomenon is conceivably exacerbated in a state of increased adiposity. Shi et al. demonstrated that free fatty acids could activate the IKK/NFkB pathway and stimulate macrophage and adipocyte production of TNF-α and IL-6 (75). Importantly, it was shown that this was dependent on TLR4 signaling. Adipocytes isolated from C57BL6/J mice which contain a global knockout of TLR4 ($TLR4^{-/-}$) displayed an attenuated IL-6 and TNF-α response to fatty acid exposure (75).

This hypothesis was further tested in vivo using an acute lipid infusion to induce insulin resistance in peripheral tissues in $TLR4^{-/-}$ mice and wild-type counterparts. Eight-hours of continuous lipid infusion induced insulin resistance in wild-type mice as evidenced by inhibited insulin-stimulated IRS-1 phosphorylaton. This effect was substantially attenuated in $TLR4^{-/-}$ mice. Utilizing a hyperinsulinemic-euglycemic clamp, the authors were able to examine the insulin sensitivity to glucose metabolism in both lines of mice infused with either saline or lipid. In wild-type mice, the glucose infusion rate required to

maintain euglycemia was reduced by 65% when infused with lipid as opposed to saline suggesting that lipid infusion was sufficient in disrupting peripheral glucose homeostasis and inducing insulin resistance. The mice lacking TLR4 did not exhibit the same response to lipid exposure and appeared to be somewhat protected from lipid-induced insulin resistance.

The long-term physiological role of TLR4 was demonstrated via chronic high-fat feeding in $TLR4^{-/-}$ mice (75). Thirty-nine weeks of high fat feeding resulted in similar degrees of adiposity in $TLR4^{-/-}$ and wild-type mice however; mice lacking TLR4 were protected from insulin resistance as evidenced by insulin tolerance tests. Additionally, high-fat fed wild-type mice presented with significantly higher adipose tissue expression of TNF-α, IL-6, and the macrophage recruiter MCP-1. The expression levels of these proinflammatory genes where attenuated in high fat fed $TLR4^{-/-}$ mice. These effects in adipose tissue have also been revealed to occur in skeletal muscle in similar studies utilizing chronic high fat feeding in animals lacking TLR4. These studies together suggest that TLR4 is obligatory in realizing the insulin desensitizing effect of lipotoxicity.

There is also evidence to suggest that TLR4 signaling plays a critical role in mediating vascular responses to diet-induced obesity. Kim et al. report that vascular inflammation in response to palmitate exposure is dependent on TLR4 function (76). Mice lacking a functional TLR4 receptor and wild type controls were high-fat fed for 8 weeks at which time aortic explants were taken and acutely exposed to palmitate. In lysates from WT mice maintained on a high-fat diet, markers of vascular inflammation both upstream and downstream of NFkB were increased and associated with insulin resistance. These effects were not evident in the TLR4 -/- mice. These findings are important in establishing a vital role for TLR4 function in cardiovascular disease, as vascular inflammation and endothelial dysfunction can progress atherosclerosis in the setting of obesity.

Recently, it has been shown that adipocyte TLR4 expression levels are elevated in diet-induced obese mice as well as other obese rodent models (ob/ob and db/db) (75). In

line with this, Reyna et al. (77) reported that obese and insulin resistant humans had significantly elevated levels of TLR4 transcripts and protein in skeletal muscle and these levels were positively associated with the severity of insulin resistance. This increase in TLR4 and subsequent NFkB signaling was accompanied by elevated levels of the proinflammatory gene IL-6.

There are many factors that contribute to fatty acid induced insulin resistance, inflammation and metabolic dysfunction that have come to characterize obesity. In recent years, it has become increasingly evident that toll-like receptors, in particular TLR4 may play a large role in serving as the link by which elevated plasma lipid levels impact obesity-related inflammation. Much of the work surrounding TLR4 has focused on its role in immune cells, macrophages, and adipocytes with little attention to its function in skeletal muscle. Being that skeletal muscle is the primary target for metabolic dysfunction with obesity, the effect of fatty acids on skeletal muscle TLR4 content and signaling is an intriguing question and one which may begin to reveal a better understanding of the driving forces relating obesity, inflammation and skeletal muscle metabolism.

Chapter 3: Specific Aims

Globally, there are over one billion overweight adults, and despite growing attention aimed at resolving this epidemic, the situation is worsening rather than improving. Overweight and obesity pose an increased risk for a multitude of chronic diseases including type 2 diabetes, hypertension, and cardiovascular disease. Skeletal muscle, by virtue of its role in regulating substrate disposal, is a primary target for the metabolic abnormities that complement obesity. Obese skeletal muscle is characterized by reduced fatty acid oxidation and a consequential elevated accumulation of lipids within the muscle fibers. This phenomenon occurs in concert with reduced insulin-stimulated glucose transport and impaired insulin signal transduction.

Obesity is linked to an underlying, chronic, low grade inflammatory state. Toll-like receptors are transmembane receptors important in the activation of pro-inflammatory transcription factors responsible for the innate immune response. Recent data from our lab indicate that 1) expression of proteins important to the TLR signaling pathway are significantly elevated in obese humans; and 2) TLR4 is positively related to intramuscular lipid accumulation and body mass index. Furthermore, we show that 3) TLR4 activation regulates substrate metabolism in a manner consistent with that observed in obesity; a metabolic phenotype favoring glycolytic pathways while compromising fat oxidation. However, the precise mechanisms by which TLR4 is regulated in the obesogenic, lipotoxic state are less clear.

Our working hypothesis is that a high lipid environment up-regulates TLR4 gene expression and protein levels. Since fatty acids are known to be elevated with obesity it is conceivable suspect that fatty acids serve as the critical link between TLR4, inflammation and obesity.

The specific aims of this project are:

SPECIFIC AIM 1: To investigate the *in-vitro* consequence of an acute and chronic lipotoxic environment to the regulation, expression and metabolic implications of TLR4 signaling in skeletal muscle cell cultures.

Hypothesis: Fatty acid exposure increases TLR4 expression and activation. Pre-treatment with fatty acids will result in an exacerbated TLR4 response to LPS.

Objective: Utilize cell culture techniques to determine the effect of a pre-treatment of differing fatty acid compositions on TLR4 expression, protein content and subsequent regulation of skeletal muscle substrate metabolism.

SPECIFIC AIM 2: To observe the *in-vivo* effect of chronic hyperlipidemia to the regulation, expression and metabolic implications of TLR4 signaling in C57BL/6 mice exposed to either a high saturated fat or high monounsaturated fat diet.

Hypothesis: Chronic high saturated fat feeding will result in enhanced TLR4 expression and protein levels. This will be associated with an increased sensitivity to LPS and subsequent metabolic dysfunction compared to control and high monounsaturated fat diets.

Objective: Four and ten-week high fat feeding in C57BL/6 mice will be undertaken to assess the long-term physiological relevance of fatty acid specificity to TLR4 expression, content and regulation of substrate metabolism.

Chapter: 4 Research Design

SPECIFIC AIM 1: To investigate the in-vitro consequence of a lipotoxic environment on the regulation, expression and metabolic implications of TLR4 signaling in skeletal muscle cell cultures.

Objective: Utilize cell culture techniques to determine the effect of a pre-treatment of differing fatty acid compositions on TLR4 expression, protein content and subsequent modulation of skeletal muscle substrate metabolism.

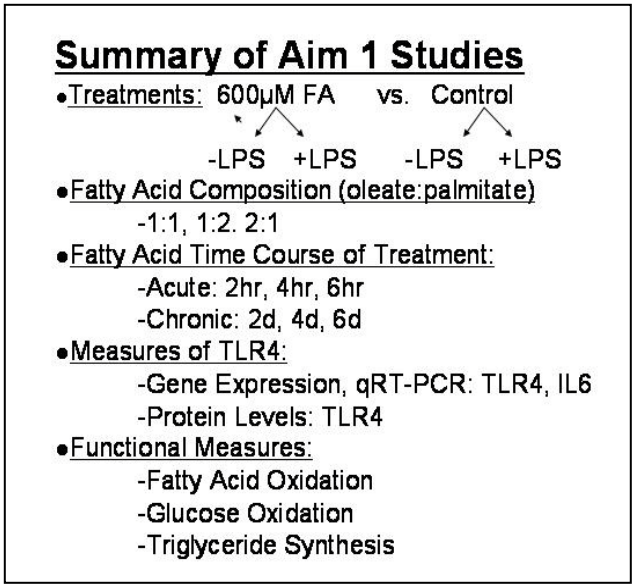
Experimental Strategy: C2C12 (murine) muscle cells will be used in experiments conducted in Specific Aim 1. The Hulver laboratory has extensive experience with these cell lines and they provide an ideal and widely used model to understand skeletal muscle function (78).

Cells will be grown to confluence in 10% fetal bovine serum in Dubelcco's modified eagles medium (DMEM) and then differentiated in 2% horse serum in DMEM for 4-7 days. In all experiments, cells will be exposed to a pretreatment of fatty acids + BSA or BSA alone followed with and without a 2hr LPS challenge. Following a pretreatment with or without fatty acids and LPS, cells will be collected for gene expression analysis of TLR4 and IL-6, a proinflammatory cytokine whose expression is tightly correlated with TLR4 activation (79). Cells will also be collected to perform Western Blots to measure protein levels of TLR4. Separate plates of cells will be grown to characterize the metabolic effects of the fatty acid pretreatment and subsequent LPS stimulation of TLR4. For the metabolic experiments, following treatment, cells will be incubated for 3hr with exposure to radiolabled substrates from which glucose and fatty acid oxidation, and lipid synthesis will be assessed, as previously described (80).

Saturated vs. Monounsaturated Fatty Acids. The concentrations of oleate and palmitate will be manipulated (1:1, 2:1, 1:2) to determine if the regulation of TLR4 is differentially effected by the saturation nature of the fatty acid mixture. For free fatty acid

treatments, a mixture of monounsaturated (oleate, 18:1) and saturated (palmitate, 16:0) fatty acids will be used. The use of palmitate alone induces a strong apoptotic effect (81) and to control for this effect oleate will be used in combination. Oleate is shown to protect the cell from the apoptotic effects of palmitate (82) and in addition, this simulates a more physiological setting. 600μM of this fatty acid mixture+BSA (or BSA alone) will be used as this concentration best confine to normal physiological levels.

Expected Outcomes: Work from other labs have shown the capacity of palmitate to elicit a robust inflammatory response (56), which is hypothesized to operate through the TLR4 signaling pathway. These findings combined with evidence that fatty acid levels are elevated in obese humans (83), suggests that is conceivable that fatty acids serve as the critical link between TLR4, obesity, and inflammation. It is predicted that a pretreatment with a palmitate:oleate cocktail will increase TLR4 and IL-6 gene expression and protein levels, in a saturation-dependent manner. In addition, a pretreatment with fatty acids is expected to exacerbate the LPS-stimulated TLR4 effect on metabolism compared to a pretreatment absent of fatty acids. Specifically, in this lipotoxic condition, we expect to observe a metabolic profile similar to what is seen with obesity, with fatty acids serving as the driving force behind TLR4-associated metabolic dysregulation. Also, it is expected that the proposed studies will discern the specificity of fatty acids in this role with increasing saturation expected to exacerbate the cellular response to LPS.



SPECIFIC AIM 2: To observe the in-vivo effect of chronic hyperlipidemia to the regulation, expression and metabolic implications of TLR4 signaling in C57BL/6 mice exposed to either a high saturated fat or high monounsaturated fat diet.

Objective: Four and ten-week high fat feeding in C57BL/6 mice will be

undertaken to assess the long-term physiological relevance of fatty acid specificity on TLR4 expression, content and regulation of substrate metabolism.

Experimental Strategy: Male C57BL/6 mice purchased from Jackson Labs will be used for the experiments proposed in Specific Aim 2. Mice will be purchased at ~5 weeks of age and fed a standard chow diet until 8 weeks of age. At 8 weeks, one third of the animals will be placed on a control diet, one third will begin a high saturated fat diet (HSF) and the remaining will be placed on a high monounsaturated diet (HMF) and fed ad libitum for 4 or 10 weeks. See Table 1 for the macronutrient breakdown. The HMF condition will contain the same overall fat content as the HSF, only modified to resemble an unsaturated profile. At 3 and 9 weeks of feeding, animals will undergo a glucose tolerance test via an i.p. injection of glucose (1g/kg BW) following a 4 hour fast. Blood glucose levels will be measured at baseline and then every 30 minutes for 120 minutes following the glucose challenge. Animals will be given 7 days rest before undergoing an insulin tolerance test. For insulin tolerance testing, animals will be injected with 0.65 U/kg of insulin; and blood glucose measurements will be performed before, and 15, 30, and 60 minutes following injection. At 4 and 10 weeks of experimental feeding, the animals will undergo carbon dioxide asphyxiation. Skeletal muscle will be harvested (gastrocnemius and quadriceps) and red and white muscle will be manually separated and either snap frozen in liquid nitrogen for gene expression and western blotting, or placed in ice-cold buffer for metabolism experiments. For metabolic experiments, radiolabeled glucose and palmitate will be used to assess substrate oxidation.

Table 1. Dietary Macronutrient Breakdown

Diet	Fat (%)	Carbohydrate (%)	Protein (%)
Control	10	70	20
HSF	60	20	20
HMF	60	20	20

Design:

Male C57BL/6 = 84 animals

↓

Control Diet (28)	HSF diet (28)	HMF diet (28)
↓	↓	↓
4, 10 wk fed	4, 10 wk fed	4, 10 wk fed
(14 per group)	(14 per group)	(14 per group)

Expected Outcomes: The experiments proposed in Specific Aim 2 are designed to better understand how chronic hyperlipidemia regulates TLR4 signaling in an in-vivo setting. It is expected that a diet consisting of greater fat content will increase free fatty acid levels in HSF and HMF mice compared those mice on the control diet. It is also anticipated that the fat saturation of the diets will impact the effect on TLR4. As such, it is expected that 4 and 10 weeks of HSF feeding will upregulate TLR4 expression and protein levels. Upon subsequent LPS challenge the effects of TLR4 signaling and subsequent IL-6 production will be enhanced in the HSF group. Also, it is expected that the LPS-induced metabolic effect on fat and glucose oxidation will be exacerbated in the HSF animals in comparison to animals fed a control diet.

Statistical Analysis. A two-way ANOVA with Tukeys post-hoc analysis will be used to compare results from C57BL/6 mice in response to diet. All cell culture studies will be repeated at least twice to establish enough replication to observe potential significant differences. T-tests will be two-tailed and the level of significance will be set at $P<0.05$.

Endpoint Measures for Animal Studies

- Measures of TLR4:
  - -Gene Expression, qRT-PCR: TLR4, IL6
  - -Protein Levels: TLR4
- Functional Measures:
  - -Fatty Acid Oxidation
  - -Glucose Oxidation
  - -Triglyceride Synthesis
- Glucose and Insulin Tolerance
- Fasting Glucose/Insulin/FFA
- Body Weight

Project Time Line:

Year 1	Year 2

←----------**Specific Aim 1**----------→

←----------**Specific Aim 2**----------→

-FA treatments: C2C12, L6, human primary cells
  FA Specificity:
    -palmitate:oleate (2:1, 1:1, 1:2)

-500 ng/ml LPS treatment

-Gene Expression Analysis:
  TLR4
  IL-6
  TNF-α

-Western Blotting
  TLR4
  IL-6

-Substrate Metabolism
  ^{14}C -Glucose oxidation
  ^{14}C -Palmitate oxidation
  Lipid Synthesis

-Enzyme Activity
  Beta-hydroxylcoA dehydrogenase
  Citrate Synthase
  Phosphofructokinase

-Data Compilation and Analysis

-Begin all animals on chow
-Begin treatment diets (CON, HSF, HMF)
-Glucose tolerance
-Insulin tolerance
-Plasma metabolites
  Fasting Insulin, Glucose, FFA

-Sacrifice animals (+/- LPS injection)
  Extract tissue (red/white gastroc)

-Gene Expression Analysis
  TLR4
  IL-6
  TNF- α

-Western Blotting
  TLR4
  IL-6

-Substrate Metabolism
  ^{14}C-Glucose oxidation
  ^{14}C-Palmitate oxidation
  Lipid Synthesis

-Enzyme Activity
  Beta-hydroxylcoA-Dehydrogenase
  Citrate Synthase
  Phosphofructokinase

-Data Compilation and Analysis

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Chapter 6: Manuscript

Toll-like receptor 4 modulates skeletal muscle substrate metabolism.

Madlyn I Frisard, [1#], Ryan P McMillan[1#], Julie Marchand[1], Kristin Wahlberg[1], Yaru Wu, [1], Kevin Voelker[1], Leonie Heilbronn[3], Brendan Muoio[1], Liwu Li[2], and Matthew W Hulver[1*]

[1]Department of Human Nutrition, Foods, and Exercise, Virginia Polytechnic Institute and State University, Blacksburg, VA. USA

[2]Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA. USA

[3]Diabetes and Obesity Program, The Garvin Institute of Medical Research, Darlington, NSW, Australia

Running title: TLR4 and Skeletal Muscle Substrate Metabolism

*Corresponding Author:
Matthew W. Hulver, Ph.D.
Virginia Polytechnic Institute and State University
Department of Human Nutrition, Foods, and Exercise
Corporate Research Center
1981 Pratt Drive
Integrated Life Science Building, Room 1012
Blacksburg, VA 24061
540-231-7354
hulvermw@vt.edu

# Authors contributed equally to this work.

Abstract

Toll-like receptor4 (TLR4), a protein integral to innate immunity, is elevated in skeletal muscle of obese and type 2 diabetic humans and has been implicated in the development of lipid-induced insulin resistance. The purpose of this study was to examine the role of TLR4 as a modulator of basal (non-insulin stimulated) substrate metabolism in skeletal muscle with the hypothesis that its activation would result in reduced fatty acid oxidation and increased partitioning of fatty acids towards neutral lipid storage. Human skeletal muscle, rodent skeletal muscle, and skeletal muscle cell cultures were employed to study the functional consequences of TLR4 activation on glucose and fatty acid metabolism. Gain and loss of function models were used to assess TLR4 dependence in lipopolysaccharide (LPS)-mediated effects in skeletal muscle. Herein, we report that transcript levels of toll-like receptor 4 (TLR4) are elevated and associated with reduced fatty acid (FA) oxidation in the skeletal muscle of obese humans. In skeletal muscle cell cultures and skeletal muscle from mice, acute stimulation of TLR4 with LPS results in increased glucose oxidation, reduced FA oxidation, and preferential partitioning of FA to intracellular neutral lipid depots. These changes in substrate oxidation are accompanied by increased enzyme activity of phosphofructokinase with reductions in citrate synthase and β-hydroxyacyl-CoA dehydrogenase activities. The LPS-induced modulations of substrate metabolism are TLR4-dependent as they are exacerbated or abolished with gain or loss of TLR4 function, respectively. In conclusion, these data establish a novel link between innate immunity and the regulation of skeletal muscle substrate metabolism.

Key Words: toll-like receptor 4, skeletal muscle, glucose metabolism, fatty acid metabolism, human, and mouse

Introduction

Obesity is associated with a low-grade, chronic activation of pro-inflammatory pathways (10, 30, 32, 37, 41). Toll-like receptors are transmembrane receptors that play an important role in innate immunity and the induction of pro-inflammatory responses (52). Toll-like receptor 4 (TLR4) was identified as the first human homologue of the drosophila Toll gene (35) and is well known as the receptor for lipopolysaccharide (LPS) (38). In addition to its location on immune cells, TLR4 is also abundant in adipose tissue, liver, and skeletal muscle (15, 19, 49). Expression in these tissues suggests a role for the TLR4 pathway in cellular functions apart from innate immunity. Frost and colleagues were amongst the first to show that TLR4 is present in skeletal muscle and when activated induces a local inflammatory response (16, 31). More recently, Reyna et al. (40) have reported increased expression and protein content of TLR4 in skeletal muscle of obese and type 2 diabetic humans, which was associated with insulin resistance. Radin et al. and Shi et al. (39, 46) have shown that TLR4 is important to the development of FA-induced insulin resistance in skeletal muscle.

The purpose of this study was to discern if TLR4 is a modulator of basal glucose and fatty acid metabolism in skeletal muscle. Herein, we confirm and extend the findings of Reyna et al. by demonstrating that transcript levels of TLR4 are elevated in skeletal muscle of obese humans and are associated with reduced FA oxidation and elevated intracellular lipid synthesis. Moreover, when TLR4 signaling is initiated in skeletal muscle by acute LPS treatment, there is a resultant shift in substrate metabolism favoring the oxidation of glucose relative to FA and increased lipid accumulation.

Methods

Human subjects. An Affymetrix Micro-Array (Human Genome U133A and U133B GeneChips, Affymetrix, Santa Clara, CA) that has been previously described (21) was revisited for this study. Subjects included in this analysis provided written informed consent under an approved protocol by East Carolina University IRB and were described previously (21, 22).

Animals. Animal studies were performed under an approved protocol by the Institutional Animal Care and Use Committee at Virginia Polytechnic and State University. Two separate studies were conducted using eight-week old male C3H/HeJ (TLR4-mutant) and C3HeB/FeJ mice (Control) that were purchased from the Jackson Laboratory (Bar Harbor, Maine). The TLR-mutant mice possess a point mutation in the TLR4 receptor and do not have functional TLR4 signaling. The control mice possess functional TLR4 signaling and served as genetic background controls. The first experiments assessed basal fatty acid oxidation (FAO) citrate synthase (CS) activity, and β-hydroxyacyl-CoA dehydrogenase (βHAD) activity in gastrocnemius whole-muscle homogenates from overnight fasted, Control (n=5) and TLR4-mutant (n=5) mice. The second experiment assessed FAO, glucose oxidation, and enzyme activities of CS, βHAD, and phosphofructokinase (PFK) in whole-muscle homogenates prepared from red and white portions of gastrocnemius muscle harvested from Control and TLR4-mutant mice 4 h following a single IP injection of either saline (Control, n=6; TLR4-mutant) or LPS (Control, n=6; TLR4-mutant, n=6; 25μg per mouse, *Escherichia coli* 0111:B4, Cat # L2630, Sigma-Aldrich, St. Louis, MO). Mice for both studies were maintained on a normal chow diet and a 12-hour light/dark cycle.

Cell culture. Cell culture studies were conducted using C2C12 mouse, L6 rat, and human primary myotubes. C2C12 and L6 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown to ~80% confluence in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin, (50 μg/ml) (Invitrogen, Carlsbad, CA)and sub-cultured in either 6 or 12-well plates (Becton Dickinson, Franklin Lakes, NJ). Cells were grown to

confluence and then differentiated into myotubes in DMEM containing 2% horse serum, penicillin (50 U/ml), and streptomycin (50 μg/ml) (Invitrogen, Carlsbad, CA). All experiments were performed between days 4-6 of differentiation.

Cultures of primary human muscle cells were performed as previously described (21) and were obtained from subjects who provided written informed consent under an approved protocol by the Pennington Biomedical Research Center IRB. LPS experiments were performed on day 7 of differentiation.

A C2C12 cell line with stable over expression of TLR4/MD2 was generated by transiently transfection at ~50% confluence with LyoVec transfection media (InVivoGen, San Diego, CA) and pDUO plasmid which co-expresses mouse TLR4/MD2 (InVivoGen, San Diego, CA). Positively transfected cells were selected and maintained using blasticidin (InVivoGen, San Diego, CA) at dosages of 30 and 10 μg/mL, respectively. TLR4 over expression was verified by qRTPCR and western blotting as described below.

LPS treatment in cell culture experiments. All cell culture experiments were performed with 500 ng/ml of LPS (*Escherichia coli* 0111:B4, Cat # L2630, Sigma Aldrich, St. Louis, MO) unless stated otherwise stated. The treatment time was 2 hours and cell culture plates were washed three times with PBS prior to initiating assessment of glucose and fatty acid metabolism or collection for any other assays described below. To ensure there were no confounding effects of impure LPS, a series of experiments were performed with ultra pure LPS (500 ng/ml, *Escherichia coli* 0111:B4, InVivoGen, San Diego, CA) and Lipid A (1 μg/ml**,** synthetic monophosphoryl lipid A from *E. coli*, InVivoGen, San Diego, CA).

FA Metabolism. Fatty acid oxidation was assessed in cell culture (C2C12, L6, and human primary myotubes) and whole-muscle homogenates by measuring and summing $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of [1-^{14}C]-palmitic acid as previously described (9, 21). Neutral lipids were extracted as

previously described (21, 22) and incorporation of [1-^{14}C]-palmitic acid was measured using an AR 2000 TLC plate scanner (Bioscan, Inc., Washington, D.C.).

Glucose Oxidation. Glucose oxidation was assessed in cell culture (C2C12, L6, and human primary myotubes) and whole-muscle homogenates by measuring $^{14}CO_2$ production from the oxidation of [U-^{14}C]-glucose (Perkin Elmer, Waltham, MA) as previously described (9, 21) with the exception that glucose was substituted for BSA-bound palmitic acid.

Glucose Uptake. Basal (non-insulin-stimulated) glucose uptake was assessed in C2C12 cells in Krebs-Ringer HEPES buffer (in mM, 136 NaCl, 4.7 KCL, 1.25 MgSO4, 1.2 Cal, 20 Hepes, pH 7.4) with the addition of 10μM 2-deoxyglucose (1μCi/mL 2-deoxy-[3H]glucose) and 10μM cytochalasin B (control for extra cellular binding). After 10 min incubation, plates were placed on ice, washed three times with ice-cold PBS and harvested in 400ul of 0.2M NaOH for cell lysis. Glucose uptake was calculated based on specific activity and expressed relative to protein content.

*RNA extraction and qRT-PCR.*RNA was extracted using an RNeasy Mini Kit (Qiagen) 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 manufacturer's specifications (Applied Biosystems, Inc., Foster City, CA). Target gene expression was normalized to β-actin RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as pre-validated assays (ABI). Relative quantification of target genes was calculated using the $2^{-\Delta CT}$ method, which was validated for each primer/probe set using a 6 point serial standard curve as described previously (59). Derivation of the $2^{-\Delta CT}$ 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, St Louis, MO). Proteins (30 μg) were

separated using a 10% Criterion-Tris- HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, CA). Blots were probed with primary antibodies against β-actin (Cell Signaling, Danvers, MA; 1:1000), PPARα (Abcam, Cambridge, MA; 1:1000) and PPARδ (Santa Cruz Biotechnology, Santa Cruz, CA; 1;1000), LXRα and LXRβ (Both Novus Biologicals, Littleton, CO; 5µg/µl), C/EBPβ and C/EBPδ (Both Cell Signaling, Danvers, MA; 1:1000), and TLR4 (Cell Signaling, Danvers, MA; 1:1000) followed by anti-rabbit, mouse, or goat secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA; 1:10,000). Proteins were visualized using Super-Signal Chemiluminescent Substrate (Pierce, Rockville, IL) and a ChemiDoc XRS Imaging System (BioRad, Hercules, CA).

Enzyme Activity. Enzyme activities were assessed in cell lysates (5-fold dilution) and muscle homogenates (20-fold dilution). Sample buffer consisted of 0.1 mol/l KH_2PO_4/Na_2PHO_4 and 2 mmol/EDTA, pH 7.2. Phosphofructokinase, citrate synthase, and ß-hydroxyacyl-CoA dehydrogenase (BHAD) activities were determined spectrophotometrically as previously described (20).

Statistics. Results were analyzed with 2-tailed Student's t tests or 2-way ANOVA with Tukey post-hoc tests (multiple comparisons). Results were expressed as mean ± SEM. The level of significance was set at $P<0.05$.

Results

Transcript levels of TLR4 are elevated in skeletal muscle from obese humans.

In the current study, a previously described (21) Affymetrix data set from analysis of rectus abdominus muscle from nonobese and obese individuals ranging in BMI from 22 to 70 kg/m^2 [n=24, 8 nonobese (BMI=23.8±0.58), 8 overweight/obese (BMI=30.2±0.81), and 8 extremely obese (BMI=53.8±3.5)] was revisited to examine TLR4 mRNA levels and the relationships between TLR4 mRNA and various indices of metabolism previously determined in incubated skeletal muscle strips from these same subjects. Toll-like receptor 4 mRNA levels were significantly higher in skeletal muscle from obese individuals relative to nonobese controls (Figure 1A) and were significantly associated with body mass index (Figure 1B; r=0.58, P=.004), partitioning of FA toward neutral lipid synthesis (Figure 1C; r=0.42, P=0.04), and FA oxidation (Figure 1D; r=-0.46, P=0.03)].

Activation of TLR4 in skeletal muscle has functional consequences on glucose and FA metabolism. The observance of elevated transcript levels in skeletal muscle from obese humans prompted functional experiments to assess glucose and FA metabolism in response to TLR4 activation. Radio-labeled substrates (glucose and palmitate) were used to measure glucose oxidation, FA oxidation, and neutral lipid synthesis in C2C12 cells following overnight serum starvation and 500 ng/mL of LPS treatment for 2 hours. Glucose uptake and oxidation were both increased by 33 and 25% (both P<0.05, Figure 2A and B), respectively, and accompanied by significant increases in lactate concentrations in the incubation media (P<0.05, Figure 2C). FA oxidation was reduced by 20% (P<0.05, Figure 2D) and partitioning of FA toward neutral lipid synthesis was increased by 26% (P<0.05, Figure 2E). Decreases in FA oxidation were accompanied by reductions in CS (P=0.053, Figure 2F) and βHAD (P<0.05, Figure 2G) enzyme activities. To ensure that TLR4-mediated changes in substrate metabolism were not isolated to C2C12 muscle cells, LPS experiments were repeated using L6 and human primary skeletal muscle cells. The identical shift in substrate metabolism was observed in both L6 (Figure 3A-C) and human primary muscle cell lines (Figure 3D-F) with TLR4 activation. Additional studies in C2C12 cells with LPS concentrations as low at 1.0 ng/mL were completed and resulted in significant

increases and decreases in glucose and fatty acid oxidation, respectively (glucose oxidation, Control = 11.98±0.63 vs. LPS = 16.0±0.40, P=0.03; FA oxidation, Control = 9.64±0.13 vs. LPS= 7.38±0.26, P=0.02). To eliminate the possibility of any confounding effects of impure LPS, experiments were performed with ultra pure LPS (500 ng/mL, InVivogen, San Diego, CA) and Lipid A (1μg/mL, InVivogen, San Diego, CA) in C2C12 and L6 cells. Lipid A is the active constituent of LPS that is responsible for binding and activating TLR4. Treatments with ultra pure LPS, relative to control treatment (PBS), significantly increased glucose oxidation (+22-27%) and partitioning of FA to neutral lipid pools (+19-27%), and decreased fatty acid oxidation (-32-56%) in both C2C12 and L6 cells (data not shown). Treatments with Lipid A, relative to control treatment (PBS), also significantly increased glucose oxidation (+27-33%) and partitioning of FA to neutral lipid pools (+21-25%), and decreased fatty acid oxidation (-25-35%) in both C2C12 and L6 cell (data not shown).

Absence of TLR4 signaling is associated with increased capacity for basal fatty acid oxidation in skeletal muscle. Eight week old Control (C3HeB/FeJ, n=5) and TLR4-mutant (C3H/HeJ, n=5) male mice were overnight fasted, euthanized, gastrocnemius muscles harvested, and whole homogenates prepared for measures of fatty acid oxidation, CS activity, and βHAD activity. The TLR4-mtuant animals, which lack functional TLR4 signaling, possessed significantly higher rates of palmitic acid oxidation, CS activity, and βHAD activity relative to control mice (Figure 4A-C). These data support the notion that increased TLR4 activity in skeletal muscle may contribute to a reduced capacity for FA oxidation in skeletal muscle.

Gain or loss of TLR4 function exacerbates or abolishes, respectively, the LPS-induced shift in skeletal muscle substrate metabolism. To determine if the LPS-induced shift in substrate metabolism was heightened with increased protein content of TLR4, a C2C12 muscle cell line with a stable over expression of TLR4/MD2 (C2C12-TLR4/MD2) was generated. The C2C12-TLR4/MD2 cells exhibited a several-fold increase in both TLR4 mRNA and protein levels relative to empty vector controls (Figure 5A). The increases and decreases in glucose and FA oxidation, respectively, in

response to LPS were significantly more robust in the C2C12-TLR4/MD2 cells relative to empty vector controls (Figure 5B).

To confirm the cell culture experiments in an *in vivo* model and determine if the LPS-mediated changes in metabolism were occurring through TLR4, acute (4 hour) LPS (25μg/mouse) experiments were conducted in eight week old, overnight fasted, Control (C3HeB/FeJ; saline, n=6; LPS, n=6) and TLR4-mutant (C3H/HeJ; saline, n=6; LPS, n=6) mice. Four hours following the I.P. injections of saline or LPS, mice were euthanized, gastrocnemius was harvested and separated into red and white portions, and whole homogenates were prepared for measures of fatty acid oxidation, CS activity, and βHAD activity and PFK activity. In Control animals, LPS induced significant reductions and increases in glucose (Figure 6A) and fatty acid oxidation (Figure 6B), respectively, which was abolished in TLR-mutant animals. Additionally, LPS altered PFK (Figure 6C), CS(Figure 6D), and βHAD (Figure 6E) enzyme activities in control animals with no effect in TLR4-mutant animals. These data establish TLR4 dependence in these LPS-induced metabolic perturbations.

TLR4 activation does not alter protein content of metabolic transcription factors. Previous work has demonstrated that LPS modulates lipid metabolism in cardiac myocytes (13), which was accompanied by decreases in protein content of peroxisome proliferator activated receptors alpha (PPARα) and delta (PPARδ), upstream transcription factors of FA metabolism transcripts. In contrast, we observed LPS-induced changes in FA metabolism in skeletal muscle cell cultures with no concomitant changes in PPARα or PPARδ protein content (Data not shown). TLR4-activation also did not alter protein content of Liver X Receptor (LXR α or δ) or CCAAT-enhancer-binding protein (CEBP β or δ)(Data not shown).

Discussion

There is mounting evidence that the immune response is important in the pathophysiology of various obesity-associated metabolic disorders (4, 47). The results from the current study demonstrate that transcript levels of TLR4 are abundantly expressed with obesity and associated with dysregulated fatty acid metabolism in human skeletal muscle. Moreover, when TLR4 signaling is stimulated by LPS *in vitro* and *in vivo,* there is a resultant shift in skeletal muscle metabolism favoring the oxidation of glucose over fatty acids and increased partitioning of fatty acids towards storage depots. Importantly, this modulation in substrate metabolism by LPS is TLR4 dependent as it is either exacerbated or abolished with the gain or loss of TLR4 function, respectively. Previous research has demonstrated the presence of the TLR4 pathway in skeletal muscle (16, 31) and has focused on the role of innate immunity and inflammation on the development of insulin resistance (39, 46). This study is important because it directly links TLR4 to the modulation of basal, non insulin-stimulated metabolism in skeletal muscle.

A large body of work (12, 14, 24, 42, 50) has been focused on better understanding the mechanism(s) responsible for dysregulated insulin-stimulated metabolism in skeletal muscle in disease states such obesity, type 2 diabetes, and the metabolic syndrome. A consensus has emerged linking intramuscular lipid accumulation to faulty insulin signaling and subsequent deficits in insulin-stimulated glucose uptake into the muscle cell (3, 5, 23, 26, 27). Less focus has been placed on determining the mechanism(s) that contribute to the abnormal metabolic characteristics of basal, non-insulin-stimulated metabolism that have been observed in skeletal muscle of obese humans. Although chronically exposed to high lipids loads, skeletal muscle from obese humans has been metabolically characterized as being more glycolytic and less oxidative. Support for this includes the following observations in skeletal muscle from obese humans: 1) a higher % of type 2b muscle fibers (53); 2) high glycolytic to oxidative enzyme activity ratios (48); and 3) high levels of fasting glucose oxidation relative fatty acid oxidation as observed in across the leg studies (26). All of these observations, which were made in a non-insulin stimulated state, are associated with high intracellular lipid content, insulin

resistance, and metabolic inflexibility (7, 8, 17, 50). Shi et. al. were the first to demonstrate the role of TLR4 in the development of lipid induced insulin resistance (46). Since then, others have shown that the absence of TLR4 either protects mice from diet-induced obesity (58) or the metabolic consequences of obesity (11, 29, 36, 46, 51). We are presenting evidence that TLR4 activation in skeletal muscle results in a more glycolytic and less oxidative environment and that mice with a loss of TLR4 function possess the opposite metabolic pattern with high basal rates of fatty acid oxidation, citrate synthase activity, and βHAD activity. Based on these findings, it is possible that loss of TLR4 function may partially confer the resistance to obesity or obesity related disorders as reported in the aforementioned studies. Additional studies are needed to conclusively link TLR4 to the predominant glycolytic nature of skeletal muscle as observed in obese humans; however, the evidence presented here warrants further investigation in this area.

Conditions of severe illness and/ or infection are characterized by alterations in energy expenditure and metabolism (2, 28). Lipopolysaccharide, a type of bacterial endotoxin is found in the outer membrane of gram negative bacteria and is made up of a polysaccharide and lipid A. Earlier studies conducted in the sixties and seventies used high doses of LPS for the purpose of understanding the physiological consequences of sepsis and septic shock. These studies demonstrated that LPS treatment to induce septic shock also resulted in alterations in mitochondrial respiration (33, 34, 57). Additionally, these earlier studies identified that lipid A was the active component of LPS and was required for the observed metabolic effects (25, 54). More recent studies in immune cells demonstrated that stimulation with LPS resulted in an increased reliance on glycolysis(18, 44).The current study extends these previous findings to demonstrate that, 1) the LPS-induced shift in basal metabolism away from lipid oxidation towards glucose oxidation is relevant in skeletal muscle; 2) these effects are observed using much lower doses of LPS than previously described; and 3) most importantly, the LPS induced effects on skeletal muscle are dependent on the presence of TLR4. Based on these findings, it is important to better understand the role of TLR4 in altered skeletal muscle substrate utilization not only in the obese state, but also in the progression of

other pro-inflammatory human conditions such as infection, sepsis, cancer, and cardiovascular disease. It is also equally important to study the metabolic effects of TLR4 activation in other tissues such as liver, adipose, and the pancreas.

The mechanism(s) contributing to the TLR4-mediated shifts in metabolism in skeletal muscle are not clear at this time. As reported herein, there are no changes in protein content of the transcription factors PPARα, PPARδ, LXR α, LXRδ, CEBP β, or CEBPδ following 2 hours of LPS stimulation. However, a consistent decrease in citrate synthase and βHAD activities was observed following LPS stimulation in both skeletal muscle cell cultures and skeletal muscle extracted from mice. Additionally, PFK activity was also increased in skeletal muscle extracted from mice following LPS stimulation. Because enzyme activity is measured *ex vivo* in neutral conditions, the likelihood of altered pH contributing the changes in enzyme activity is remote. Due to the acute treatment period, it is also improbable that reductions in enzyme protein content caused these changes. A plausible explanation is that oxidative damage may have contributed to altered enzyme function. Our lab has observed, and is currently confirming, increased production of hydrogen peroxide following 2 hours of LPS stimulation (Data not shown). Increased production of hydrogen peroxide is indicative of reactive oxygen species (ROS) formation and greater potential for oxidative damage. There is precedence for increased ROS production in response to TLR4 activation in cell lines other than skeletal muscle (1, 43). It is also well established that ROS interact with proteins causing modifications and malfunction (6, 45, 55, 56). Studies are currently underway in our lab to examine the role of ROS in TLR4-mediated changes in substrate metabolism.

An additional hypothesis is that hypoxia-inducible factor 1 alpha (HIF1α) may mediate the TLR4-mediated increases in glucose metabolism. Schuster et al. showed in neutrophils that LPS stimulation results in HIF1α stabilization, which prevents ubiquination and increases its transcriptional activity (44). Schuster et al. also observed increased glucose uptake with LPS stimulation and that these effects were dependent

on HIF1α activity (44). The role of HIF1α in TLR4-mediated increases in glucose metabolism in skeletal muscle warrants future investigation.

In conclusion, we report for the first time that TLR4 expression is associated with reduced FA oxidation increased lipid synthesis in skeletal muscle of obese humans. Furthermore, activation of TLR4 by LPS in skeletal muscle results in a shift in substrate metabolism favoring glucose oxidation over FA and increased partitioning of FA toward neutral lipid depots. These effects are TLR4 dependent as they are either exacerbated or blunted with the gain or loss of TLR4 function, respectively. The mechanism(s) responsible for these effects remain to be elucidated.

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Figure 1. TLR4 expression is elevated in skeletal muscle from obese individuals and is associated with BMI and fatty acid metabolism. Rectus abdominus samples were obtained from nonobese and obese humans with a BMI ranging from 22–70 kg/m^2, mRNA was extracted and an Affymetrix Gene Array was performed. (A) TLR4 mRNA levels in nonobese, obese, and extremely obese humans (mean±SEM). Scatter plots are shown comparing the relationship between TLR4 mRNA and (B) BMI, (C) FA partitioning, (D) FA oxidation. FA partitioning was calculated by dividing the rate (nmol/g wet weight/h) of palmitate esterified into TAG by the rate of palmitate oxidized. *P<0.05.

Figure 2. Activation of TLR4 in C2C12 skeletal muscle cells modulates glucose and fatty acid metabolism. Radio-labeled substrates (glucose and palmitate) were used to measure glucose and FA metabolism in C2C12 cells following 2 hours of LPS treatment (500 ng/mL). In response to LPS, there was an increase in glucose uptake (A), glucose oxidation (B), and lactate presence in the media (C). LPS treatment also resulted in a decrease in total FA oxidation[complete (CO_2) and incomplete (ASMs)] (D), increased fatty acid partitioning to neutral lipid depots (E), and reductions in citrate synthase (F) and β–hydroxyacyl-CoA dehydrogenase (G) activities. Data are presented as Mean±SEM. *P<0.05.

Figure 3. Activation of TLR4 in L6 and human primary skeletal muscle cells modulates glucose and fatty acid metabolism. Radio-labeled substrates (glucose and palmitate) were used to measure glucose and FA metabolism in L6 and human primary skeletal muscle cells following 2 hours of LPS treatment (500 ng/mL). In response to LPS, there was an increase in glucose oxidation (A, L6; and C, Human primary cells), a decrease in fatty acid oxidation (B, L6; and D, Human primary cells), and an increase partitioning of fatty acids toward neutral lipid depots (C, L6; E, human primary cells). Data are presented as Mean±SEM. *P<0.05.

Figure 4. Loss of TLR4 function is associated with an increased capacity to oxidize fatty acids in skeletal muscle. Gastrocnemius skeletal muscles were extracted from Control (C3HeB/FeJ, n=5) and TLR4-mutant (C3H/HeJ, n=5) mice and whole-muscle homogenates were prepared for measures of [1-^{14}C]-palmitic acid oxidation and enzyme activities of citrate synthase and β–hydroxyacyl-CoA dehydrogenase. Fatty acid oxidation (A), citrate synthase activity (B), and β–hydroxyacyl-CoA dehydrogenase activity (C) were all higher in the TLR4-mutant mice relative to Control mice. Data are presented as Mean±SEM. *P<0.05.

Figure 5. Over expression of TLR4 in C2C12 skeletal muscle cells results in an enhanced LPS-mediated shift in substrate metabolism. C2C12 cells were generated with a stable over expression of the TLR4/MD2, treated (500 ng/mL) with LPS for 2 hours, and oxidation of [U-^{14}C]-glucose and [1-^{14}C]-palmitic acid was assessed. Relative to empty vector controls, the stable cell line possessed higher TLR4 mRNA and protein levels (A) and the LPS-induced percent changes in glucose and fatty acid metabolism were more robust (B). Data are expressed as Mean±SEM. *P<0.05.

Figure 6. LPS induced shift in muscle substrate metabolism occurs in vivo and is TLR4 dependent. At 8 weeks of age, Control (C3HeB/FeJ, n=14) and TLR4-mutant (C3H/HeJ, n=14) were injected with either saline (n=7 per group) or 1mg/kg (~25μg per mouse) of LPS (n=7 per group) and sacrificed 4 hours post injection. Gastrocnemius skeletal muscle was harvested and dissected into red and white portions. LPS treatment resulted in robust increases and decreases in LPS-induced percent changes in glucose (A) and fatty acid (B) oxidation, respectively, which was completely block in the TLR4-mutant animals. These effects coincided with an increase in PFK activity (C) and decreased in citrate synthase (D) and βHAD (E) activities in Control mice, which were also blocked in the TLR4-mutant animals. Data is presented as Mean±SEM. *P<0.05.

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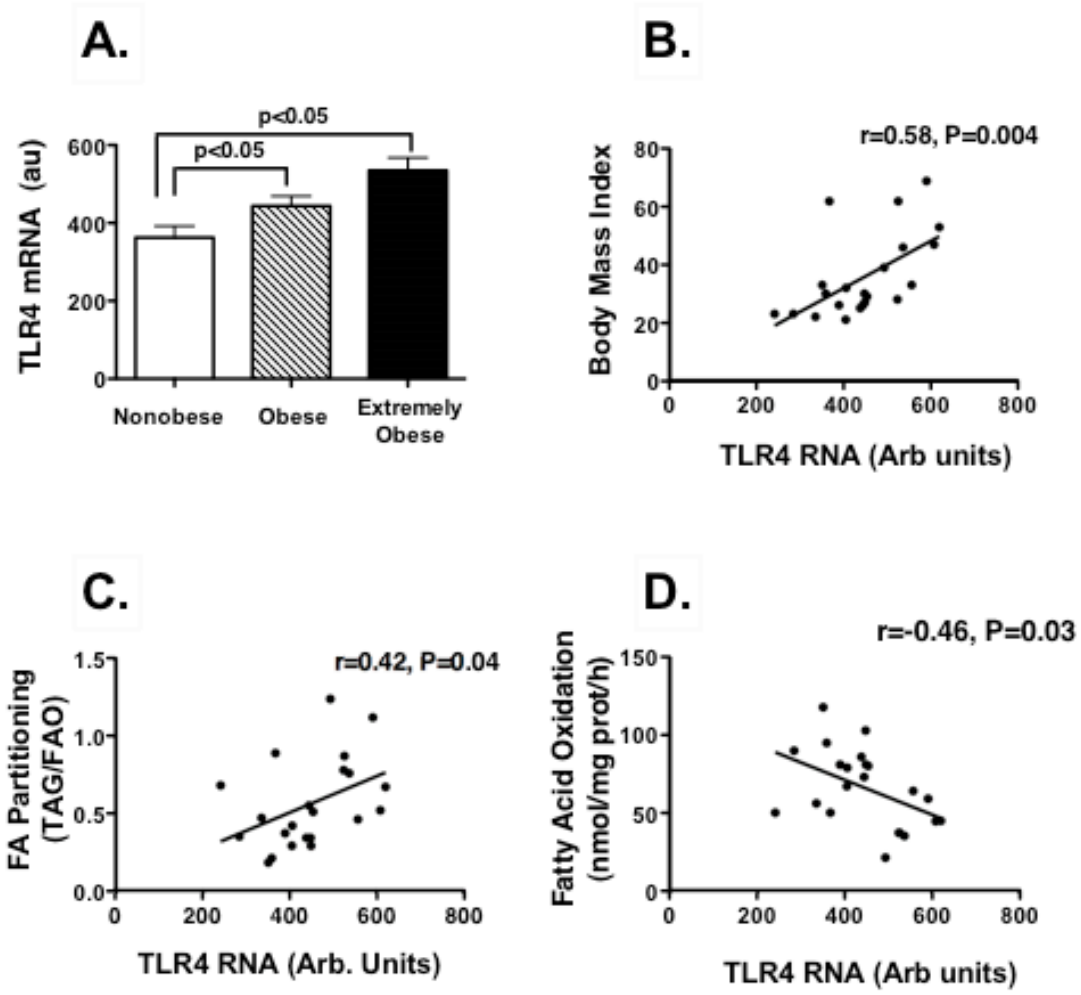
A.
p<0.05
p<0.05
TLR4 mRNA (au)
600
400
200
0
Nonobese
Obese
Extremely Obese
B.
r=0.58, P=0.004
Body Mass Index
80
60
40
20
0
0
200
400
600
800
TLR4 RNA (Arb units)
C.
r=0.42, P=0.04
FA Partitioning (TAG/FAO)
1.5
1.0
0.5
0.0
0
200
400
600
800
TLR4 RNA (Arb. Units)
D.
r=-0.46, P=0.03
Fatty Acid Oxidation (nmol/mg prot/h)
150
100
50
0
0
200
400
600
800
TLR4 RNA (Arb units)

Figure 1

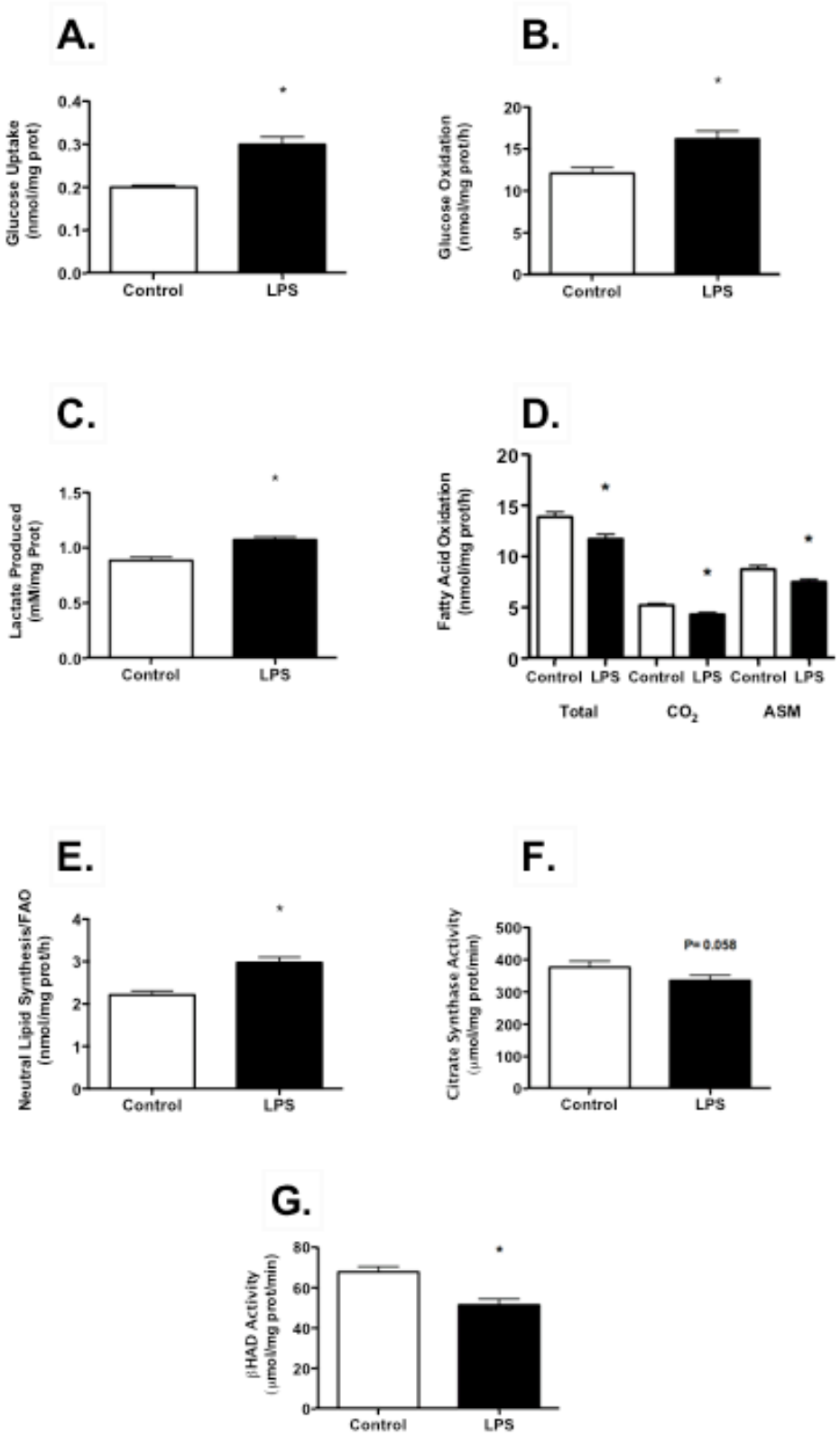
A.
Glucose Uptake (nmol/mg prot)
0.4
0.3
0.2
0.1
0.0
Control
LPS
*
B.
Glucose Oxidation (nmol/mg prot/h)
20
15
10
5
0
Control
LPS
*
C.
Lactate Produced (mM/mg Prot)
1.5
1.0
0.5
0.0
Control
LPS
*
D.
Fatty Acid Oxidation (nmol/mg prot/h)
20
15
10
5
0
Control LPS Control LPS Control LPS
Total
CO2
ASM
*
*
*
E.
Neutral Lipid Synthesis/FAO (nmol/mg prot/h)
4
3
2
1
0
Control
LPS
*
F.
Citrate Synthase Activity (µmol/mg prot/min)
500
400
300
200
100
0
P= 0.058
Control
LPS
G.
βHAD Activity (µmol/mg prot/min)
80
60
40
20
0
Control
LPS
*

Figure 2

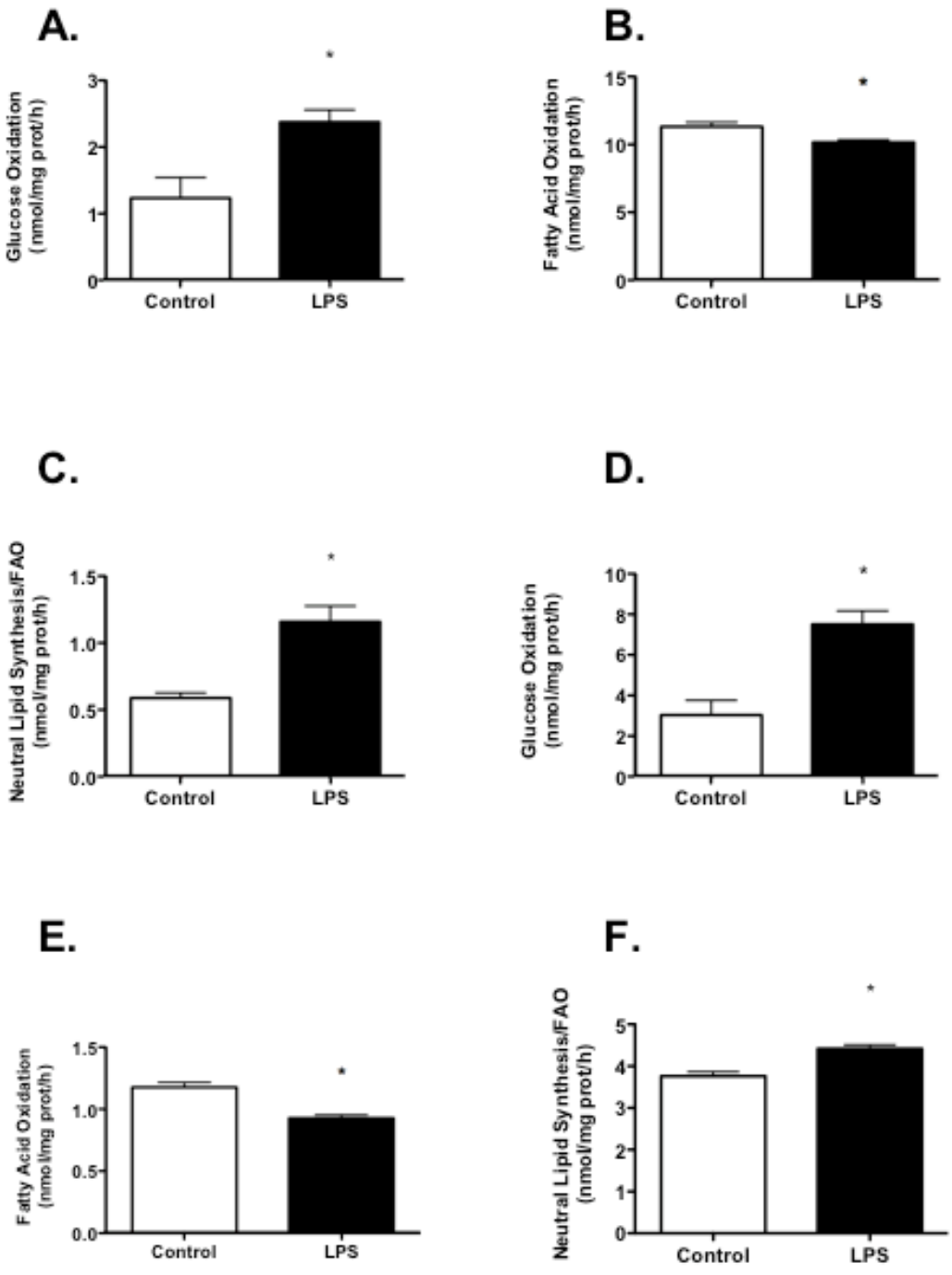
A.
Glucose Oxidation (nmol/mg prot/h)
3
2
1
0
*
Control
LPS
B.
Fatty Acid Oxidation (nmol/mg prot/h)
15
10
5
0
*
Control
LPS
C.
Neutral Lipid Synthesis/FAO (nmol/mg prot/h)
1.5
1.0
0.5
0.0
*
Control
LPS
D.
Glucose Oxidation (nmol/mg prot/h)
10
8
6
4
2
0
*
Control
LPS
E.
Fatty Acid Oxidation (nmol/mg prot/h)
1.5
1.0
0.5
0.0
*
Control
LPS
F.
Neutral Lipid Synthesis/FAO (nmol/mg prot/h)
5
4
3
2
1
0
*
Control
LPS

Figure 3

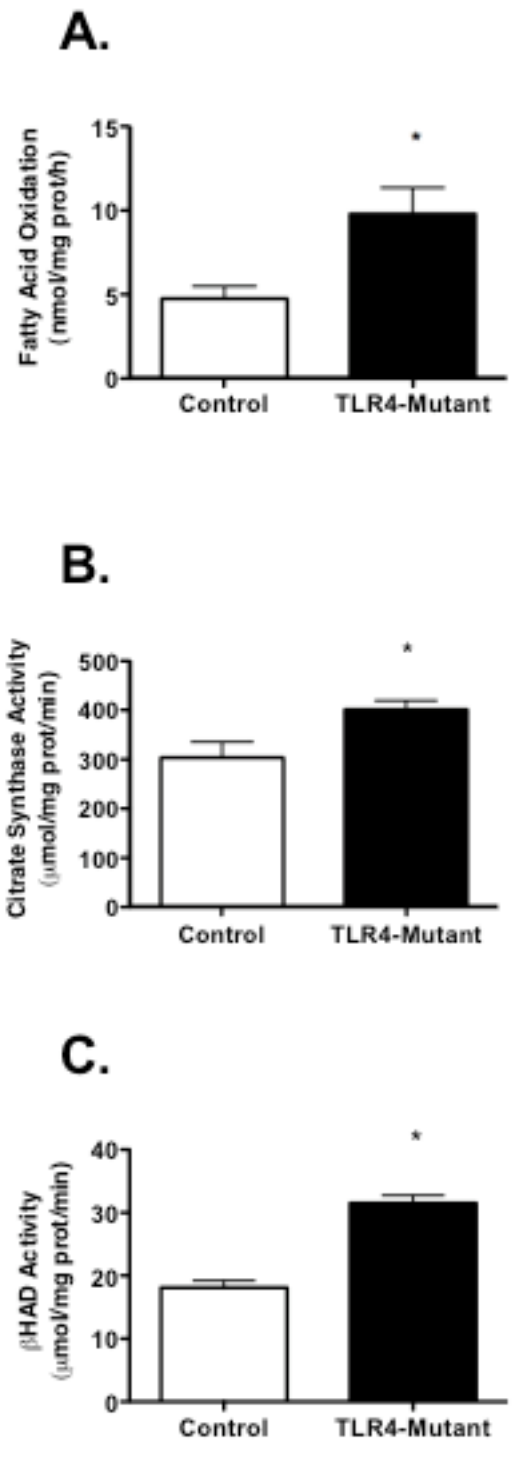
A.
Fatty Acid Oxidation (nmol/mg prot/h)
15
10
5
0
*
Control
TLR4-Mutant
B.
Citrate Synthase Activity (µmol/mg prot/min)
500
400
300
200
100
0
*
Control
TLR4-Mutant
C.
βHAD Activity (µmol/mg prot/min)
40
30
20
10
0
*
Control
TLR4-Mutant

Figure 4

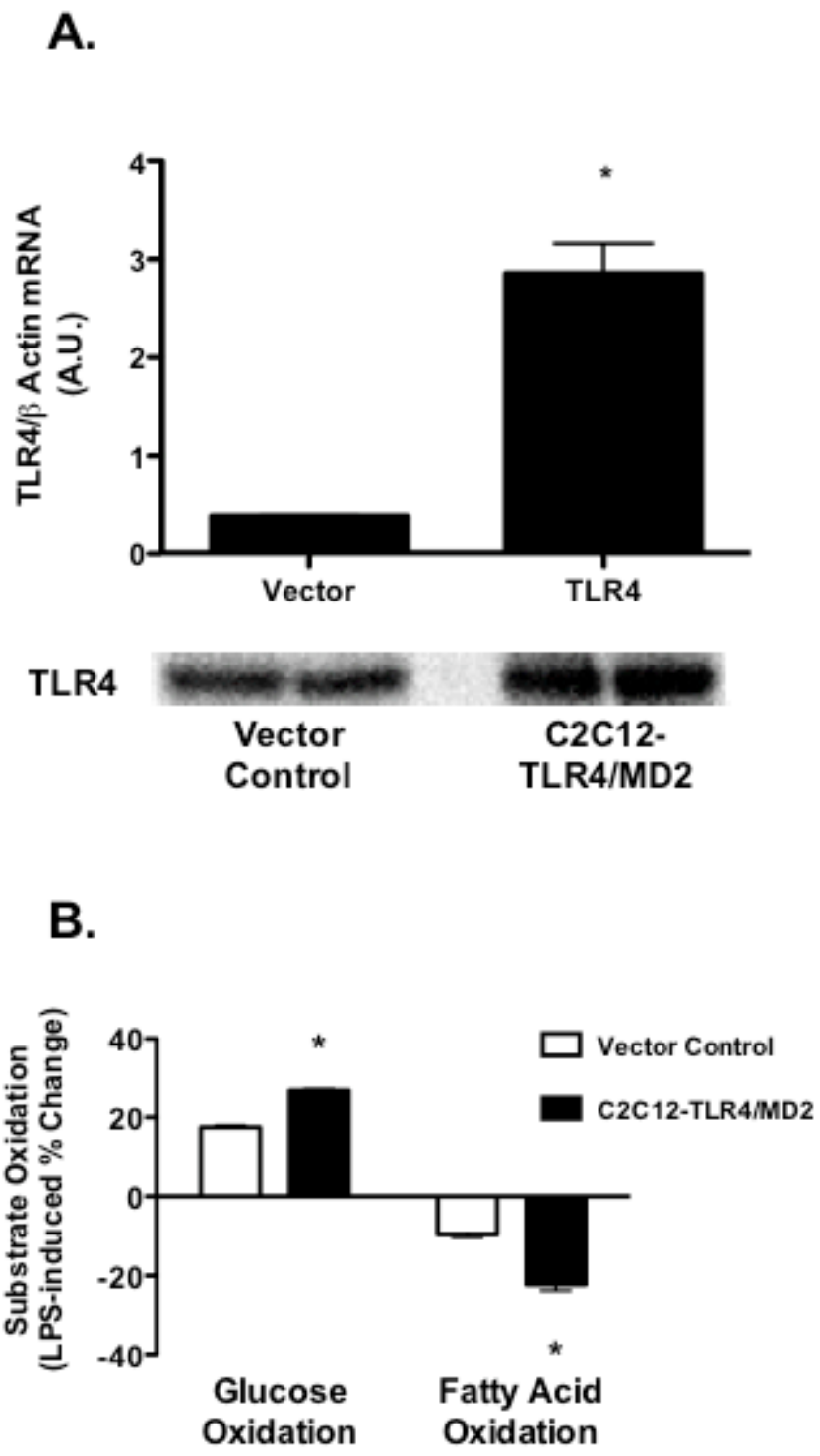
A.
TLR4/β Actin mRNA (A.U.)
4
3
2
1
0
*
Vector
TLR4
TLR4
Vector Control
C2C12-TLR4/MD2
B.
Substrate Oxidation (LPS-induced % Change)
40
20
0
-20
-40
*
*
Vector Control
C2C12-TLR4/MD2
Glucose Oxidation
Fatty Acid Oxidation

Figure 5

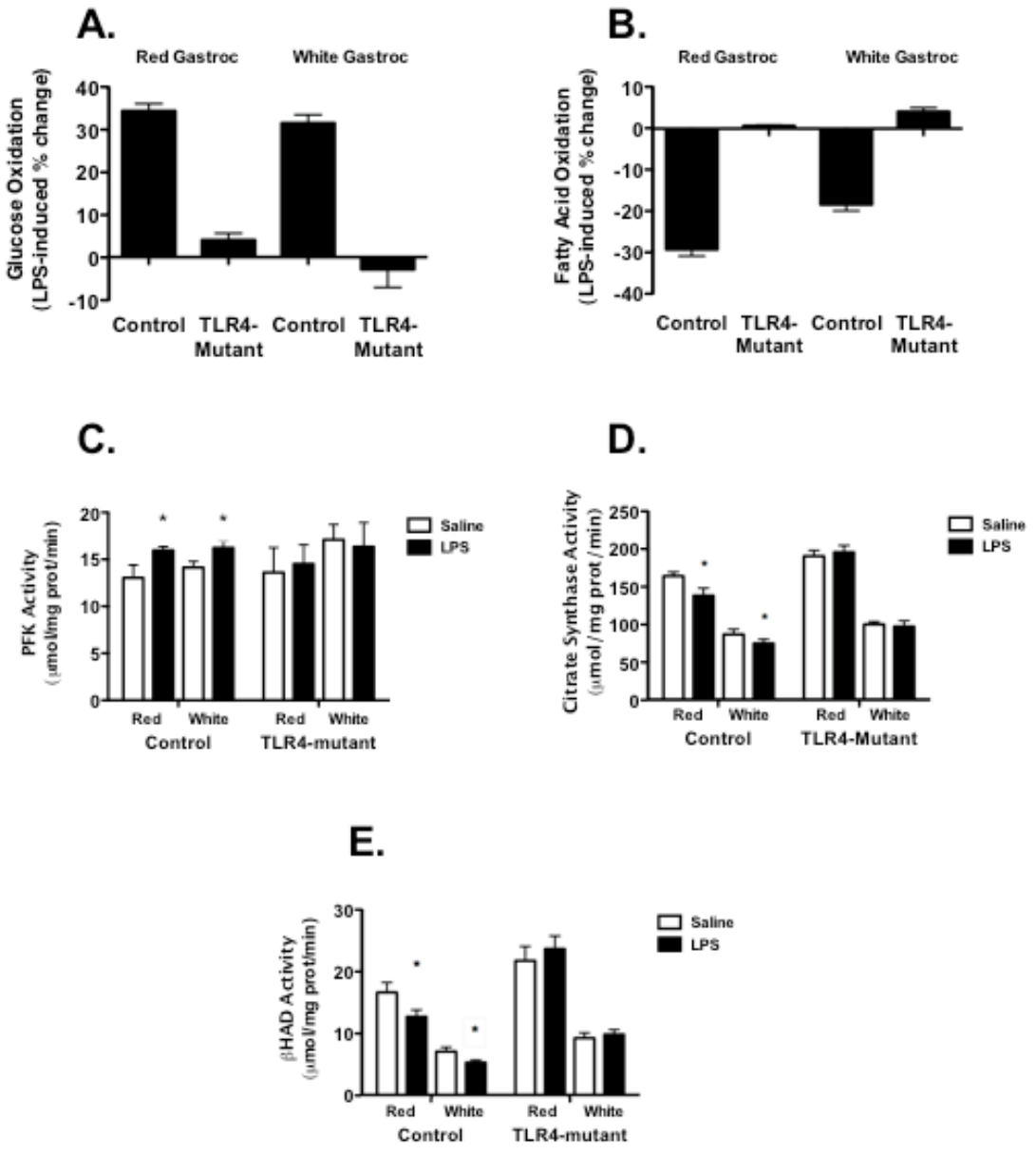
A.
Red Gastroc
White Gastroc
Glucose Oxidation (LPS-induced % change)
40
30
20
10
0
-10
Control
TLR4-Mutant
Control
TLR4-Mutant
B.
Red Gastroc
White Gastroc
Fatty Acid Oxidation (LPS-induced % change)
10
0
-10
-20
-30
-40
Control
TLR4-Mutant
Control
TLR4-Mutant
C.
PFK Activity (µmol/mg prot/min)
20
15
10
5
0
*
*
Saline
LPS
Red
White
Red
White
Control
TLR4-mutant
D.
Citrate Synthase Activity (µmol/mg prot/min)
250
200
150
100
50
0
*
*
Saline
LPS
Red
White
Red
White
Control
TLR4-Mutant
E.
βHAD Activity (µmol/mg prot/min)
30
20
10
0
*
*
Saline
LPS
Red
White
Red
White
Control
TLR4-mutant

Figure 6

Chapter 7: Manuscript

A high saturated lipid load sensitizes the toll-like receptor 4 signaling pathway in skeletal muscle.

Ryan P. McMillan, Madlyn I. Frisard, Kevin A. Voelker, Kim R. Haynie, Jeb S. Orr, and Matthew W. Hulver*

Department of Human Nutrition, Foods, and Exercise, Virginia Polytechnic Institute and State University, Blacksburg, VA. USA

Running title: Fatty acids and skeletal muscle TLR4

*Corresponding Author:
Matthew W. Hulver, Ph.D.
Virginia Polytechnic Institute and State University
Department of Human Nutrition, Foods, and Exercise
Corporate Research Center
1981 Pratt Drive
Integrated Life Science Building, Room 1012
Blacksburg, VA 24061
540-231-7354
hulvermw@vt.edu

Abstract

Growing evidence suggests that obesity and associated metabolic dysregulation occurs in concert with markers of low-grade inflammation. Toll-like receptors (TLRs) are transmembrane receptors that play an important role in innate immunity and the induction of inflammatory responses. Our laboratory has observed that TLR4 expression is elevated in the skeletal muscle of obese humans. The purpose of this study was to examine the effects of saturated vs. mono-unsaturated FA on TLR4 transcription and function using ex vivo and in vivo models. C2C12 myotubes were incubated in FA-enriched growth medium with varying ratios of palmitate and oleate for 12 hours. Following FA treatment, cells were either collected for measures of mRNA and protein levels of TLR4 or challenged with LPS (500 ng/mL) for 2 hours to assess TLR4 mediated changes in interleukin-6 (IL-6) and basal rates substrate (glucose and palmitic acid) metabolism. TLR4 mRNA and protein content were increased in stepwise fashion with higher palmitate concentration ($p<0.05$). This was associated with an exacerbated LPS effect on IL-6 mRNA and protein levels, and glucose and fatty acid metabolism. To determine if these effects are translated to an in-vivo model, C57BL/6 mice were fed high saturated fat (HSF), high monounsaturated fat (HMF), and control diets for 10 weeks. Following the dietary intervention, animals were challenged with I.P. injections of either saline or LPS (1 mg/kg), sacrificed 4 h post-injection, and red and white gastrocnemius muscle were harvested for measures of TLR4, TLR2, IL-6, TNF alpha, MCP1 and basal rates of substrate (glucose and palmitic acid) metabolism. TLR4 mRNA and protein levels were not altered with either the HSF or HMF diets. However, there was a heightened LPS response with regards to increases in transcription of TLR2, IL-6, TNF-α, MCP-1, and changes in substrate metabolism following the HSF diet ($p<0.05$). These effects were not observed in response to the HMF diet. In conclusion, these data demonstrate that a milieu of high saturated fatty acids results in elevated sensitization of the TLR4 pathway in skeletal muscle. These results provide insight into how a westernized diet, one enriched in saturated fat, may link chronic inflammation with obesity-associated metabolic abnormalities.

Key Words: toll-like receptor 4, inflammation, skeletal muscle, high fat diet, saturated fatty acids, and monounsaturated fatty acids

Introduction

An emerging pattern of increased food availability and over-nutrition combined with reduced physical activity has resulted in a dramatic increase in weight gain in the United States (1). Obesity is tightly associated with a variety of metabolic disorders such as Type 2 diabetes (T2D), hyperlipidemia, and cardiovascular disease (2-5). Skeletal muscle comprises approximately 40% of total body mass and is the predominant site for substrate disposal, and therefore; is primarily responsible for much of the metabolic dysregulation that accompanies obesity (6). The skeletal muscle of obese humans is highly glycolytic, and characterized by a reduced proportion of type 1 muscle fibers and a reduced capacity to oxidize fat (7-9). Hyperlipidemia, a condition associated with obesity, is known to induce a myriad of complications to skeletal muscle physiology. Lipid over-exposure is shown to be detrimental to skeletal muscle glucose homeostasis and promotes the progression of insulin resistance to T2D (10-14).

Growing evidence suggests that obesity and metabolic disorders, including insulin resistance and T2D, occur in concert with chronic low-grade inflammation (15, 16). Toll-like receptor 4 (TLR4), a transmembrane receptor integral to the innate immune response, is particularly involved in the recognition of lipopolysaccharide (LPS), a product of the outer membrane of gram-negative bacteria (17, 18). TLR4 plays an important role in promoting pro-inflammatory responses through activation of transcription factors such as nuclear factor kappa B (NFkB) (19). The receptor is expressed ubiquitously; located not only on immune cells such as macrophages, but is also present in skeletal muscle (19). This suggests a potential role for the TLR4 pathway in cellular functions apart from innate immunity and a potential link between immunity, chronic inflammation and obesity.

There is evidence to suggest that TLR4 signaling plays a critical role in mediating vascular responses to diet-induced obesity. Kim et al. report that vascular inflammation in response to prolonged fatty acid exposure is dependent on TLR4 function (20). In addition, TLR4 has been implicated in the development of fatty acid induced insulin

resistance (21). Shi et al demonstrated that free fatty acids could activate the receptor in both macrophages and adipose tissue which results in impaired insulin signaling.

TLR4 is present in skeletal muscle and when activated, via LPS, induces an inflammatory response (22). Whether TLR4 plays a role in other cellular processes in skeletal muscle, in particular those associated with obesity, has yet to be fully elucidated. There is evidence reporting increased TLR4 expression and protein content in the skeletal muscle of obese humans (23). Our laboratory has shown that activation of this pathway causes a partitioning of fatty acids toward storage as triglyceride and away from oxidative pathways, and increased glycolytic activity (in review). However, the relationship between the obesogenic environment, particularly lipid-oversupply to skeletal muscle, and TLR4 regulation is not well understood. Therefore, the central purpose of the present study was to investigate the role of fatty acid surplus on the regulation of skeletal muscle TLR4 and associated TLR4-dependent metabolic defects.

Methods

Cell Culture. Mouse C2C12 myoblasts (ATTC, Manassas, VA) were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum, 50 U/mL penicillin and 50 µg/mL streptomycin (Invitrogen, Carlsbad CA). Cells were maintained in either 6 or 12-well plates (Becton Dickinson, Franklin Lakes, NJ) and upon confluency were differentiated into myotubes in media containing 2% horse serum, 50 U/mL penicillin and 50 µg/mL streptomycin. Experiments were conducted, following overnight serum starvation, between days 4 and 7 of differentiation.

Fatty acid and LPS treatment in cell culture. Lipid-containing media were prepared by conjugating the free fatty acids palmitate (16:0) and oleate (18:1) with fatty acid-free, endotoxin-free bovine serum albumin (BSA). Briefly, 600 µM of fatty acids were dissolved in ethanol in a boiling water bath followed by binding to fatty acid-free, endotoxin-free BSA at 37°C for 30 min. Solutions were diluted into warmed (37°C) DMEM to achieve to a concentration of 600 µM of FA/300 µM BSA (2% BSA wt/vol, 2:1 FFA:BSA) with appropriate concentrations of palmitate and oleate added to achieve ratios 2:1, 1:1, and 1:2 (palmitate:oleate). Fully differentiated myotubes were incubated in varying ratios of palmitate and oleate or BSA alone for 12 hours and following fatty acid pretreatment, cells were washed with PBS and treated with or without 500 ng/mL LPS (*Escherichia coli* 0111:B4, Cat # L2630, Sigma Aldrich, St. Louis, MO) for 2 hours. After 2 hours of LPS treatment, cells were washed three times with PBS followed by collection for gene expression analysis, western blotting, or assessment of glucose and fatty acid metabolism.

Real-time Quantitative PCR Analysis. Following treatment, cells were scraped and collected in TRIzol (Invitrogen, Carslbad, CA) and total RNA was isolated using the RNeasy Mini Kit (Qiagen) 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 manufacturer's specifications (Applied Biosystems, Inc., Foster City, CA).

Primer and probes sets for all target genes were purchased as pre-validated assays (ABI). Relative quantification of target genes was calculated using the $2^{-\Delta CT}$ method, which was validated for each primer/probe set using a 6 point serial standard curve as described previously (24). Derivation of the $2^{-\Delta CT}$ equation has been described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). Target gene expression was normalized to β-actin RNA levels.

Protein Analysis. Protein analysis was performed using cell lysates harvested in Mammalian Cell Lysis Buffer (Sigma Aldrich, St Louis, MO). Proteins (30-60 µg) were separated using a 10% Criterion Tris-HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, CA). Blots were probed with a TLR4 primary antibody (Cell Signaling, Danvers, MA; 1:1000) with β-actin Cell Signaling, Danvers, MA; 1:1000) serving as a loading control. This was followed by anti-rabbit secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA; 1:10,000). Proteins were visualized using Super-Signal Chemiluminescent Substrate (Pierce, Rockville, Il.) and a ChemiDoc XRS Imaging System (BioRad, Hercules, CA). For the determination of IL-6 and p-IκBα, a Bio-Plex suspension array system (BioRad, Hercules, CA) was used as previously described (25). To detect TLR4 protein levels from mouse tissue homogenates a TLR4 ELISA (Cat #E0753, USCN Life Sciences, Wuhan China) was performed according to the manufacturer's instructions.

Cell Culture Substrate Oxidation. Fatty acid oxidation was assessed in cell culture by measuring and summing $^{14}CO_2$ production and ^{14}C-labeled acid-soluble metabolites from the oxidation of [1-^{14}C]-palmitic acid (Perkin Elmer, Waltham, MA), respectively, as previously described (26, 27). Glucose oxidation was assessed in cell culture by measuring $^{14}CO_2$ production from the oxidation of [U-^{14}C]-glucose (Perkin Elmer, Waltham, MA) as previously described (26, 27) with the exception that glucose was substituted for BSA-bound palmitic acid.

Animal Husbandry. Animal studies were performed under an approved protocol by the Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State

University. Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 5 weeks of age, were kept on a 12 hour light/dark cycle, and fed a standard chow diet until 8 weeks of age. At 8 weeks of age, animals were divided into three groups (n=14/group) and fed either a high saturated fat (HSF) diet (60% fat), a high monounsaturated fat (HMF) diet (60 % fat), or a control diet (10% fat) ad libitum for 10 weeks. All diets were custom prepared by Research Diets, Inc. (New Brunswick, NJ.) and the composition of the diets is presented in Supplemental Data Table 1. At 10 weeks of age, animals underwent an i.p. injection of LPS (1mg/kg BW, ~25µg per animal) or saline (n=7/group), euthanized 4 hours post-injection, and gastrocnemius and quadriceps skeletal muscle were harvested and divided into red and white portions for assays performed as described below.

Glucose and Insulin Tolerance Testing. At 8 weeks of feeding, animals were fasted for 4 hours and a glucose tolerance test was performed. Briefly, animals received an i.p. injection of glucose (1g/kg) and blood was collected, via tail vein, at baseline and then every 30 minutes for 120 minutes for assessment of blood glucose concentrations. Animals were given 7 days to recover before undergoing an insulin tolerance test. For insulin tolerance testing, animals were fasted for 4 hours and then subjected to an i.p. injection of 0.65 U/kg of insulin. Blood glucose measurements were obtained, via tail vein, at baseline, and 15, 30, and 60 minutes following injection.

Tissue Preparation. Approximately 50 mg fresh white and red quadriceps muscle samples were immediately placed into 0.2 mL of a modified sucrose EDTA medium (SET) on ice containing 250 mM sucrose, 1 mM EDTA, 10 mM tris-HCl, and 10 mM tris-HCl, pH 7.4. Muscle samples were then minced thoroughly with scissors and then SET buffer was added to a 20-fold diluted (wt:vol) suspension. The minced samples were homogenized in a Potter-Elvehjem glass homogenizer at 10 passes across 30 seconds at 1,200 rpm with a motor-driven teflon pestle.

Tissue Homogenate Substrate Oxidation. Palmitate and glucose oxidation rates were determined in the fresh muscle homogenates prepared as described above. The

oxidation rate in muscle homogenates were measured by counting the $^{14}CO_2$ produced from palmitic acid or glucose during incubation. Eighty μl of a 20-fold (wt:vol) diluted muscle homogenates were incubated with 320 μl of reaction media (pH 7.4). Final concentrations of the reaction media were in mmole per liter: sucrose, 100; Tris-HCl, 10; potassium phosphate, 5; potassium chloride, 80; magnesium chloride, 1; L-carnitine, 2; malate, 0.1; ATP, 2; coenzyme A, 0.05; dithiothreitol, 1; EDTA, 0.2; and bovine serum albumin, 0.3%. The substrates used were 0.2 mM palmitate-1-^{14}C (1.0μCi/ml) or ^{14}C-glucose (1.0μCi/mL). After 60 minutes of incubation at 37°C, 200 μl of 70% perchloric acid was injected to stop the reaction and evolve CO_2 from the reaction media. CO_2 produced during the 60 minutes incubation was trapped with 400 μl of 1M sodium hydroxide. Trapped $^{14}CO_2$ was determined by liquid scintillation counting by use of 5 ml EcoLite liquid scintillation cocktail (MP Biomedicals, Santa Ana CA).

Enzyme Activity. Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloactetate. CoASH reduces DTNB and citrate synthase activity was determined from the reduction of DTNB over time as described previously (28). Briefly, ten microliters of a 1:100 (wt:vol) diluted muscle homogenate was added, in triplicate, to 170 μl of a solution containing Tris buffer (0.1M, pH 8.3), DNTB (1 mM, in 0.1 M in Tris buffer) and oxaloacetate (0.01 M, in 0.1 M Tris buffer). Following a 2 minute background reading, the spectrophotometer (SPECTRAmax ME, Molecular Devices Corporation, Sunnyvale California) was calibrated and 30 μl of 3 mM acetyl CoA was added to initiate the reaction. Absorbance was measured at 405 nm at 37°C every 12 seconds for 7 minutes. Maximum CS activity was calculated and reported as μmol/mg/min. For the determination of β-hydroxyacyl-CoA dehydrogenase (BHAD), oxidation of NADH to NAD was measured (28). In triplicate, 35 μl of 1:20 (wt:vol) muscle homogenate was added to 190 μl of a buffer containing 0.1 M liquid triethanolamine, 5 mM EDTA tetrasodium salt dihydrate, and 0.45 mM NADH. The spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) was calibrated and 15 μl of 2 mM acetoacetyl CoA was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 6 minutes at 37°C. Maximum BHAD activity was calculated and reported as μmol/mg/min. To assess

phosphofructokinase (PFK) activity, 30 µl of 1:20 (wt:vol) muscle homogenate was used to measure the oxidation of NADH to NAD in the presence of fructose-6-phosphate (29). In triplicate, 35 µl of 1:20 (wt:vol) muscle homogenate was added to 140 µl of an assay buffer consisting of, in mg/ml (0.0025 antimycin, 0.05 aldolase, 0.05 GAPDH) combined with, in mM (100 TRIS HCL, 12 MgCl2, 400 KCL, 2 AMP, 1 ATP, and 0.17 NADH). Following a 2 minute backround reading, the spectrophotometer (SPECTRAmax PLUS 384, Molecular Devices Corporation, Sunnyvale California) was calibrated and 20 µl of 2.9 mM fructose-6-phosphate was added to initiate the reaction. Absorbance was measured at 340 nm every 12 seconds for 6 minutes at 37°C. Maximum PFK activity was calculated and reported as µmol/mg/min.

Statistics. A two-way ANOVA with Tukey post-hoc analysis (multiple comparisons) was used to compare results from C57BL/6 mice in response to diet. All cell culture studies were repeated at least twice to establish enough replication to observe potential significant differences. Student's t-tests are two-tailed and the level of significance was set at $P<0.05$. Results are expressed as mean ± SEM.

Results

Saturated but not monounsaturated fatty acids increase TLR4 transcription and protein in C2C12 myotubes. We examined the notion that exposure to elevated FA levels may contribute to increased TLR4 expression in skeletal muscle. To this end, we treated fully differentiated C2C12 myotubes with 600 μM fatty acid cocktails containing varying ratios of palmitate:oleate (2:1, 1:1, and 1:2) bound to bovine serum albumin (FFA:BSA molar ratio, 2:1). By varying the relative contribution of palmitate and oleate to the treatment we were able to assess fatty acid specificity and their degree of saturation on TLR4 expression and content. Following 4 hours of FA exposure, the monounsaturated treatment resulted in a decrease in TLR4 mRNA compared to the control condition containing BSA alone. As the concentration of palmitic acid in the media increased, TLR4 expression levels were increased in step-wise fashion with the most saturated condition resulting in a greater than 30% increase in TLR4 compared to control, $p<0.05$ (Figure 1A). Consequently, protein content of TLR4 was assessed in response to the 2:1, palmitate: oleate cocktail. Figure 1B illustrates a significant increase in protein content of TLR4 in response to this FA treatment.

Saturated fatty acid treatment enhances the IL-6 response to LPS in C2C12 myotubes. The observation that saturated FA increase TLR4 mRNA and protein content prompted us to investigate whether this treatment sensitizes the TLR4 pathway to LPS. C2C12 myotubes were incubated in the presence of 600μM of 2:1 palmitate: oleate, or BSA alone for 4 hours, washed with PBS, and then challenged with 500 ng/mL of LPS for 2 hours to activate TLR4. Cells were then collected and IL-6 mRNA was assessed using qRT-PCR. IL-6 mRNA was increased 40-fold in response to LPS following the control treatment of BSA alone. Following exposure to the saturated FA cocktail, the LPS-stimulated increase in IL-6 mRNA was ~80-fold, indicating a heightened sensitization of TLR4 to LPS following FA treatment (Figure 2).

Saturated fatty acid treatment exacerbates the LPS-mediated effects on substrate metabolism. Our laboratory has reported that activation of TLR4 in skeletal muscle alters substrate metabolism in a manner characterized by increased glucose oxidation,

decreased fatty acid oxidation, and a preferential partitioning of fatty acids into intramyocellular lipid depots. Based on our observations that specifically saturated FA increase TLR4 content and in-turn sensitizes the receptor signaling pathway, we sought to determine whether this FA effect on TLR4 is translated into a functional metabolic consequence. Accordingly, we exposed C2C12 cells to the FA conditions described above then treated with and without LPS, and measured CO2 production from radiolabeled palmitate and glucose. Consistent to what has been observed before, LPS activation of TLR4 increased glucose oxidation by 25% ($p<0.05$) and decreased palmitate oxidation by 18% ($p<0.05$). The pre-treatment with saturated FA (2:1, palmitate: oleate) significantly enhanced the TLR4-mediated effects on glucose and palmitate metabolism, as evidenced by a larger increase in glucose oxidation (35% vs. 25%) and further depression in fat oxidation (30% vs. 18%) in response to LPS, $p<0.05$ (Figure 3). The pre-treatment containing the monounsaturated cocktail (1:2, palmitate:oleate) blocked the LPS-mediated effects on both glucose and palmitate oxidation. This suggests that the saturation nature of the FA pre-treatment determines the TLR4-mediated effect on metabolism.

10-weeks of high fat feeding does not alter skeletal muscle TLR4 gene expression and protein content in C57BL/6 mice. To examine whether these specific effects of FA on skeletal muscle TLR4 are also evident chronically and in-vivo, we fed male C57BL/6 mice CON, HSF, and HMF diets for 10-weeks. The composition of these diets is shown in Table 1. Both high fat diets resulted in significant increases in body weight compared to the control diet (mean increase in body weight ± SEM) was 16.8 ± 1.37 in mice fed the HSF, 16.0 ± 1.37 in mice fed HMF, and 5.2 ± .51 gm in mice fed CON, $p<0.0001$) (Figure 4A). The body weights did not differ between HSF and HMF. HSF and HMF animals also exhibited impaired glucose homeostasis as evidenced by significantly greater area under the curve for both insulin and glucose tolerance tests (Figure 4B, 4C) At 10-weeks of experimental feeding, animals were challenged with an I.P. injection of saline or LPS (~25 µg/mouse), sacrificed 4hours post-injection, and red and white muscle was harvested for measures of gene expression and protein analysis, and glucose and fat metabolism. Quadriceps TLR4 mRNA did not differ across diets

(Figure 5A). An ELISA was conducted to assess TLR4 protein levels and no differences were detected between diet conditions (Figure 5B).

High fat feeding sensitizes the TLR4 signaling pathway in the skeletal muscle of C57BL/6 mice. To assess TLR4 sensitization to LPS in HSF and HMF animals, we examined the transcript and protein levels of downstream targets of TLR4 following dietary intervention and subsequent LPS or saline challenge.

IL-6. LPS challenge resulted in significant increases in both IL-6 mRNA and protein across all diet conditions when compared to saline, ($p<0.05$). In red quadriceps, in comparison to CON, LPS induced a significantly greater change in mRNA in HMF and HSF, respectively (Figure 6A). In white quadriceps, the LPS-induced increase was 1.9-fold greater in HMF and 16.0-fold greater in HSF in comparison to the CON diet condition. Meanwhile, LPS increased IL-6 protein levels in red quadriceps by 1.8, and 2.6-fold greater in HMF and HSF, respectively compared to CON (Figure 6B).

MCP-1. Monocyte chemo-attractant protein-1 mRNA was significantly increased in response to LPS across all diet conditions ($p<0.05$). Increases in MCP-1 in red quadriceps were 1.3 and 3.2-fold in HMF and HSF, respectively, in comparison to CON (Figure 6C). This effect with saturated fat feeding was further enhanced in the white quadriceps as we observed a 2.8, and 10.7-fold change in MCP-1 RNA with LPS in HMF and HSF conditions respectively, in comparison to CON.

P-IκBα. Phophspho-IkappaB alpha was significantly increased in response to LPS across all diet conditions. In white gastrocnemius, the LPS-induced fold change was 1.9, 1.4, and 3.1 in the CON, HMF, and HSF groups respectively (Figure 6D). The response to LPS was significantly greater in the HSF group compared to the HSF and HMF animals. In red gastrocnemius, LPS induced a significant increase in p-IκBα although the degree to which these changes occurred were similar across all diet conditions (data not shown).

TLR2. TLR2 mRNA levels were increased significantly following LPS administration. LPS induced a 5.8, 6.2, and 12.9-fold increase in the CON, HMF, and HSF diets, respectively, in red quadriceps muscle (Figure 7A). In white quadriceps, LPS induced a

5.1, 6.3, and 10.1-fold increase in CON, HMF, and HSF fed animals, respectively (Figure 7B).

LPS-induced alterations in substrate metabolism are exacerbated with high saturated fat feeding. Glucose and fatty acid metabolism were measured in red and white gastrocnemius whole homogenates following a 4hour LPS or saline administration. LPS induced a 15% percent decline in palmitate oxidation in CON animals (Figure 8). LPS-mediated impairment in fat oxidation was exacerbated in HSF mice, a 50% reduction compared to 26% reduction in CON, ($p<0.05$), which was not observed in HMF mice. Figure 10 shows that HSF mice displayed an exacerbated increase in glucose oxidation in response to LPS compared to CON animals, a 40% increase compared to 28% in CON, $p<0.05$. This effect was not evident in HMF mice. PFK activity trended to increase ($p=0.08$) in CON red gastrocnemius but only reached statistical significance in the HSF animals where LPS elicited a 28% increase ($p=0.001$) (Figure 9A). In white gastrocnemius, there was a 24% increase in PFK activity in HSF animals, a 25% decrease in HMF animals and no change in response to LPS in CON animals. There was a significant diet x treatment interaction between CON and HSF diets in both red and white gastrocnemius with regards to LPS-induced changes in PFK ($p=0.01$). In red gastrocnemius, BHAD activity was reduced by 8% in CON animals in response to LPS, and this effect was enhanced to 35% ($p<0.05$) in HSF, (Figure 9B). LPS had no effect on BHAD in HMF mice. Citrate synthase activity was significantly reduced in response to LPS in the red muscle from the HSF animals. This effect was not significant in CON muscle (Figure 9C).

Discussion

Over the past decade, a large body of literature characterizing toll-like receptors and their role in the innate immunity has accumulated (19, 30), and TLRs are believed to play an integral role in the relationship between systemic, chronic inflammation and obesity (31-33). TLR4 has been implicated in the development of fatty acid induced insulin resistance in peripheral tissues (20, 21). TLR4 -/- mice are protected from lipid-induced serine phosphorylation of IRS-1 and show a reduced expression of pro-inflammatory mediators TNFα, IL-6, MCP-1 and SOCS3 in response to both acute lipid infusion and chronic high-fat feeding compared to pair-fed counterparts (20). In addition, our laboratory, in conjunction with other groups, has shown that TLR4 expression is elevated in the skeletal muscle of obese and insulin-resistant humans (23). Taken together these latest discoveries suggest that TLR4 may provide an alluring solution to the longstanding question of the mechanisms linking inflammation with obesity and insulin resistance.

The primary goal of our current study was to discern the role of fatty acids and their saturation nature on skeletal muscle TLR4 content and signaling. We found that incubating C2C12 myotubes with saturated fatty acid-enriched cocktail results in increased TLR4 gene expression and protein content. This was associated with a more robust expression of the proinflammatory gene IL-6 in response to an LPS challenge. Palmitate, a saturated fatty acid, is known to regulate the expression of TNF-α and activate NFkB, which has been linked to fatty-acid induced inflammation and insulin resistance (34, 35). Incubation of human myotubes in the presence of palmitate alone induces both rapid as well as chronic increases in NFkB nuclear translocation and DNA binding (34) and this is associated with concomitant increases in IL-6 mRNA and protein. It is important to note that our study utilized a physiologically relevant saturated fatty acid condition; one containing a 2:1 ratio of palmitate to oleate. Palmitate exposure alone has been shown to be highly toxic and pro-apoptotic (36-38) and most importantly does not truly represent a circumstance that would be encountered in-vivo. Our ex-vivo findings suggest that this skeletal muscle-generated pro-inflammatory effect of saturated fatty acids is caused by increased transcriptional activation of TLR4 and

resulting increases in receptor content. As the fatty acid pretreatment was manipulated to mimic a more unsaturated nature, the effect on TLR4 was significantly attenuated. Increasing the oleate concentration relative to palmitate, while maintaining 600μm total fatty acid levels, resulted in a step-wise reduction TLR4 expression levels. These observations suggest that the effect of fatty acids on skeletal muscle TLR4 transcripts operate via a saturation-dependent mechanism.

The anti-inflammatory properties of unsaturated fatty acids have been well documented (39-41). While saturated fatty acids have been shown to induce insulin resistance (42-44), monounsaturated fatty acids are linked to improved whole-body insulin sensitivity in diabetic humans (39, 41). Monounsaturated fatty acids have in fact; have been shown to be protective to the pro-inflammatory effect of saturated fatty acids. Co-incubation of oleate in the presense of palmitate has been shown to reverse the palmitate-induced pro-inflammatory/insulin desensitizing effects in skeletal muscle (45). Coll and colleagues propose that this effect is due, in part, to oleate preventing a rise in intracellular DAG levels by: 1) elevating the expression levels of the mitochondrial fatty acid transporter carnitine palmitoyl transferase-1 (CPT-1), thus increasing β-oxidation and 2) channeling excess palmitate into triglycerides as opposed to the more toxic DAG by modulation of the triglyceride synthesizing enzyme Dgat2.

The results from our cell culture studies provide a novel mechanism by which skeletal muscle inflammatory responses are mediated by fatty acids. The effect of fatty acids on the inflammatory response to LPS appears to be mediated by the differential effect of saturated and monounsaturated fatty acids on TLR4 transcription and content. Saturated fatty acids increased TLR4 gene expression and protein and those myotubes were hypersensitive to the inflammatory effect of LPS; whereas monounsaturated fatty acids reduced TLR4 levels and those myotubes exhibited a blunted inflammatory response to LPS.

To determine if these lipid-induced effects on TLR4 were also evident in-vivo we fed C57BL/6 mice a high saturated fat, a high monounsaturated fat, or a control diet for 10

weeks. Both high fat fed groups gained significantly more weight compared the control animals and were also glucose and insulin intolerant while control animals exhibited normal glucose homeostasis. Interestingly, we observed no differences in skeletal muscle TLR4 gene expression or protein content across diet. However, when challenged with LPS, the HSF animals responded with an exacerbated inflammatory response as evidenced by significantly greater increases in IL-6, MCP-1, and greater shifts in LPS-induced alterations in substrate metabolism. We show that chronic exposure to saturated fatty acids in vivo has the same sensitization effect on TLR4 signaling that is evident in cultured myotubes acutely exposed to a high saturated fat milieu. However, since TLR4 transcripts and protein content were unaltered by diet, this suggests that this sensitization is elicited likely via different mechanisms. Based on reports of elevated TLR4 levels in the skeletal muscle of obese and insulin resistant humans (23) it was hypothesized that diet-induced obesity, particularly that generated by high saturated fat feeding, would result in similarly increased TLR4 levels in C57BL/6 mice. Since this was not observed in our study this suggests that the increased levels of TLR4 reported in obese humans are likely not the direct result of weight gain or increased adiposity. This discrepancy between observed obese human and obese mouse levels of TLR4 raises a few questions. In our model, obesity was strictly diet-induced whereas human obesity develops via a much more complex path. Factors such as physical activity, genetics, and environmental factors all play a part in contributing to overweight and obesity in humans. Studies examining the effect of high-fat feeding on skeletal muscle TLR4 levels have not been undertaken in humans and thus inherent difficulties arise when comparing the weight gain in our C57BL/6 high fat-fed mice with that observed in a cross-section sampling of obese humans. It is conceivable that multiple and different mechanisms exist by which in vivo lipotoxicity sensitize the TLR4 pathway in humans and rodents. Similarly, the fact that we observed elevated expression of TLR4 in myotubes exposed to a fatty acid milieu but not following chronic high fat feeding, yet a similar heightened sensitization to LPS, raises other mechanistic questions. In an ex-vivo environment, the circulating hormonal effects of lipid oversupply and the potential for multiple organ cross-talk that occur in a whole-body milieu are

removed. Thus, there is much work to be done in this area to fully understand how lipids mediate skeletal muscle TLR4-induced inflammation and metabolic dysregulation.

TLR4 activation by LPS has been shown to induce the expression of TLR2 in endothelial cells (46). We found that LPS injection increased TLR2 mRNA and this effect was markedly enhanced in HSF animals. TLR2 activation, by peptidoglycan, elicits a robust IL-6 and MCP-1 inflammatory response, similar to that seen with LPS activation of TLR4 (22). Thus, it is conceivable that the heightened sensitivity to LPS exhibited in the HSF animals was, in part, due to TLR4-induced increases in TLR2 levels. Although TLR4 is the most widely known receptor for LPS, TLR2 has been shown to be responsive to LPS (47) and thus, TLR2 may operate in conjunction with TLR4 to potentiate the LPS effect we observed in the HSF animals. Our study does not provide any evidence that TLR2 is contributing to the fatty acid-induced effects on TLR4 signaling and future work with TLR2 knockdown studies would be necessary to clearly identify its role in the sensitization effects of LPS-mediated effects observed here.

Dietary saturated fatty acids have been reported to bind TLR4 and initiate its' inflammatory signaling. However, it is unlikely that the enhanced sensitization to LPS that we observed in the presence of saturated fatty acids is due to a direct ligand/binding effect. The IL-6 response to TLR4 activation is rapid and robust. If saturated fatty acids were binding TLR4 to directly initiate TLR4 signaling we would have expected to observe elevated IL-6 levels in the HSF animals independent of LPS treatment. Despite the fact that the HSF animals exhibited significantly higher body weights and impaired glucose homeostasis compared to the control animals, we did not detect any difference in basal levels of IL-6 mRNA or protein content among diet treatments. Our data is in agreement with previous reports in adipocytes (48) which utilized radiolabeled binding assays to conclude that neither saturated nor monounsaturated fatty acids bind directly to the TLR4/MD-2 receptor complex.

TLR4 must be membrane bound to adequately respond to LPS with inflammatory signaling (49). TLR4 is localized in the endoplasmic reticulum following translation

where it becomes physically associated with MD-2. TLR4 trafficking from intracellular compartments to the plasma membrane is shown to be regulated by the chaperone proteins glycoprotein 96 (gp96) and protein associated with Toll-like receptor 4 (PRAT4A) (50, 51). LPS fails to activate TLR4 signaling in cells deficient of gp96 or PRAT4A (51). It is not inconceivable to suggest that the heightened response to LPS in HSF animals could be, in part, due to saturated fatty acids affecting one or more of these chaperone proteins or another aspect of the translocation process. Experiments are currently underway to determine if saturated fatty acids influence cell surface TLR4 or the trafficking processes that lead to its surface expression.

Our laboratory has recently reported that skeletal muscle, when challenged with LPS, adopts a TLR4-dependent, obese metabolic phenotype. TLR4 activation results in a partitioning of lipids away from oxidative pathways and into intramyocellular storage depots. Excessive accumulation of lipids within skeletal muscle fibers is a common defining feature of obesity and is linked to insulin resistance (10-14). In the current study we found that i.p. injections of LPS indeed resulted in reduced fatty acid oxidation and a preference for glucose as a substrate. This was associated with reductions in the maximal activities of BHAD and CS, two key enzymes regulating fatty acid beta-oxidation and the tricarboxylic acid cycle, respectively. LPS/TLR4-induced reductions in fatty acid oxidation were exacerbated under conditions of elevated saturated fat. HSF animals exhibited a more profound decrease in fat oxidation following LPS challenge, an effect that was not observed in the HMF animals despite being similarly obese. This metabolic phenomenon was also evident in cultured myotubes pretreated with fatty acid cocktails. These data have important implications in understanding the relationships between diet-induced obesity, inflammation, and the metabolic phenotype that often characterizes the obese state. The observation that saturated fatty acids sensitize the TLR4 pathway provides a unique mechanism by which acute and chronic lipotoxicity induce metabolic dysregulation in obese skeletal muscle. In today's society, a culture of increased saturated fat consumption has become more and more commonplace**.** These results provide insight into how a westernized diet, one enriched in saturated fat, may link chronic inflammation with obesity and its associated metabolic abnormalitie

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Figure 1. TLR4 mRNA and protein levels are increased in C2C12 myotubes in a step-wise fashion with increasing concentrations of palmitic acid exposure. C2C12 myotubes were incubated in 600μM fatty acid cocktails with varying ratios of palmitic acid: oleic acid. Cells were washed with PBS and assayed for gene expression using qRT-PCR (A) and Western Blotting (B). Gene expression data are presented as means±SEM.

Figure 2. TLR4-mediated increases on IL6 expression are exacerbated following saturated FA treatment in C2C12 myotubes. C2C12 myotubes were incubated in 600 μM FA:BSA (2:1 palmitate:oleate) or BSA alone for 12hr, washed with PBS and then challenged with 500 ng/mL of LPS for 2 hr. Cell were harvested in 1 mL TRIzol for analysis of gene expression using qRT-PCR. Data is presented as LPS-induced fold change relative to β-actin.

Figure 3. Saturated FA treatment exacerbates TLR4-mediated effects on glucose and fatty acid metabolism in C2C12 myotubes. C2C12 myotubes were incubated in 600 μM FA:BSA (2:1 palmitate:oleate) or BSA alone for 12hr, washed with PBS and then challenged with 500 ng/mL of LPS for 2 hr. Following LPS challenge, radiolabeled substrates (glucose and palmitate) were used to measure glucose and FA metabolism. Cells were harvested in 0.05% sodium dodecyl sulfate and oxidation values were normalized to total protein content. Data is presented as LPS-induced percent change.

Figure 4. 10 weeks of high saturated fat and high monounsaturated fat feeding significantly increased body mass and disrupted glucose homeostasis. Male C57BL/6 were fed a 60% high fat diet or control diet for 10 weeks and body weight was measured weekly (A). Data are presented as means±SEM. Glucose (B) and insulin tolerance (C) tests were performed at weeks 8 and 9 of high fat feeding, respectively. Blood glucose levels were measured via tail vein at indicated intervals.

Figure 5. High fat feeding does not alter TLR4 mRNA or protein content in C57BL/6 mice. Following 10 weeks of high fat feeding, animals were challenged with I.P. injections of saline or LPS 1 mg/kg (~25ug per mouse), sacrificed 4h post-injection, and quadriceps were harvested in TRIzol for qRT-PCR or lysis buffer for TLR4 protein analysis by Bio-Plex suspension array system (Bio-Rad, Hercules, CA). Quadriceps muscle was manually separated in to red (A) and white (B) portions. Gene expression analysis for TLR4 is shown relative to β-actin and TLR4 protein content is shown relative to total protein content. Data are presented as means±SEM.

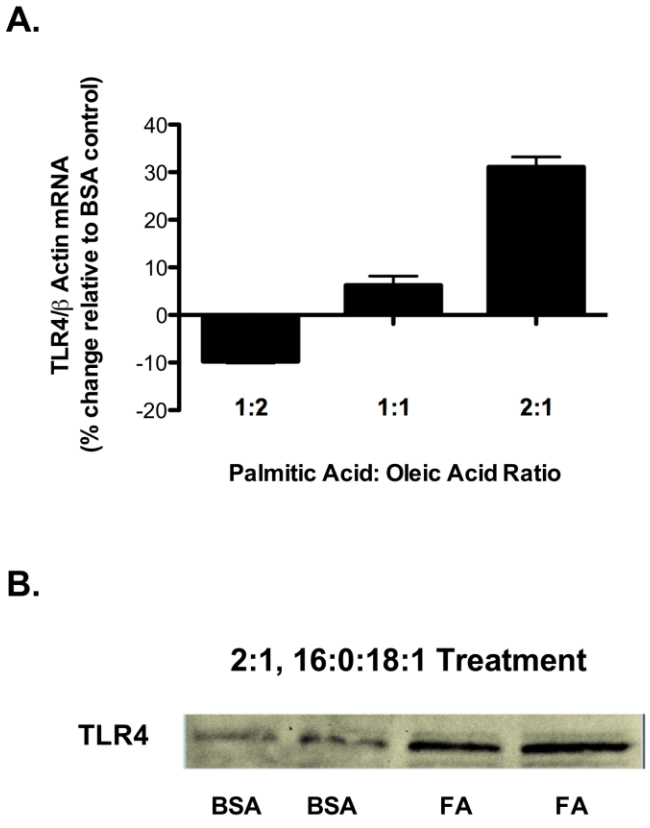
Figure 6. **High saturated fat feeding exacerbates the inflammatory to LPS.** Following 10 weeks of high fat feeding, animals were challenged with I.P. injections of saline or 1mg/kg LPS (~25ug per mouse), sacrificed 4h post-injection, and quadriceps were harvested in TRIzol for IL-6 (A), MCP-1 (C) and mRNA quantification by qRT-PCR or lysis buffer for IL-6 (B) and p-IκBα (D) protein analysis by Bio-Plex suspension array system. Gene expression is shown relative to β-actin and protein content is shown relative to total protein content. Data are presented as means±SEM. Statistical significance was established at $P<0.05$.

Figure 7. High saturated fat feeding increases the TLR2 mRNA response to LPS. Red (A) and white (B) gastrocnemius muscle was extracted and processed in TRIzol. mRNA analysis was performed by qRT-PCR and expression levels are shown relative to β-actin mRNA. Data are presented as means±SEM or LPS-induced fold change in TLR2 mRNA.

Figure 8. High saturated fat feeding enhances the TLR4-mediated shift in substrate metabolism. Following 10wk of high fat feeding animals were challenged with LPS for 4hr, sacrificed, and gastrocnemius muscles were separated into red and white portions. Substrate oxidation was assessed using [U-^{14}C]-glucose or [1-^{14}C]-palmitic acid (A). Results were corrected for mg of total protein and presented as percent change in total oxidation in response to LPS. Enzyme activity (B-D) was assessed in red and white gastrocnemius muscle homogenates and measured

spectrophotometrically. Data was corrected for mg total protein and presented as µmol/mg protein/minute.

Figure 1.



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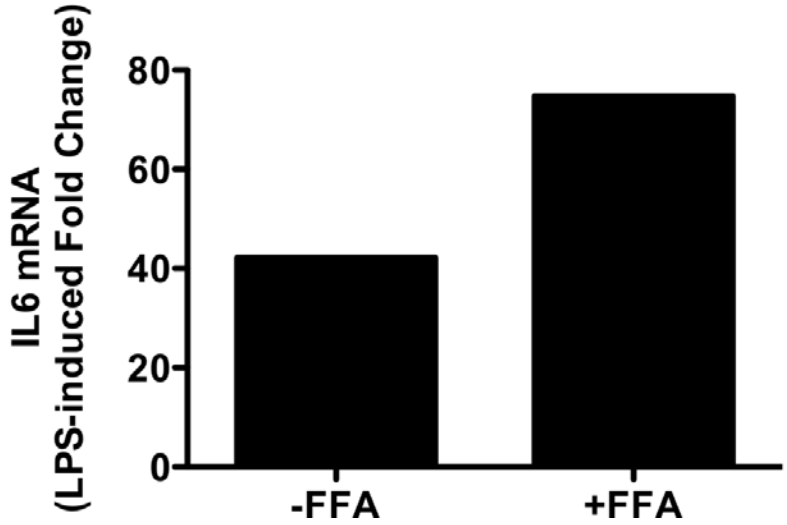

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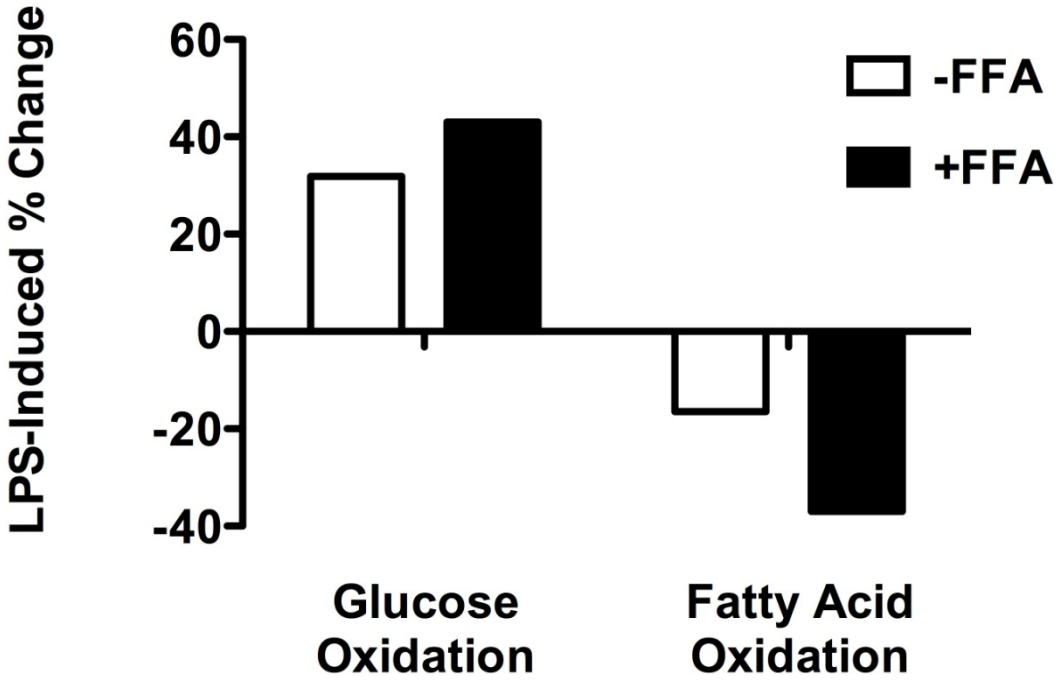

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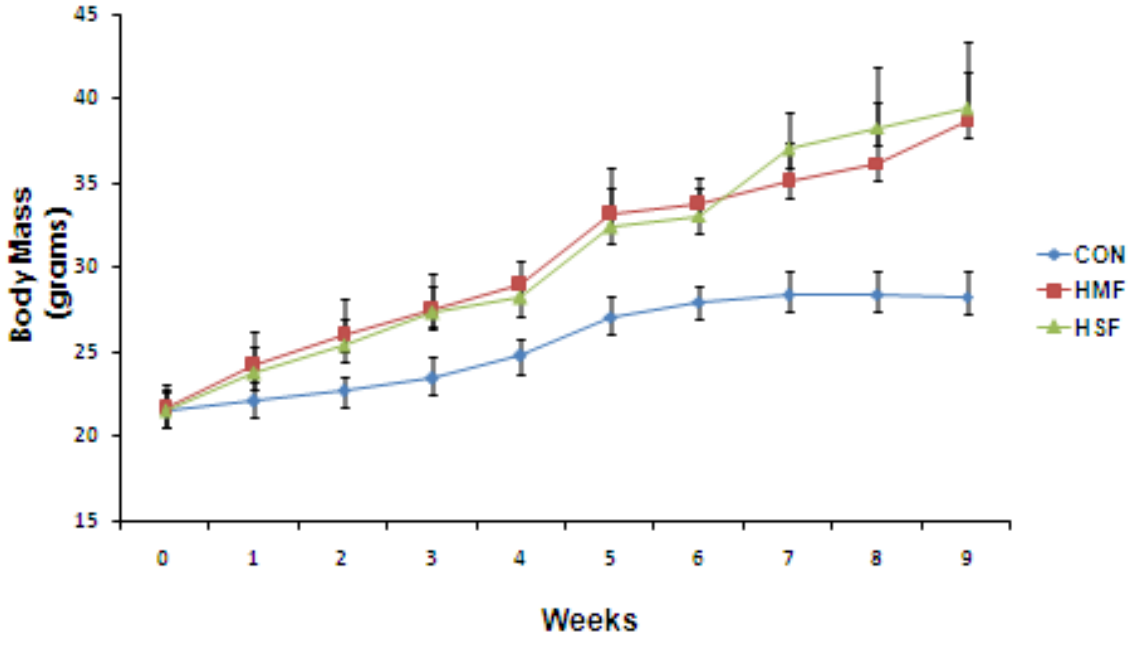

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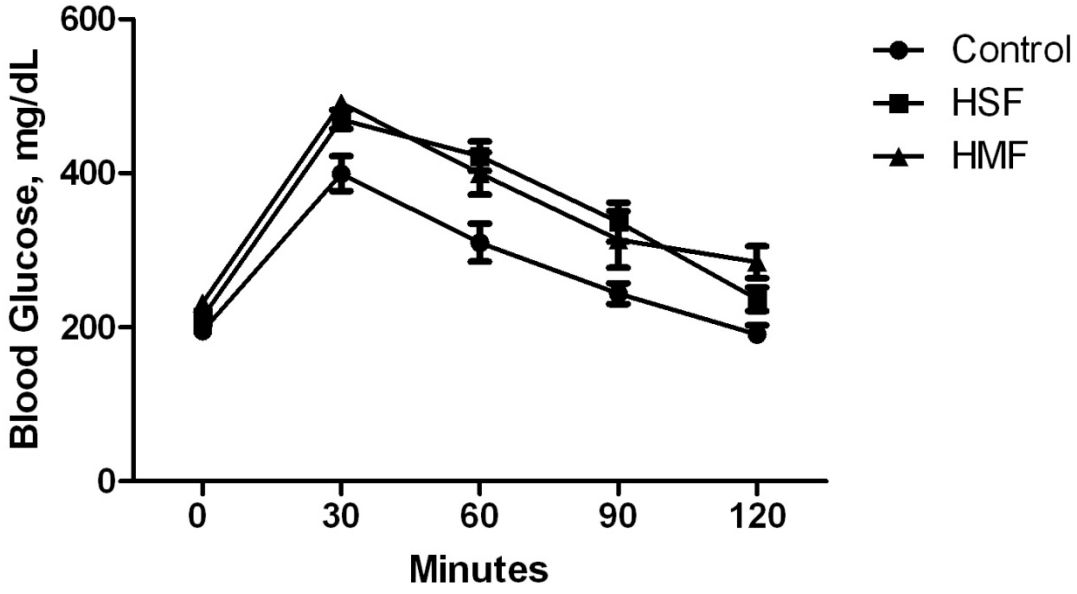

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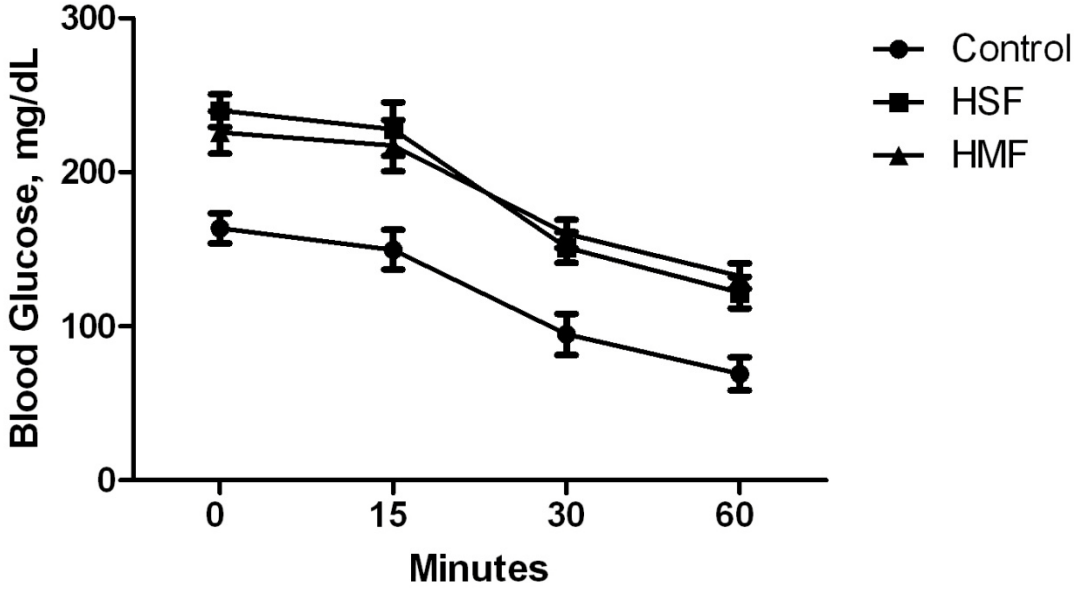

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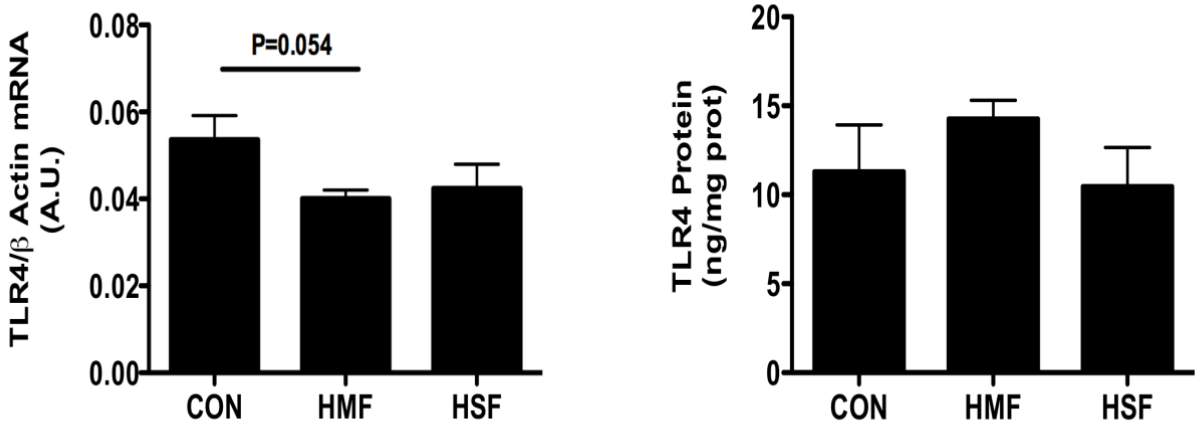

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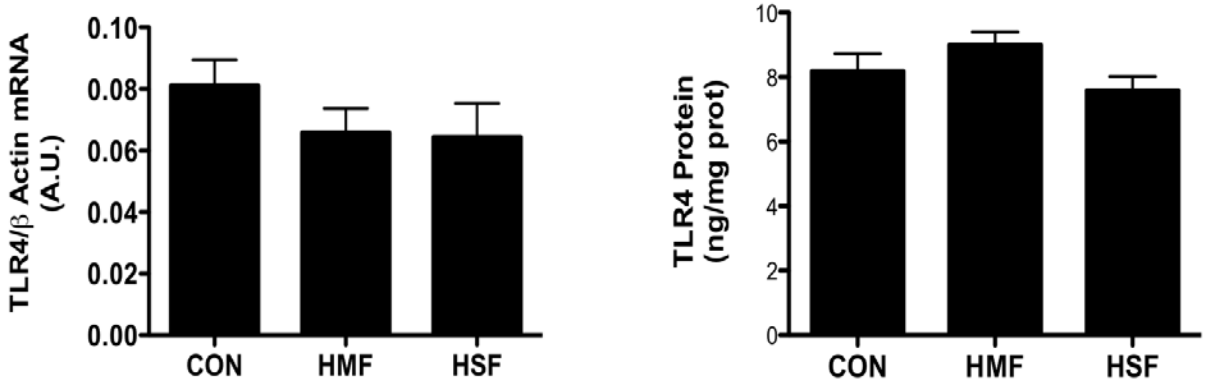

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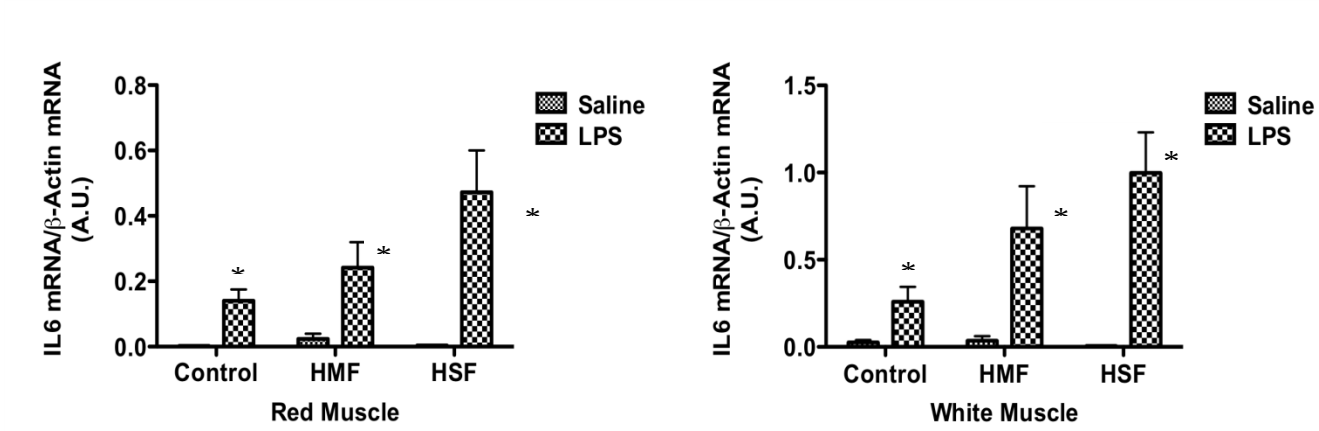

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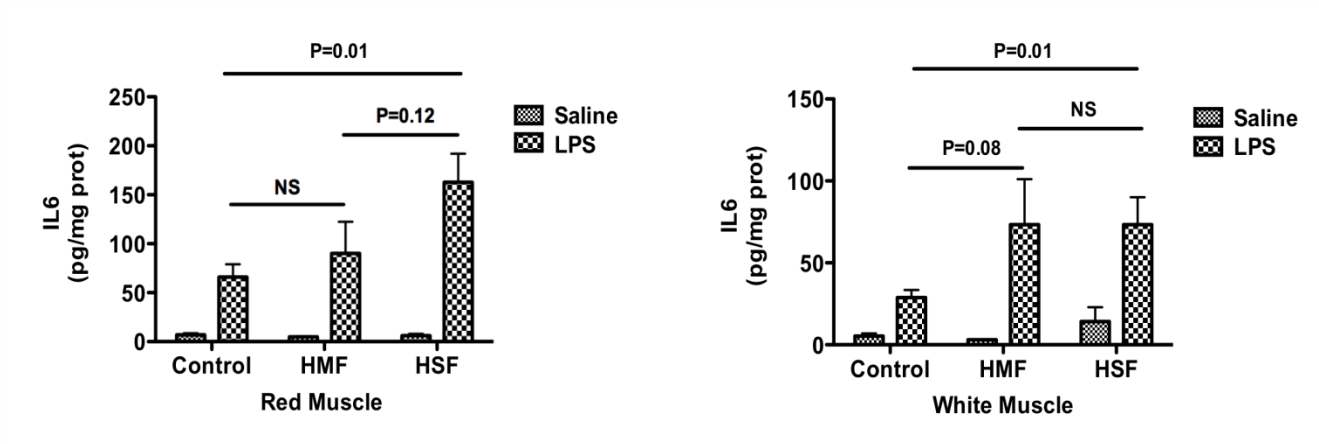

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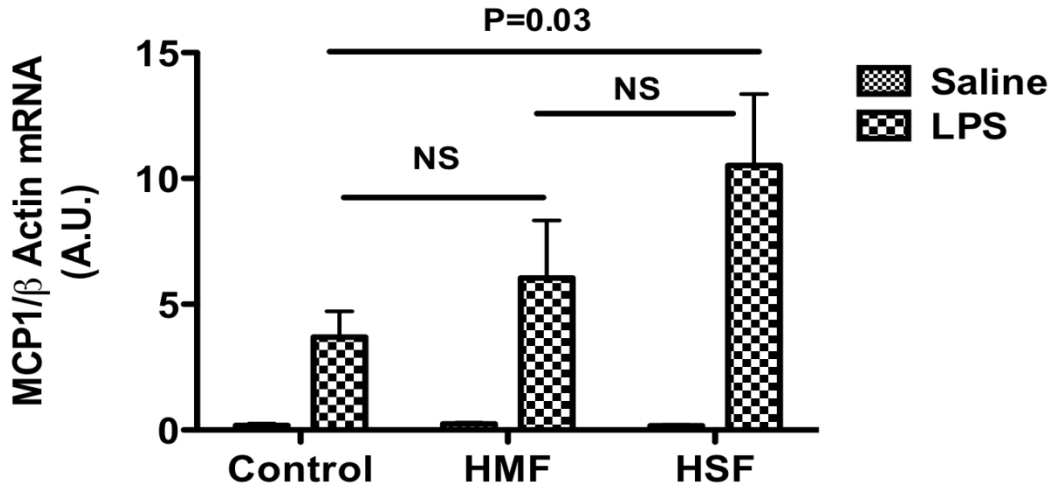

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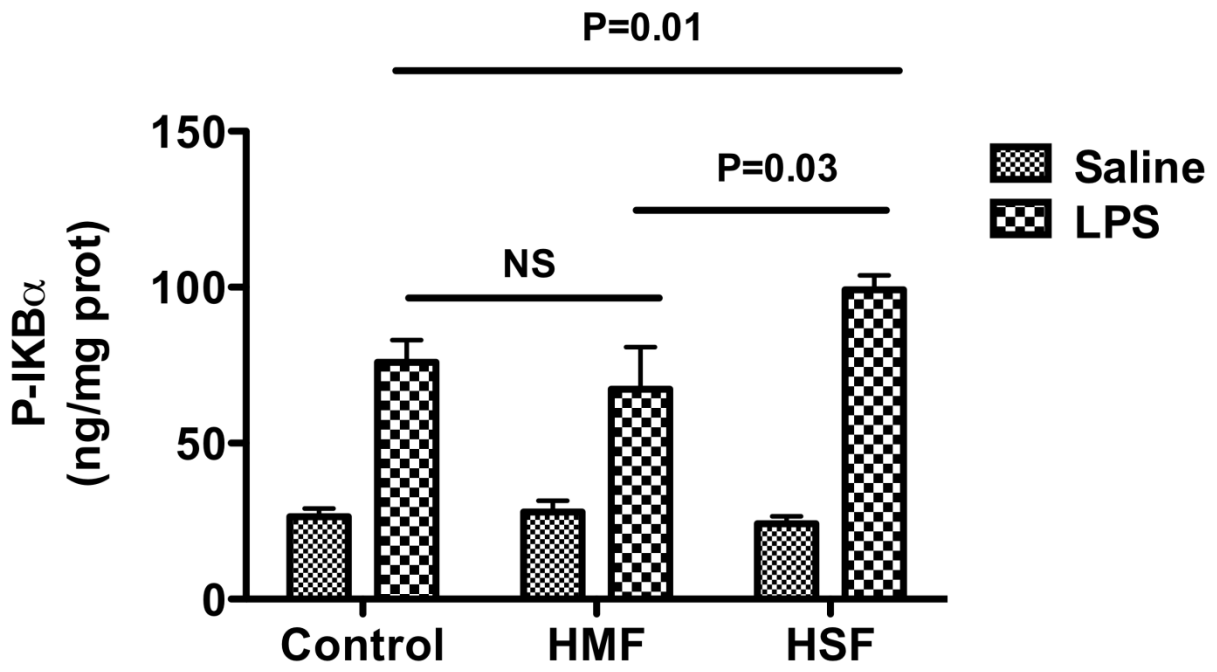

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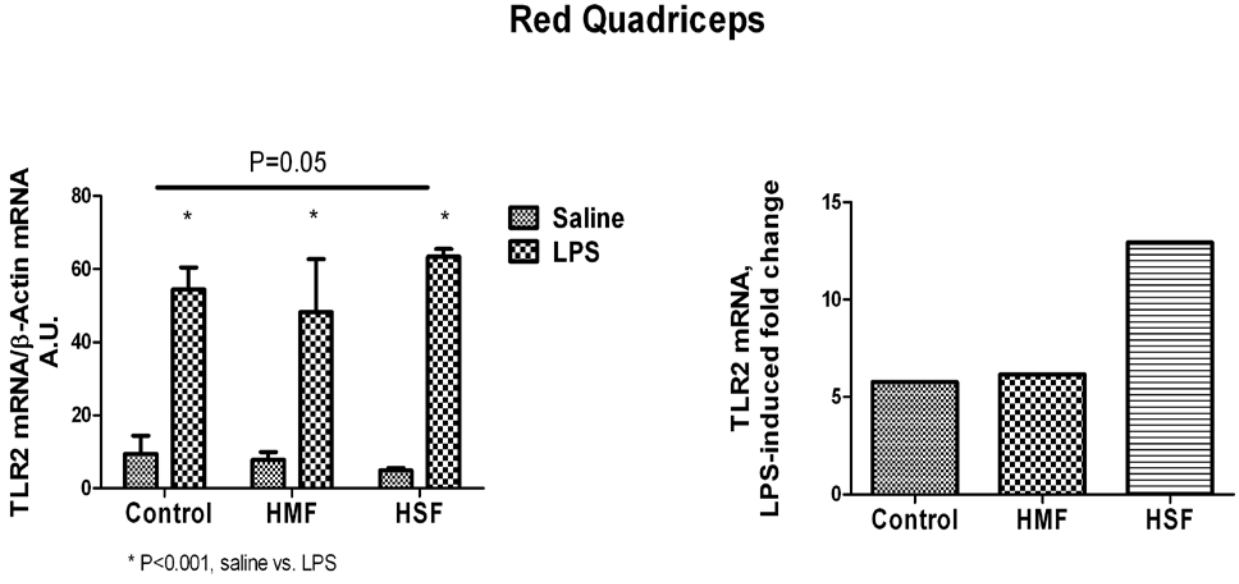

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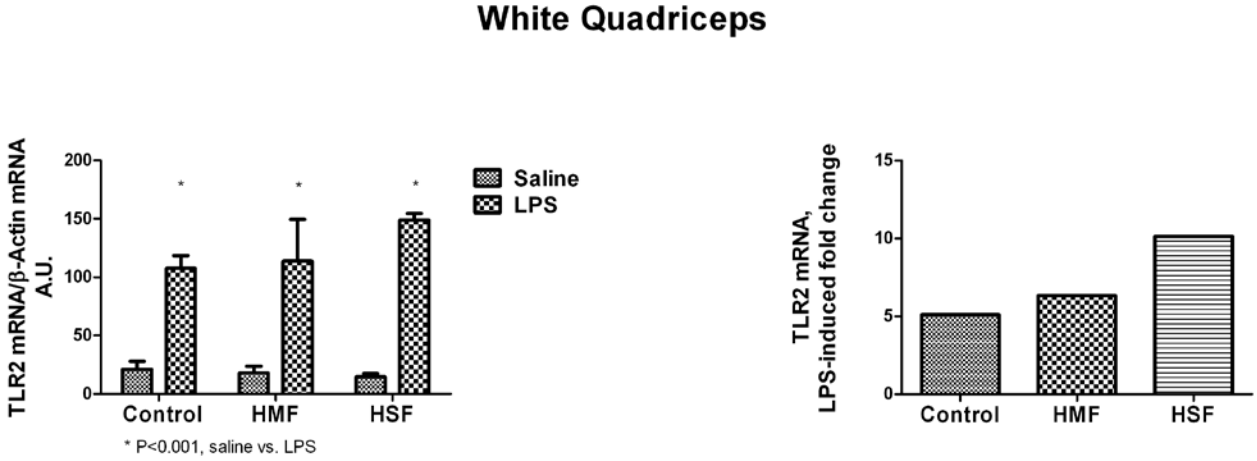

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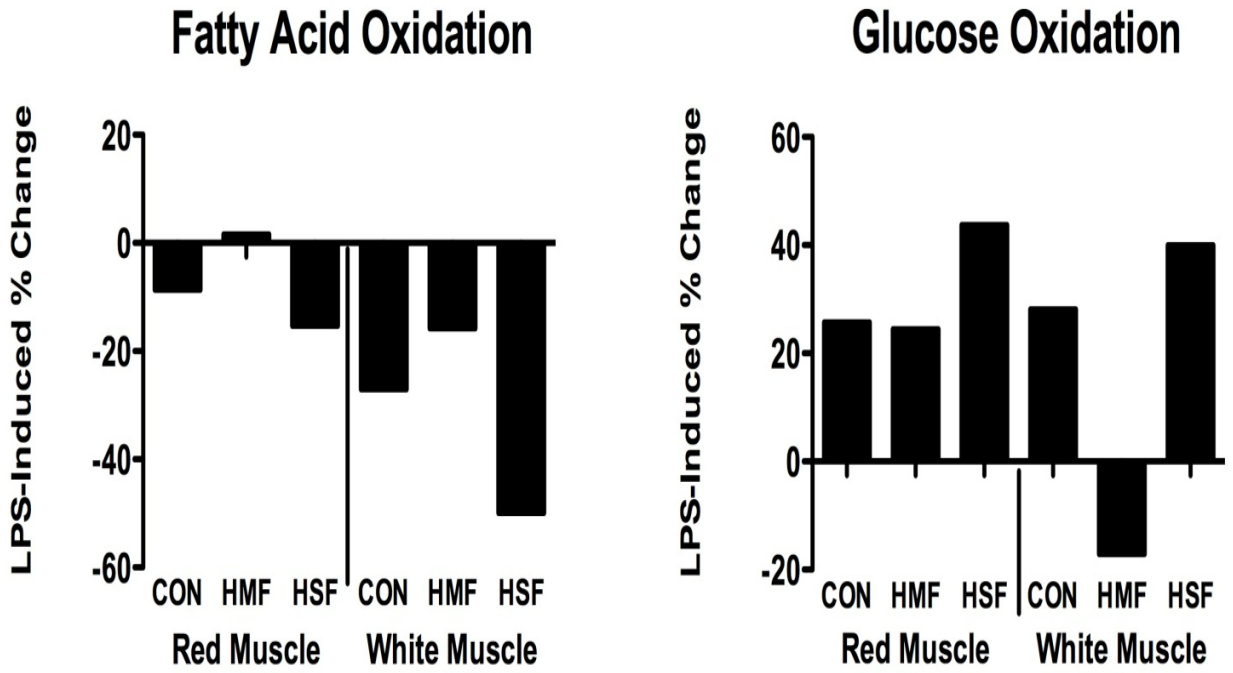

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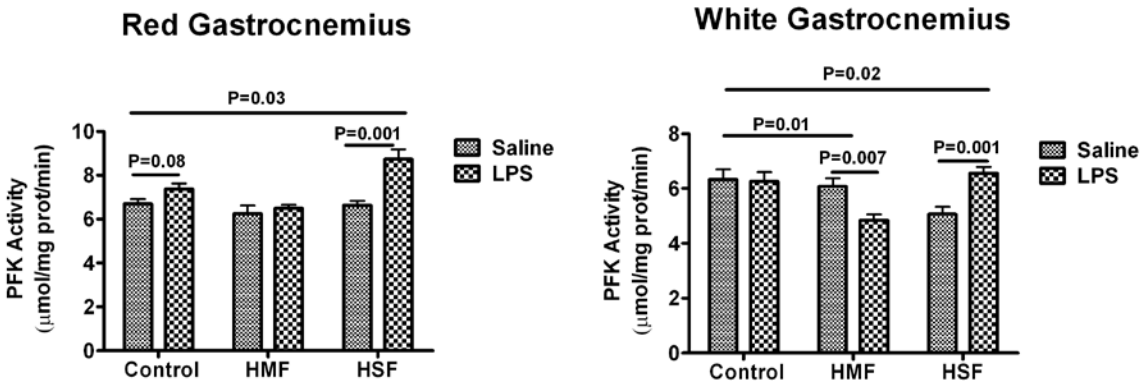

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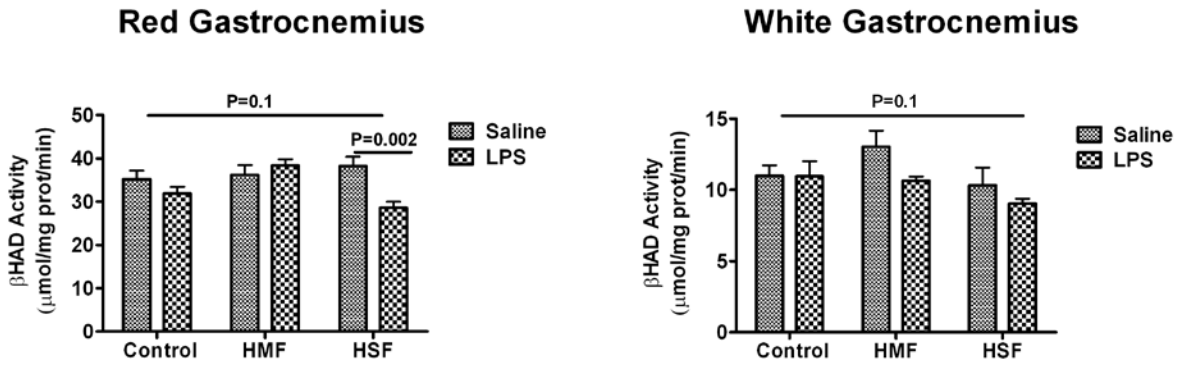

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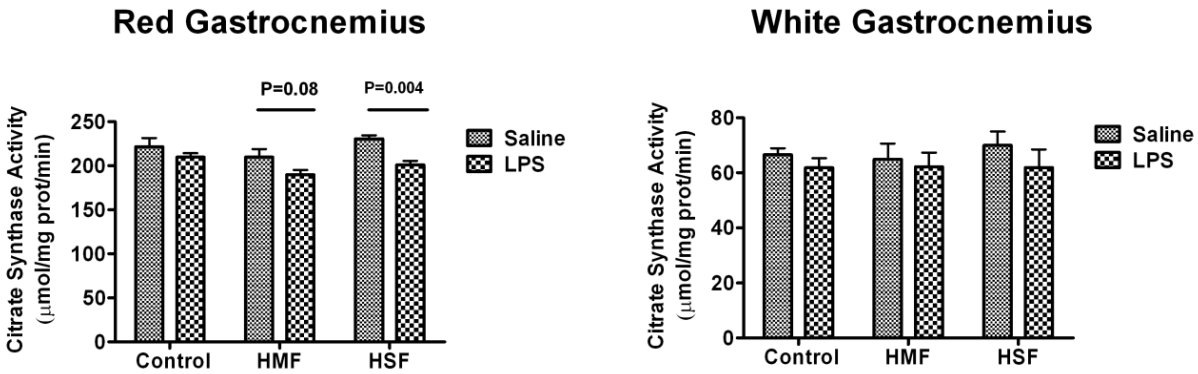
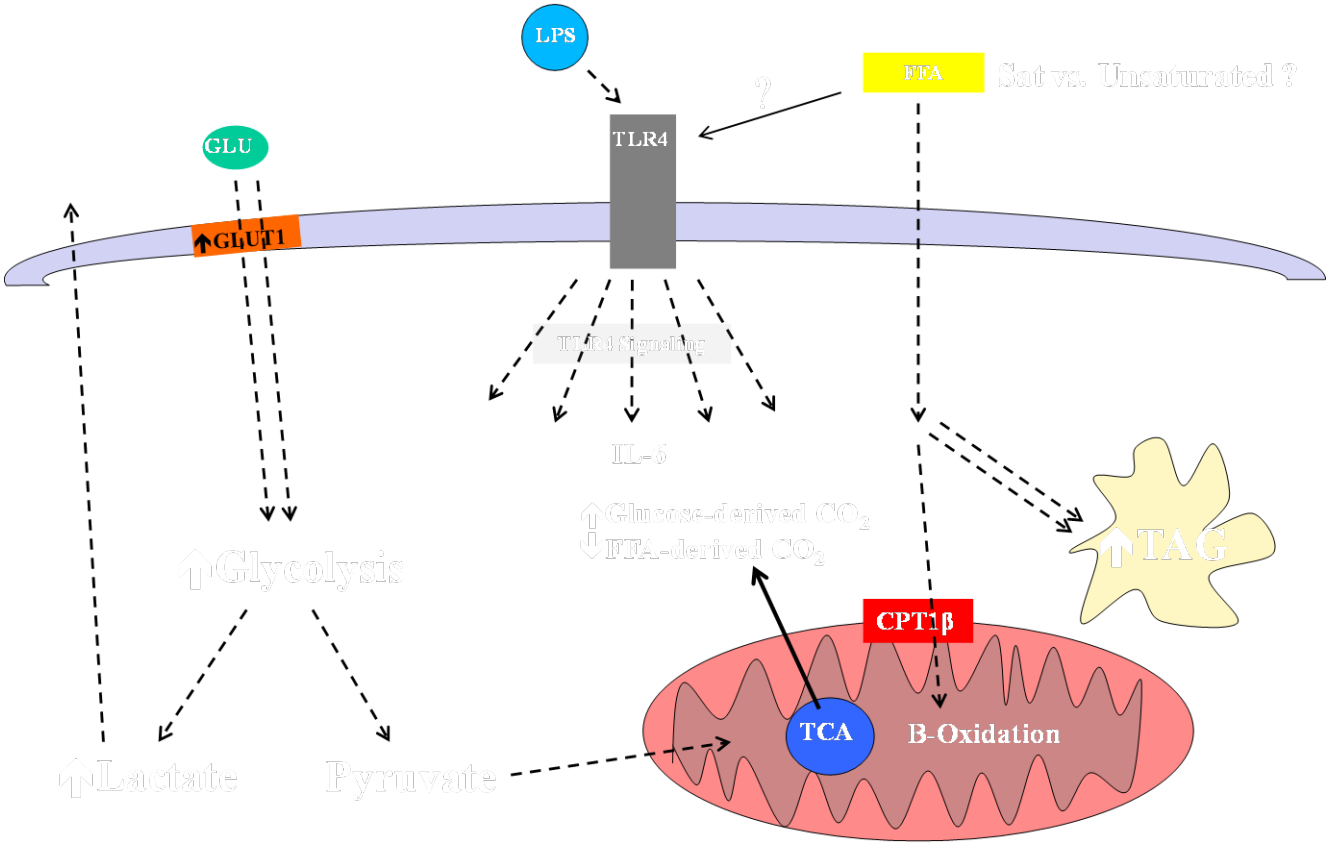


Supplemental Data

Table 1. Dietary Composition of 10-week fed C57BL/6 mice.

Ingredient	Control-D12450B	HMF-D08091701	HSF-D08061904
Fat Source			
Butter	---	85g	245g
Lard	20g	---	---
Olive Oil	---	185g	---
Soybean Oil	25g	---	25g
Total	45g	270g	270g
Protein Source			
Casein	200g	200g	200g
L-Cystine	3g	3g	3g
Carbohydrate Source			
Corn Starch	315g		
Maltodextrin 10	35g	125g	125g
Sucrose	350g	69g	69g
FAT %	10	60	60
CHO %	70	20	20
PRO %	20	20	20
Saturated Fat %	25	30	60
Monounsaturated Fat %	35	60	30
Polyunsaturated Fat %	40	10	10

Figure 1. Schematic of TLR4 signaling.



Chapter 8: Implications/Future Directions

The central objective of this project was to elucidate the role of free fatty acids on TLR4 regulation of skeletal muscle metabolism in the context of obesity. Treatments in cell culture and high fat feeding studies in C57BL/6 mice were undertaken to assess the effect of fatty acids on TLR4 content and signaling. A secondary objective was to discern the role of the saturation nature of fatty acids on these effects.

We found that exposure of cultured myotubes to fatty acids increased both TLR4 mRNA and protein content in a saturation-dependent manner. This was associated with a heightened inflammatory response to LPS and a resultant exacerbation of TLR4-dependent alterations in lipid and glucose metabolism. Sensitization to LPS was also evident in mice fed a high saturated fat diet. LPS challenge in these animals resulted in a more robust inflammatory response compared to control animals despite no changes in skeletal muscle TLR4 expression or protein levels. It was hypothesized that chronic exposure to saturated fat would exacerbate the response to LPS via upregulation of TLR4 transcript and receptor protein content. Although we observed a heightened sensitization to LPS in response to saturated fatty acids this effect was not due to increased receptor content suggesting an alternate mechanism(s). One such mechanism may involve differential regulation of TLR4 trafficking and cellular localization. Perhaps, saturated fatty acids merely affect TLR4 translocation from the ER to the plasma membrane, altering the cytosolic/surface ratio of TLR4, and thus making more of the receptor readily available to recognition by LPS. Experiments are currently underway in our laboratory using immunohistochemistry staining techniques and membrane isolation in skeletal muscle tissue to investigate this question.

Widely documented is the association of obesity with chronic, low grade inflammation. It was under this premise, along with gene array data from our laboratory indicating elevated TLR4 levels in obese humans, which prompted the studies performed herein to better understand how lipotoxicity influences the inflammatory response in skeletal muscle. However, obesity is characterized not only by hyperlipidemia but also

hyperinsulinemia. It is possible that chronic high levels of circulating insulin either directly or indirectly interact with the TLR4 receptor. At the time of these studies, there is no evidence that insulin influences TLR4 action in skeletal muscle; however, hyperinsulinemia is associated with increased cytokine production and inflammatory responses either or both of which may influence TLR4 expression.

In addition, heat shock proteins (HSP) have been shown to bind TLR4 and there is evidence suggesting that HSP-60 and HSP-70, in particular, are elevated in obese humans with BMI>30kg/m^2. In agreement with this, a gene microarray conducted in our laboratory comparing nonobese and obese skeletal muscle confirm that HSP-70 expression is significantly higher in obese humans compared to their lean counterparts. The role of these stress-induced proteins in TLR4 regulation with obesity is unclear and may be another area worthy of study.

Future studies are also planned to determine the mechanisms whereby TLR4 exhibits its metabolic effects. One possible explanation for an LPS-stimulated increase in glucose oxidation is regulation by hypoxia-inducible factor 1 alpha (HIF1). Studies performed in our laboratory have shown that the use of a HIF1 inhibitor abolishes the TLR4 dependent effect on glucose oxidation and mediates the concomitant decrease in fatty acid oxidation. Further studies are planned to expand upon these findings.

Chapter 9: Appendix

IACUC Approval Letter

VirginiaTech

Institutional Animal Care and Use Committee
Josep Bassaganya-Riera, Chair
2000 Kraft Drive, Suite 2000 (0497)
Blacksburg, Virginia 24061
540/231-7421
Email: jbasaga@vbi.vt.edu
www.acc.vt.edu

PHS/OLAW Assuramce A-3208-01
USDA Research Facility 52-R-0012

October 29, 2008

Approval date: 10/28/2008
Expiration Date: 10/27/201

MEMORANDUM

TO: Matthew Hulver

FROM: Josep Bassaganya-Riera

SUBJECT: Review of Protocols Involving Animals

The purpose of this memo is to verify that the Virginia Tech Institutional Animal Care and Use Committee has reviewed and granted approval of protocol **#08-228-HNFE**, entitled **"The effects of a high lipid enviroment on pro-inflammatory pathways in skeletal muscle."**, submitted by **Matthew Hulver**. The funding source for this protocol is NIH (OSP# 431509).

Period of Approval

This research protocol is approved for a period of three years, from **October 28, 2008 to October 27, 2011**. If the research experiments offered under this protocol will be conducted on a continuing basis throughout the three-year approval period, the protocol must undergo continuing review on an annual basis. In such cases, the principal investigator must submit an annual continuing review form prior to the one-and two-year anniversaries of the approval date. If the reseaarch conducted under this protocol will continue to be conducted after the end of the three-year approval period, a new protocol must be submitted and approved prior to the three-year anniversary of the original approval date. The principal investigator is responsible for submitting all paperwork required to maintain IACUC approval.

Changes to Approved Protocols

Any changes in animal numbers, species, procedures/treatments, or pain category must be submitted to the IACUC for review and approval **before** those changes are implemented. Failure to seek IACUC approval for amending approved protocol procedures may result in withdrawal of permission to conduct the research.

Federal Compliance Assurance

All proposals involving the use of living vertebrates are reviewed by the Virginia Tech Institutional Animal Care and Use Committee to assure humane care and treatment of the animals involved. Approved proposals comply with:

1. "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training"
2. The Animal Welfare Act, As Amended
3. The Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals
4. "Virginia Tech Policies Governing the Use of Animals in Research and Teaching"

Virginia Tech has a written, approved Animal Welfare Assurance on file with the PHS Office of Laboratory Animal Welfare (OLAW). The university's Animal Welfare Assurance number is A-3208-01, expiration date 3 -31-2010.