The role of Toll-like Receptor 4 in the Modulation in Skeletal Muscle Metabolism

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Abstract

Toll-like receptor 4 (TLR4) is a transmembrane receptor, which upon activation by lipopolysaccharide (LPS) from Gram-negative bacteria, plays an important role in the induction of the innate immune response. Our lab has previously demonstrated that activation of TLR4 in skeletal muscle results in the preferential oxidation of glucose for ATP production over that of fatty acids. Currently, the exact mechanism(s) for TLR4-induced modulation of metabolism are not known.

The purpose of this project was to test the hypothesis that activation of TLR4 pathway causes increased ROS production, which contributes to deceased fatty acid oxidation and altered mitochondrial respiration in skeletal muscle. To this end, skeletal muscle cells were studied following acute and chronic treatments with LPS, and a mouse model with muscle-specific over expression of TLR4 (mTLR4) was studied under chow fed conditions and following 16 weeks of high fat feeding.

Acute LPS treatment of C2C12 cells resulted in mitochondrial uncoupling as evidenced by higher levels of state IV respiration, reduced maximally simulated respiration, and a robust induction of uncoupling protein 3. These observations occurred in conjunction with increased pyruvate dehydrogenase activity. The LPS-induced changes in substrate preferences and maximally-stimulated mitochondrial respiration were prevented in the presence of the antioxidants, N-acetyl-L-cyteine (NAC) and catalase. Using isolated

flexor digitorum brevis (FDB) muscle fibers from C57BL/6J mice, we showed that LPS treatment results in significant increases in ROS production that are evident at 15 min and still increasing at 45 min following the addition of LPS to incubation media. Hyperpolarization of mitochondrial membrane potential was also evident at 15 min post LPS treatment in FDB fibers.

Fatty acid oxidation measured in skeletal muscle whole homogenates from the mTLR4 mice was significantly reduced compared to wild-type littermates on a standard chow diet. Following a 16 week high fat diet, the mTLR4, compared to wild-type mice, gained more weight and fat mass, were glucose intolerant, and displayed elevated production of mitochondrial-derived reactive oxygen species (ROS) from complex III.

In conclusion, these data show that TLR4 activation elicits a change in mitochondrial substrate preference in that acetyl-CoA derived from pyruvate oxidation is the preferred substrate for the TCA cycle over that derived from β-oxidation of fatty acids. These data also lend strong support to the idea that the TLR4-mediated change in substrate preference is dependent on the production of ROS.

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Chapter 1: Introduction

Obesity and metabolic disorders, including insulin resistance and type 2 diabetes mellitus, are tightly associated with inflammation, production of reactive oxygen species (ROS), and oxidative stress. Mitochondria are both a source and target of ROS, which are generated as a natural byproduct of metabolic reactions and can cause subsequent damage to respiratory chain complexes and lipid membranes located within the organelle.

Toll-like receptors (TLR) are transmembrane receptors that, upon activation, play an important role in the induction of the inflammatory response. Toll-like receptor 4 (TLR4), originally discovered as the receptor for lipopolysaccharide (LPS) and its role in an innate immune response, has recently received increased attention for its role in high fat diet-induced obesity and insulin resistance. Our laboratory has shown that TLR4 expression is elevated in skeletal muscle of obese humans, and upon activation with LPS results in increased glucose oxidation and decreased fatty acid oxidation. It is well documented that TLR4 activation in immune cells causes increased ROS production, which is a normal process to immune defense. However, aberrant ROS production in non-immune cells can lead to oxidative damage of DNA and proteins and subsequent cellular dysfunction. To date, it is not known if TLR4 activation in skeletal muscle causes increased ROS production and oxidative stress. The hypothesis of this project is that activation of TLR4 pathway causes increased ROS production, which contributes to deceased fatty acid oxidation and altered mitochondrial respiration in skeletal muscle.

Chapter 2: Review of Literature

1. Introduction

Metabolic syndrome is a major public health burden affecting a substantial number of people worldwide. The increased prevalence of obesity is tightly associated with metabolic syndrome, and obesity contributes to the development of a variety of acute and chronic diseases, such as cardiovascular disease, insulin resistance, diabetes and cancer.

Skeletal muscle is the predominant site of whole body substrate disposal, and therefore, is primarily responsible for the metabolic disorders that accompany obesity. In recent years, an abundance of evidence has emerged demonstrating a close link between obesity and a state of chronic low-level inflammation. Toll-like receptors are transmembrane receptors that play an important role in promoting proinflammatory responses in skeletal muscle. Additionally, increased reactive oxygen species (ROS) production in mitochondria is a widely accepted participant in the development and progression of insulin resistance and its complications.

This review aims to summarize the most recent information on the molecular and cellular underpinnings of inflammatory mediated oxidative stress and metabolic derangements with a focus on the area of obesity and metabolic syndrome.

2. Metabolic Syndrome

2.1 Definition of Metabolic Syndrome

Metabolic syndrome is a common metabolic disorder that is linked to obesity and increased cardiovascular disease and other health problems such as diabetes and stroke. It is identified by the presence of three or more of the following: enlargement of the waist

circumference, higher levels of arterial pressure, low density lipoprotein cholesterol and glycemia, and reduction of high density lipoprotein cholesterol [1].

Metabolic syndrome is also known as metabolic syndrome X, syndrome X, and insulin resistance syndrome [2]. According to the National Cholesterol Education Program's Adult Treatment Panel III report (ATP III), there are six risk factors that can lead to metabolic syndrome. The following list contains those factors that are generally accepted as being characteristic of this syndrome [3]:

- Abdominal obesity
- Atherogenic dyslipidemia
- Raised blood pressure
- Insulin resistance ± glucose intolerance
- Prothrombotic state
- Proinflammatory state

Currently, there is disparity in the definition of the metabolic syndrome depending on the source. The World Health Organization (WHO) [4], the European Group for the study of Insulin Resistance (EGIR) [5], the American Heart Association/National Heart, Lung and Blood Institute (AHA/NHLBI) [6] have all defined it differently. These various definitions include different factors and criteria, but most of them do agree on the essential components such as, glucose intolerance, insulin resistance, obesity, hypertension, and dyslipidemia.

2.2 Mechanisms underlying Metabolic Syndrome

Due to the complexity of the metabolic syndrome, the precise mechanisms and signaling pathways are not yet known. The most accepted and unifying hypothesis to describe the pathophysiology of the metabolic syndrome is insulin resistance [2].

Insulin resistance is the condition in which the cells of the body become resistant to physiological levels of insulin. Excess caloric intake and lack of physical exercise, combined with abdominal obesity, play a key role in the development of a pro-inflammatory insulin-resistant state that generates the clinical features of the metabolic syndrome [7]. Although abundant data place a greater priority on insulin resistance in the pathogenesis of the metabolic syndrome, the relationship between insulin resistance and the symptoms of metabolic syndrome is complex.

Insulin resistance and obesity are closely associated; most people with categorical obesity (body mass index [BMI] $\geq$ 30 kg/m^2) have postprandial hyperinsulinemia and relatively low insulin sensitivity [8]. However, it had also been reported that 16% of insulin resistant patients were of normal weight (BMI < 25 kg/m2) [9]. Nevertheless, the absence of insulin resistance in a substantial proportion of patients with the metabolic syndrome indicates that other important causative factors exist [10]. Besides insulin resistance, obesity may be viewed as another factor that is responsible for the rising prevalence of metabolic syndrome by contributing to the development of hypertension, dyslipidemia, and insulin resistance [11].

Beyond insulin resistance and obesity, there are still other factors that also directly or indirectly contribute to the development of metabolic syndrome, such as ageing, chronic

inflammation, and hormonal changes [11, 12].Therefore, the mechanisms that lead to the metabolic syndrome are complex and require further clarification.

2.3 Studies relating Metabolic Syndrome with Obesity and Other Disease

The metabolic syndrome is a major public health burden due to its high prevalence in the general population and its association with other disease such as diabetes, cardiovascular disease, and cancer. The frequency of metabolic syndrome among US adults is approximately 22%, which increases with age from 6.7% in the 20 to 29 y of age range to 43.5% in the 60 to 69 y of age range [12].

Metabolic syndrome is present in a majority of obese patients with type 2 diabetes [13]. The study [14] reported that men who met the WHO definition of the metabolic syndrome in which adiposity was defined as waist-hip ratio > 0.90 or body mass index ≥ 30 kg/m^2 had a nearly nine-fold greater likelihood of developing diabetes than men without the metabolic syndrome. This finding is similar to the report which indicates that a large fraction of persons who developed type 2 diabetes had the metabolic syndrome during the past 8 years [15].

Since two components of metabolic syndrome, hypertension and dyslipidemia, have been shown to be the main predictors of cardiovascular disease, it is not surprising that the metabolic syndrome has a strong association with cardiovascular disease. The meta-analysis from the Galassi group indicated that individuals with the metabolic syndrome have a 61% increased risk of cardiovascular disease compared to individuals without the metabolic syndrome [16]. The prevalence of metabolic syndrome has increased with the

increasing incidence of not only diabetes and cardiovascular disease, but also various types of cancers such as breast and colon cancers [17, 18].

These findings highlight the value in understanding the mechanism(s) of metabolic syndrome, as this could lead to proper and aggressive therapeutic interventions to prevent further progression in associated disease states.

3. Obesity-Epidemiology, Association or Connection with Diseases

3.1 Obesity Epidemiology

In 1948, obesity was first included in the international classification of diseases [19]. From then on, the epidemic of overweight and obesity has spread all over the world and it appears this trend will continue in almost all countries (WHO, 2007).

According to the WHO (2009), the prevalence of overweight and obesity is usually assessed by using body mass index (BMI), defined as the mass in kilograms divided by meters squared (kg/m^2). A BMI over 25 kg/m^2 is defined as overweight. The definition of obesity is a very high amount of body fat in relation to lean body mass, or BMI of 30 kg/m^2 or higher. Globally, there are more than 1 billion adults that are overweight and at least 200 million of them who are obese (WHO, 2003). The prevalence of obesity is not only a serious health concern among adults, but also affects children. In 2007, it was estimated that globally 22 million children under 5 years of age were overweight [19].

The rapid increase in obesity prevalence is evident in most developed countries, especially the USA. The rates of obesity have more than doubled in less than 50 years in the United States [20]. The data collected through the Centers for Disease Control and Prevention (CDC)'s Behavioral Risk Factor Surveillance System (BRFSS) shows that, in

1985, eight states had a prevalence of obesity between 10-14%, and no states had a prevalence equal to or greater than 15%. By 2008, only one state (Colorado) had a prevalence of obesity less than 20%. Thirty-two states had prevalences equal to or greater than 25%; six of these states (Alabama, Mississippi, Oklahoma, South Carolina, Tennessee, and West Virginia) had a prevalence of obesity equal to or greater than 30%.

3.2 The role of obesity in the pathogenesis of insulin resistance

Insulin resistance is a state in which physiological levels of insulin are inadequate to produce a normal insulin response in target organs, such as adipose tissue, liver, and muscle. It is well established that obesity is associated with insulin resistance and is a major causative factor for this disorder [21]. However, the pathogenesis of obesity-induced insulin resistance has not been fully elucidated.

Evidence from several studies suggested that circulating free fatty acids play an important role in the development of insulin resistance in the obese state. Plasma free fatty acid (FFA) levels are elevated with obesity [22-24]. Although it has been established that elevated blood FFA levels can inhibit insulin action in multiple tissues including skeletal muscle, liver, adipose tissue, and vascular endothelial cells, it is not entirely clear why FFAs are elevated in obese people and how they disrupt the actions of insulin.

To address this question, Dresner et.al measured insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase (PI 3-kinase) activity in muscle biopsy samples. They found that increased concentrations of plasma FFAs induce insulin resistance in humans through inhibition of glucose transport activity, and the inhibiton may be caused by decreased IRS-1 dependent PI 3-kinase activity [25]. Activation of PKC-θ may also

contribute to IκB kinase (IKK) and c-Jun N-terminal kinase (JNK) activation by FFAs; moreover, IKK and JNK mediate protein kinase (PKC)-θ signals for IRS-1 serine phosphorylation and degradation. [26]. Elevated plasma FFAs during a euglycemic-hyperinsulinemic clamp was associated with increases in intracellular fatty acyl-CoA and diacylglycerol (DAG) concentrations, which are known to contribute to activation of PKC-θ [27]. PKC is a serine/threonine kinase which leads to decreased IRS-1 tyrosin phosphorylation and decreased activation of IRS-1 associated PI3-kinase activity resulting in reduced insulin-stimulated glucose transport activity, thereby causing insulin resistance [28].

Emerging data indicate that several adipocyte-derived cytokines or hormones are also involved in obesity-induced insulin resistance. Adipose tissue it is now considered a very active endocrine organ in addition to its role for energy storage [29]. As an endocrine tissue, adipose tissue secretes different kinds of cytokines (adipocytokines) into the circulation which have a variety of biological functions, including the regulation of energy balance, lipid and glucose metabolism, insulin secretion and activation of proinflammatory pathways (Fig. 2.1).

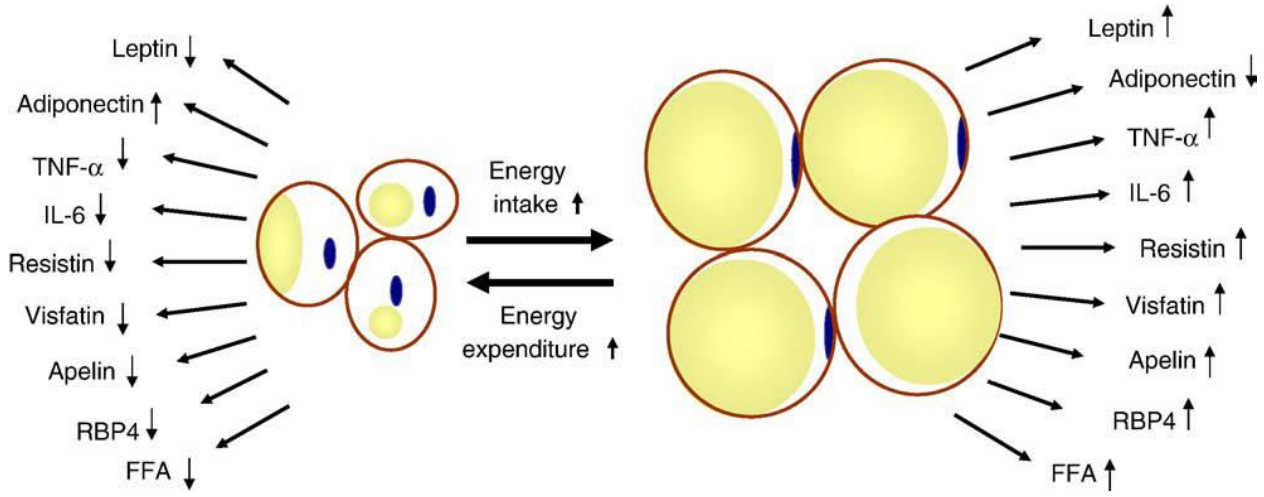

Figure 2. 1 An overview of secretion of adipocytokines in adipose tissue under normal and obesity conditions (used with permission)[30].

Elevated blood levels of several adipocytokines, including TNF-α, interleukin-6 (IL-6), and resistin have been observed in obese humans and several have been postulated to be responsible for the obesity-associated increase in insulin resistance [31, 32].

Since plasma FFAs have recently been shown to activate inflammatory pathways and to raise plasma adipocytokines levels, Boden has postulated that elevated plasma adipocytokines levels in obesity could be a consequence of elevated plasma FFA levels [33].

3.3 The associations between obesity and other disease states

The worldwide epidemic of obesity, boosted by the modern lifestyle characterized by a lack of physical activity and an energy-dense diet, is associated with an increase in all

cause mortality [34]. It is estimated that there are over 100,000 excess deaths annually caused by obesity in the United States [35].

There are numerous diseases associated with obesity that contribute to excess mortality. For example, obesity is a major risk factor for type 2 diabetes. Obese adults are at increased risk of adverse metabolic outcomes including increased blood pressure, cholesterol, triglycerides, and insulin resistance (WHO, 2009). Obesity is also associated with hypertension and heart disease, which could cause serious morbidity [36, 37].

The other major disease group associated with obesity is cancer. Several studies indicated that there is an association between obesity and prostate cancer [38], gallbladder cancer [39], breast cancer [40], and colorectal cancer [41].

Obesity is also associated with a variety of gastrointestinal disorders. Central adiposity may be the most important risk for the development of acid reflux and related complications such as Barrett's esophagus and esophageal adenocarcinoma [42].

It is well established that the prevalence of obesity is increasing worldwide, especially in Westernized societies. Obesity is associated with multiple disease states and metabolic disorders. To date the precise mechanism(s) linking obesity to these disorders is not completely clear.

4. Inflammation, Obesity, Insulin resistance, and Substrate Metabolism

4.1 The connection between inflammation and obesity

In recent years, it has become clear that obesity is linked with a chronic state of inflammation which may contribute to the development of a variety of acute and chronic diseases, including metabolic syndrome, cardiovascular disease, diabetes, and cancer.

The link between inflammation and obesity was first addressed by Hotamisligil et al. in 1993, who found that the inflammatory cytokine tumor necrosis factor (TNF) – α was elevated in obese rats and contributed to the development of insulin resistance [43]. Similarly, serum levels of TNF- α were also increased in the adipose as well as muscle tissues of obese humans but were decreased after weight loss [44-46].

Recent studies have revealed there are a number of inflammation-related substances elevated in adipose tissue with obesity. Interleukin-6 (IL-6) is a proinflammatory cytokine secreted from multiple cell types including activated macrophages and lymphocytes [47]. It has been demonstrated that adipose tissue releases large amounts of IL-6 in vivo and the production of IL-6, as well as systemic concentrations, increase with adiposity [48]. Moreover, visceral adipose tissue released 2-3 times more IL-6 than subcutaneous adipose tissue [49], which indicated that the accumulation of visceral adipose tissue plays a key role in chronic inflammation in obese individuals.

C-reactive protein (CRP) is one of the acute phase proteins, the levels of which rise in response to systemic inflammation. It has been suggested that in obese individuals, CRP concentration was significantly correlated with BMI [50]. Furthermore, IL-6 secreted by adipocytes is one of the chief inducers of CRP production and stimulates hepatic synthesis of CRP in the obese state [47, 51].

Adipose tissue in obese subjects is characterized by macrophage infiltration and the accumulation of macrophage in adipose tissue is directly proportional to adiposity in both mice and humans [52, 53]. This discovery gives us a better understanding of the obesity-induced inflammation, since the presence of macrophage is a major characteristic of

inflammation [54]. Increased accumulation of proinflammatory T-lymphocytes are also present in visceral adipose tissue and associated with obesity [55, 56]. Therefore, obesity-induced inflammation appears to involve the disorders in both innate and adaptive immune systems.

There are other adipokines linked to inflammation in obesity. Adiponectin, an anti-inflammatory adipokine is a key mediator of obesity-induced inflammation, which is inversely associated with obesity [57]. Resistin, leptin and visfatin, which linked to proinflammatory response, are increased in adipose tissue with obesity [58- 60].

Elevated plasma free fatty acids (FFAs) levels are a distinguishing feature of obesity, infection, and other inflammatory states [61]. Free fatty acids activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in adipocytes and macrophages via toll-like receptor 4 (TLR4) and the induction of inflammatory response is weakened in the absence of functional TLR4 [62]. These findings indicate that FFA and TLR4 might act as a direct link between obesity and inflammation.

4.2 Inflammatory pathways to insulin resistance

The studies discussed above strongly suggest that obesity is associated with a state of chronic, low-grade inflammation. This inflammatory state is a major etiologic factor in the development of insulin resistance, impaired glucose tolerance and even diabetes. However, the molecular basis and physiological significance are not yet fully understood. Recent research has identified a link between specific inflammatory pathways and obesity-associated insulin resistance, which has provided important insights into the underlying mechanisms.

Insulin binding to its receptor induces a complex process including multiple pathways. After simulation of the insulin receptor, phosphorylation of tyrosine residues of insulin receptor substrate (IRS)-1 is a key step in the transmission of the insulin signal to downstream events [63]. Inflammatory pathways could interact with insulin signaling and inhibit signaling downstream of the insulin receptor, which then leads to insulin resistance [61].

The NF-κB pathway has been linked to the proinflammatory effects of obesity and insulin resistance [53]. As we discussed above, NF-κB is activated by FFAs. Palmitate has been shown to increase NF-κB gene and protein expression in skeletal muscle cells via phosphorylation of protein kinase C (PKC)-ε which causes defects in insulin signaling [64]. Other PKC family members are also activated by FFAs and may also involve inhibition of insulin signaling [65]. Another study demonstrated that TNF-α is correlated with protein expression of IκB kinase (IKK)-β to induce NF-κB activation; they also treated diabetic mice with IKK-β inhibitor, which contributed to lower plasma glucose levels and a reversal of whole-body insulin resistance [66]. Notably, inhibition of IKK-β prevents NF-κB activation and the subsequent production of numerous pro-inflammatory cytokines and chemokines.

Similarly to NF-κB, c-Jun N-terminal kinases (JNKs) are also activated by TNF-α, as well as by FFAs [67]. JNK1 knockout mice present with significantly improved insulin sensitivity and enhanced insulin receptor signaling capacity in liver, muscle and adipose tissue [68]. JNKs phosphorylate IRS-1at the ser-307 site thereby inhibiting tyrosine phosphorylation to prevent further transmission of the insulin signal in Chinese hamster

ovary cells [69]. These findings indicate that JNKs are a crucial component of the inflammatory pathway and responsible for obesity-induced insulin resistance.

4.3 Regulation of Inflammation pathways and substrate metabolism

The systemic effects of obesity induced inflammation are potentially regulated by multiple mechanisms. Sarah et al. suggested that Wnt pathway plays a distinct role in the regulation of inflammation and immunity and are also involved in the sustained inflammation in sepsis [70].

As discussed above, lipids stimulate inflammatory cascades and inhibit insulin signaling. However, the role of lipids in the regulation of inflammatory pathway is complex as they may also act in an anti-inflammatory capacity. The liver X receptor (LXR) is a lipid-dependent regulator of inflammatory gene expression. LXRs and their ligands inhibit the expression of inflammatory mediators and reduce inflammation in mice [71]. Joseph et al. confirmed that LXR agonists were effective inhibitors of IκBα, IL-1β, MCP-1, IL-6 and IL1-receptor antagonist expression.

To understand the role of sustained hyperglycemia on the response of proinflammatory mediators in sepsis, Jacob et al. measured the plasma levels of lactate, IL-6, IL-10 and endotoxin in rats. Their data suggest that pronounced hyperglycemia during sepsis exacerbates inflammatory responses in rats [72].

There are many other pharmacological manipulations which regulate inflammatory pathways by inhibition of inflammatory kinases and are agonists of relevant transcription factors [61]. The inhibition of inflammatory pathways is likely due to other relevant mechanism, which have yet to be elucidated.

5. Toll-like Receptors

5.1 What are Toll-like Receptors

Toll-like receptors (TLRs) are type I transmembrane proteins that function as pattern recognition receptors (PRRs) of the innate immune system that detect invasion of microbial pathogens [73]. To date, 10 different human TLRs have been identified (figure 2.2).

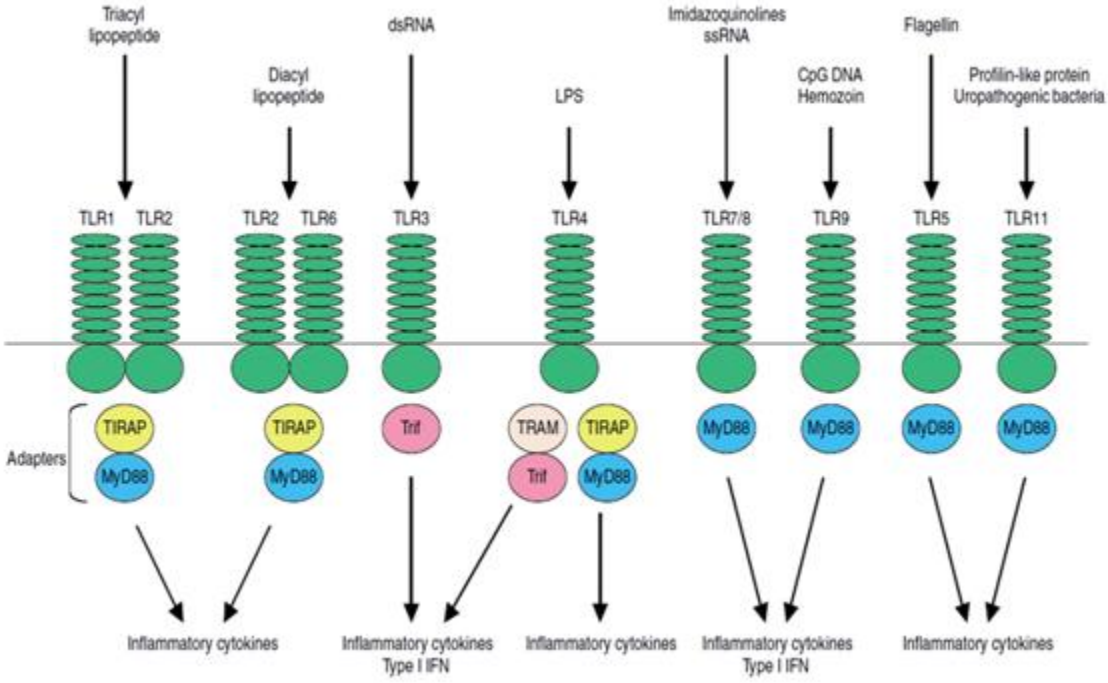

Figure 2. 2 Toll-like Receptors (used with permission) [74]

Toll-like receptor 4 is a member of the Toll-like receptor (TLR) family which plays a fundamental role in the innate immune system and pathogen recognition [75]. Toll-like receptor 4 has been characterized as the first mammalian TLR of Drosophila Toll [76]. TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria and is the LPS sensor in both mice and humans [77]. In addition, TLR4 is also involved in the

recognition of taxol and some endogenous ligands, but requires very high concentrations to be active [78].

5.2 TLR4 signaling

Toll like receptor 4 plays an important role in the induction of inflammatory responses by transcriptionally activating NF-κB. Both CD14 (cluster of differentiation 14) and MD-2 (the protein encoded by lymphocyte antigen 96) are required for TLR4-mediated recognition of LPS and induce the activation of the NF-κB signaling pathway [73].

TLR4 signaling involves an adaptor protein MyD88 (Myeloid differentiation primary response gene 88). Upon LPS recognition, TLR4 recruits MyD88 through interactions with the TIR (Toll-interleukin-1 receptor) domains [79]. Besides MyD88, there are other four TIR-domain containing adaptor proteins involved in the TLR4 signaling known as MAL (MyD88-adaptor-like), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule) and SARM (sterile α and HEAT-armadillo motifs-containing protein) [80].

Two pathways have been demonstrated to initiate downstream TLR4 signaling, namely the MyD88-dependent and MyD88-independent (TRIF-dependent) pathways (figure 5.2). In the MyD88-dependent pathway, after LPS binds to TLR4 and its co-receptors CD14 and MD-2, the TIR adaptor MAL recruits MyD88 to the receptor complex through a TIR-TIR homotypic protein interaction that leads to the auto-phosphorylation of IL-1R-associated kinase (IRAK) [81]. Upon activation, IRAK interacts with tumor necrosis factor (TNF)-associated factor 6 (TRAF6), leading to the stimulation of the kinase complex IκB kinase (IKK) which, in turn, phosphorylates inhibitor κB (IκB), and finally

activates NF-κB, which subsequently transcriptionally regulate proinflammatory cytokines, chemokines, and oncogenes [82-84].

In the MyD88-independent pathway TRIF, via a bridging adaptor TRAM, plays an important role in the delayed NF-κB response [85]. Yamamoto et al. [86] reported that mice deficient in both MyD88 and TRIF showed complete loss of NF-κB activation in response to TLR4 stimulation, which demonstrated that TRIF is essential for the MyD88-independent pathway.

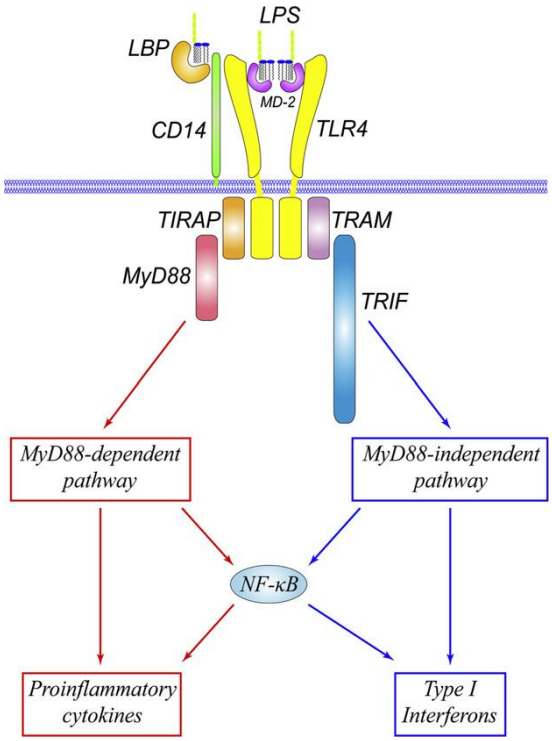

Figure 2. 3 MyD88-dependent and MyD88-independent pathways (used with permission) [79]

5.3 The Role of TLR4 in Insulin Resistance in Skeletal Muscle

Growing evidence indicates that obesity and insulin resistance are closely associated with inflammation. TLR4 is activated by LPS and plays a critical role in innate immunity and is linked to the development of insulin resistance. However, the cellular mechanisms by which TLR4 contributes to insulin resistance are not yet fully understood.

To directly address whether TLR4 is involved in insulin resistance, Song et al. examined the mRNA level of TLR4 in obese mice. They observed that during adipocyte differentiation the mRNA level of TLR4 was elevated and remarkably enhanced in fat tissues. Moreover, they found that TLR4 activation in 3T3-L1 adipocytes provoked insulin resistance [87]. A similar finding was reported by Poggi et al., who used TLR4 mutant mice as an animal model and provided evidence that TLR4 inactivation prevented the development of insulin resistance in white adipose tissue in response to a high fat diet [88].

Since skeletal muscle is a major target tissue contributing to whole-body insulin sensitivity, it is important to know whether TLR4 is also functional in skeletal muscle. Indeed, there is a growing body of evidence in both animal and human studies that have demonstrated that abnormal TLR4 expression and signaling may contribute to the pathogenesis of insulin resistance in skeletal muscle [83, 89]. The effects of TLR4 inhibition/deletion on lipid-induced insulin resistance were investigated in rodent skeletal muscle. They found that the activation of NFκB was blocked by inhibiting TLR4 in L6 myotubes and that the absence of TLR4 in skeletal muscle protected against lipid-induced insulin resistance [90].

Since the modulation of TLR4 signaling by dietary fats may partly explain the observed connection between high fat dietary intake and insulin resistance. It is important to identify whether free fatty acids (FFAs) are the ligands of TLR4 responsible for promoting inflammatory signaling in these conditions. To determine whether FFAs activate TLR4 signaling in macrophages with endogenous TLR4 expression, Shi et al. isolated peritoneal macrophages from TLR4-knock-out and wild type mice and treated the cells with FFAs. They found FFAs potently induced TNF-α and IL-6 mRNA in wild type mice but not in TLR4-knock-out mice, which indicate that FFAs are capable of utilizing TLR4 signaling to induce an inflammatory response in macrophages [91]. Moreover, it was reported that saturated fatty acids (SFAs), but not unsaturated fatty acids, induce NFκB activation and expression of other inflammatory markers mediated through TLR4 in monocyte/macrophage cells [92]. However, a recent study found that SFAs alone did not stimulate TLR-dependent signaling pathways or expression of TLR-target genes in HEK-293 cells or in macrophages, endothelial cells, smooth muscle cells, adipocytes, skeletal muscle cells, and human peripheral blood mononuclear cells, which suggests there might be alternative mechanisms link dietary fat intake with TLR-associated pathologies [93].

In summary, a better understanding of the mechanisms responsible for abnormal TLR4 signaling in obese subjects may lead to new pharmacological treatments that could abolish this disorder and provide additional strategies for treating insulin resistance and type 2 diabetes.

6. Oxidative Stress/ROS, Mitochondrial Dysfunction and TLR4

6.1 Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS), also known as oxygen-free radicals (OFR), are an inevitable natural by-product of normal cellular metabolism and lead to the oxidation of lipids, nucleic acids, and proteins [94]. ROS are produced through a number of mechanisms both exogenously and endogenously. Since ROS are oxygen-containing molecules can either accept or donate a free electron, they are unstable and easily react with other molecules. Some of the most common ROS are hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\bullet}$) and hydroxyl radicals (HO•) [95]. ROS are classically defined as oxygen-containing radicals capable of independent existence with one or more unpaired electrons and it is now expanded to include reactive oxygen-containing compounds without unpaired electrons, such as hydrogen peroxide (H_2O_2) [96].

ROS present a paradox, since they are both beneficial and harmful to living organisms [97]. On one hand, ROS are important to essential cellular functions including cell signaling, growth factor response, and cell growth and apoptosis [98]. On the other hand, when present at high concentrations, ROS can cause severe damage to important macromolecules in cells, such as DNA, proteins and lipids, and therefore, contribute to the development of some prevalent disease and disorders such as cancer and cardiovascular disease (CVD) [99].

Normally, cells can defend themselves against ROS damage, as there are many defense systems that have evolved to prevent the accumulation of ROS and reduce their

damaging effects including various non-enzymatic molecules (glutathione, vitamins A, C, and E) as well as enzymatic scavengers of ROS (superoxide dismutases, catalase, and glutathione peroxide) [100]. However, these defense systems do not always work effectively and this yields a state which is commonly described as “oxidative stress”. Oxidative damage may arise from ROS overproduction and/or the attenuated ability for clearance of ROS by defense mechanisms.

Oxidative stress is thought to play a major role in the overall pathophysiology of aging and is associated with inflammation based chronic degenerative diseases [101]. Several amino acids are modified in their structure, such as sulphydryl oxidation and disulphide formation, by oxidizing reactions thus resulting in functional and structural damage to proteins [102]. Recent study shows that after the application of the natural antioxidant esculetin, which scavenges ROS, Chinese hamster lungs fibroblast cells were protected from lipid peroxidation, protein carbonyl, and DNA damage induced by H_2O_2 [103].

The development of cancer is associated with high oxidative stress. Lorkowska et al. confirmed that superoxide anion production is increased in oral squamous carcinoma cells and they also found that the oxidative stress generated by both NADPH oxidase and nitric oxide synthase may be related to the immune response [104].

Oxidative stress has been observed in both experimental hypertension and spontaneous hypertension in rodents [105]. The anti-hypertensive drug, Benidipine, was found to inhibit ROS production in human polymorphonuclear leukocytes. Moreover, it prevented high-salt induced oxidative stress and renal dysfunction in stroke-prone spontaneously

hypertensive rats [106]. However, the exact mechanism by which ROS regulate signaling molecules in hypertension remains unclear.

6.2 ROS production and mitochondrial dysfunction

A mitochondrion is a membrane-enclosed organelle involved in a range of vital cellular processes, such as energy production, apoptosis, pyrimidines biosynthesis, fatty acid metabolism, as well as calcium homeostasis [107]. One of the most important functions of mitochondria is oxidative phosphorylation for the generation of cellular energy [108]. Mitochondrial dysfunction could be induced by a numbers factors such as oxidative stress, genetic factors, mitochondrial biogenesis (or defects in biogenesis), and aging [109]. It was reported that aging causes rat heart mitochondrial complex I deficiency, which could result from ROS induced cardiolipin peroxidation[110]. Mitochondrial dysfunction is suspected to contribute to many diseases and disorders such as Parkinson's disease [111], cancer [112], Alzheimer's disease [113], insulin resistance [114], and cardiovascular disease [115].

The main ROS source under physiologic conditions in somatic cells is thought to involve electron leakage from the mitochondrial electron transport chain (ETC) during cellular respiration [116]. Although the intermembrane space and mitochondrial matrix have antioxidant factors to scavenge ROS and prevent oxidative damage, the mitochondria are thought to contribute about 90% of the ROS production even under normal conditions [117]. Evidence has implicated complex I and III of the electron transport chains as the predominant source of mitochondrial ROS production [118, 119].

Mitochondria are vulnerable to ROS. High levels of ROS in mitochondria may lead to oxidative phosphorylation impairment, which may result in increased ROS production, thus causing even more mitochondrial and cellar damage [120]. It was reported that increased intracellular ROS production contributed to mitochondrial dysfunction, which involved the impairment of mitochondrial activity and the depolarization of the mitochondrial membrane potential, and significant inhibition of proliferation in human hepato-blastoma G2 cells [121]. Moreover, Koppers et al. showed that disruption of mitochondrial electron transport flow in human spermatozoa resulted in generation of ROS from complex I or III; they also found that the induction of ROS on the matrix side of the inner mitochondrial membrane at complex I resulted in peroxidative damage to the midpiece and a loss of sperm movement, that could be prevented by antioxidant α-tocopherol [122].

6.3 Potential linking with insulin resistance

The data from Gonzalez's lab clearly show that women with polycystic ovary syndrome exhibit increased mononuclear cell derived ROS generation and P^{47phox} expression in response to physiologic hyperglycemia that is independent of obesity, which suggest that the resultant oxidative stress may contribute to a proinflammatory state that induces insulin resistance [123].

Since mitochondria are the primary cellular site for fatty acid oxidation and utilization, there is an increased interest on the role of altered mitochondrial function in skeletal muscle lipid accumulation and development of insulin resistance.

Recent evidence shows that saturated fatty acids, palmitic and stearic acids, decrease insulin-induced glycogen synthesis, glucose oxidation, and lactate production, and at the same time, decrease both mitochondrial hyperpolarization and ATP generation in C2C12 cells as well as primary cultures of rat skeletal muscle cells [124]. These results indicated that saturated free fatty acid-induced mitochondrial dysfunction is associated with impaired glucose metabolism in muscle cells. Magnetic resonance spectroscopy studies in humans demonstrated that reduced mitochondrial function may predispose individuals to intramyocellular lipid accumulation and insulin resistance [114]. In a human intervention study, researchers found insulin sensitivity was improved after weight loss, and also observed significant increases in skeletal muscle mitochondrial density, cardiolipin content, and mitochondrial oxidation enzymes [125].

However, whether mitochondrial dysfunction is a cause or consequence of insulin resistance is not yet clear. Data from Bonnard's lab indicated that mitochondrial dysfunction does not precede the onset of insulin resistance since no changes were observed in a prediabetic state, but rather is a complication of diet-induced ROS production in skeletal muscle in diabetic mice [126]. Another study found that feeding rats a high-fat diet caused muscle insulin resistance in concert with a gradual increase in muscle mitochondria by activating peroxisome proliferator-activated receptor (PPAR) δ [127]. This finding is counter to a study that demonstrated fewer mitochondria in offspring of insulin resistant parents that was attributed to decreased expression of nuclear-encoded genes that regulate mitochondrial biogenesis [128].

6.4 Role of TLR4 in ROS production

Reactive oxygen species are vital to the proper function of the innate immune system. TLR4 expression in human pulmonary artery endothelial cells was down-regulated by hypoxia at both the mRNA and protein levels, and this was thought to be due to a ROS-mediated decrease in activator protein 1 (AP-1) binding activity [129]. ROS are essential for bacterial killing and in this context are mostly generated from polymorphonuclear neutrophils (PMN) and macrophages in response to stimuli [130].

Recognition of bacterial components such as lipopolysaccharide (LPS) by Toll-like Receptor 4 (TLR4) is important in eliminating bacterial infection by the recruitment of multiple pro-inflammatory cytokines; however, the activation of TLR4 can also contributes to a serious human disease known as septic shock [131]. Recent data show that TLR4 and NAD(P)H oxidase (Nox)2 as well as Nox4 are essential to early LPS-induced ROS production and cell necrosis in human umbilical vein endothelial cells and this response is independent from pro-inflammatory cytokine synthesis [132]. Another study has shown that apoptosis signal-regulating kinase 1 (ASK1) is involved in TLR4-mediated mammalian innate immune responses. In ASK1-deficient mice, ASK1 was found necessary for the TLR4-mediated induction of proinflammatory cytokines but not through other TLRs, because activation of TLR4 but not TLR2 led to ROS production [133].

To further investigate the role of TLR4 in ROS production, Ryan et al. detected LPS-induced ROS generation in macrophage cells from mice that have a functional TLR4 receptor. However, in cells from mice that lack functional TLR4, LPS did not induce generation of ROS. These authors also found that antioxidants such as N-acetyl-L-

cysteine and dimethyl sulfoxide blocked LPS-induced IL-8 expression at both the protein and mRNA levels and also blocked the nuclear translocation of NF-κB, which suggested that ROS may regulate immune signaling through TLR4 via their effects on NF-κB [134]. Similar findings were also reported by Park et al., who demonstrated that a direct interaction of TLR4 with Nox4 is essential for LPS-mediated ROS production and NF-κB activation in HEK293T cells [135].

7. Skeletal Muscle Metabolism and ROS

7.1 Skeletal muscle metabolism in obesity and insulin resistance

Skeletal muscle constitutes the primary site of fatty acid and carbohydrate oxidation, is the largest insulin utilizing tissue in the body, and is implicated in the development of insulin resistance. Insulin resistance is associated with obesity and is a risk factor for the development of type 2 diabetes and cardiovascular disease. Therefore, it is important to have a better understanding of skeletal muscle metabolism in the obese and insulin resistance condition.

Skeletal muscle contains different types of fibers. The type I fibers (slow twitch fibers) are primarily suited for aerobic conditions, whereas type IIb fibers (fast twitch fibers; IIx in humans) are more dependent on anaerobic metabolism [136]. Compared to healthy controls, He et al. observed reduced oxidative enzyme activity and increased lipid content in muscle obtained from human obese and diabetic subjects [137].

It is well established that intramyocellular lipids accumulation is associated with skeletal muscle insulin resistance in both rodents and humans [138]. Elevated triglyceride content in insulin resistant muscle may contribute to increased diacylglycerol concentration,

which activates protein kinase C (PKC), and in turn, blunts the insulin signaling cascade by inhibiting tyrosine kinase activity of the insulin receptor substrate 1 [139, 140]. A study in human demonstrated that there is a defect in skeletal muscle fatty acid oxidation with extreme obesity which coincides with the accumulation of intramyocellular long-chain fatty acyl-CoAs [141]. Stearoyl-CoA desaturase 1 (SCD1), a lipogenic gene, is robustly up-regulated in skeletal muscle from extremely obese humans, which may contribute to reduced fatty acid oxidation [142]. Data also indicate that the defects in free fatty acid metabolism can be corrected by treatment with peroxisome proliferator-activated receptor-γ (PPARγ) agonists in human skeletal muscle cells in vitro, which may involve effects on both glucose and lipid metabolism [143]. However, the mechanisms responsible for PPARγ-mediated changes in skeletal muscle substrate metabolism are not yet known.

Obesity and type 2 diabetes are associated with changes in protein phosphorylation and gene expression in skeletal muscle. In a study conducted in streptozotocin-induced-diabetic mice, 235 genes were identified as changed in diabetes and half of them could be corrected toward normal after insulin treatment and euglycemic conditons [144]. Short-term consumption of a high-fat diet in humans, results in increased fatty acid translocase (FAT/ CD36) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) in human skeletal muscle, which indicate these fatty acid-sensitive genes encoding proteins are necessary for fatty acid transport and β-oxidation in skeletal muscle [145].

7.2 Contribution of ROS production to insulin resistance in skeletal muscle

There is evidence that reactive oxygen species (ROS) production plays an important role in the development of insulin resistance. It has been reported that production of ROS by

NADPH oxidase activation is associated with the development of insulin resistance and is related to complications in vascular tissue [146]. There is also evidence that ROS production by NADPH oxidase is markedly enhanced by angiotensin II (Ang II) in L6 myotubes, and impairs insulin-induced activation of insulin receptor substrate 1 (IRS1), activation of protein kinase B (Akt), and glucose transporter-4 (GLUT4) membrane translocation in skeletal muscle cells [147].

To explore the potential mechanism of the linkage between ROS production and insulin resistance in skeletal muscle, Wei et al. employed transgenic rats with elevated tissue Ang II levels. Compared with control rats, the transgenic rats exhibited significantly increased NF-κB activation and TNF-α expression, which were dependent on NADPH oxidase-derived ROS. The authors also showed that, NF-κB activation and nuclear translocation are required for Ang II-induced insulin resistance in skeletal muscle [148]. These findings provide solid evidence indicating that NF-κB plays an important role in Ang II/ROS-induced skeletal muscle insulin resistance. These findings warrant further investigation into the role of Ang II in mitochondrial-derived ROS.

Mitochondria play a central role in ROS production in most tissues [149]. MacLellan et al. demonstrate that mitochondrial ROS production decreased significantly with increased uncoupling protein (UCP) 3 expression in muscle cells which also resulted in increased fatty acid oxidation [150]. However, a recent study in human skeletal muscle biopsies demonstrates that the maximal capacity for mitochondrial ROS production was not increased in insulin resistant and type 2 diabetic participants [151].

In summary, this is an evolving field of research and further investigation is required to elucidate the role of ROS production in the development of insulin resistance in skeletal muscle.

7.3 Role of Exercise

Endurance exercise training induces many adaptations in skeletal muscle including increased capacity for oxidative metabolism of fatty acids and carbohydrates [152]. The adaptations of mitochondrial enzymes in skeletal muscle occur rapidly in response to endurance exercise training in humans, as well as in rats [153]. However, the molecular mechanisms initiated by increased physical activity that enable the skeletal muscle adaptations are undoubtedly complex. The increase in fat oxidation after exercise is most likely associated with the genes involved in the regulation of fatty acid uptake across the plasma membrane (plasma membrane-associated FA-binding protein) and the mitochondrial membrane (beta-hydroxyacyl-CoA dehydrogenase) [152]. During prolonged exercise, gene expression of mitochondrial fusion and fission proteins in skeletal muscle respond rapidly to increased metabolic demand, which could significantly affect the efficiency of oxidative phosphorylation [154].

Skeletal muscle fibers continually produce reactive oxygen species (ROS) at a resting state and this production is increased substantially with contraction [155]. Muscular contraction has been shown to produce several specific ROS including nitric oxide, superoxide, hydrogen peroxide and hydroxyl radical [156].

ROS production in skeletal muscle may play an important role in affecting redox-regulated processes that control the adaptive responses during exercise. In a study of a

single period of contractile activity in mouse muscle, increased ROS production caused a rapid, transient reduction in muscle protein thiol content, increased protein content of heat shock proteins, and elevated activities of superoxide dismutase and catalase. All of which was blocked with pre-supplementation with vitamin C [157, 158]. These processes are modified during ageing and in some disease states, providing the potential that interventions affecting ROS production may influence muscle function or viability in these situations [159].

8. Conclusion

Increased efforts to control metabolic syndrome including obesity and insulin resistance constitute one of the main public health burdens today. Recent studies show that obesity is associated with a state of low-level chronic inflammation which is thought to contribute to the development of other metabolic disorders. Understanding the mechanism(s) that link obesity with inflammation and insulin resistance will open novel strategies for treatment and prevention of metabolic syndrome. Multiple pathways have been implicated in the development of insulin resistance, and increased production of ROS appears to plays an important role in the pathogenic process. However, the precise mechanisms that contribute to inflammatory-mediated ROS production and subsequent insulin resistance remain unclear.

Chapter 3: Specific Aims

SPECIFIC AIM 1: Test the hypothesis that acute and chronic inflammation in cultured skeletal muscle cells results in mitochondrial dysfunction, which is dependent on the production of reactive oxygen species.

Objective 1: To determine the effects of acute and chronic inflammation via TLR4 activation on the production of reactive oxygen species, mitochondrial respiration, and mitochondrial oxidative capacity.

Objective 2: To demonstrate that mitochondrial dysfunction in response to TLR4 activation is dependent on the production of reactive oxygen species.

SPECIFIC AIM 2: Test the hypothesis that muscle-specific over-expression of TLR4 in transgenic mice results in decreased fatty acid oxidation, increased production of reactive oxygen species and mitochondrial dysfunction in skeletal muscle.

Objective: To determine the effects of increased TLR4 expression on fatty acid oxidation, production of reactive oxygen species, and mitochondria function in skeletal muscle.

SPECIFIC AIM 3: Test the hypothesis that muscle-specific over-expression of TLR4 exacerbates the effects of high fat feeding on fatty acid oxidation, production of reactive oxygen species and mitochondrial function.

Objective: To determine the effects of increased TLR4 expression on fatty acid oxidation, production of reactive oxygen species, and mitochondria function following 16 weeks of high fat feeding.

Chapter 4: Research Design

SPECIFIC AIM 1: Test the hypothesis that acute and chronic inflammation in cultured skeletal muscle cells results in mitochondrial dysfunction, which is dependent on the production of reactive oxygen species.

Objective 1: To determine the effects of acute and chronic inflammation via TLR4 activation on the production of reactive oxygen species, mitochondrial respiration, and mitochondrial oxidative capacity.

Objective 2: To demonstrate that mitochondrial dysfunction in response to TLR4 activation is dependent on the production of reactive oxygen species.

Research Models:

Cell Culture: C2C12 muscle cells and human primary muscle cells were used to determine if acute and chronic inflammatory insult increases oxidative stress and results in mitochondrial dysfunction in skeletal muscle. C2C12 cells were purchased from the American Type Culture Collection (Manassas, VA). C2C12 cells are a stable muscle cell line derived from mice, which are capable of differentiation and have been used widely to study skeletal muscle function and metabolism [160-163]. C2C12 cells are used in Dr. Hulver's laboratory, routinely to establish mechanism(s), which are then translated to human primary muscle cells to establish physiological relevance in human skeletal muscle. Human primary skeletal muscle cells were obtained from non-obese (BMI < 25) and obese (BMI > 40) humans under an IRB protocol approved at the Pennington

Biomedical Research Center and Virginia Tech. Primary muscle cells cultured from non-obese humans were used in the experiments.

Experimental Strategy:

Acute and chronic LPS studies: C2C12 and human primary muscle cells were treated with either 50pg/ml (low dose) or 500ng/ml (high dose) of LPS from Escherichia coli 0111:B4 (Sigma, St. Louis, MO) for 2, 12, or 24 hours. Immediately following treatment, cells were collected. Measurements of mitochondrial function, substrate metabolism, and production of ROS and oxidative stress were assessed (Table 1).

NAC studies: C2C12 and human primary cells were treated with 500ng/ml of LPS in the absence and presence of 20mM N-acetyl-L-cyteine (NAC), a cysteine derivative and precursor in the formation of the anti-oxidant glutathione, to determine if the effects of inflammatory activation in skeletal muscle cells are dependent on the production of ROS. Measures were assessed as described in Table 4.1.

Table 4.1 Endpoint Measures for cell culture Studies

Measurement
• Respirometry of skeletal muscle cells or isolated mitochondria was performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). • Fatty Acid Oxidation. • Glucose Oxidation. • Gene expression analysis of ATP Synthase, Catalase, Drp1, IL-6, MCP1, Mitofilin, NFkB, OPA1, SOD2, and UCP3. • Western Blot to measure protein contents of ATP Synthase, Catalase, Drp1, Mitofilin, Mitofusin2, OPA1, SOD2, Thioredoxin, and UCP3. • Enzyme Activity of citrate synthase was performed spectrophotometrically. • Mitochondrial DNA copy number.

NOTES:

- Catalase is a H_2O_2 scavenger.
- Drp1 (Dynamin-related protein 1) is a key regulator of mitochondrial fission.
- IL-6 (Interleukin-6) is a pro-inflammatory cytokine.
- MCP 1 (Monocyte chemotactic protein-1) plays a role in the recruitment of monocytes to sites of injury and infection.
- Mitofilin, also known as mitochondrial inner membrane protein, is important in mitochondrial morphology and regulates cristae formation.
- Mitofusin 2 is a regulator of mitochondrial fusion.
- NF-kB (Nuclear Factor-KappaB) is a transcription factor that controls gene expression involved in immune and inflammatory responses.
- OPA1 (optic atrophy type 1), dynamin-related GTPase required for mitochondrial fusion and regulation of apoptosis.
- SOD2 (Superoxide dismutase 2) scavenges superoxide radical.
- Thioredoxin is one of a class of small redox proteins that act as antioxidants.
- UCP3 (Mitochondrial uncoupling protein 3), a member of mitochondrial anion carrier proteins, is an uncoupling protein.

SPECIFIC AIM 2: Test the hypothesis that muscle-specific over-expression of TLR4 in transgenic mice results in decreased fatty acid oxidation, increased production of reactive oxygen species and mitochondrial dysfunction in skeletal muscle.

Objective: To determine the effects of increased TLR4 expression on fatty acid oxidation, production of reactive oxygen species, and mitochondria function in skeletal muscle.

Research Models:

Animal Models: C57BL/6 mice with muscle-specific over-expression of the TLR4 gene were used for the experiments proposed in Specific Aim 2. This model demonstrates increased expression of IL-6, a marker of inflammation, in skeletal muscle compared to wild type controls. Animal studies were performed under an approved protocol by the IACUC at Virginia Tech. Immediately following a 12 hour fast, the animals were sacrificed using carbon dioxide asphyxiation. Skeletal muscle was harvested (gastrocnemius and quadriceps). Mitochondria were isolated from red and white muscle individually for measures of mitochondrial respiration, fatty acid oxidation, ROS production and enzyme activity.

SPECIFIC AIM 3: Test the hypothesis that muscle-specific over-expression of TLR4 exacerbates the effects of high fat feeding on fatty acid oxidation, production of reactive oxygen species and mitochondrial function.

Objective: To determine the effects of increased TLR4 expression on fatty acid oxidation, production of reactive oxygen species, and mitochondria function following 16 weeks of high fat feeding.

High Fat Feeding Studies: C57BL/6 mice with muscle-specific over-expression of TLR4 and their wildtype littermates were fed either a control diet or a high fat diet for 16 weeks. Diets were designed and purchased from Harlan (Madison, WI) (Table 4.2).

Table 4.2 Formula of Teklad Diets

Formula	TD. 10453 Lower Sucrose Control Diet (10% Fat Kcal) (g/Kg)	TD. 10505 45% Fat Kcal Diet (9% Sucrose) (g/Kg)
Casein	185	236
L-Cystine	3	3.6
Corn Starch	485.25	225
Maltodextrin	100	115
Sucrose	90	90
Cellulose	47.15	42.35
Lard	20	195
Soybean Oil	20	30
Mineral Mix, AIN-93G-MX (94046)	35	44.8
Calcium Phosphate, dibasic	2	2.6
Vitamin Mix, AIN-93-VX (94047)	10	12.8
Choline Bitartrate	2.5	2.75
Food Color	0.1 (Yellow)	0.1 (Red)

Body weight was recorded weekly during high fat feeding. At 16 weeks of high fat feeding, following a 12 hour fast, the animals were sacrificed using carbon dioxide asphyxiation. Skeletal muscle was harvested (gastrocnemius and quadriceps). Mitochondria were isolated from red and white muscle individually for measure of mitochondrial respiration, fatty acid oxidation, and ROS production.

Statistical Analysis

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 $P < 0.05$.

Chapter 5: Literature Cited

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Chapter 6: Manuscript

Toll-like Receptor 4 Modulates Skeletal Muscle Mitochondrial Substrate Preference in a Reactive Oxygen Species Dependent Manner

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Abstract

We have previously demonstrated that activation of toll-like receptor 4 (TLR4) in skeletal muscle results in increased reliance on glucose as an energy source under non-insulin stimulated conditions, and a concomitant decrease in fatty acid oxidation. Herein we examined the effects of lipopolysaccharide (LPS) on mitochondrial function and the dependence on the production of reactive oxygen species (ROS) in TLR4-mediated changes in substrate metabolism. To this end, studies were conducted using LPS, at concentrations synonymous with metabolic endotoxemia, as the TLR4 ligand. Pyruvate oxidation, mitochondrial respiration, ROS production and membrane potential were assessed. LPS exposure (50 pg/mL) resulted in a significant reduction in maximal (carbonyl cyanide 4-(trifluoro-methoxy) phenylhydrazone (FCCP)-stimulated) respiration and increased oligo-induced state 4 respiration in C2C12 cells, both of which suggest mitochondrial uncoupling. These observations were observed in conjunction with increased protein levels of UCP3 and pyruvate dehydrogenase activity. The LPS-mediated changes in substrate oxidation and maximal mitochondrial respiration were prevented in the presence of the antioxidants, NAC and catalase. LPS injections in C57BL/6J mice resulted in declines in respiratory control ratio, ADP-stimulated state 3, and FCCP-stimulated maximal respiration in isolated mitochondria. Finally, using isolated flexor digitorum brevis (FDB) muscle fibers from C57BL/6J mice, we showed that LPS treatment results in significant increases in ROS production that are evident at 15 min and still increasing at 45 min following the addition of LPS to incubation media. Hyperpolarization of mitochondrial membrane potential was also evident at 15 min post LPS treatment in FDB fiber. In conclusion, TLR4 activation in skeletal muscle alters

mitochondrial substrate preference in a ROS dependent manner. Based on the results reported herein, we speculate that TLR4 may be important in the process of glucose disposal under acute conditions of endotoxin exposure, but under chronic conditions may prove deleterious and contribute to metabolic inflexibility in skeletal muscle.

Key Words: Toll-like receptor 4, lipopolysaccharide, skeletal muscle, mitochondria, substrate metabolism

Introduction

Toll-like receptor 4 (TLR4) is a transmembrane protein integral to innate immunity [1]. Upon activation by lipopolysaccharide (LPS), TLR4 initiates an inflammatory response through a variety of downstream targets including interleukin 6 (IL-6) and monocyte chemotactic protein 1 (MCP1) [2]. Recently, TLR4 has been implicated in a number of diseases including type 2 diabetes and cardiovascular disease. Mutations resulting in a nonfunctional TLR4 receptor protect from obesity, insulin resistance, and cardiovascular disease in rodent models [3, 4]. In humans, polymorphisms in the TLR4 gene are associated with reduced incidence of heart disease and diabetes [3, 4]. We and others have observed increased expression of TLR4 in skeletal muscle from obese and type 2 diabetic individuals, relative to nonobese, healthy controls [5-7]. Furthermore, our group recently demonstrated that activation of TLR4 in skeletal muscle results in increased reliance on glucose as an oxidative substrate under non-insulin-stimulated conditions, and a concomitant decrease in fatty acid oxidation [6].

An emerging body of evidence supports a role for ROS in the development of diseases including obesity, type 2 diabetes and cardiovascular disease [8, 9]. ROS are not only byproducts of aerobic metabolism, but are integral to the immune response [10]. In fact, activation of TLR4 in immune cells results in the production of superoxide and other reactive oxygen species, which are required for downstream signaling [11, 12]. Whether TLR4 activation results in increased ROS production in skeletal muscle is currently unknown, as are the mechanism(s) responsible for TLR4-mediated modulation in substrate preference. The purpose of the studies presented herein was to test the

hypothesis TLR4 modulates mitochondrial substrate handling in a ROS-dependent manner.

Methods

Cell culture. C2C12 murine myoblasts were purchased from the American Type Culture Collection (Manassas, VA) and cultured as previously described [6]. Experiments were performed between days 4-6 of differentiation.

Animal studies. Studies were performed under an approved protocol by the Institutional Animal Care and Use Committee at Virginia Tech, and mice for all studies were maintained on a normal chow diet and a 12:12-h light-dark cycle. In the first study, 8-wk-old male C57BL/6J mice were injected with saline (control, n=2) or LPS (LPS, n=2; 1 μg/kg body weight, ~0.025μg per mouse) following an overnight fast. LPS from *Escherichia coli* 0111:B4 was used for all studies (L2630; Sigma-Aldrich, St. Louis, MO). Animals were sacrificed 4-hour post injection and respiration was measured in isolated mitochondria prepared from the red portion of gastrocnemius muscle. In the second study, mitochondria were isolated from the red portion of gastrocnemius muscle harvested from one C57BL/6J mouse. The mitochondria were then exposed to either saline, (n=3), 50 pg/mL LPS (n=3) for 1 hour while mitochondrial function was assessed. The purpose of the second study was to determine if LPS had direct effects on mitochondria function independent of TLR4 signaling.

Mitochondrial isolation from red gastrocnemius muscle. Mitochondria were isolated from red gastrocnemius muscle as previously described with modifications [13]. Tissue samples were collected in buffer containing 67mM sucrose, 50mM Tris/HCl, 50mM KCl,

10mM EDTA/ Tris, and 10% bovine serum albumin (all from Sigma-Aldrich, St. Louis, MO). Samples were minced and digested in 0.05% trypsin (Invitrogen, Carlsbad, CA) for 30 minutes. Samples were homogenized and mitochondria were isolated by differential centrifugation.

LPS treatment in cell culture studies. C2C12 muscle cells were treated 50pg/mL of LPS from Escherichia coli 0111:B4 (Sigma-Aldrich, St. Louis, MO) for 2, 12, or 24 hours, as described below. This dose was selected as it is synonymous with LPS levels reported in a state of metabolic endotoxemia [14] and our previous work [6] showed that this concentration resulted in a substrate preference for the oxidation of glucose over that of fatty acids following the above specified treatment time course in C2C12 cells.

Antioxidant treatment in cell culture studies. C2C12 cells were co-treated with either 50pg/mL of LPS with or without 20 mM of N-acetyl-L-cyteine (NAC) (A7250; Sigma-Aldrich, St. Louis MO) or 25 U/mL of Catalase (C1345; Sigma-Aldrich, St. Louis MO) for 2 hours. Cells were pretreated with Catalase for 30 minutes before the addition of LPS as previously described [15].

TLR4 antibody treatment in cell cutlure. C2C12 cells were co-treated with either 50 pg/mL of LPS with or without 10 μg/mL of TLR4 neutralization antibody (sc-13591, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours. Cells were pretreated with the TLR4 antibody for 30 minutes before the addition of LPS.

Mitochondrial respiration in cultured cells. Assessment of mitochondrial respiration in C2C12 muscle cells was performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA) as described by Gerencser et al with modifications [16].

Cells were seeded into 20 wells of a XF24 V7 microplate at a density of 5,000 cells per well. Cells were grown to 80% confluence and differentiated into myotubes. Experiments were conducted in serum free media on day 4 of differentiation immediately following treatments. Experiments consisted of 3-minute mixing, 2-minute wait, and 3-minute measurement cycles. Oxygen consumption was measured under basal conditions, in the presence of the mitochondrial inhibitors oligomycin (0.5 μM, O4876; Sigma-Aldrich, St. Louis, MO), rotenone (0.25 μM, R8875; Sigma -Aldrich, St. Louis, MO), and in the presence of the mitochondrial uncoupler, FCCP (0.3 μM, C2920; Sigma-Aldrich, St. Louis, MO) to assess maximal respiration. Mitochondrial energy metabolism can be divided into three modules, substrate oxidation, proton leak, and ATP turnover [17]. Changes in mitochondrial function will be reflected by a change in one or more of the three modules [18]. Through the use of various mitochondrial uncouplers and inhibitors, experiments were conducted to assess basal respiration rate, proton leak (oligomycin induced state 4 rate), ATP synthesis rate (basal rate- oligomycin rate), maximal respiration rate (FCCP stimulated, maximal capacity of electron transport chain and Kreb's cycle), coupling efficiency (ATP synthesis rate/ basal rate) and cellular respiratory control ratio (cRCR; FCCP rate/ oligomycin rate) to assess mitochondrial uncoupling and leakiness. Together, these parameters provide an overall assessment of mitochondrial function in cultured cells as well as provide insight into the cause of dysfunction. All experiments were performed at 37 ℃.

Respiration in isolated mitochondria. Respirometry measures of isolated mitochondria were performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Immediately following mitochondrial isolation and protein quantification,

mitochondria were plated on Seahorse cell culture plates at a concentration of 5 ug/ well in the presence of 10 mM pyruvate (P5280; Sigma-Aldrich, St. Louis, MO) and 5 mM malate (P5280; Sigma-Aldrich, St. Louis, MO). Experiments consisted of 25 second mixing and 4-7 minute measurement cycles. Oxygen consumption was measured under basal conditions, ADP (5 mM, Sigma-Aldrich, St. Louis, MO) stimulated state 3 respiration, oligomycin (2μM) induced state 4 respiration, and uncoupled respiration in the presence of FCCP (0.3 μM) to assess maximal respiration. Respiratory control ratio was calculated as the ratio of state 3 and oligomycin induced state 4 respiration. All experiments were performed at 37 ℃.

Fatty acid, glucose, and pyruvate oxidation. Palmitate ([1-14C]-palmitic acid), glucose ([U-14C]-glucose), and pyruvate ([1-14C]-pyruvate) oxidation were assessed in C2C12 cells as previously described [6, 19].

Confocal microscopy imaging for mitochondrial heterogeneity, membrane potential (ΔΨ), and ROS production. Muscle fibers from flexor digitorum brevis muscle (FDB) were harvested from C56Bl/6J mice as previously described [20]. Myofibers were enzymatically isolated in DMEM with 2% FBS, 1 μl/ml gentamicin (Invitrogen, Carlsbad, CA) and 2 mg/ml type I collagenase (C0130; Sigma-Aldrich, St. Louis, MO) for 1–3 h at 37 ℃. Myofibers were then plated on extracellular matrix (ECM; E1270; Sigma-Aldrich, St. Louis, MO)-coated imaging dishes (P35G-1.0-14-C; Matek, Ashland, MA)) and rested for 12 h before imaging.

Imaging experiments with FDB muscle fibers were conducted as previously described with modifications [21]. Mitochondrial heterogeneity experiments were performed on

plated FDB fibers using a Zeiss LSM 510 Meta microscope (Carl Zeiss, Oberkochen, Germany) with a 100× oil immersion objective. Fibers were stained with 7nM tetramethylrhodamine, ethyl ester, perchlorate (TMRE) and 1uM of mitotracker green (MTG), both from Invitrogen (Carlsbad, CA) for 30 minutes at 37°C. Fibers were then washed and TMRE was added and remained on the fibers during imaging. TMRE was excited with a 543-nm helium/neon laser and emission recorded through a band-pass 650-nm to 710-nm filter. Mitotracker green was excited using a 488-nm argon laser, and emission was recorded through a band pass 500- to 550-nm filter. The Nernst equation allows conversion of fluorescent intensity (FI) values, which reflect dye concentration distributions over the mitochondrial membranes, into absolute millivolt values. Further, an adapted Nernst equation was used to determine the SD of potentials derived at each and every pixel in an image, thereby giving a value of $\Delta\Psi$ heterogeneity [21]. Immediately following the addition of the stains, fibers were imaged, exposed to 50 pg/mL of LPS for 15 minutes, and imaging was repeated.

Intracellular ROS levels were measured in FDB fibers using 5-(and-6)-carboxy-2',7'-difluorodihydrofluorescein diacetate (carboxy-H2DFFDA, Molecular Probes, Invitrogen, Carlsbad, CA) as previously described with modifications [22]. Fibers were incubated with 10 μM of carboxy-H_2DFFDA for 30 minutes at 37°C prior to imaging. Fibers were then exposed for 400 ms, and 1 image was recorded 15 minutes post exposure. Fibers were then treated with 50 pg/mL of LPS in the absence or presence of NAC, and imaged at 30 and 45-minute time points. Imaging for intracellular ROS levels was conducted on a Nikon T2000-E inverted microscope with a 10x air objective to allow for maximum number of fibers per image. Images were taken using a 488 nm excitation filter and a

560 nm long pass emission filter. Mitochondrial ROS production occurs within seconds/ minutes and therefore 45 minutes is a sufficient time frame to assess ROS production directly due to LPS exposure.

Western blotting. Western analysis was performed using cell lysates harvested in Mammalian Cell Lysis Buffer (Sigma-Aldrich, St Louis, MO) as previously described [6, 19]. Blots were probed with primary antibodies against β-actin, caspase 3, thioredoxin, mitochondrial superoxide dismutase (SOD2), and catalase (all, Cell Signaling, Danvers, MA; 1:1000),

Mitochondrial isolation from cells for UCP3 protein quantification. Mitochondria were isolated from cell extracts following a 2 h treatment with 50 pg/mL of LPS as previously described with modifications [23]. Cells were washed, trypsinized to lift, and collected. Cells were then pelleted and resuspended in isolation buffer (0.3 M Mannitol, 0.2 mM EDTA, 10 mM HEPES, and 0.1% BSA, all Sigma-Aldrich, St. Louis, MO). The sample was homogenized and mitochondria were extracted by differential centrifugation. Western blotting was performed on isolated mitochondrial protein as described above. Blots were probed for uncoupling protein 3 (1 μg/ml; Abcam, Cambridge, MA) and were visualized as described above.

Mitochondrial DNA copy number. Mitochondrial copy number was assessed according to the method of He et. al. [24]. Immediately following 2 hours of 50 pg/ml of LPS exposure, DNA was extracted using a commercially available kit (Invitrogen, Carlsbad, CA). TaqMan primers and probes were designed using Primer Express version 2.1 and real-time PCR was carried out in an ABI PRISM 7900 sequence detector (Applied

Biosystems, Carlsbad, CA). Murine skeletal muscle genomic DNA copy number was measured at the UCP2 gene and mtDNA copy number was measured from the cytochrome C oxidase II (COX II) mtDNA gene. mtDNA copy number was calculated by the absolute value of ΔCt values between groups (control vs. LPS). Because amplification occurs exponentially (increasing twofold with each cycle of PCR), log base 2 of ΔCt is the copy number for each sample.

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

Acute TLR4 activation leads to mitochondrial uncoupling in C2C12 skeletal muscle cells. To determine the role of TLR4 activation on mitochondrial function, C2C12 myotubes were exposed to 50 pg/mL of LPS for 2 hours and oxygen consumption under basal, state 3, oligomycin-induced state 4, and FCCP-stimulated (maximal) conditions was measured using a Seahorse Bioscience Extracellular Flux 24 analyzer (XF24). An LPS dose of 50 pg/mL was used because we have previously shown this concentration to elicit changes in substrate oxidation in C2C12 cells [6]; and moreover, this concentration of LPS equates to levels reported under conditions recently termed 'metabolic endotoxemia', which have been observed following a high fat meal in humans or high fat feeding in mice [14, 25, 26]. Two hours of exposure to LPS resulted in a significant decline in cellular respiratory control ratio (cRCR) in (Figure 6.1A). These changes were a direct result of higher rates of respiration in the presence of oligomycin (inhibitor of ATP synthesis) and lower rates of FCCP-stimulated (mitochondria uncoupler) maximal

respiration (Figure 6.1 B & C). In conjunction with these changes in cellular bioenergetics, there was a significant induction of UCP3 protein content as measured in mitochondrial isolated from C2C12 cells under identical LPS treatment conditions (Figure 6.1 D). To determine the effects of chronic TLR4 activation on mitochondrial function, experiments were performed with 50 pg/mL of LPS for 12 and 24 hours. The more chronic treatment with LPS treatment resulted in a significant decline in FCCP-stimulated maximal respiration (12h: Control, 166.5±16.9 vs. LPS, 118.2±12.3, P=0.03; 24h: Control, 132.2±5.2 vs. LPS, 103.4.2±3.3, P=0.0001) with no significant effect on respiration in the presence of oligomycin or cRCR (data not shown).

These results show that acute treatment with LPS results in mitochondrial uncoupling; however, more chronic exposure to LPS (12 and 24 hours) only affects maximally stimulated mitochondrial respiration (via FCCP). These results imply that longer term TLR4 activation may be compromising mitochondrial function, whereas in the short term, the mitochondria are not dysfunctional per se, but more so responding to physiological cues (perhaps ROS production) which lead to uncoupling, subsequent increases in respiration, and ultimately, a switch to a more rapidly metabolized substrate for ATP production, e.g., glucose.

The effect of LPS on mitochondrial function in C2C12 cells is dependent on TLR4. As an approach to confirm that the effects of LPS on mitochondrial substrate handling were TLR4 dependent, C2C12 cells were exposed to 50 pg/mL of LPS for 2 hours with and without the TLR4 neutralizing antibody, MTS510 (Santa Cruz Biotechnology, Santa Cruz, CA). As a result, the LPS-induced decline in maximal respiratory capacity (via FCCP) was completely blocked with the neutralization of TLR4 (Figure 6.2). These

findings support our previous work showing that LPS-mediated changes in skeletal muscle substrate metabolism were prevented in mice lacking TLR4 function [6].

The TLR4 effect on metabolism in C2C12 cells is blocked by antioxidant treatment. To determine if TLR-mediated effects on mitochondrial function are dependent on the ROS production, FCCP-stimulated maximal respiration was assessed in C2C12 cells following 2 hours of exposure to 50 pg/mL of LPS in the presence and absence of NAC and catalase. The presence of both antioxidants prevented the LPS-induced decline FCCP-stimulated maximal respiration (Figures 6.3 A & B). To determine whether the TLR4-mediated effects on substrate metabolism were also dependent on ROS, fatty acid and glucose oxidation were assessed following 2 hours of exposure to 50 pg/mL of LPS with and without NAC. The LPS-induced changes in fatty acid oxidation and glucose oxidation were also blocked in the presence of NAC (Figure 6.3, C & D).

Pyruvate dehydrogenase activity is increased in C2C12 cells following TLR4 activation. [1-14C]-pyruvate was used as assess the activity of pyruvate dehydrogenase (PDH), the enzyme that catalyzes the oxidation of pyruvate resulting in the provision of glucose derived acetyl CoA to the TCA cycle. This is a direct measure of pyruvate dehydrogenase activity as the number one carbon of pyruvate is liberated as CO_2 in the oxidation of pyruvate to acetyl-CoA. Following a 2 h treatment of 50 pg/mL of LPS, PDH activity was significantly increased compared to controls (Figure 6.4). These data support our recent report that activation of TLR4 by LPS results in an increase in glucose oxidation [6] and suggest that increased PDH activity along with enhanced glucose uptake [6] are likely mechanisms for this affect.

The LPS-induced effects in C2C12 cells are independent of changes mitochondrial DNA copy number. Genomic DNA and mitochondria DNA copy numbers were assessed by measuring the content of uncoupling protein 2 (UCP2) and cytochrome C oxidase II (COX II), respectively, in DNA samples extracted from C2C12 following 2 hours of treatment with 50 pg/mL of LPS. mtDNA copy number was determined by calculating the ratio of the absolute value of ΔCt values of COX2 and UCP2. Not surprising due to the acute treatment, LPS had no effect on mitochondrial copy number (Figure 6.5).

TLR4 activation by LPS alters the bioenergetics in mitochondria isolated from skeletal muscle. As a follow-up to the cell culture studies, male C57BL/6J mice were injected i.p. with either saline or LPS (1 μg/kg body weight, ~0.025μg per mouse), euthanized 4h post injection, and mitochondrial function was studied in mitochondrial preparations isolated from red gastrocnemius skeletal muscle. Compared to saline controls, LPS resulted in significant declines in respiratory control ratio (RCR), ADP-stimulated state 3 respiration, and FCCP-stimulated maximal respiration (Figure 6.6, A-C). No effect on basal respiration or respiration in the presence oligomycin was observed (Figure 6.6 D & E).

Previous research has demonstrated that direct LPS exposure to isolated mitochondria resulted in significant reductions in state 3 respiration [27, 28]. However, these earlier studies were conducted using very high doses (>100 μg/mL) of LPS and therefore may have been toxic to the mitochondria. To test whether physiologically relevant doses of LPS directly altered mitochondrial function, mitochondria were isolated from male C57BL/6J mice and treated with 50 pg/mL of LPS. Direct LPS exposure to isolated mitochondrial had no effect on any aspects of mitochondrial respiration (Figure 6.7, A-E).

Furthermore, it has been established that in order for TLR4 signaling to be initiated by LPS, it must bind to the TLR4/MD2/CD14 receptor complex [29]. While, it is possible that LPS can be internalized into the cell, the LPS/TLR4/MD2/CD14 receptor complex remains intact throughout the internalization process. Thus, it is unlikely, in the context of an intact cell, that unbound LPS would ever directly interact with intracellular organelles, including mitochondria. As such, these results show that LPS-mediated declines in mitochondrial function are TLR4 dependent and not a function of LPS alone.

TLR4 activation by LPS results in increased mitochondrial membrane potential and ROS production. Based on the results reported above showing that the LPS-induced changes in mitochondrial function and substrate oxidation were ROS dependent, we directly measured mitochondrial membrane potential and ROS production in flexor digitorum brevis (FDB) muscle fibers isolated from a male C57Bl/6 mouse in response to treatment with 50 pg/mL LPS. We chose the isolated FDB muscle fiber model for these assays because it allows for greater ease in tracking the effects of LPS treatment in an individual muscle cell over time. In C2C12 cells, this was problematic due to high a degree of variability across cells in the permeability of fluorescent dyes and intense fluorescent background. Using the FDB muscle fibers, hyperpolarization of the mitochondrial membrane potential was evident at fifteen minutes after the addition of LPS to incubation media (Figure 6.8A). These changes in membrane potential were accompanied by significant increase in ROS production that were evident at 15 min and continued up to 45 minutes following the addition of LPS to incubation media (Figure 6.8B).

Assessing the heterogeneity of mitochondrial membrane potential across the population of mitochondria within a cell is a relatively new approach to assess mitochondrial health. In cell types other than skeletal muscle, increased heterogeneity has been associated with cell pathology. For example, mouse pancreatic beta cells display increased heterogeneity when they have been stressed with high concentrations of glucose and cardiomyocytes demonstrate increase heterogeneity following ischemia-reperfusion injury [21, 30]. We assessed mitochondrial heterogeneity in FDB muscle fibers in response to 50 pg/mL of LPS by calculating the standard deviation of the mean membrane potential of the mitochondrial population in the entire muscle fiber. We observed significant increases in the heterogeneity of mitochondrial membrane potential at 15 min after the addition of LPS to the incubation media (Figure 6.8C). Although the implications of these observations are not completely understood, Wikstrom et al have suggested that increased mitochondrial heterogeneity may suggest increased fragmentation of the mitochondrial network [31].

Discussion

Our group has previously reported that acute TLR4 activation in skeletal muscle results in the preferential oxidation of glucose over that of fatty acids. The purpose of the work described here was to assess the role of mitochondrial function in TLR4-induced modulation in skeletal muscle substrate handling. The important observations reported herein are that acute TLR4 activation results in increased ROS production, mitochondrial uncoupling, both of which occur in the context of with increased reliance on pyruvate as an oxidative substrate for the mitochondria. Moreover, the LPS-induced increases in glucose oxidation and FCCP-induced maximal respiration in the mitochondria are

prevented in the presence of the antioxidant NAC. Based on these findings, we speculate that acute TLR4 activation in skeletal muscle results in increased ROS production and mitochondrial uncoupling, which leads to the relieved inhibition of pyruvate dehydrogenase and subsequently, increased reliance on glucose as the preferred oxidative substrate for ATP production in the mitochondria.

It has been well established in non-muscle cells, that activation of TLR4 via LPS increases ROS production [32]. This response is a normal physiological event in immune cells [33]. Here, we report for the first time that the activation of TLR4 with LPS also results in the rapid production of ROS in skeletal muscle. The source of ROS production in immune cells has been predominantly linked to NADPH oxidase [34]. In the context of skeletal muscle, the predominant site of ROS production is not clear. However, based on the respirometry data in response to LPS reported here, which occurs in conjunction with robust increases in protein levels of UCP3, we speculate that the increase in ROS production is mitochondria derived. Future studies using inhibitors of either NADPH oxidase, xanthine oxidase, or the mitochondrial electron transport chain in combination with LPS treatment are warranted to gain further insight into the source of TLR4-mediated ROS production in skeletal muscle. Additionally, studies using quanosine diphosphate (GDP), a potent inhibitor of mitochondrial uncoupling, is a logical next step to determine if the observed changes in mitochondrial substrate preference is indeed dependent on increased activity of UCP3.

In addition to the production of ROS leading to mitochondrial uncoupling as antioxidant defense, it has also been shown that ROS stimulates glucose uptake in skeletal muscle [35]. It was reported that contraction-induced increases in ROS production and glucose

uptake in isolated mouse EDL muscles are reduced by 50% in the presence of the antioxidants NAC and ebselen [36]. Moreover, insulin-stimulated glucose uptake in mouse EDL muscles was not significantly attenuated by NAC [36]. We recently reported in Frisard *et al*, 2010 [6], that 2 hours of LPS exposure caused a significant increase in basal (non-insulin stimulated) glucose uptake and lactate production in skeletal muscle cells, suggesting an increase in glycolytic flux. Based on these observations, we speculate that in the context of acute TLR4 activation, ROS production not only causes mitochondrial uncoupling and subsequent increases PDH activity, but also leads to an increase in glucose uptake, hence providing increased flux of substrate to meet the increased demand for ATP production.

In support of the notion that the LPS-induced increase in ROS production contributes to increased glucose uptake, we show that the TLR4-mediated increase in glucose oxidation is blocked in the presence of the antioxidant NAC. Future studies to determine if these antioxidants have the same effect on TLR4-mediated glucose uptake are warranted.

The current work also demonstrates for the first time that TLR4 activation in skeletal muscle results in a significant increase in mitochondrial membrane potential heterogeneity. Heterogeneity in mitochondrial membrane potential is the degree of variability, or standard deviation, in the membrane potential of the mitochondria within a cell. It is well known that mitochondria are heterogeneous [21, 37]. Wikstrom, *et.al* demonstrated that β-cell mitochondria exhibit membrane *potential* heterogeneity that can be altered by changes in fuel levels and that an in vitro model of diabetes is characterized by an increase in mitochondrial membrane potential heterogeneity [21]. As such, this increased heterogeneity may indicate fragmentation of the mitochondrial network, an

increase in damaged mitochondria, or both [38]. The impact is evident when understood in terms of ATP production potential. As Nicholls [39] previously demonstrated, within a physiologically relevant range of mitochondrial membrane potential ($\Delta\Psi$), the maximum ATP/ ADP ratio that can be maintained by mitochondria decreases by 10-fold with every 14mV decrease in $\Delta\Psi$, and thus represents the metabolic capacity of the mitochondrial population as a whole. To our knowledge, mitochondrial membrane potential heterogeneity has not previously been examined in skeletal muscle. Herein we show increased heterogeneity following TLR4 activation, which suggests that an acute inflammatory insult results in a disparity of membrane potential between mitochondria within the muscle cell. These effects can ultimately result in disorganization and fragmentation of the mitochondrial network, which can have a lasting impact on the energy producing potential of the cell.

The results reported here, beg the question: What is the physiological relevance of TLR4 in skeletal muscle and how might it be linked to metabolic derangements in skeletal muscle? As we and others have established, TLR4 is present in skeletal muscle and, upon activation, alters substrate preference [6]. It is also known that endotoxin, the primary ligand for TLR4, increases in the blood along with chylomicron transport from the small intestine following a meal [40]. As such, one could speculate that blood endotoxin levels rise and fall over the course of day with repeated transitions between post-prandial to post-absorptive states. If this assertion were to hold true, a reasonable hypothesis would be that TLR4 activation assists in the physiological response to a meal to adequately dispose of glucose by promoting greater uptake and utilization at the level of skeletal muscle. Along these lines, we have generated preliminary evidence in our laboratory

showing that acute LPS exposure (2 hours) enhances insulin-stimulated glycogen synthesis in C2C12 cells. However, exposing C2C12 cells to LPS for 12 and 24 hours results in insulin resistance and the inability for insulin to stimulate glycogen synthesis (unpublished observations). These data imply that acute increases in endotoxin may serve a beneficial purpose, but chronic activation of pro-inflammatory pathways in skeletal muscle becomes deleterious. It has been well established that skeletal muscle of obese [41-43] and type 2 diabetic [44, 45] humans preferentially uses glucose as a substrate for ATP production under fasting conditions, despite the high levels of circulating fatty acids. Because of this metabolic derangement with fasting, skeletal muscle in these disease states fails to respond to insulin with a further increase in glucose utilization following a meal. These characteristics have been termed 'metabolic inflexibility', which is defined as the inability to adequately respond to regulatory cues and switch to the preferential metabolism of the most abundant substrate. It has now been proposed that an obese state is associated with chronic metabolic endotoxemia, a condition characterized by modest elevations in blood endotoxin levels [14]. More evidence is needed to confirm that human obesity is associated with elevated levels with endotoxin; however, if this were to prove correct, another plausible hypothesis would be that chronic activation of TLR4 in skeletal muscle contributes to metabolic inflexibility and insulin resistance. Future studies in humans exposed to low levels of endotoxin both acutely and chronically would glean much needed information in this regard.

In conclusion, we report that activation of TLR4 in skeletal muscle cells by doses of LPS that are synonymous with levels observed with metabolic endotoxemia results in altered substrate metabolism in the mitochondria with glucose being preferred over that of fatty

acids. This observation occurs in conjunction with increased ROS production, mitochondria uncoupling, and increased PDH activity. Additionally, the mitochondria's preference for glucose as a substrate under TLR4-stimulated conditions is dependent on ROS production.

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Figure Legends

Figure 6.1: Acute TLR4 activation leads to mitochondrial uncoupling in C2C12 skeletal muscle cells. C2C12 myotubes were exposed to 50 pg/mL LPS for 2 hours. Immediately following exposure, oxygen consumption was measured. (A) RCR, (B) oligomycin respiration rate, (C) FCCP-stimulated maximal respiration, and (D) UCP3 protein content as measured in mitochondrial isolated from C2C12 cells following 2 hours 50 pg/mL LPS exposure. Data are presented as means + SE. *P < 0.05, control vs. LPS.

Figure 6.2: The effect of LPS on mitochondrial function in C2C12 cells is dependent on TLR4. C2C12 cells were exposed to 50 pg/mL of LPS for 2 hours with and without the TLR4 neutralizing antibody, MTS510 (Santa Cruz Biotechnology, Santa Cruz, CA). Immediately following exposure, FCCP-stimulated maximal respiration was measured. Data are presented as means + SE. *P < 0.05, control vs. LPS.

Figure 6.3: The TLR4 effect on metabolism in C2C12 cells is blocked by antioxidant treatment. C2C12 cells were treated with 50 pg/mL of LPS in the absence and presence of NAC and catalase for 2 hours. (A) FCCP- stimulated maximal respiration following acute LPS exposure in the absence and presence of NAC. (B) FCCP- stimulated maximal respiration following acute LPS exposure in the absence and presence of catalase, (C) Glucose oxidation following acute LPS exposure in the absence and presence of NAC, (C) Fatty acid oxidation following acute LPS exposure in the absence and presence of NAC. Data are presented as means + SE. *P < 0.05, control vs. LPS.

Figure 6.4: Pyruvate dehydrogenase activity is increased in C2C12 cells following TLR4 activation. [1-14C]-pyruvate was used as assess the activity of PDH. C2C12

myotubes were exposed to 50 pg/mL LPS for 2 hours. Immediately following exposure, PDH activity was measured. Data are presented as means + SE. *P < 0.05, control vs. LPS.

Figure 6.5: The LPS-induced effects in C2C12 cells are independent of changes mitochondrial DNA copy number. Mitochondrial copy number in C2C12 cells following 2 hours of 50 pg/mL LPS exposure. Data are presented as means + SE. No significant effects of LPS were observed.

Figure 6.6: TLR4 activation causes mitochondrial dysfunction in skeletal muscle from C57BL/6J mice. C57BL/6J mice were injected with LPS at a dose of 1 μg/kg body mass. Four hours post injection, red gastrocnemius muscle mitochondria were isolated, and oxygen consumption was measured. (A) RCR, (B) ADP-stimulated state 3 respiration, (C) FCCP-stimulated maximal respiration, (D) basal respiration, and (E) oligomycin respiration rate following LPS injection. Data are presented as means + SE. *P < 0.05, control vs. LPS.

Figure 6.7: Direct LPS exposure to isolated mitochondria from red gastrocnemius muscle does not cause mitochondrial dysfunction. Mitochondria were isolated from red gastrocnemius muscle from C57BL/6J mice. Mitochondria were directly exposed to 50 pg/mL LPS for 1 hour while oxygen consumption was measured. (A) RCR, (B) ADP-stimulated state 3 respiration, (C) FCCP-stimulated maximal respiration, (D) basal respiration, and (E) oligomycin respiration rate following LPS exposure. Data are presented as means + SE. No significant effects of LPS were observed.

Figure 6.8: TLR4 activation by LPS results in increased mitochondrial membrane potential and ROS production. (A) mitochondrial membrane potential in FDB muscle fibers isolated from a male C57Bl/6J mouse in response to treatment with 50 pg/mL LPS (B) ROS production in FDB muscle fibers following 50 pg/mL LPS for 30 and 45 minutes. (C) Mitochondrial heterogeneity in FDB muscle fibers before and after 15 minutes of 50 pg/mL LPS exposure. Data are presented as means + SE. *P < 0.05, control vs. LPS.

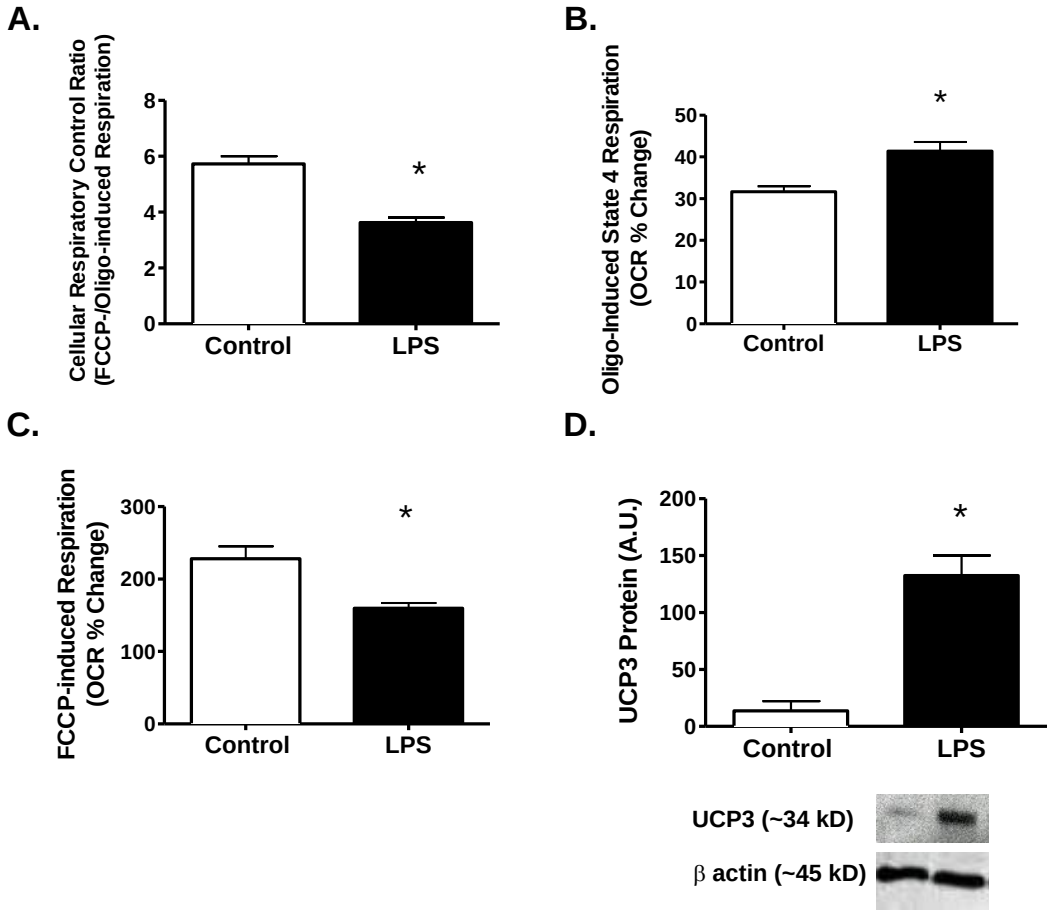

Figure 6. 1

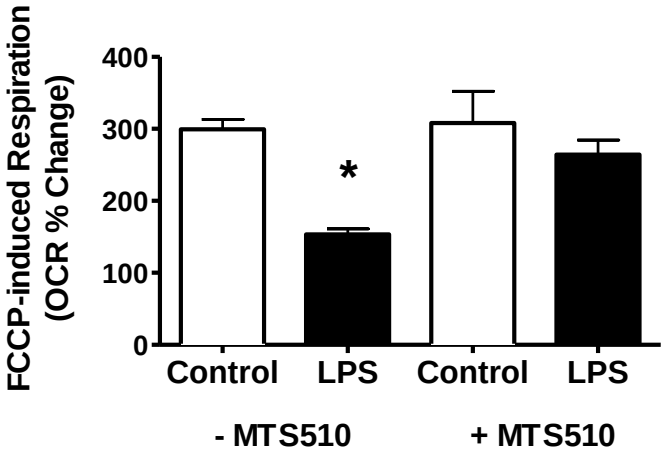

Figure 6. 2

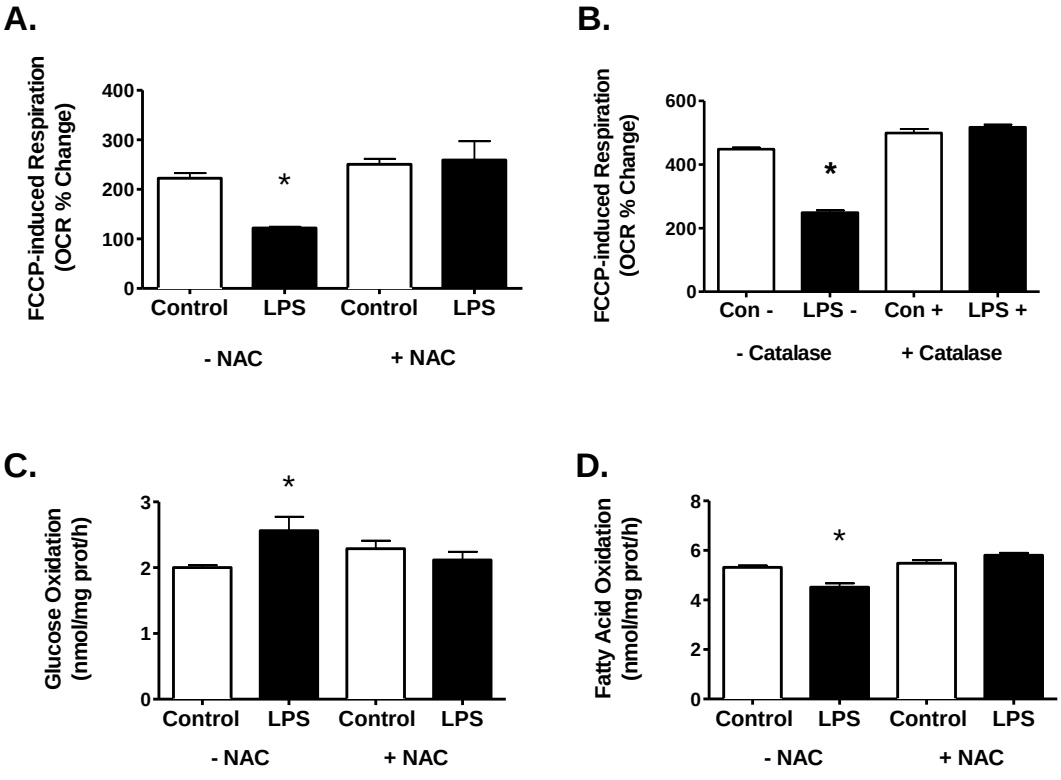

Figure 6. 3

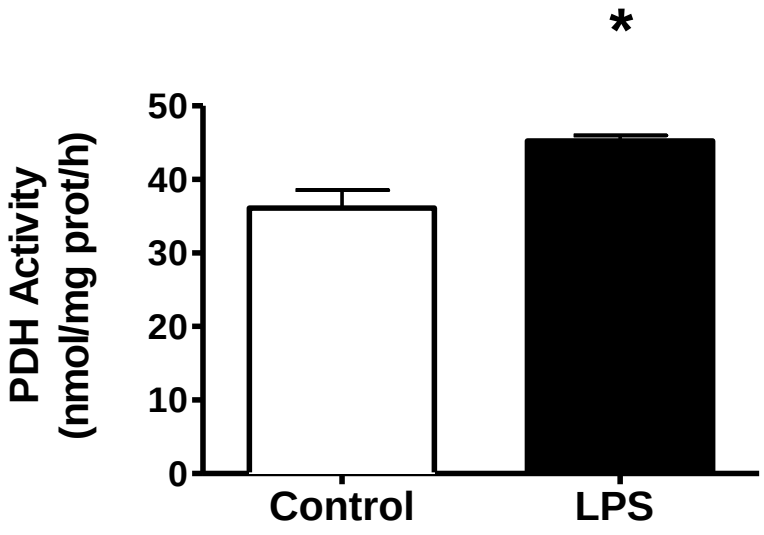

Figure 6. 4

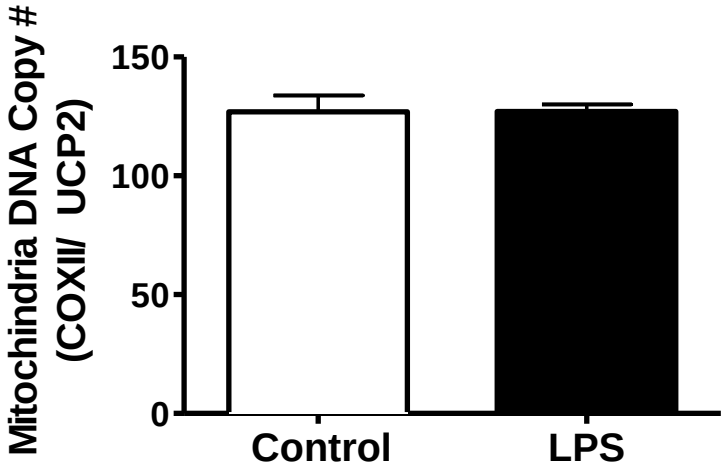

Figure 6. 5

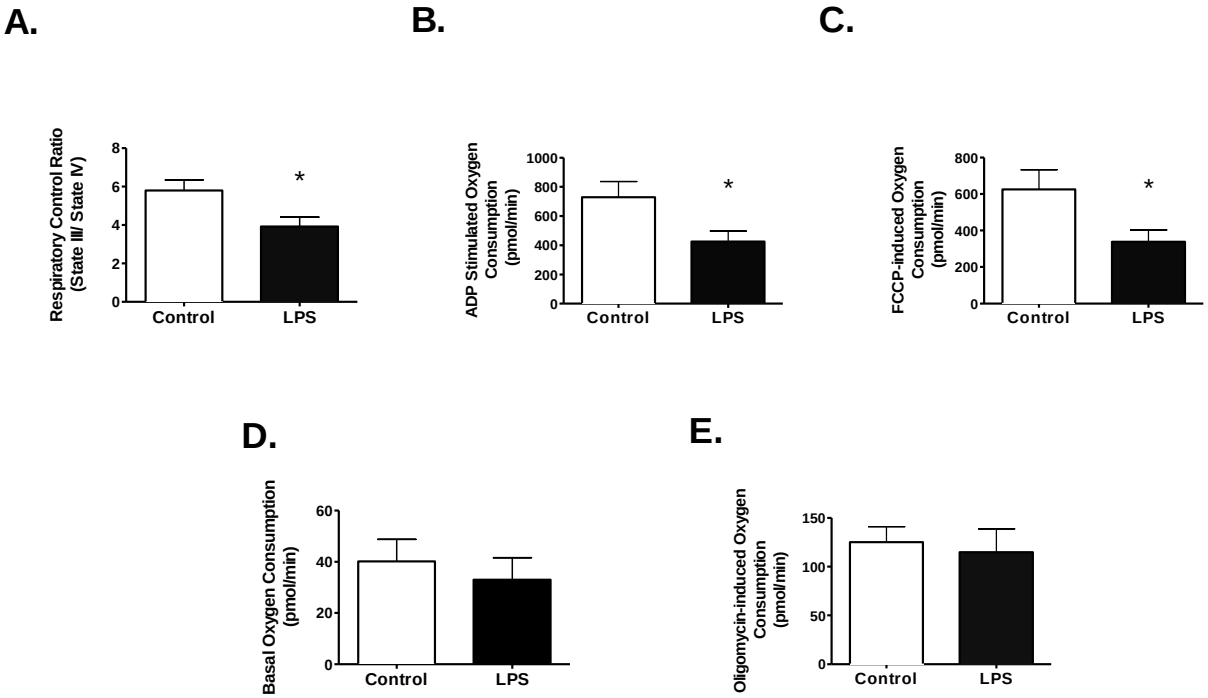

Figure 6. 6

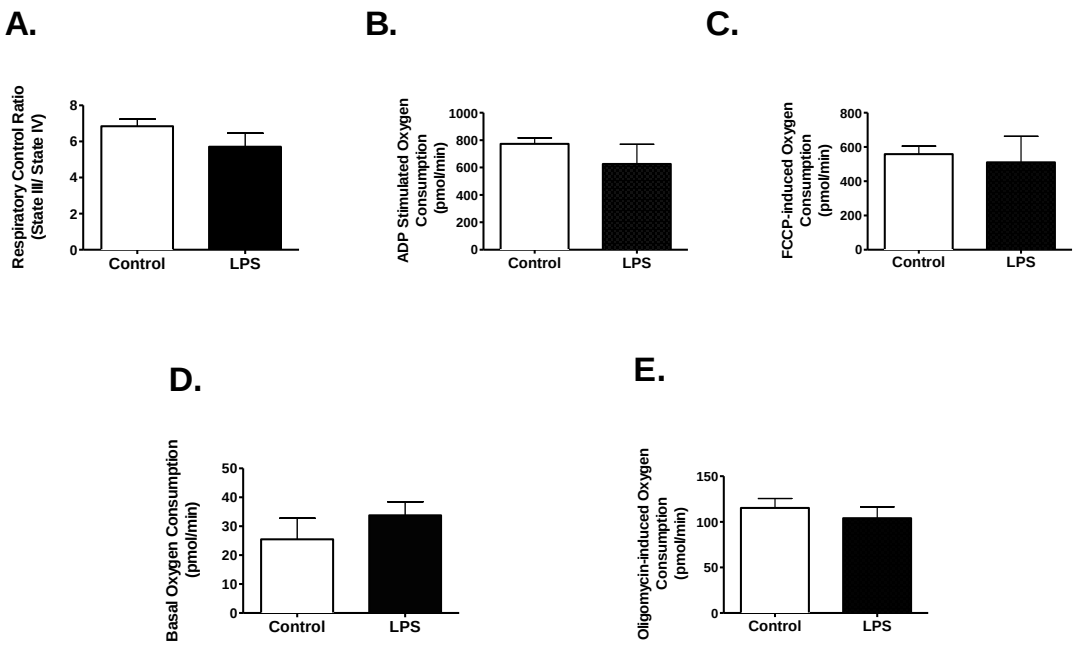

Figure 6. 7

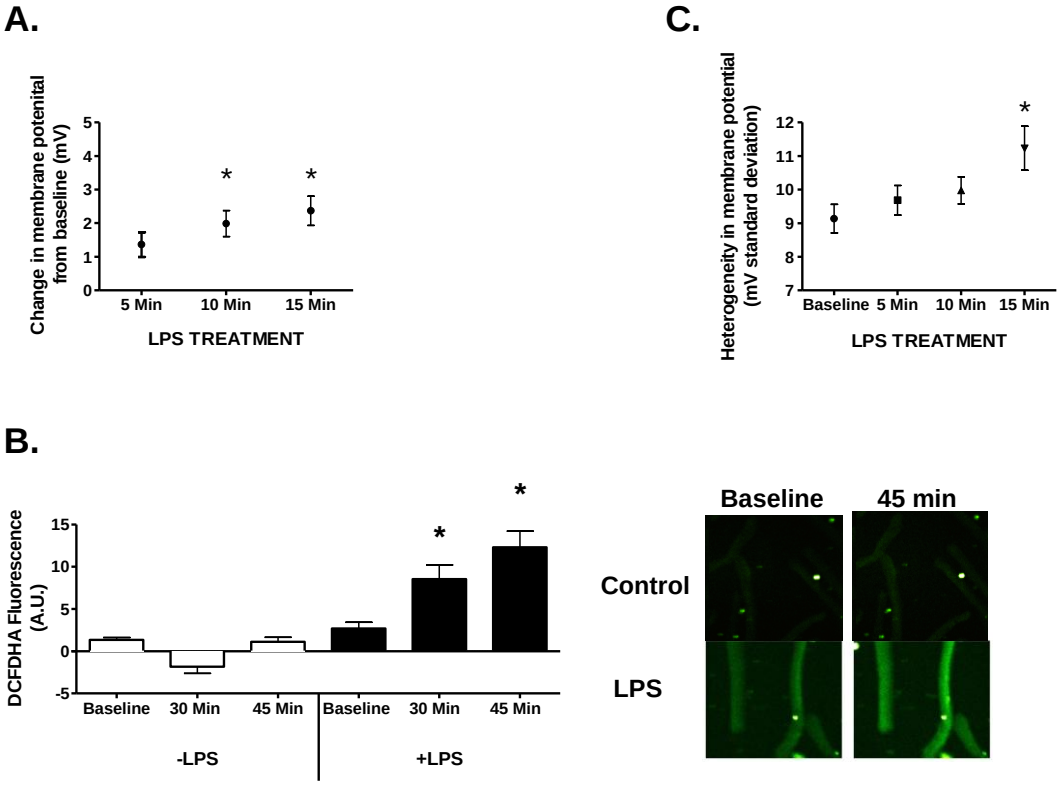

Figure 6. 8

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Chapter 7: Manuscript

Transgenic Mice Expressing High Levels of Toll-like Receptor 4 Modulates Fatty Acid Metabolism in Skeletal Muscle

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Abstract

Toll-like receptor 4 (TLR4) is elevated in skeletal muscle of obese humans, and data from our lab showed that activation of TLR4 in skeletal muscle with lipopolysaccharide (LPS) results in decreased fatty acid oxidation. The purpose of this study was to determine if over-expression of TLR4 specific in skeletal muscle alters mitochondrial function and reduces fatty acid oxidation (FAO). C57BL/6 mice with muscle-specific over-expression of the TLR4 (mTLR4) gene were used for all experiments. Isolated mitochondria and whole homogenates from rodent skeletal muscle (gastrocnemius and quadriceps) were studied. TLR4 over-expression resulted in a significant reduction in FAO in muscle homogenates; however, mitochondrial respiration and ROS production do not appear to be affected on a standard chow diet. To determine the role of TLR4 over-expression in skeletal muscle in response of high fat challenge, mTLR4 mice and WT control mice were fed high fat and low fat diets for 16 weeks. The high fat diet significantly decreased FAO in isolated mitochondria in mTLR4 mice, which was observed in concert with an increase in production of ROS in complex 3, and more body fat and greater area under the curve for the glucose tolerance test. These findings suggest that TLR4 plays an important role in the metabolic dysfunction in skeletal muscle.

Key Words: toll-like receptor 4, skeletal muscle, mitochondria, high fat diet, and fatty acid oxidation

Introduction

Growing evidence indicates that obesity and metabolic dysregulation are closely associated with inflammation [1-3]. Toll-like receptor 4 (TLR4) is activated by lipopolysaccharide (LPS) and plays a critical role in the innate immune system and the induction of inflammatory responses [4]. Skeletal muscle comprises ~ 40% of total body mass and is a major site of postprandial, insulin-stimulated glucose disposal, as well as fatty acid oxidation under fasting conditions. Since skeletal muscle is a major target tissue contributing to whole-body metabolism, it is important to understand TLR4's functional significance in skeletal muscle. Indeed, there is a growing body of evidence in both animal and human studies that have demonstrated that abnormal TLR4 expression and signaling may contribute to the pathogenesis of insulin resistance in skeletal muscle [5-7]. Moreover, it has been reported that TLR4 activation in skeletal muscle both *in-vitro* and *in-vivo* results in increased glucose oxidation and reduced fatty acid oxidation [8]. These findings provide critical insight into the role of TLR4 in skeletal muscle metabolism linking a proinflammatory state with obesity.

A mitochondrion is a membrane-enclosed organelle involved in a range of vital cellular processes, such as energy production, apoptosis, pyrimidine biosynthesis, fatty acid metabolism, and calcium homeostasis [9-11]. One of the most important functions of mitochondria is oxidative phosphorylation for the generation of cellular energy [12]. Mitochondrial dysfunction could be induced by a numbers factors such as oxidative stress, genetic factors, mitochondrial biogenesis (or defects in biogenesis), and aging [13-16]. Since mitochondria are the primary cellular site for fatty acid oxidation and utilization, there is an increased interest on the role of altered mitochondrial function in skeletal muscle lipid accumulation and development of insulin resistance. Recent work from our lab showed that activation of TLR4 in skeletal muscle causes

mitochondrial dysfunction, which is partially dependent on the production of reactive oxygen species (paper in review). However, the mechanism that contributes to altered mitochondrial function and the inability to handle fatty acid overload in skeletal muscle is still unknown.

In this study, a transgenic mouse model with over-expression of TLR4 specific in skeletal muscle (mTLR4) was generated to directly assess the role of TLR4 in mediating mitochondrial function and skeletal muscle substrate metabolism. Mitochondrial function, substrate metabolism, and enzyme activities of the mTLR4 transgenic mice on a standard chow diet and on a high fat (HF) diet were assessed. Our hypothesis was that muscle-specific over-expression of TLR4 would result in mitochondrial dysfunction and reduced fatty acid oxidation in skeletal muscle, both of which would be exacerbated by a high fat diet.

Methods

Animal husbandry. Male C57BL/6 mice with muscle-specific over-expression of the TLR4 (mTLR4) gene were used for all experiments. A muscle creatine kinase promoter was used to drive the muscle-specific over-expression. Animal studies were performed under an approved protocol by the Institutional Animal Care and Use Committee at Virginia Tech. Mice for all studies were maintained on a standard chow diet (or high fat diet) and a 12hr light-dark cycle. All experiments were performed after an overnight fast (10-12h), and following euthanasia, the gastrocnemius and quadriceps were harvested, mitochondria were isolated, and measures of mitochondrial respiration, fatty acid oxidation, ROS production, and enzyme activity were performed.

LPS injection in mTLR4 mice. Male mTLR4 mice and their wild type (WT) littermates were injected with saline or LPS (1 mg/kg body weight, ~25 μg/mouse) following an overnight fast

(n=2). LPS from Escherichia coli 0111:B4 was used for all studies (L2630;Sigma-Aldrich, St. Louis, MO). To determine whether LPS response is higher in mTLR4 mice, the animals were sacrificed and soleus muscle was collected 4 hours post injection to assess IL6 mRNA expression.

Real-time quantitative PCR (RTQ-PCR). Tissue was collected in Trizol, homogenized and RNA was extracted. Total RNA was prepared using TriZol reagent according to the manufacturer's protocol (Life Technology), treated with DNase I (Ambion, Austin, TX), and quantified using an Agilent Bioanalyzer. Real-time quantitative PCR (RTQ-PCR) was performed using an ABI 7900 Fast HT RTQ-PCR Instrument and software (PE Applied Biosystems, Foster City, CA). IL6 and TLR4 gene was used with results normalized to beta-actin (mouse) RNA levels by RTQ-PCR.

Western Blot Analysis. Western blot analysis was performed as previously described[17], using skeletal muscle preparation in 50 mM Hepes, (pH7.5), 15 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 2 mM EDTA, 10% glycerol, 1 % Triton x-100, 10 mM NaP2O7, 100 mM NaFl, 10 mM PMSF, and 10 g/mL aprotinin. Proteins (30-70g) loaded in duplicate was separated using a 10% Criterion-Tris-HCl gel (Bio-Rad, Hercules, CA) and subsequently transferred to PVDF membrane (Bio-Rad, Hercules, CA). Blot was probed with primary antibodies against GAPDH and TLR4 followed by anti-rabbit. Proteins were visualized using Super-Signal Chemiluminescent Substrate (Pierce, Rockville, IL) and a ChemiDoc XRS Imaging System (BioRad, Hercules, CA).

High fat feeding studies. mTLR4 mice and their WT littermates were fed either a control low fat diet (Teklad TD.10453) or a high fat diet (Teklad, TD.10505) for 16 weeks at the age between 6 to 8 month old (n=11). Diets were designed and purchased from Harlan (Madison, WI).

Following 16 weeks of high fat feeding, mice were sacrificed after an overnight fast, and gastrocnemius and quadriceps were harvested, mitochondria were isolated, and measures of mitochondrial respiration, fatty acid oxidation, ROS production, and enzyme activity were performed.

Glucose Tolerance Test. 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.

Body composition and whole body calorimetry assessment. Body composition was assessed via the Bruker mini spec LF90. Energy expenditure and respiratory exchange ratio were measured over 48-hours using indirect calorimetry (TSE Systems, Chesterfield, MO)

Skeletal muscle whole homogenate preparation. Approximately 50 mg fresh 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 over 30 seconds at 1,200 rpm with a motor-driven teflon pestle, and measures of fatty acid oxidation, and enzyme activity were performed.

Mitochondrial isolation from skeletal muscle. Mitochondria were isolated from red and white skeletal muscle from quadriceps femoris and gastrocnemius muscle as previously described with modifications [18]. Tissue samples were collected in buffer containing 67mM sucrose. 50mM Tris/HCl, 50mM KCl, 10mM EDTA/ Tris, and 10% bovine serum albumin (all from Sigma-

Aldrich, St. Louis, MO). Samples were minced and digested in 0.05% trypsin (Invitrogen, Carlsbad, CA) for 30 minutes. Samples were homogenized and mitochondria were isolated by differential centrifugation.

Measures of respiration in isolated mitochondria. Respirometry of isolated mitochondria was performed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, MA). Immediately following isolation and protein quantification, mitochondria were plated on Seahorse cell culture plates at a concentration of 5ug/well in the presence of pyruvate (10mM)/ malate (5mM). Experiments were conducted in sucrose/ mannitol solution to maintain the integrity of the mitochondria. Experiments were consisting of 25 second mixing and 4-7 minute measurement cycles, unless otherwise stated. Oxygen consumption were measured under basal conditions, ADP (5mM) stimulate state 3 respiration, oligomycin (2μM) induce state 4 respiration, and uncoupled respiration in the presence of the mitochondrial uncoupler FCCP (0.3μM) to assess maximal oxidative capacity. All experiments were performed at 37 ℃.

Fatty acid oxidation. Palmitate oxidation was assessed in isolated mitochondria and 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 (Perkin Elmer, Waltham, MA), respectively, as previously described [17, 19].

Glucose Oxidation. Glucose oxidation was assessed in muscle homogenates measuring $^{14}CO_2$ production from the oxidation of [U-14C]-glucose (Perkin Elmer, Waltham, MA) as previously described [17, 19] with the exception that glucose was substituted for BSA-bound palmitic acid.

Enzyme activity. Enzyme activities were assessed in isolated mitochondria and muscle homogenates and were determined spectrophotometrically as previously described [20]. Citrate

synthase activity was determined spectrophotometrically by the rate of DNTB reduction upon exposure to acetyl coA (412nm). β-3-hydroxyacyl coenzyme A dehydrogenase (BHAD) and Malate dehydrogenase (MDH) activity were determined by the rate of NADH oxidation in the presence of acetoacetyl coA or oxaloacetate, respectively (340nm).

Pyruvate dehydrogenase (PDH) activity. PDH plays an important role in linking the glycolytic metabolic pathway to the citric acid cycle. To evaluate its activity in mTLR4 mice, [1-14C]-pyruvic acid oxidation to $^{14}CO_2$ was assessed at 16-weeks of high fat feeding as previously described [21]. Isolated mitochondria from red skeletal muscle was incubated in 1.5 μCi/mL of [1-14C]-pyruvic acid for 3 hours. The incubation media was then acidified with 45% perchloric acid and $14CO_2$ was trapped and in 1M NaOH and quantified upon addition of scintillation fluid and scintillation counting.

ROS measures in isolated mitochondrial. Amplex Red Hydrogen Peroxide/Peroxidase assay Kit was used for measures of ROS production. To measure ROS production from complex 1, complex 3, and reverse electron transfer (REV), isolated mitochondria were plated on a 96-well black plate at a concentration of 5ug/well under three different conditions, respectively. The three conditions were pyruvate (20mM)/malate (10mM)/oligomycin (2μM)/rotenone (200nM) for complex 1, pyruvate (20mM)/malate (10mM)/oligomycin (2μM)/SOD (400U/ml)/antimycin A (2μM) for complex 3, and succinate (20mM)/oligomycin (2μM) for reverse electron flow to complex 1 (REV). Experiments were conducted in sucrose/mannitol solution to maintain the integrity of the mitochondria. Amplex Red working solution then was loaded into the plate to begin the reactions. Experiments consisted of 1 minute delay and 1 minute reading cycles, followed by a 5 second mixing cycle performed every third reading. All experiments were performed at 37℃. Measures for ROS levels were conducted on a microplate reader (Biotek

synergy 2, Winooski, VT). Fluorescence of Amplex Red was measured using a 530nm excitation filter and a 560nm emission filter.

Statistical analysis. Results were analyzed with Student's t tests or 2-way ANOVA with Tukey's post-hoc analysis. Results are expressed as mean ± SEM. The level of significance was set at $P < 0.05$.

Results

Muscle-specific TLR4 transgenic mice. C57BL/6 mice with muscle-specific TLR4 over-expression were generated via the muscle creatine kinase promoter. Protein content of TLR4 was higher in mTLR4 mice compare to WT mice from skeletal muscle homogenates (Figure 7.1A). To evaluate a potential role for TLR4 in the induction of an inflammatory response in our animal model, both mTLR4 mice and their WT littermates were injected with either saline or LPS (1 μg/kg body weight) and mRNA levels of IL-6, a marker of a pro-inflammatory response, and TLR4 were assessed in soleus muscle. As expected, a significant increase in IL-6 mRNA level was induced in response to LPS in both mTLR4 and WT mice (Figure 7.1B). Moreover, there was a significant genotype x treatment interaction between mTLR4 and WT mice in LPS induced IL-6 mRNA level ($p=0.003$). mRNA level of TLR4 was significant higher in mTLR4 mice after both saline and LPS injection (Figure 7.1C).

Metabolic phenotype of mTLR4 mice on chow diet. On chow diet, there were no significant changes in body weight and body fat between mTLR4 and WT mice, although a trend toward increased body weight ($p=0.08$) and body fat ($p=0.12$) in mTLR4 mice was observed. Additionally, muscle-specific TLR4 over-expression did not alter respiratory exchange ratio (RER) or energy expenditure (Data not shown). Despite no significant differences in body mass,

WT mice respond slightly better to glucose challenge compare to mTLR4 mice. Blood glucose was significant higher in mTLR4 mice at 90 min following injection (135.7±4.2 vs. 154.2±10.8, WT vs. mTLR4, P=0.042). Area under glucose tolerance curve was 337.3±9.4 vs. 367.3±15.3, WT vs. mTLR4 (P=0.044).

Skeletal muscle substrate metabolism in mTLR4 mice. To determine if muscle-specific TLR4 over-expression altered substrate metabolism, we assessed glucose and fatty acid oxidation in homogenates and/or isolated mitochondria of skeletal muscle. In mice that were fed a standard chow diet, mTLR4 mice have decreased fatty acid oxidation (FAO) compare to their wild type littermates in homogenates from both red (Figure 7.2C) and white muscle (Figure 7.2F). There is a similar effect in isolated mitochondria from red muscle (red mitochondria), a trend toward decreased FAO in mTLR4 mice (126.7 ± 15 vs. 155.5 ± 10.7; mTLR4 vs. WT, p=0.07), indicating an attenuated capacity to oxidize FA in muscle from mTLR4 mice. No differences were observed in glucose oxidation between mTLR4 and WT mice (Data not shown).

Moreover, oxidative enzyme activities were measured in both red and white skeletal muscle whole homogenates, as well as in isolated mitochondria from red skeletal muscle. Citrate synthase (CS) activity was significantly lower in isolated mitochondria in mTLR4 mice compare to WT mice (Figure 7.3); no difference observed in CS or BHAD in muscle homogenates (Data not shown).

Mitochondrial function in mTLR4 mice. To examine whether increased muscle TLR4 expression affects mitochondrial function in skeletal muscle, mitochondria were isolated for measures of mitochondrial respiration and ROS production. There were no significant differences between mTLR4 and WT mice in respiratory control ratio (RCR), basal respiration,

ADP-stimulated state 3 respiration, or FCCP-stimulated maximal respiration rate (Data not shown). ROS production was similar in complex 1, complex 3, and REV for both mTLR4 and WT mice (Data not shown). Thus, these results indicate that mitochondrial respiration and ROS production do not appear to be affected by muscle-specific over-expression of TLR4 on standard chow diet.

The effects of high fat feeding on metabolic phenotype in mTLR4 mice. To examine whether muscle-specific over-expression of TLR4 exacerbate the effects of a HF diet on mitochondrial function and substrate metabolism we fed male mTLR4 mice and their WT littermates low fat (LF, 10% fat) and high fat (HF, 45% fat) diets for 16-weeks. Body weight was recorded weekly. High fat feeding significantly increased body mass in both groups of mice (Figure 7.4A). mTLR4 mice trended to gain more weight compare to WT mice on the HF diet after high fat feeding (Figuure 4B-C).

After 16 weeks HF diet, mTLR4 mice have significantly more body fat compared to WT mice ($p=0.01$, Figure 7.6A). Furthermore, there was a significant genotype x diet interaction between mTLR4 and WT mice in body fat mass ($p=0.044$). RER was significantly decreased in both WT and mTLR4 mice after HF diet (Figure 7.6B) with no significant genotype effect. Energy expenditure did not change in either WT or mTLR4 mice in response to the HF diet (Figure 7.6C-D). The area under the curve for glucose tolerance test was significantly higher in mTLR4 mice compared to WT mice (535.6 ± 21.6 vs. 643.2 ± 43.2, $P=0.019$).

Mitochondrial function from mTLR4 mice skeletal muscle after HF diet. Reduced mitochondrial function has been observed in response to high fat feeding [22, 23]. Following HF diet, both WT and mTLR4 mice responded by increasing basal respiration in red mitochondria.

High fat diet resulted in a significant decrease in ADP-stimulated state 3 respiration (Figure 7.7D) and FCCP-stimulated maximal respiration (Figure 7.7G) in isolated mitochondria from white skeletal muscle (white mitochondria) from WT mice compared to the low fat diet, however we did not observe the same effect in mTLR4 mice. Respiratory control ratio, an index of the functional integrity of prepared mitochondria, did not change in response to HF diet in either WT mice or mTLR4 mice (Figure 7.7F, 7.7H).

We performed measurements of ROS production in mitochondria isolated from skeletal muscle. These experiments showed that the high-fat diet resulted in slightly increased levels of ROS in both WT and mTLR4 mice in red mitochondria (Figure 7.8, A-C). High fat diets resulted in significant increase of ROS production in complex 3 in mTLR4 mice compare to low fat diet. However the similar diet effects in WT mice did not reach statistical significance. Furthermore, ROS production of complex 3 in mTLR4 mice on the HF diet was significantly higher compared to WT mice. These results suggested that muscle TLR4 over-expression aggravates the HF diet induced increases in ROS production.

FA metabolism in isolated mitochondria from mTLR4 mice skeletal muscle after HF diet. Our studies demonstrated that TLR4 over-expression results in significant decrease in FAO on a standard chow diet in homogenates, but only show the trend in isolated mitochondria. The same effect was observed on HF diet with a significantly decreased FAO in mTLR4 mice in red mitochondria compare to WT mice (genotype x diet interaction, p=0.099, Figure 7.9C, 7.9G).

Numerous reports have shown that fat-rich diets decrease carbohydrate oxidation and PDH activity [24-27]. In line with this, we observed a decline in PDH activity in response to HF diet in WT mice (p=0.09). However, this decline was not evident in mTLR4 mice (Figure 7.10F).

Finally, we sought to examine the response of oxidative enzymes in to high fat feeding in mTLR4 and WT mice. MDH activity was significantly increased (634±39 vs. 539.4±31, HF vs. LF, $p<0.05$) in WT mice while this adaptation to high fat feeding was not evident in mTLR4 (511.5±36 vs. 512.1±18, HF vs. LF). No changes were observed with CS and or BHAD activity (Figure 7.10A-B, 7.10D-E).

Discussion

Our lab successfully generated mTLR4 mice with a higher TLR4 protein content in skeletal muscle. These mice are characterized by over-expression of TLR4 gene specific in skeletal muscle. In this study, we demonstrate that TLR4 over-expression causes decreased fatty acid oxidation in skeletal muscle on both standard chow diet and HF diet. In addition, mTLR4 mice have abnormal GTT alone with more fat and gain more weight compare to WT mice when put on a HF diet. Increased ROS production from mTLR4 mice were also observed after HF diet.

HF diet is extensively used in rodents to investigate the impact of diet induced obesity [28-30]. It is not surprising that there was a significant weight gain after 16 weeks of HF diet from both groups of mice. mTLR4 mice gain more weight and have more body fat compare to WT mice, which we did not observe on a standard chow diet. Interestingly, it has been reported that the mice that have a loss-of-function mutation in TLR4 have similar fat weights compare with control mice on a chow diet; but there was a 40% decreased fat weights in mutant TLR4 mice after HF diet [5]. These data suggested an important role of TLR4 in the development of obesity induced from HF diet. It is well know that obesity is closely associated with inflammation. Cani et al. recently reported that HF diet induced inflammation was linked to metabolic endotoxemia which defined as increased plasma LPS levels [31]. TLR4 recognizes LPS of Gram-negative bacteria and is the LPS sensor in both mice and humans [32]. In addition, TLR4 plays an

important role in the induction of inflammatory responses by transcriptionally activating NF-κB, which has been linked to the development of insulin resistance [6, 33]. In the high fat feeding study, weight gain and more body fat in mTLR4 mice were linked to a larger area under the curve for glucose during the glucose tolerance test. There is a growing body of evidence in both animal and human studies that have demonstrated that abnormal TLR4 expression and signaling may contribute to the pathogenesis of insulin resistance in skeletal muscle [6, 7]. Recently, A study was reported by Tsukumo et al., who used TLR4 mutant mice as an animal model and provided evidence that TLR4 inactivation prevented the development of insulin resistance in response to a HF diet [5]. Altogether, these results demonstrate that over-expression of TLR4 in skeletal muscle results in enhanced fat accumulation on a HF diet, which is associated with impaired glucose tolerance.

With human obesity, skeletal muscle has been characterized as less oxidative and insulin resistant compared to non-obese controls [34]. As a result of this, fatty acid oxidation in a fasted state and post-prandial glucose disposal are blunted. The cause of skeletal muscle insulin resistance is not entirely clear but a prevailing theory is that over exposing skeletal muscle to fatty acids that cannot be met with adequate oxidation results in mitochondrial dysfunction and the accumulation of intracellular lipid metabolites, all of which are known to disrupt multiple points in the insulin signaling cascade[35]. To date, the mechanisms that contribute to reduced oxidative capacity and the inability to handle fatty acid overload in skeletal muscle is unknown. Our previous research has demonstrated that activation of TLR4 results in a shift in substrate metabolism in skeletal muscle that favors glucose oxidation over fatty acids [36]. We have also found that this shift in substrate metabolism is partially dependent on the production of ROS and occurs in concert with mitochondrial uncoupling and increased PDH activation (in review).

Evidence from the current study demonstrates that over-expression of TLR4 leads to a decrease in FAO on a standard chow diet in homogenate from skeletal muscle. However, FAO is similar compare with WT mice in isolated mitochondria. We also show that there is no difference between mTLR4 mice and WT mice in oxygen consumption and ROS production from isolated mitochondria. Altogether, these data indicate that the reduced FAO in mTLR4 mice on chow diet may be a result of reduced mitochondria number in skeletal muscle and not due to inherent impaired mitochondrial function.

Converse to effects of chow diet, the current study demonstrates that, when put on a HF diet, there was a significant reduction FAO in isolated mitochondria in mTLR4 mice. Moreover, mTLR4 mice produce more ROS compare to WT mice in isolated mitochondria on a HF diet indicating that these mice developed impaired mitochondria through high fat challenge. As we know, mitochondria are vulnerable to ROS. High levels of ROS in mitochondria may lead to oxidative phosphorylation impairment which may result in increased ROS production, thus causing even more mitochondrial and cellular damage [37, 38]. To further investigate the role of TLR4 in ROS production, Ryan et al. detected LPS-induced ROS generation in macrophage cells from mice which have a functional TLR4 receptor; however, in cells from the lack of functional TLR4 receptor mice, LPS did not induce the generation of ROS [39]. Similar findings were also reported by Park et al., where they demonstrated that a direct interaction of TLR4 with Nox4 is essential for LPS-mediated ROS production and NF-κB activation in HEK293T cells [40]. The present study, coupled with previous observations, reveals an important role of TLR4 in fatty acid metabolism and mitochondria function. Indeed, our data suggest that TLR4 is involved in the alterations of fatty acid metabolism and increased ROS production induced by HF diet,

which may lead eventually to mitochondrial malfunction. Thus, TLR4 seems to provoke potentially deleterious changes in substrate homeostasis and mitochondrial function after HF diet.

PDH plays an important role in linking glycolysis metabolic pathway to the citric acid cycle. It has been demonstrated that both adult and neonatal muscles from rat are able to adjusting their enzyme activity in response to HF diet [41]. Our data showed that WT mice tend to adapt to HF diet by increase FAO as well as decrease PDH activity, whereas this adaption was totally blunted in the mTLR4 mice. Consistent with these observations, mTLR4 mice did not alter MDH activity in response to HF diet. However, mTLR4 mice have similar CS activity and β-HAD activity compare with WT mice on a HF diet. Evidence from our study indicates that TLR4 plays an important role in the adaption of mitochondrial enzymes involved in substrate metabolism in response to the HF diet. These observations deserve further investigation.

In summary, first, we have demonstrated that TLR4 over-expression is associated with reduced FAO in skeletal muscle. Second, we found that in mTLR4 mice, oxidative enzymes fail to adapt to a HF diet compare with WT mice. Altogether, we demonstrate that TLR4 plays an important role in the metabolic dysfunction in skeletal muscle. Muscle-specific over-expression of TLR4 in mice may provide unique models to examine the link between fatty acid utilization, obesity, and mitochondria function.

Figure Legends

Figure 7.1. IL6 and TLR4 mRNA levels are increased to a greater extent in mTLR4 mice after LPS injection. mTLR4 and WT mice were injected with either saline or LPS at a dose of 1mg/kg body weight. The animals were sacrificed and soleus muscle was collected 4 hours post injection. mRNA of IL6 and TLR4 were assessed. (A) Protein content of TLR4 from skeletal muscle homogenates. (B) mRNA levels of IL6. (C) mRNA levels of TLR4. Data are expressed as mean ± SEM. *P<0.05.

Figure 7.2. FAO is suppressed in muscle homogenates from mTLR4 mice on a standard chow diet. mTLR4 mice and their WT littermates were sacrificed after an overnight fast. Fatty acid oxidation was measured in homogenates by assessing [1-14C]-palmitic acid breakdown to 14-CO_2 and acid soluble metabolites. (A) CO_2 production in red muscle. (B) Acid-soluble metabolites (ASM) in red muscle. (C) Total FAO in red muscle. (D) CO_2 production in white muscle. (E) ASM in white muscle. (F) Total FAO in white muscle. Data expressed as mean ± SEM, * p<0.05.

Figure 7.3. CS activity decreased in isolated mitochondria from mTLR4 mice compared to WT mice on a standard chow diet. mTLR4 mice and their WT littermates were sacrificed after overnight fast. CS activity was measured in isolated mitochondria (red mitochondria). Data expressed as mean ± SEM, * p<0.05.

Figure 7.4. mTLR4 mice gain more mass on a high fat diet compared to WT littermates. Mice were maintained on 12hr light-dark cycles and fed either a 45% high fat (Teklad TD.10505) or control (TD.10453) diet for 16 weeks. Body mass are recorded weekly. (A) Body weight. (B) Body weight response to HF diet, percent change. (C) Body weight gain after HF diet. Data expressed as mean ± SEM. * P<0.05.

Figure 7.5. mTLR4 mice become more significantly glucose intolerant on a high fat diet compared to WT mice. Following an overnight fast, mice were injected with 1g/kg glucose and blood was measured via the tail vein every 30 minutes for 2 hours. Data is expressed as mean ± SEM. * P<0.05.

Figure 7.6. mTLR4 mice gain more body fat on a high fat diet compared to WT littermates. Mice were maintained on 12hr light-dark cycles and fed on HF diet. Body composition was assessed via the Bruker mini spec LF90. Energy expenditure was measured over 48-hours using indirect calorimetry (TSE Systems, Chesterfield, MO). (A) Body fat mass. (B) Respiratory exchange ratio. (C) Energy expenditure per lean mass. (D) Energy expenditure per lean mass response to HF diet, percent change. (E) GTT AUC (F) GTT AUC response to HF diet, percent change. Data is expressed as mean ± SEM. * P<0.05.

Figure 7.7. Mitochondrial function in mTLR4 mice after HF diet. Mitochondria were isolated and oxygen consumption was measured using a Seahorse Bioscience extracellular flux analyzer. (A) Basal respiration in red mitochondria. (B) ADP-stimulated state 3 respiration in red mitochondria. (C) Basal respiration in white mitochondria. (D) ADP-stimulated state 3 respiration in white mitochondria. (E) FCCP-stimulated maximal respiration in red mitochondria. (F) RCR in red mitochondria. (G) FCCP-stimulated maximal respiration in white mitochondria. (H) RCR in white mitochondria. Data are expressed as mean ± SEM. *P<0.05.

Figure 7.8. ROS production in isolated mitochondria after HF diet. ROS production was measured using Amplex Red Hydrogen Peroxide/Peroxidase assay Kit. (A) Complex 1 in red mitochondria. (B) Complex 2 in red mitochondria. (C) REV in red mitochondria. (D) Complex 1

in white mitochondria. (E) Complex 2 in white mitochondria. (F) REV in white mitochondria. Data are expressed as mean ± SEM. *P<0.05.

Figure 7.9. WT mice tend to adapt to HF diet by increasing FAO in red mitochondria; no adaptation is existent in mTLR4 mice. Fatty acid oxidation was measured in homogenates by assessing [1-14C]-palmitic acid breakdown to $14CO_2$. (A) CO_2 production in red mitochondria. (B) ASM in red mitochondria. (C) Total FA oxidation in red mitochondria. (D) CO_2 production in white mitochondria. (E) ASM in white mitochondria. (F) Total FA oxidation in white mitochondria. (G) Total FA oxidation response to HF diet in red mitochondria, percent change. Data expressed as mean ± SEM, * p<0.05.

Figure 7.10. Enzyme activity in isolated mitochondria after HF diet. Enzyme activities were measured in skeletal muscle mitochondria. (A) BHAD activity in red mitochondria. (B) CS activity in red mitochondria. (C) MDH activity in red mitochondria. (D) BHAD activity in white mitochondria. (E) CS activity in white mitochondria. (F) PDH activity in red mitochondria. Data expressed as mean ± SEM, * p<0.05.

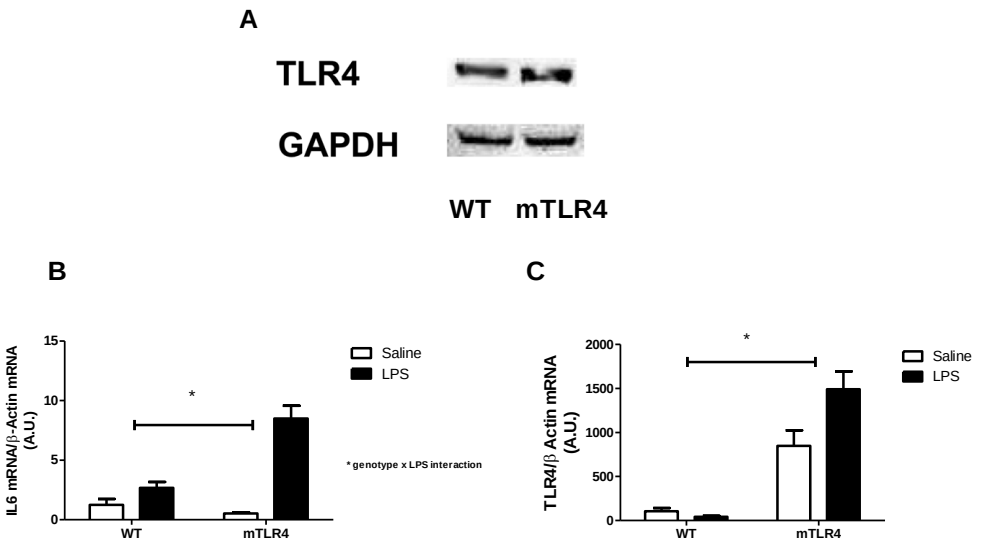


Figure 7. 1

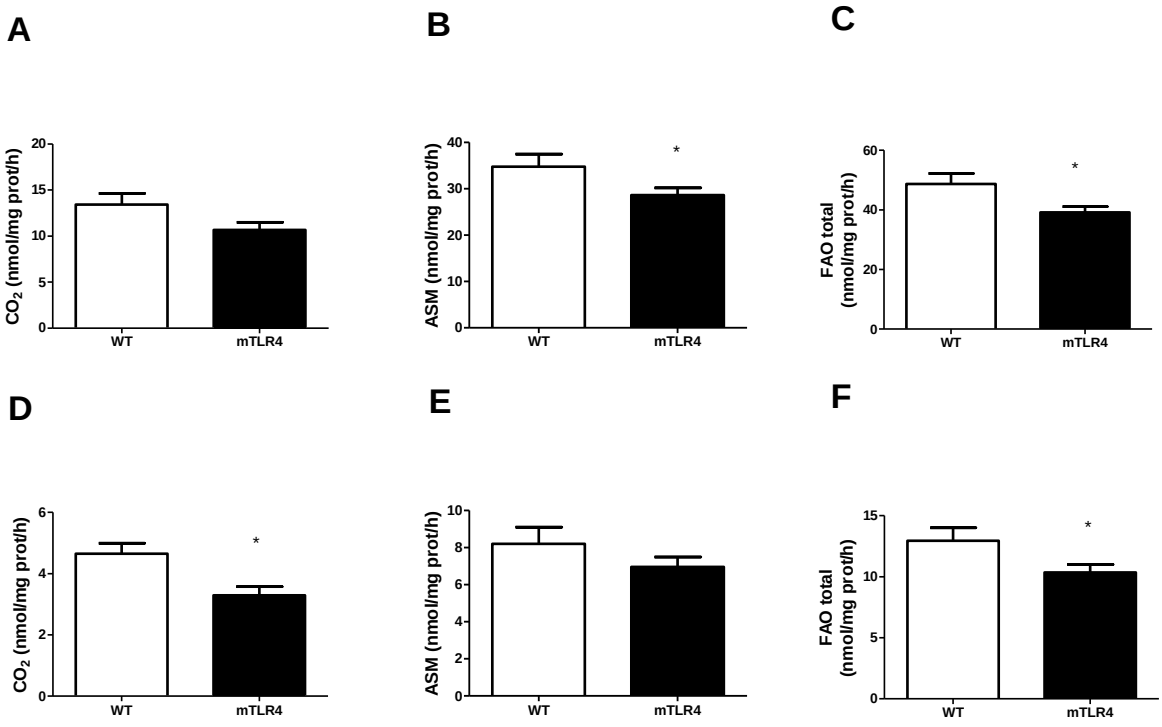
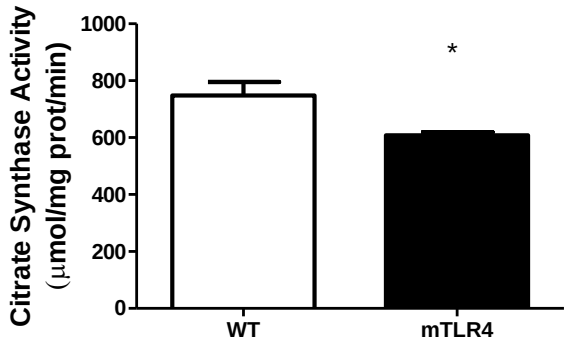


Figure 7. 2



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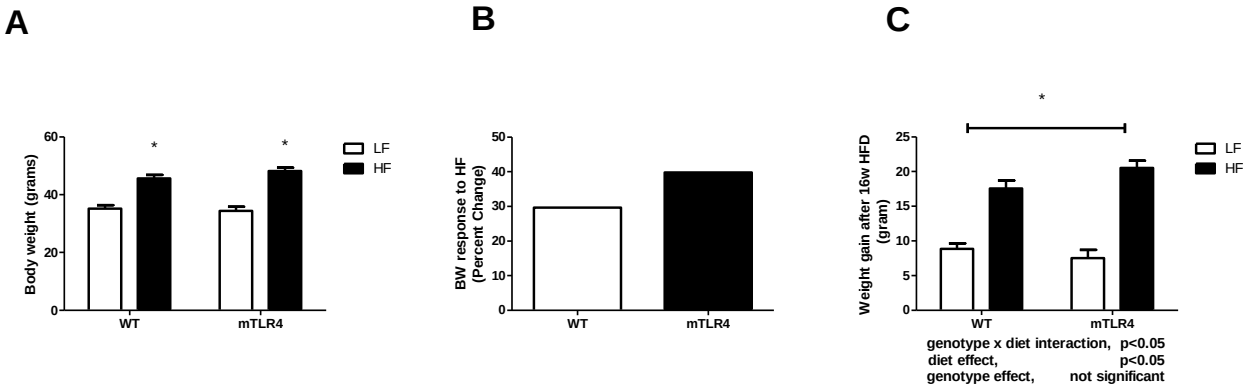

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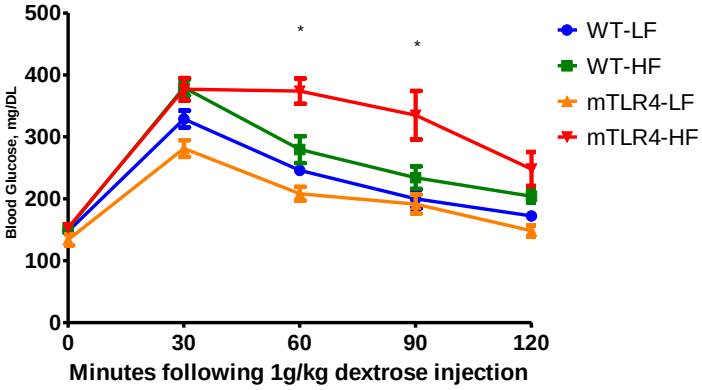

Figure 7. 5

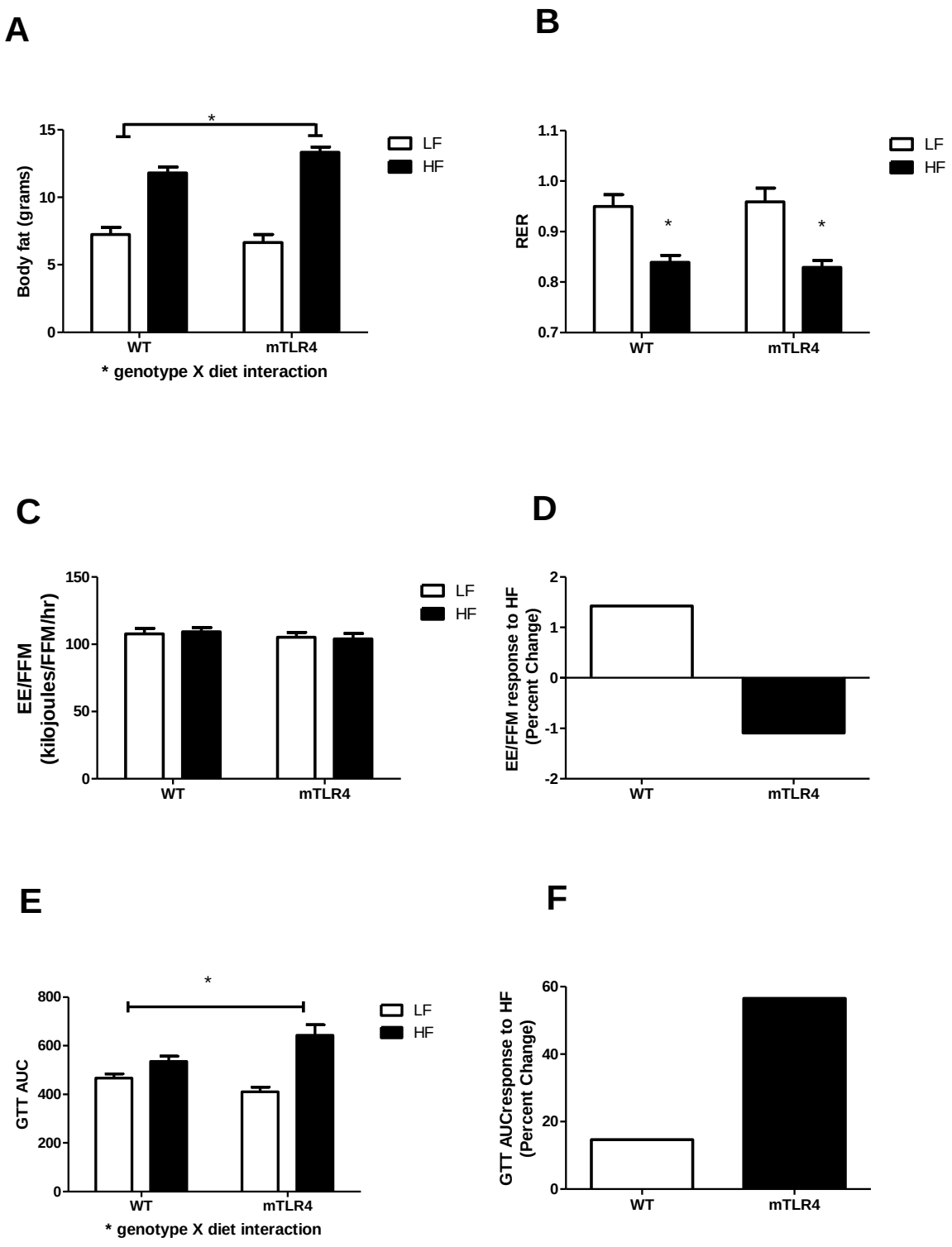
A
Body fat (grams)
15
10
5
0
*
LF
HF
WT
mTLR4
* genotype X diet interaction
B
RER
1.1
1.0
0.9
0.8
0.7
*
*
LF
HF
WT
mTLR4
C
EE/FFM (kilojoules/FFM/hr)
150
100
50
0
LF
HF
WT
mTLR4
D
EE/FFM response to HF (Percent Change)
2
1
0
-1
-2
WT
mTLR4
E
GTT AUC
800
600
400
200
0
*
LF
HF
WT
mTLR4
* genotype X diet interaction
F
GTT AUCresponse to HF (Percent Change)
60
40
20
0
WT
mTLR4

Figure 7. 6

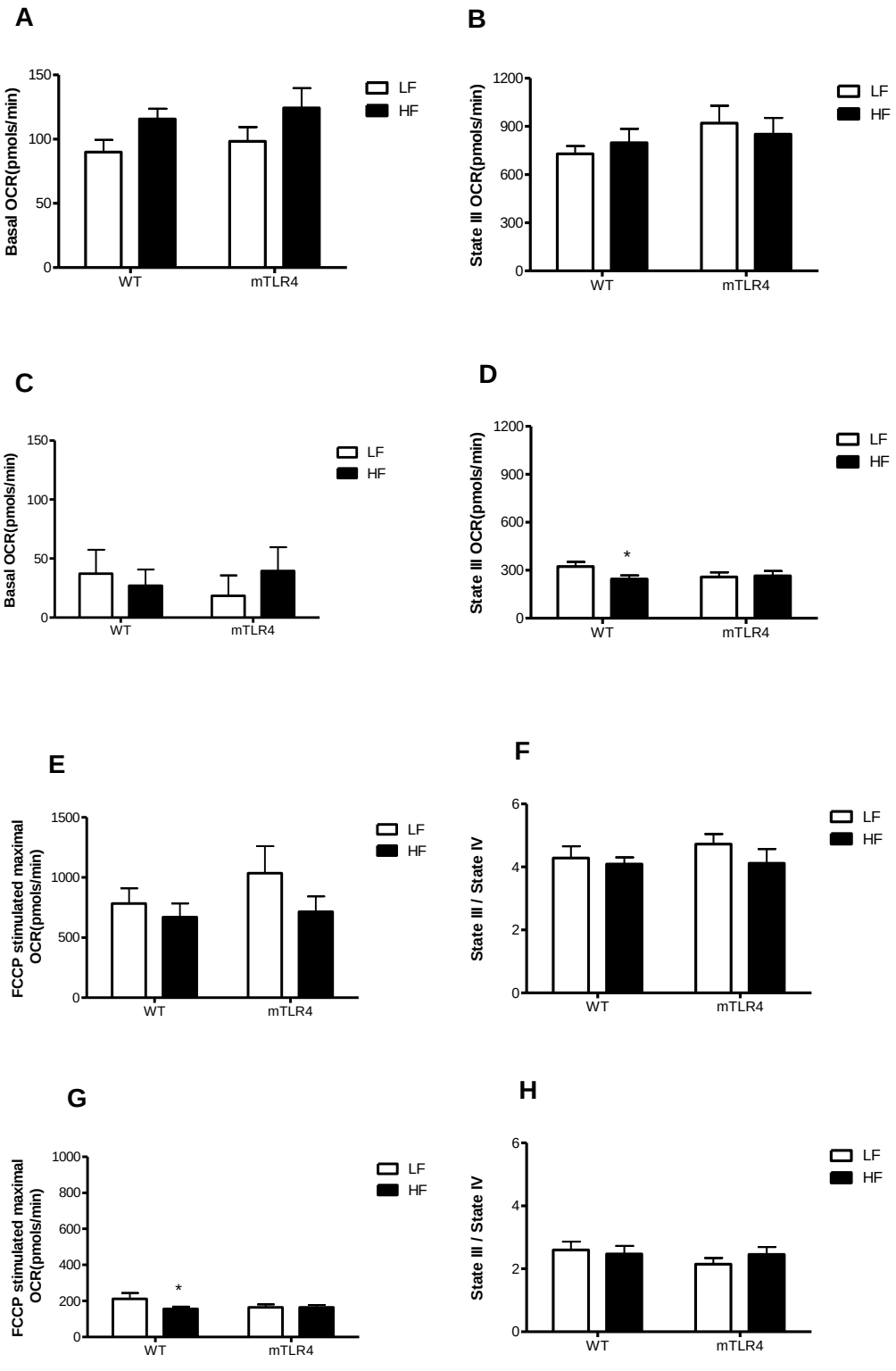
A
Basal OCR(pmols/min)
150
100
50
0
WT
mTLR4
LF
HF
B
State III OCR(pmols/min)
1200
900
600
300
0
WT
mTLR4
LF
HF
C
Basal OCR(pmols/min)
150
100
50
0
WT
mTLR4
LF
HF
D
State III OCR(pmols/min)
1200
900
600
300
0
*
WT
mTLR4
LF
HF
E
FCCP stimulated maximal OCR(pmols/min)
1500
1000
500
0
WT
mTLR4
LF
HF
F
State III / State IV
6
4
2
0
WT
mTLR4
LF
HF
G
FCCP stimulated maximal OCR(pmols/min)
1000
800
600
400
200
0
*
WT
mTLR4
LF
HF
H
State III / State IV
6
4
2
0
WT
mTLR4
LF
HF

Figure 7. 7

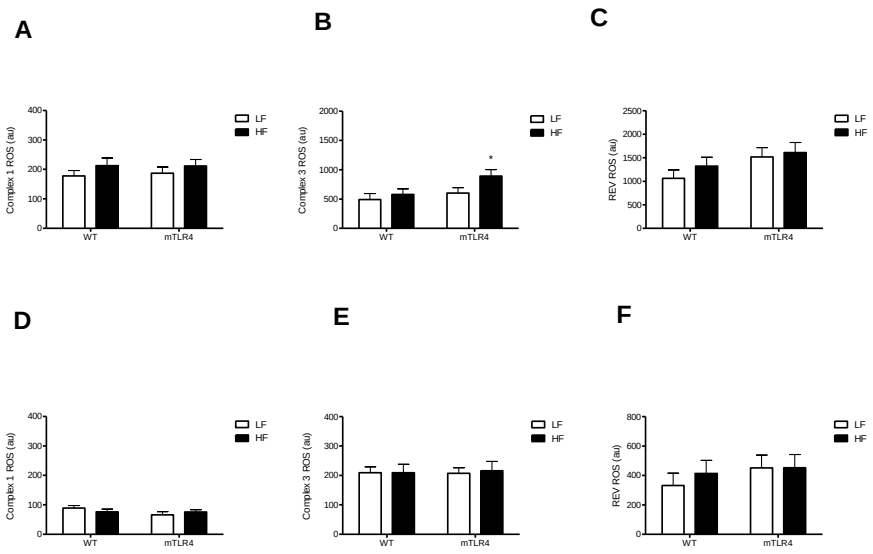

Figure 7. 8

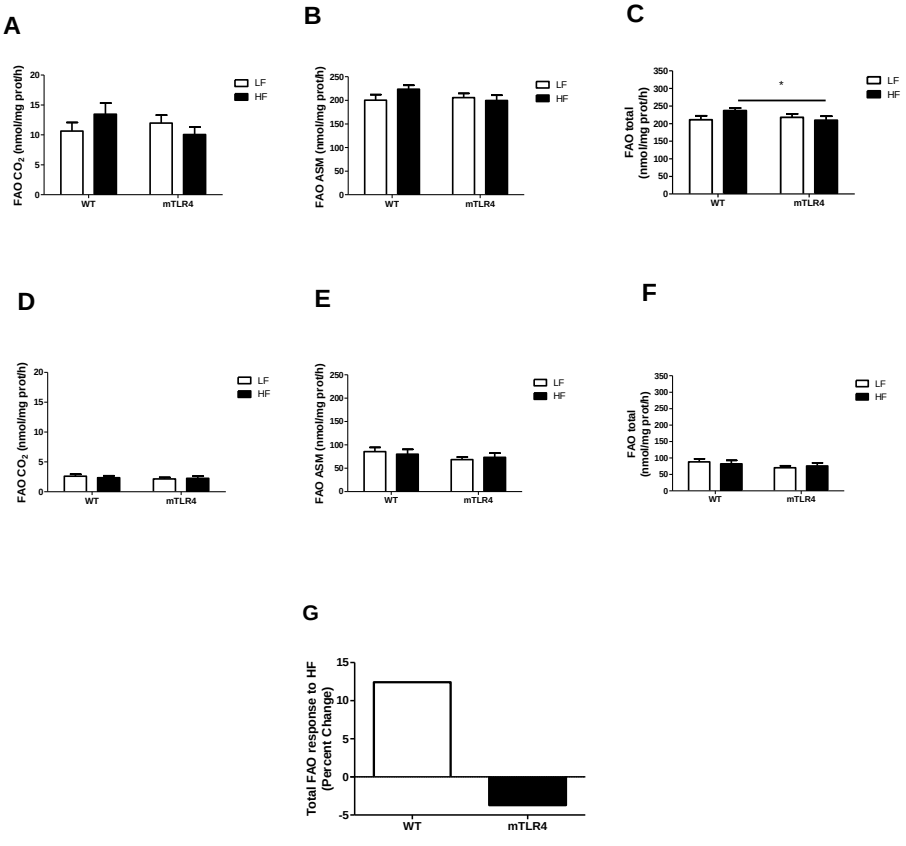

Figure 7. 9

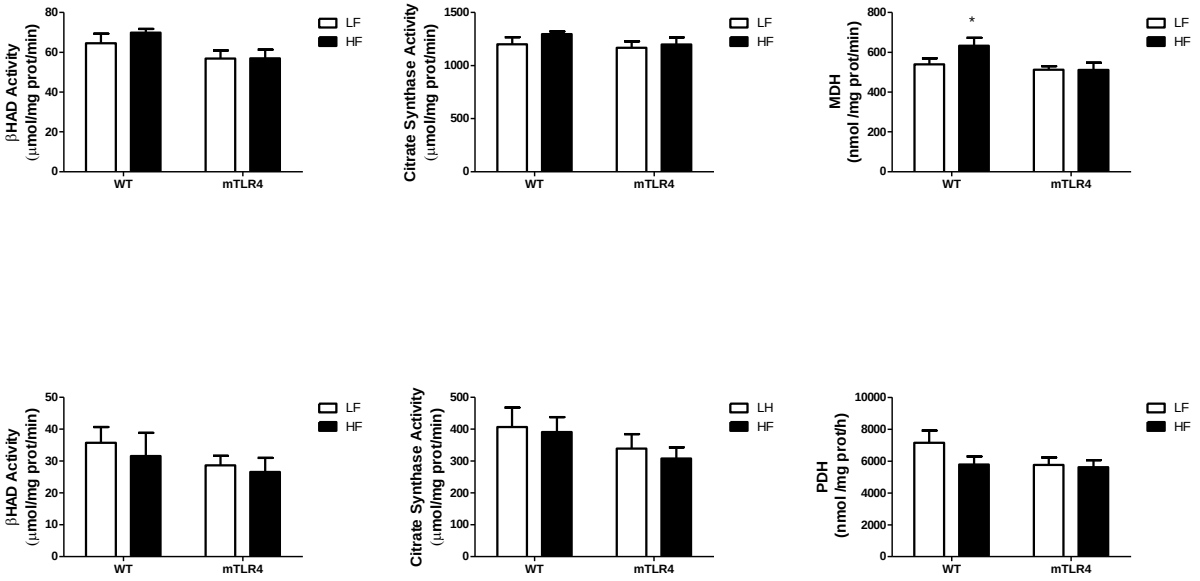
βHAD Activity (μmol/mg prot/min)
Citrate Synthase Activity (μmol/mg prot/min)
MDH (nmol /mg prot/min)
βHAD Activity (μmol/mg prot/min)
Citrate Synthase Activity (μmol/mg prot/min)
PDH (nmol /mg proth)
LF
HF
LH
WT
mTLR4
*

Figure 7. 10

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Supplementary Data

Figure 1. Muscle TLR4 over expression does not alter body weight, body fat, or energy expenditure on a standard chow diet. Mice were maintained on 12hr light-dark cycles and fed on a standard chow diet. Body composition was assessed via the Bruker mini spec LF90. Energy expenditure was measured over 48-hours using indirect calorimetry (TSE Systems, Chesterfield, MO). (A) Body mass. (B) Body fat. (C) Respiratory exchange ratio. (D) Energy expenditure per lean mass. Data is expressed as mean ± SEM. * $P<0.05$.

Figure 2. Compared to mTLR4, WT mice respond slightly better to glucose challenge on a standard chow diet. Following an overnight fast, mice were injected with 1g/kg glucose and blood was measured via the tail vein every 30 minutes for 2 hours. Data is expressed as mean ± SEM. * $P<0.05$.

Figure 3. Mitochondrial function is not different between mTLR4 and WT mice on a standard chow diet. mTLR4 mice and their WT littermates were sacrificed after overnight fast. Mitochondria were isolated and oxygen consumption was measured using a Seahorse Bioscience extracellular flux analyzer. (A) Basal respiration. (B) ADP-stimulated state 3 respiration. (C) FCCP-stimulated maximal respiration. (D) RCR. Data are expressed as mean ± SEM. *$P<0.05$.

Figure 4. ROS production is not different between mTLR4 and WT mice in isolated mitochondria on a standard chow diet. ROS production was measured using Amplex Red Hydrogen Peroxide/Peroxidase assay Kit. (A) Complex 1. (B) Complex 2. (C) REV. Data are expressed as mean ± SEM. *$P<0.05$.

Figure 5. FAO trends to be suppressed in isolated muscle mitochondria from mTLR4 mice on a standard chow diet. mTLR4 mice and their WT littermates were sacrificed after overnight

fast. Fatty acid oxidation was measured in isolated mitochondria (red mitochondria) by assessing [1-14C]-palmitic acid breakdown to 14-CO_2 and acid soluble metabolites. (A) CO_2 production. (B) ASM. (C) Total FAO. Data expressed as mean ± SEM, * $p<0.05$.

Figure 6. Muscle TLR4 over expression does not alter glucose oxidation on a standard chow diet. mTLR4 mice and their WT littermates were sacrificed after overnight fast. Radio-labeled glucose was used to measure glucose metabolism in skeletal muscle homogenates. (A) Glucose oxidation in red homogenates. (B) Glucose oxidation in white homogenates. Data expressed as mean ± SEM, * $p<0.05$.

Figure 7. Oxidative enzyme activities are not different in mTLR4 mice compared to WT mice in muscle homogenates on a standard chow diet. mTLR4 mice and their WT littermates were sacrificed after overnight fast. Oxidative enzyme activities were measured in skeletal muscle homogenates. (A) CS activity in red homogenates. (B) CS activity in white homogenates. (C) BHAD activity in red homogenates. (D) BHAD activity in white homogenates. Data expressed as mean ± SEM, * $p<0.05$.

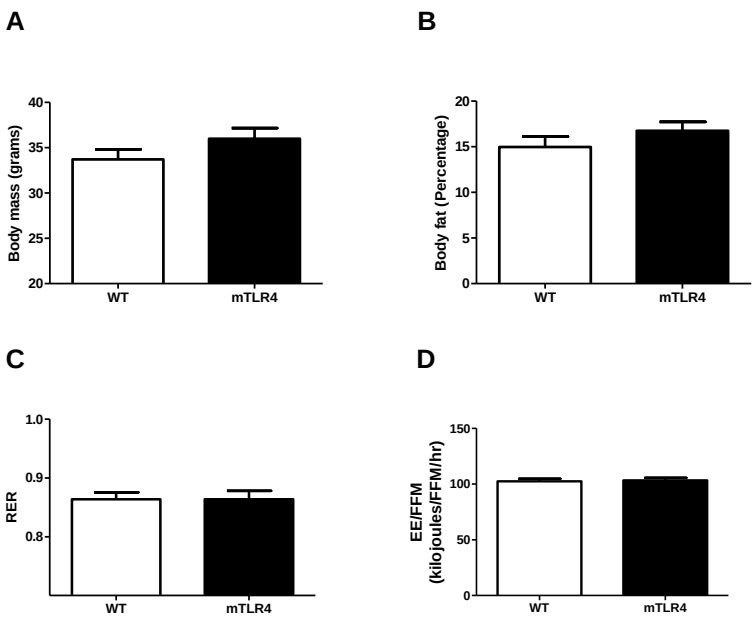
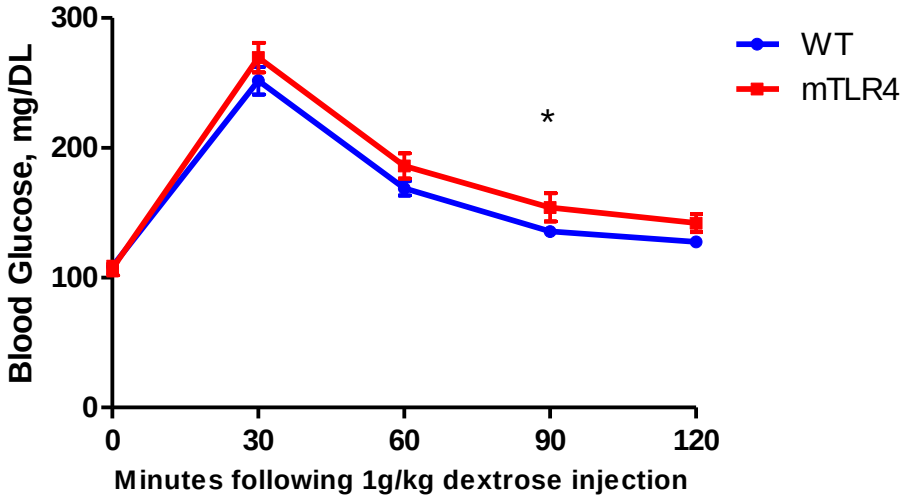


Figure 1



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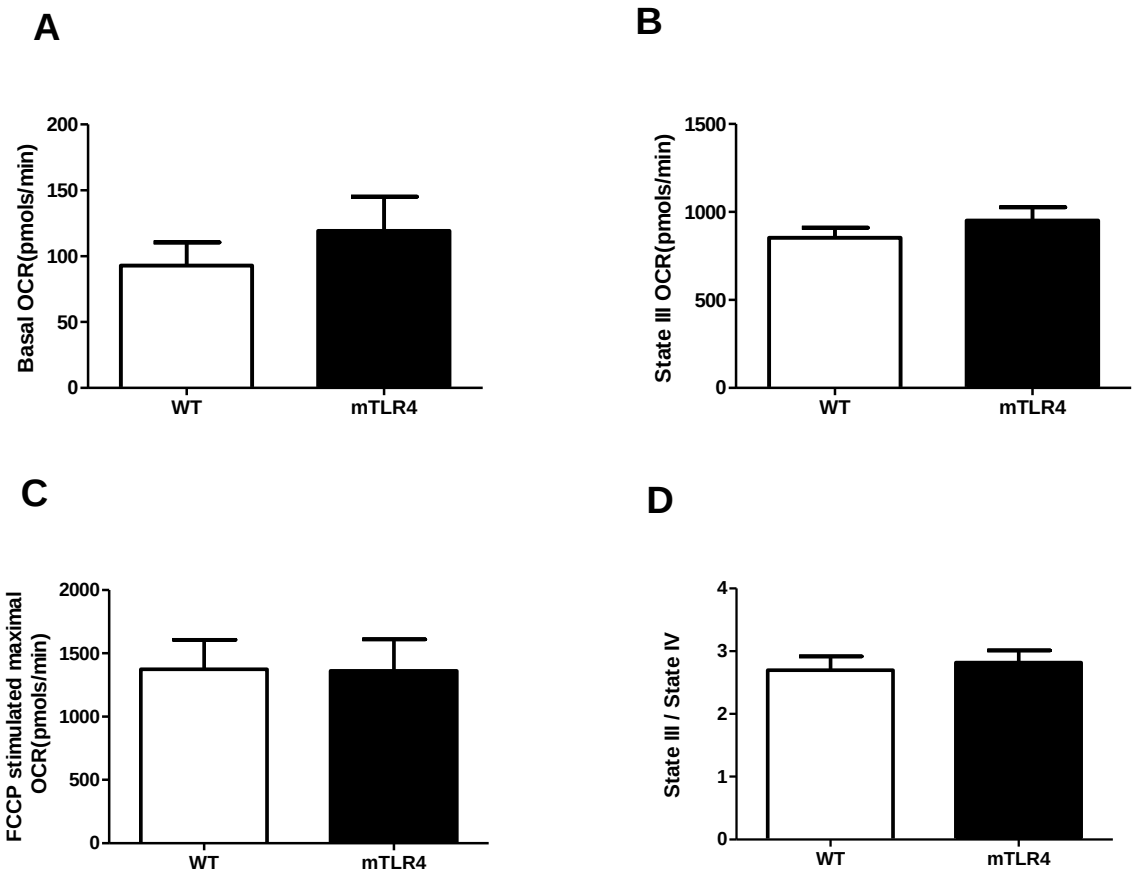
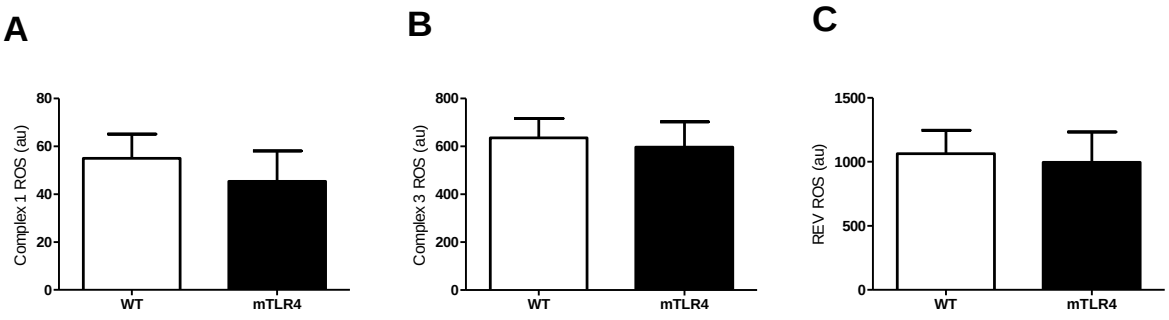


Figure 3



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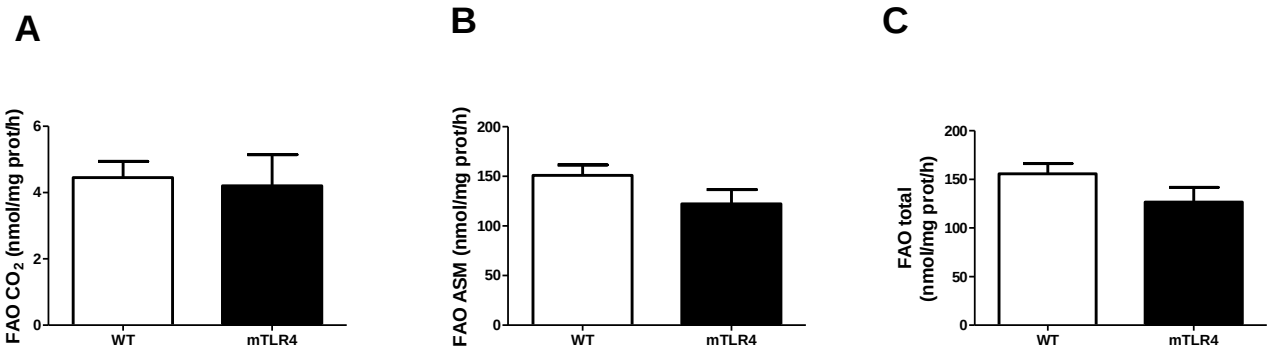

Figure 5

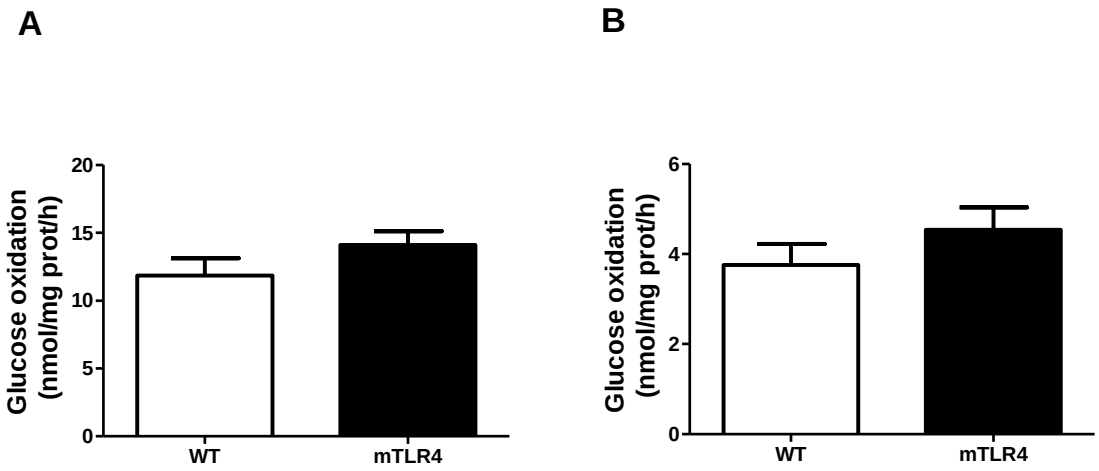

Figure 6

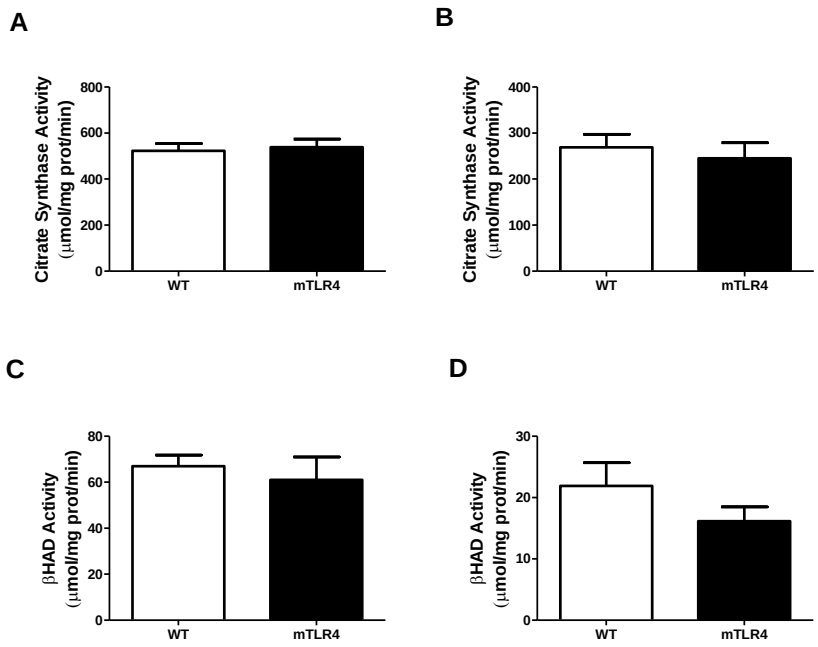
A
B
C
D
Citrate Synthase Activity (µmol/mg prot/min)
βHAD Activity (µmol/mg prot/min)
WT
mTLR4
0
200
400
600
800
100
300
10
20
30
40
60
80

Figure 7

Chapter 8: Conclusions/Future Directions

The central objective of this project was to examine the metabolic effects of TLR4 in skeletal muscle.

- The cell culture studies highlight close links between TLR4 activation and mitochondrial uncoupling. Two hours of LPS (50pg/mL) treatment in C2C12 cells resulted in deceased cellular respiratory control ratio, FCCP-stimulated maximal respiration, and increased respiration in the presence of oligomycin, which are all indicative of mitochondrial uncoupling.
- Mitochondria isolated from skeletal muscle of C57BL/6 mice 4 hours following an intraperitoneal injection of LPS (1 µg/kg) displayed reduced respiratory control, ADP-stimulated respiration, and maximal respiration.
- There was no effect on any aspects of mitochondrial respiration in isolated mitochondria treated directly with LPS, suggesting that LPS must bind to TLR4 to initiate changes in mitochondrial function. To further confirm the role of TLR4 in mitochondrial substrate metabolism, TLR4 neutralizing antibody was used in the acute TLR4 activation in C2C12 cells. As expected, the LPS-induced effects in mitochondrial substrate handling were completely blocked with the neutralization of TLR4.
- The current work also demonstrated that TLR4 activation in skeletal muscle results in production of ROS in concert with mitochondrial uncoupling and altered substrate metabolism. These effects are either completely or partially blocked in the presence of antioxidant.
- To directly assess the role of TLR4 in handing substrates metabolism in skeletal muscle, C57BL/6 mice with muscle-specific over-expression of the TLR4 gene were generated.

Fatty acid oxidation was significantly decreased in muscle homogenates from mTLR4 mice on a standard chow diet, but not isolated mitochondria. Mitochondrial respiration and ROS production do not appear to be affected by over-expression of TLR4 on standard chow diet.

- 16 weeks of high fat feeding significantly increased body mass in both mTLR4 and WT mice. mTLR4 mice gained significantly more weight and body fat on a high fat diet compared to WT littermates.
- Following 16 weeks high fat diet, there was a significant reduction of fatty acid oxidation in isolated mitochondria in mTLR4 mice. In addition, increased ROS production and higher area under the curve for glucose tolerance test were also observed in mTLR4 mice.

In this study we have identified several critical areas where additional research is needed.

- Determining the source of ROS. Future studies utilizing inhibitors of either NADPH oxidase, xanthine oxidase or the mitochondrial electron transport chain in combination with LPS treatment are needed.
- Determining the source of mitochondrial uncoupling is also imperative. Based on UCP3 western blot results, uncoupling protein might be the source of mitochondrial uncoupling. GDP binding studies would help us to evaluate whether uncoupling protein is the real source of mitochondrial uncoupling.
- Investigating the mechanism(s) for increased PDH activity after acute TLR4 activation. Whether it is due to: a) increased pyruvate levels as a result of increased glycolytic flux, or b) reduced acetyl-CoA and/or NADH levels in the mitochondrial matrix, is not currently known. Future measurements of mitochondrial levels of these metabolites may shed some light on this topic.

- Evaluating the effects of activating TLR4 pathway on substrate metabolism and mitochondrial function in mTLR4 mice, both on standard chow diet and after high fat feeding. As our research has suggested that over-expressed TLR4 gene in skeletal muscle may not equate to the activation of TLR4.
- Monitoring cage activity and food intake of mTLR4 mice during high fat feeding would provide valuable information to determine the mechanism that mTLR4 mice gain more weight on high fat diet as compared to that of WT control mice.