The Metabolic Effects of Low Grade Inflammation on Postprandial Metabolism Following a High Fat Meal

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Abstract

Inflammation is a central feature of various metabolic diseases including obesity and type-II diabetes. For this study, we hypothesized postprandial metabolism following an acute, high fat (HF) meal to be impaired in mice pre-injected with an inflammatory agonist. To this end, C57BL/6J mice were injected with saline or lipopolysaccharide (LPS, 1µg/kg_{bw}) following an overnight fast and gavaged 2hr post-injection with water or a HF meal in liquid form (5kcal; 21.4%SF, 40.8%UF, 27.1%CHO, 10.7%PRO). Blood and muscle samples taken 3hr post-gavage underwent *ex vivo* analysis. Overall, results demonstrated a metabolic response to a HF meal that was blocked in the presence of LPS. Metabolic flexibility, though unchanged following the HF meal alone, was reduced following the HF meal in the presence of LPS. Additionally, state-4 uncoupled mitochondrial respiration, which was increased following the HF meal, was also reduced following the HF meal in the presence of LPS. Similar near-significant trends were also observed with total palmitate oxidation. Although no independent response to a HF meal or LPS exposure was observed, a unique interaction between treatments significantly diminished ADP dependent, state-3 and maximal respiration. These effects do not appear to be dependent on the production of reactive oxygen species (ROS) since neither the HF meal nor LPS exposure resulted in increased production of ROS. In conclusion, these results demonstrate that acute activation of inflammatory pathways results in alterations in metabolic response to a HF meal in skeletal muscle from mice, although the mechanism underlying these effects is not yet understood.

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Chapter 1: Introduction

Introduction

Comparative risk assessment of American fatalities indicated that complications from being overweight or obese lead to 1 in 10 deaths in 2005 (Danaei et al., 2009). Today, more than 35% of the United States adult population is obese with a body mass index (BMI) of at least 30 kg/m^2 and trends are not improving (Flegal, Carroll, Kit, & Ogden, 2012). Further complications of obesity include the development of type II diabetes (T2D) with prevalence increasing from 23%, to 33%, to 43% in *class I, II, and III obesity*, respectively (Nguyen, Nguyen, Lane, & Wang, 2011). Dysregulated metabolism in skeletal muscle has been implicated in the development of obesity and associated comorbidities (Hulver et al., 2005; Hulver et al., 2003). However, the mechanism(s) underlying these defects are not entirely understood.

Inflammation has been associated with obesity, T2D, and a variety of other chronic diseases (Arkan et al., 2005; Yudkin, 2007; Yudkin, Kumari, Humphries, & Mohamed-Ali, 2000). Inflammation, while broadly recognized as a critical component of the innate immune system, has been shown to disrupt normal metabolism (Feingold et al., 1992; Grunfeld & Feingold, 1991; Kitagawa, Yamaguchi, Imaizumi, Kunitomo, & Fujiwara, 1992). For example, hypertriglyceridemia and disrupted insulin signaling have been attributed to activation of pro-inflammatory pathways in response to endotoxins and other inflammatory agonists (Feingold, et al., 1992; Kitagawa, et al., 1992; Lang, Cooney, & Vary, 1996; Memon, Grunfeld, Moser, & Feingold, 1993). Reciprocally, treatment with the anti-inflammatory agents is able to restore insulin signaling following inflammatory activation (Jung et al., 2008; Wang, Wu, Siegel, Egan, & Billah, 1995).

In 2007, Cani et al. provided a physiological context for endotoxin-induced inflammation in the development of metabolic disease through an association between

diet, the intestinal microbiota, and circulating endotoxin in mice (Cani et al., 2007). By treating mice with the chronic, low doses of endotoxin observed in the circulation of mice with high fat diet (HFD)-induced obesity, Cani et al. was able to cause weight gain, promote hepatic lipid storage, and disrupt whole body insulin signaling in a toll-like receptor 4 (TLR4) dependent manner (Cani, et al., 2007). As the exclusive receptor for the bacterial endotoxin lipopolysaccharide (LPS), the innate immune receptor TLR4 promotes the transcription factor-regulated expression of inflammatory cytokines in a variety of immune and non-immune cells (Beutler, Du, & Poltorak, 2001).

Skeletal muscle possesses, in addition to functional TLR4, higher TLR4 expression in obese humans than non-obese controls (Frisard et al., 2010; Lang, Silvis, Deshpande, Nystrom, & Frost, 2003). Furthermore, a glycolytic shift in metabolism is observed in both rodent and cell culture models in response to low dose LPS treatments (Frisard, et al., 2010). This glycolytic shift features increased activity of phosphofructokinase along with decreased activity of citrate synthase (CS) and β-hydroxyacyl-Coenzyme A dehydrogenase (βHAD) (Frisard, et al., 2010). As CS and βHAD are key enzymes of the Kreb's cycle and β-oxidation, respectively, this suggests that there may be defects in mitochondrial function during low-grade inflammation (Frisard, et al., 2010). This report supports the implication of mitochondrial dysfunction in a variety of diseases including obesity, diabetes, and metabolic syndrome where inflammation is also implicated (Finocchietto et al., 2008; Hernandez-Aguilera et al., 2013; Ritov et al., 2005). The suppression of oxidative metabolism induced by a low grade inflammatory state may lead to the inability to adjust fuel oxidation to fuel availability, a condition known as metabolic inflexibility (Kelley & Mandarino, 2000). The inability to match substrate oxidation with availability is characteristic of and a potential

cause of obesity particularly in an environment where overconsumption of fat and sugar is evident. Therefore, the goal of the current study is to investigate the effects of inflammation on skeletal muscle metabolic flexibility and mitochondrial function during postprandial metabolism following a high fat meal.

Statement of the Problem

Obesity and T2D are strongly associated chronic diseases characterized by inflammation and mitochondrial dysfunction, with a 49.1% prevalence of obesity among diabetics (Finocchietto, et al., 2008; Hernandez-Aguilera, et al., 2013; Ritov, et al., 2005). Though HFD-induced elevation of plasma endotoxin has been shown to induce obesity and T2D while decreasing skeletal muscle mitochondrial function, the specific effects of inflammation on postprandial metabolism in skeletal muscle has not been determined (Cani, et al., 2007; Frisard, et al., 2010). This study aims to investigate the effects of low dose endotoxin pre-injection on postprandial metabolism following a high fat meal.

Significance of the Study

Obesity is a condition affecting more than 35% of the United States' population (Flegal, et al., 2012). Exhaustive attempts to combat obesity by improving energy balance have been taken, however, the factors involved in the regulation of energy balance are not entirely understood (Hill, Wyatt, & Peters, 2012). As inflammation has been shown to powerfully affect energy balance, a thorough investigation of inflammation's effects on skeletal muscle's contribution to energy balance is necessary (Cani, et al., 2007; Frisard, et al., 2010). Gaining a better understanding of how skeletal

muscle responds to the inflammatory microenvironment of metabolic diseases could lead to novel treatment options in the future.

Specific Aim

Determine the effects of acute activation of inflammatory pathways on postprandial metabolism and mitochondrial function in skeletal muscle in mice following a high fat meal.

Hypothesis

Activation of inflammatory pathways will impair postprandial metabolism and mitochondrial function in skeletal muscle in mice following a high fat meal.

Objective

Prior administration of exogenous LPS (endotoxin) will impair postprandial fat oxidation, metabolic flexibility, mitochondrial respiration and increase reactive oxygen species production in skeletal muscle of mice following a high fat meal.

Basic Assumptions

1. The *in vivo* physiological characteristics of C57Bl/6J mice are comparable to those of humans.
2. Homogeneity of physiological characteristics within the C57Bl/6J strain.

Limitations

1. Limited amount of skeletal muscle available per mouse.

Chapter 2: Review of the Literature

Skeletal Muscle and Metabolism

Skeletal muscle is a tissue widely recognized for its capacity to exert voluntary, contractile forces on the skeletal system to produce movement, stabilize joints, and maintain postures and positions (Hamill & Knutzen, 2009). In addition, skeletal muscle supports and protects visceral and internal organs, regulates cavity pressure, produces heat, and provides voluntary control over oral consumption and waste excretion (Dunford & Doyle, 2008; Hamill & Knutzen, 2009). Contractile activity and the other physiological functions of human skeletal muscle require energy from the hydrolysis of adenosine triphosphate (ATP) (Dunford & Doyle, 2008). To meet this demand for ATP, skeletal muscle utilizes anywhere between 20 to 87% of systemic oxygen supply at a given point of the day to break down fuel macronutrients (Rolfe & Brown, 1997). While ATP demand is continuous throughout the day, the availability of carbohydrates and lipids is typically at flux (Gropper, Smith, & Groff, 2009).

In response to the ever-changing availability of macronutrients in the body, skeletal muscle substrate preference between carbohydrates and lipids continuously shifts throughout the day in healthy humans (Kelley & Mandarino, 2000). Unlike carbohydrates and lipids, proteins are not primarily used as fuel except during periods of intense physiological stress (Elia, Stubbs, & Henry, 1999). As macronutrients consumed through the diet enter circulation, they can either remain in circulation for rapid systemic availability or, if circulating concentrations are too high, be taken up and stored in the liver, adipose tissue, or skeletal muscle for later use. In skeletal muscle, glycogen and triglycerides are utilized as the stored forms of carbohydrates and lipids, respectively. However, macronutrients stored in the liver and adipose can be released into circulation to also meet systemic demand. With the ability to utilize carbohydrates

and lipids from a variety of sources, skeletal muscle demonstrates a varying preference for carbohydrates and lipids dependent on momentary physiological conditions (Kelley & Mandarino, 2000; Krogh & Lindhard, 1920; Randle, 1998; Randle, Garland, Hales, & Newsholme, 1963).

Respiratory quotient (RQ), though directly a gas-exchange ratio of CO_2 produced to O_2 consumed at the cellular level, is utilized as an indirect measure of the body's relative reliance upon carbohydrates and lipids (Krogh & Lindhard, 1920; Romijn et al., 1993). RQ reports exclusive oxidation of carbohydrates and lipids as 1.0 and ~0.7, respectively, with values in between linearly derived to express a proportional combination of fuel source consumption (Gropper, et al., 2009; Krogh & Lindhard, 1920). Respiratory exchange ratio (RER) is a similar metric used at rest to determine systemic substrate preference using the same scale (Gropper, et al., 2009). Unlike RQ, however, RER is a ratio of CO_2 exhaled to O_2 inhaled at the mouth providing opportunity for CO_2 contributed from bicarbonate buffering to inflate RER values during exercise (Gropper, et al., 2009). In the early 20th century, scientists were first able to describe substrate preference in a variety of physiological conditions using these methods (Krogh & Lindhard, 1920).

In the fasted state, skeletal muscle metabolism of healthy humans is characterized by the predominant uptake and subsequent oxidation of lipids and therefore a lower RQ (Gropper, et al., 2009; Krogh & Lindhard, 1920). Lipids are released by adipose tissue into circulation to satisfy fasting energy requirements, sparing circulating glucose for the brain (Randle, 1998). The fasted-state suppression of glycolytic activity has been attributed to end-product inhibition by acetyl-coenzyme A (CoA), a product of both aerobic glycolysis and β-oxidation (Randle, Kerbey, & Espinal,

1988). While fasting, pyruvate concentrations diminish due to reduced glucose availability and acetyl-CoA is increasingly produced from lipids instead of pyruvate (Gropper, et al., 2009). Pyruvate dehydrogenase (PDH), the enzyme responsible for converting the glycolytic end-product pyruvate into the Kreb's cycle substrate acetyl-CoA, is a critical regulatory point of skeletal muscle metabolism (Pilegaard, Saltin, & Neufer, 2003; Spriet et al., 2004)

In addition to allosteric and transcriptional regulation, the activity of PDH is dependent upon its phosphorylation status, a property regulated by PDH phosphate phosphorylase (PDHP) and PDH kinase (PDK) (Gropper, et al., 2009). PDK inhibits PDH activity via phosphorylation when pyruvate concentrations are reduced by limited glucose availability and acetyl-CoA concentrations are elevated by increased fatty acid oxidation (Gropper, et al., 2009). Correspondingly, transcription and activity of PDK4, the PDK isozyme most prevalent in skeletal muscle, is highly up-regulated in skeletal muscle upon fasting and being fed a HFD (Peters et al., 2001; Pilegaard, et al., 2003; Spriet, et al., 2004). Upon refeeding, increased glucose availability drives a shift in substrate preference from lipids to carbohydrates (Gropper, et al., 2009; Pilegaard, et al., 2003).

In the fed state, the glycolytic shift in skeletal muscle metabolism is marked by an elevation in RQ (Krogh & Lindhard, 1920; Randle, et al., 1963). The blood glucose responsive hormone insulin restores PDH activity and ultimately glycolytic flux through promotion of PDH phosphatase via Ca^{2+} signaling (Gropper, et al., 2009; Macaulay & Jarett, 1985). In addition to up regulating glycolytic flux via reactivation of PDH, insulin stimulates the translocation of glucose transporter 4 (GLUT4) to the cell membrane to drastically accelerate skeletal muscle glucose uptake (Furtado, Somwar, Sweeney, Niu,

& Klip, 2002; Gropper, et al., 2009; Larner et al., 1989). The insulin initiated shift toward carbohydrate oxidation also includes a reciprocal effect on lipolytic metabolism with the inhibition of fatty acid uptake into the mitochondria (Furtado, et al., 2002; Witters & Kemp, 1992).

Under fed conditions, insulin signaling, along with AMP-activated protein kinase (AMPK) dephosphorylation, activates acetyl-CoA carboxylase (ACC) causing the conversion of acetyl-CoA into malonyl-CoA (Witters & Kemp, 1992). This intermediate of *de novo* lipogenesis is also a potent competitive inhibitor of lipid oxidation's rate-limiting carnitine palmitoyltransferase-1 (CPT1) (McGarry, 1995). As the insulin-stimulated uptake of circulating glucose normalizes glucose concentrations, there is a matched decline in circulating insulin (Jacot, Defronzo, Jequier, Maeder, & Felber, 1982). As glucose and insulin concentrations decline, lipolytic activity reciprocally elevates to meet energy demands (Jacot, et al., 1982). Elevations in AMP concentrations activate AMPK to block glycogen synthesis, *de novo* lipogenesis, and glycolytic flux while promoting β-oxidation (McGarry, 1995). The dynamic response of skeletal muscle metabolism to varying availabilities of oxidative substrates throughout fed and fasted metabolic states demonstrates a key trait of skeletal muscle: metabolic flexibility (Kelley & Mandarino, 2000).

Metabolic flexibility, a term coined by Kelley and Mandarino in 2000, describes the capacity to adapt substrate specific oxidative metabolism to match substrate availability (Galgani, Moro, & Ravussin, 2008; Kelley & Mandarino, 2000). The robust metabolic flexibility of human skeletal muscle is characterized by a fasted dependence on the oxidation of lipids and a preference for carbohydrate oxidation under fed, insulin-stimulated conditions (Andres, Cader, & Zierler, 1956; Kelley, Reilly, Veneman, &

Mandarino, 1990). To quantify metabolic flexibility, studies compare the difference (Δ) between RQ or RER at the fasted state and the fed/insulin-stimulated state (Kelley & Mandarino, 2000). Large ΔRQ reveals metabolic flexibility while low values demonstrate metabolic inflexibility (Kelley & Mandarino, 2000). Rather than demonstrate a highly lipolytic fasted metabolism and a highly glycolytic fed metabolism, metabolically inflexible subjects are unable to adjust substrate oxidation to macronutrient availability with overall impairments to lipid oxidation during the fasted state (Randle, 1998; Smith et al., 2000). Such metabolic inflexibility has often been associated with cardiovascular disease (CVD), obesity, T2D, and metabolic syndrome (Kelley, Goodpaster, Wing, & Simoneau, 1999; Randle, et al., 1963; Stump, Henriksen, Wei, & Sowers, 2006; Thyfault, Rector, & Noland, 2006).

While there appears to be a genetic predisposition for an individual to become metabolically (in)flexible, a number of interventions have been shown to improve metabolic flexibility (Aucouturier, Duche, & Timmons, 2011; Carvalho-Filho, Ueno, Carvalheira, Velloso, & Saad, 2006; Corpeleijn, Saris, & Blaak, 2009; De Pergola et al., 2003; Galgani et al., 2008; Kelley & Goodpaster, 2001; Kelley & Mandarino, 2000). In 2000, Smith et al. demonstrated that certain individuals had an impaired ability to adjust lipolytic activity to match increases in dietary fat intake (Smith et al., 2000). However, the described metabolic inflexibility was ameliorated by increased physical activity (Smith et al., 2000). In addition to increasing physical activity, low glycemic index diets, thiazolidinedione (TZD) treatments, and dietary supplementation with either Ω3 fatty acids or antioxidants have all been shown to improve metabolic flexibility (Haber et al., 2003; Horakova et al., 2012; Solomon, Haus, Cook, Flask, & Kirwan, 2013). Further investigation of the pathways involved in metabolic flexibility or inflexibility should yield

novel, improved interventions targeted against the various diseases associated with the condition.

Inflammation and Metabolism

Inflammation is a critical component of innate, or nonspecific, immunity (Coico, 2003). While adaptive immunity functions through delayed, antigen-specific responses to environmental intrusions, innate immunity uses rapid, generalized defenses ranging from acidic skin pH to the phagocytosis of foreign particles to protect the host (Coico, 2003). The classic symptoms of inflammation: pain, heat, redness, swelling, and loss of function; are observed in response to mechanical, physical, chemical, biologic, and immunologic injury (Coico, 2003). These symptoms are the result of the inflammatory response to recruit immune cells to the site of injury/infection, isolating tissue damage, and restoring health through phagocytosis of pathogens and debris (Coico, 2003). While the classical symptoms of inflammation are significant, the cellular and molecular mechanisms of this innate immune response are the priority of current research initiatives ("Aims and scope," 2013).

Inflammation is initiated and propagated through various cell- and plasma-derived mediators of the innate immune response to establish a unique microenvironment optimized for isolating damage, recruiting immune cells, eliminating pathogens and damaged cells, and restoring homeostasis (Coico, 2003). In this inflammatory microenvironment, cytokine expression is regulated by detection of various pathogen-associated molecular patterns (PAMP) and danger-associated molecular patterns (DAMP) by the pattern recognition receptors (PRR) of immune and non-immune cells (Chandel, Trzyna, McClintock, & Schumacker, 2000; Coico, 2003;

Seong & Matzinger, 2004). Toll-like receptors (TLR) are a class of PRRs found to be expressed in a variety of immune and non-immune cells (Coico, 2003). These TLRs are responsible for detecting PAMPs like bacterial endotoxins and viral nucleic acids (Coico, 2003). NFκB is the transcription factor that regulates cytokine expression downstream of PRR activation (Chandel, et al., 2000; Lang, et al., 2003). In addition to PAMPs and DAMPs, NFκB-regulated cytokine expression is also promoted by the binding of certain cytokines, including interleukein-1 (IL-1) and tumor necrosis factor-α (TNFα), to their respective receptors (Siebenlist, Franzoso, & Brown, 1994). In addition to promoting further cytokine expression, cytokines activate a number of signal transduction pathways and transcription factors to alter metabolism, cellular growth, and microenvironment (Grunfeld & Feingold, 1991; Kim et al., 2004; Krogh-Madsen, Plomgaard, Moller, Mittendorfer, & Pedersen, 2006). In general, the inflammatory response is responsible for restoring homeostasis to damaged tissues through various systemic and cellular mechanisms (Coico, 2003). While the inflammatory response is a vital component of the innate immune system, unresolved or chronic low grade inflammation has been shown to act to the detriment of health (Coico, 2003; Reid, Rurak, & Harris, 2009).

Inflammation has been implicated in the development of a number of chronic diseases including obesity, T2D (type II diabetes), metabolic syndrome, atherosclerosis, chronic obstructive pulmonary disease, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and cancer (Belov & Lakshina, 2010; Blasko et al., 2004; Donath & Shoelson, 2011; Gilli et al., 2011; Hotamisligil, 2006; Pradhan & Andreasson, 2013; Rosenquist, 2008). Furthermore, inflammation has emerged as a significant player in the skeletal muscle metabolic dysfunction observed in subjects diagnosed with obesity,

T2D, and metabolic syndrome (Mauvais-Jarvis, 2013; Yokota et al., 2013). For example, TNFα, an acute phase cytokine known to potently promote pro-inflammatory cytokine expression, is expressed 4-fold higher in the skeletal muscle of insulin-resistant (IR) subjects compared to insulin-sensitive (IS) subjects (Saghizadeh, Ong, Garvey, Henry, & Kern, 1996). Krogh-Madsen et al. reduced whole body glucose uptake rates through rhTNF infusion during a euglycemic hyperinsulinemic clamp (Krogh-Madsen, et al., 2006). This is in conjunction with the early findings of Hotamisligil et al. demonstrating TNFα impairment of insulin signaling in the liver, adipose tissue, and skeletal muscle (Hotamisligil, Budavari, Murray, & Spiegelman, 1994). For skeletal muscle in particular, an inverse linear relationship between glucose disposal rates and TNF expression is observed (Krogh-Madsen, et al., 2006; Saghizadeh, et al., 1996). In obese humans, weight loss reduced circulating pro-inflammatory cytokines TNFα and IL-6 while elevating circulation of the anti-inflammatory cytokine IL-10 (Jung, et al., 2008). In fact, Kim et al. demonstrated the capacity of IL-10 infusion to rescue both IL-6 and lipid induced defects in insulin signaling during a euglycemic hyperinsulinemic clamp (Kim, et al., 2004).

TLR4 is a PRR expressed in a wide variety of cell types throughout the body and has been implicated in a number of chronic metabolic diseases (Beutler, et al., 2001; Reyna et al., 2008; Schilling et al., 2011; Tsukumo et al., 2007). The ligand responsible for TLR4 activation is the lipid A chemotoxic domain of lipopolysaccharide (LPS), an endotoxin and component of the cell wall of Gram-negative bacteria (Beutler, et al., 2001; Raetz & Whitfield, 2002). The binding of LPS to TLR4 leads to activation of NFκB, a transcription factor regulating the expression of pro-inflammatory cytokines (Lang, et al., 2003). In skeletal muscle, LPS readily promotes the expression and secretion of

NFκB-regulated inflammatory cytokines including TNF-α, IL-1β, IL-6, and monocyte chemoattractant protein-1 (MCP1) (Frisard, et al., 2010; Lang, et al., 2003). Synergistically, inflammatory cytokines alter physiology downstream of TLR4, functioning as mediators of LPS-initiated metabolic responses (Grunfeld & Feingold, 1991; Memon, et al., 1993). Intriguingly, TLR4 expression and activation is up regulated in the adipose tissue and skeletal muscle of obese and T2D subjects (Frisard, et al., 2010; Reyna, et al., 2008; Shi et al., 2006).

Besides increasing susceptibility to infection and protection from endotoxin-induced septic shock, mice lacking TLR4 have been shown to protect against HFD-induced whole body, white adipose tissue, skeletal muscle insulin resistance in white adipose tissue and skeletal muscle (Eisenstein & Angerman, 1978; McCallum & Pontious, 1979; Poggi et al., 2007; Radin, Sinha, Bhatt, Dedousis, & O'Doherty, 2008; Shi, et al., 2006). In 2007, Cani et al. demonstrated in mice that in addition to inducing obesity and whole body insulin resistance, high fat feeding also caused a chronic, sub-septic elevation in serum endotoxin, a condition Cani termed as 'metabolic endotoxemia' (Cani, et al., 2007). After establishing a causal relationship between these metabolic impairments to LPS exposure, knocking out TLR4 function prevented development of these metabolic impairments under a HFD (Cani, et al., 2007). Further investigation by Frisard et al. revealed a TLR4-dependent metabolic response of skeletal muscle to subseptic doses of endotoxin (Frisard, et al., 2010). LPS injection in wild type mice induced a predominant reliance of skeletal muscle glucose oxidation in place of lipid oxidation, a response dependent on TLR4 and NFκB function that was blocked in the absence of TLR4 (Frisard, et al., 2010).

Overall, the metabolic inflexibility observed in response to low doses of LPS included increased weight gain, glucose uptake, insulin resistance, anaerobic glycolysis, liver triglycerides, and circulating triglycerides along with decreased activity in both the Kreb's cycle and β-oxidation pathways of the mitochondria (Cani, et al., 2007; Frisard, et al., 2010). While these findings are essential to address the obesity epidemic, a concerted, mechanistic understanding of the roles of inflammation and a HFD in the development of metabolic dysfunction is necessary. As most waking hours are spent in a postprandial state, it is critical to understand the dynamic factors regulating metabolism during this state (Lopez-Miranda & Marin, 2010).

Chapter 3: Methods

Methods

***Animal studies*.** This study was performed under a protocol approved by the Institutional Animal Care and Use Committee at Virginia Tech. Mice for this study were maintained on a normal chow diet and a 12:12-h light-dark cycle. 6-mo-old male C57BL/6J mice (n = 24) were randomly assigned to groups pre-injected with either saline (control, n = 12) or LPS (LPS, n = 12; 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 from each injection group were randomly assigned to be gavaged 2 hr post-injection with either 300 μL of water (n = 6) or a fluid, high fat meal (HF; n = 6; 5 kcal; SF = 21.4% kcal, UF = 40.8% kcal , CHO = 27.1% kcal, PRO = 10.7% kcal) made from sucrose (S7903; Sigma-Aldrich, St. Louis, MO), Crisco® Vegetable Oil (Kroger, Blacksburg, VA), and Carnation® Evaporated Milk (Kroger, Blacksburg, VA). In summary, there were four different treatment groups saline/water (n = 6), saline/HF (n = 6), LPS/water (n = 6), and LPS/HF (n = 6). Animals were sacrificed 5 hr after injection. Skeletal muscle from the gastrocnemius and quadriceps were taken and separated between red and white muscle fibers to be homogenized for oxidative and enzyme assays, isolated into mitochondrial fractions for respiratory and enzymatic measure, and flash frozen for mRNA and protein measures. Blood was collected via postmortem cardiac puncture and concentrations of insulin, interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP1), and tumor necrosis factor-α (TNF-α) were measured in the serum using the Bioplex suspension array system (Bio-Rad, Hercules, CA).

Skeletal muscle whole homogenate preparation. Approximately 50 mg of 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 1mM ATP, pH 7.4. Muscle samples were minced with scissors and then SET buffer was added to a 20-fold diluted (wt:vol) suspension. The minced samples were homogenized in a Potter-Elvehjem glass homogenizer at 10 passes across 30 sec at 1,200 rpm with a motor-driven teflon pestle, and measures of substrate oxidation and enzyme activity was performed.

Mitochondrial isolation from red muscle. Mitochondria was isolated from red muscle as previously described with modifications (Frezza, Cipolat, & Scorrano, 2007). Tissue samples were collected in buffer containing 67 mM sucrose, 50 mM Tris/HCl, 50 mM KCl, 10 mM 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 min. Samples were homogenized and mitochondria were isolated by differential centrifugation (Liu et al., 2011).

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 consisted of 25 sec mixing and 4-7 min measurement cycles, unless otherwise stated. Oxygen consumption was sequentially measured under basal conditions, ADP (5mM) stimulated, state-3 respiration, oligomycin (2μM) induced state-4 respiration, and uncoupled respiration in the presence of the mitochondrial uncoupler FCCP (0.3μM) to assess maximal oxidative

capacity. Respiratory control ratio (RCR) was calculated as the ration of ADP-stimulated, state-3 and oligomycin-induced, state-4 respiration. All experiments were performed at 37°C.

ROS measures in isolated mitochondria. Amplex Red Hydrogen Peroxide/Peroxidase assay Kit was used for measures of ROS production from complex I, III and reverse electron flow. Immediately following isolation and protein quantification, mitochondria were plated on 96 well black plate at a concentration of 5 ug/well under three different conditions. The conditions were pyruvate (20 mM)/malate (10mM)/oligomycin (2 μM)/rotenone (200 nM) 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 was then loaded onto the plate to begin the reactions. Experiments consisted of 1 min delay and 1 min reading cycles, followed by a 5 sec mixing cycle performed every third reading. All experiments were performed at 37°C. 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.

Substrate oxidation and metabolic flexibility. 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 (Cortright et al., 2006; Hulver, et al., 2005). [1-^{14}C]-pyruvic acid

was used to assess PDH activity. Metabolic flexibility was determined by comparing PDH activity in the presence and absence of 100mM palmitate.

Enzyme activity. Enzyme activities were assessed in muscle homogenates and isolated mitochondria. CS and β-HAD activities were determined spectrophotometrically as previously described (Frisard, et al., 2010). CrAT activity was determined spectrophotometrically as previously described (Muoio et al., 2012). Malate dehydrogenase (MDH) activity was measured as previously described with modifications (Bergmeyer, 1965). The reaction occured at 37°C in 0.1 M potassium phosphate (pH 7.2) assay buffer containing 6.897 μM reduced nicotinamide adenine dinucleotide (NADH) and 137.9 μM oxaloacetate. After 5 sec of shaking the reaction mix-activated samples, the change in absorbance at 340 nm was measured every 10 sec for 2 min.

Calculations and statistics

Comparisons between the four treatment groups were analyzed using a 2-way ANOVA with Tukey post-hoc tests. Results were expressed as mean ± SEM and level of significance was set at $P<0.05$.

Chapter 4: Results

Mitochondrial bioenergetics in response to a high fat meal with and without prior LPS administration

To assess whether LPS altered the mitochondrial bioenergetic response to a single high fat meal, respirometry was assessed in mitochondria isolated from skeletal muscle following the meal with prior exposure to LPS or saline. Basal oxygen consumption rate (OCR) was not different across treatments or between conditions (Figure 1A). However, there was a significant ($p < 0.05$) meal x treatment effect on state-3 (ADP-stimulated) respiration (Figure 1B). Specifically, LPS stimulation resulted in reduced state-3 respiration in response to HF meal, which was not evident with saline. There was also a significant meal x treatment interaction with state-4 respiration (Figure 1C). State-4 respiration increased with HF feeding relative to with saline treatment, but this was blocked in the presence of LPS. There were no significant differences in RCR_o, the ratio of ADP-stimulated, state-3 respiration to oligomycin-stimulated, state-4 respiration, across treatments or between conditions (Figure 1D). The meal x treatment interaction trended towards significance for FCCP-stimulated,

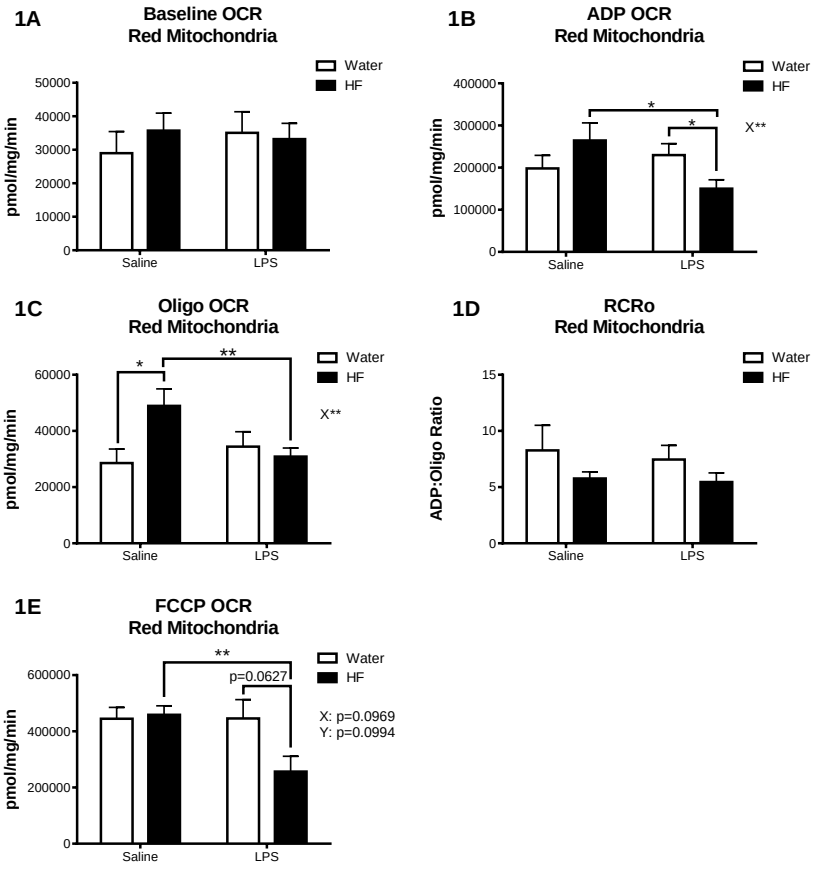

Figure 1. Mitochondrial Bioenergetics. x= LPS x HF interaction; y= LPS Effect; and z= HF Effect. Significance has been individually denoted or signified as follows: p<0.050*; p<0.025**; p<0.010***.

maximal respiration and while there was no effect of the meal in the presence of saline, there was a significant decrease following the HF meal in the presence of LPS (Figure 1E).

Fat oxidation in response to a high fat meal with and without prior LPS administration

Significant effects and interactions were not observed in palmitate oxidation rates in red or white skeletal muscle homogenates. However, trends were observed with some of the measures in response to the high fat meal and/or LPS (Figure 2).

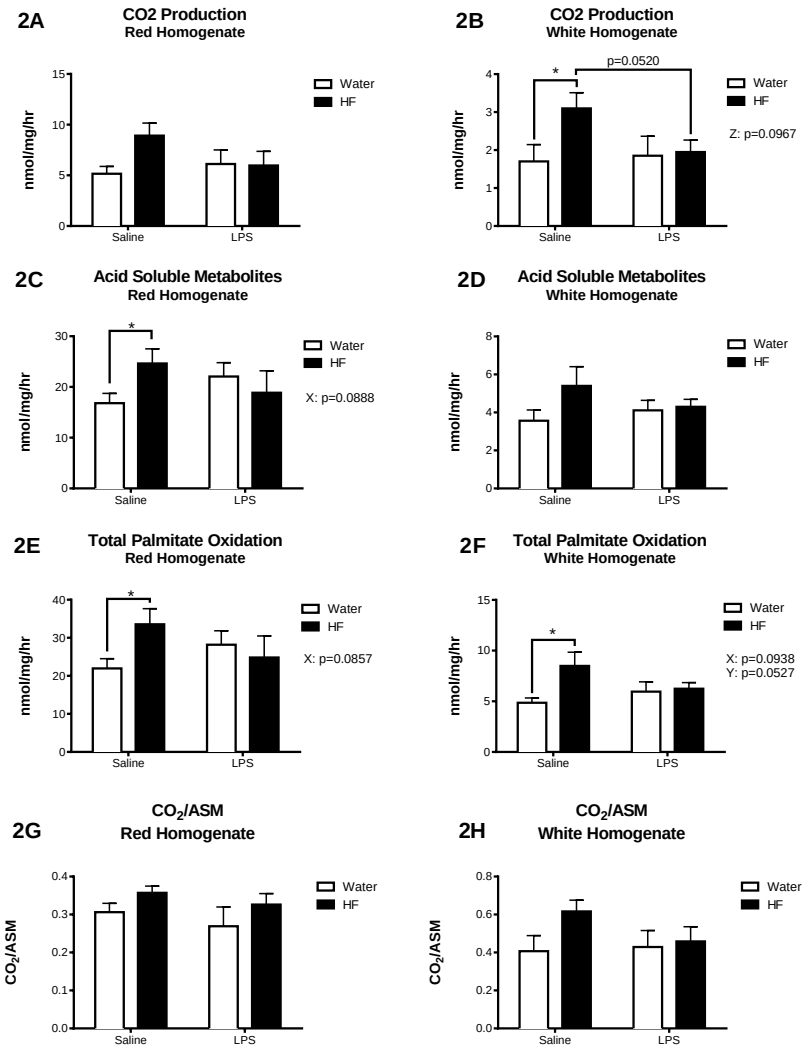

Figure 2. Palmitate oxidation. x= LPS x HF interaction; y= LPS Effect; and z= HF Effect. Significance has been individually denoted or signified as follows: p<0.050*; p<0.025**; p<0.010***.

In red skeletal muscle homogenates, there were significant increases in CO_2 production, ASMs, and total palmitate oxidation with a HF meal in control mice (Figure 2A, 2C, & 2E; respectively). No such meal response was observed in CO_2 production, ASM, and total palmitate oxidation with LPS administration. In white skeletal muscle homogenates, significant increases with a HF meal were also observed in CO_2

production and total palmitate oxidation, which was not observed in animals exposed to LPS (Figure 2B & 2F, respectively). Neither red, nor white homogenates demonstrated significant differences in efficiency of palmitate oxidation, assessed as CO_2/ASM, between treatments (Figure 2G & 2H).

Pyruvate dehydrogenase activity in response to a high fat meal with and without prior LPS administration

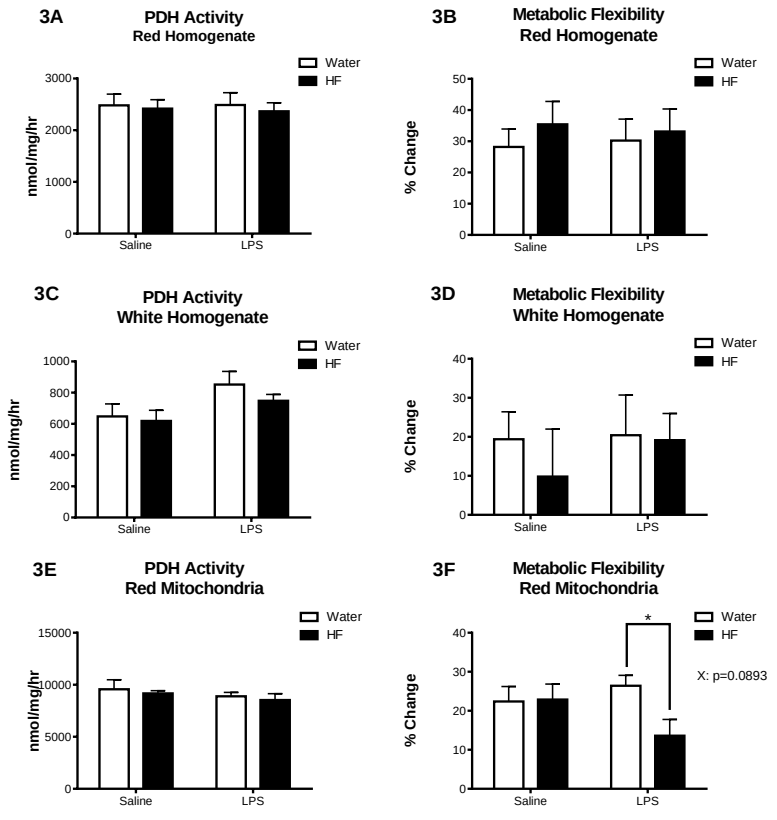

Figure 3. Pyruvate oxidation. x= LPS x HF interaction; y= LPS Effect; and z= HF Effect. Significance has been individually denoted or signified as follows: p<0.050*; p<0.025**; p<0.010***.

There were no significant differences in pyruvate oxidation following the high fat meal or with LPS administration (Figure 3A, 3C, & 3E). Although there were no significant differences in the metabolic flexibility of red and white skeletal muscle homogenates, mitochondria isolated from red skeletal muscle were significantly less flexible following a HF meal with prior LPS administration, in which the interaction trended towards significance (p = 0.0893) (Figure 3F).

Carnitine acetyltransferase, β-hydroxyacyl-coA dehydrogenase, citrate synthase, and malate dehydrogenase activity

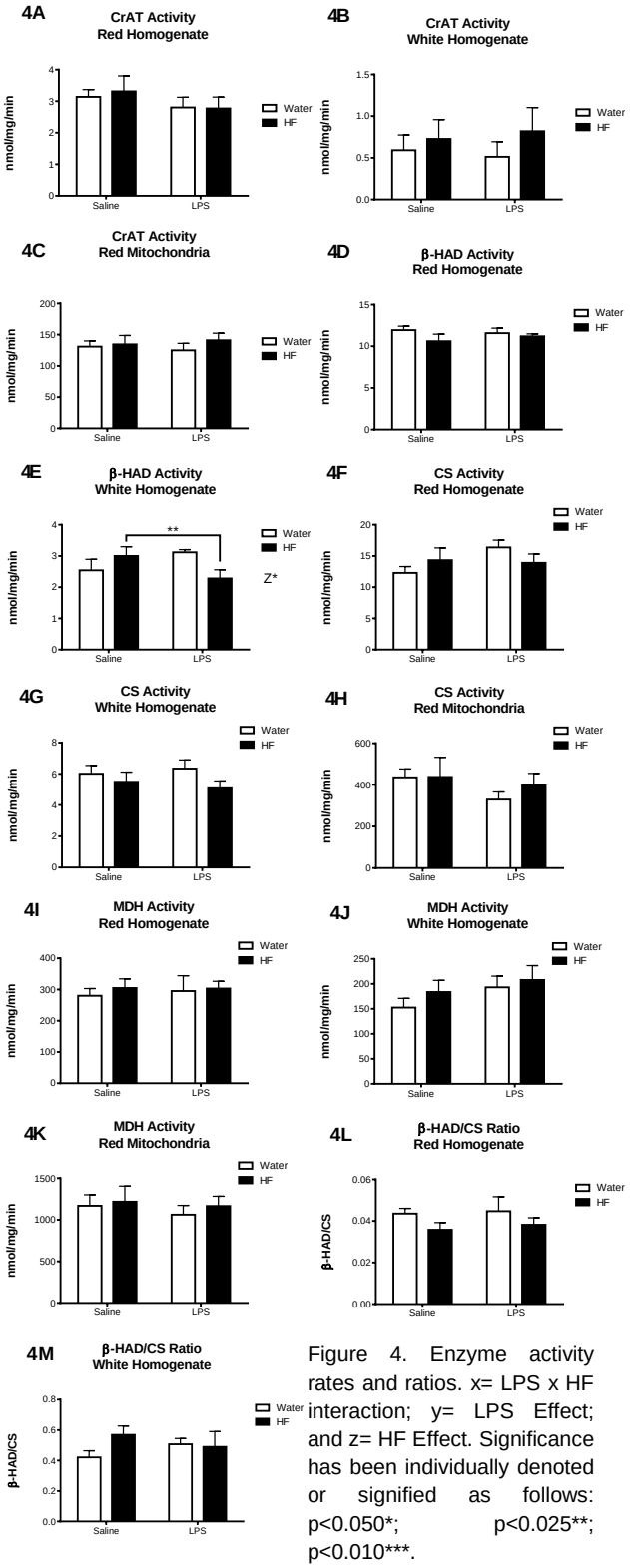

Figure 4. Enzyme activity rates and ratios. x= LPS x HF interaction; y= LPS Effect; and z= HF Effect. Significance has been individually denoted or signified as follows: p<0.050*; p<0.025**; p<0.010***.

Spectrophotometric analysis of individual metabolic enzyme activities was utilized to further elucidate the effects of inflammation on the meal response (Figure 4). The activity of CrAT, an enzyme responsible for shuttling excess acetyl moieties out of the mitochondria, was not significantly different across treatments or between conditions in homogenates or mitochondrial isolates (Figure 4A-C). Overall, there is no interaction between LPS and the HF for βHAD. In white skeletal muscle (Figure 4D), βHAD increased in response to the meal, but this appears to be blocked in the presence of LPS (Figure 4E). No significant differences were observed across treatments or between conditions in the activity rates of TCA cycle enzymes CS and MDH (Figure 4F-K). As a result, βHAD/CS activity was not significantly affected across treatments or between conditions (Figure 4L-M).

Mitochondrial ROS production in response to a high fat meal with and without prior LPS administration

No significant interactions or effects were observed in ROS production from complex I, complex III, or the reverse flow of electrons to complex I in the isolated mitochondria of red skeletal muscle (data not shown).

The concentration of serum signaling molecules in response to a high fat meal with and without prior LPS administration

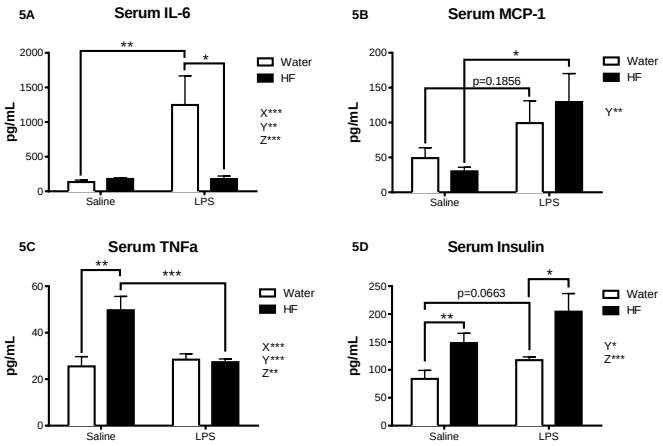

Figure 5. Serum signaling molecules. x= LPS x HF interaction; y= LPS Effect; and z= HF Effect. Significance has been individually denoted or signified as follows: p<0.050*; p<0.025**; p<0.010***.

To determine potential signaling pathways through which inflammation could regulate the metabolic response of skeletal muscle to a high fat meal, serum concentrations of insulin and a number of inflammatory cytokines were measured (Figure 5). Serum concentrations of IL6 demonstrated a significant meal x treatment effect (Figure 5A). While HF feeding did not affect IL6 concentrations, LPS treatment significantly elevated IL6 concentrations 9-fold independently. However, the combination of the HF meal in the presence of LPS completely blocked the IL6 response. While no treatment interaction or HF meal effect was observed, LPS independently increased MCP1 concentration (Figure 5B). There was a significant meal x treatment effect with TNFα (Figure 5C). Specifically, TNFα was significantly elevated with HF feeding with saline, but not LPS. Though no significant meal x treatment interaction was observed in serum insulin, significant increases in response to a HF meal under both treatments were observed (Figure 5D). A significant

LPS effect was also observed as evidenced by a LPS induced increase in fasted-state insulin concentration that trended towards significance ($p=0.0663$).

Chapter 5: Discussion

The current study was designed to investigate the effects of acute inflammation on postprandial metabolism following a high fat meal. Overall, these results demonstrate, for the first time, that postprandial oxidative metabolism following a high fat meal is impaired in skeletal muscle of mice exposed to LPS. Though postprandial lipid metabolism was upregulated in response to the HF meal challenge, activation of inflammatory pathways impaired this metabolic flexibility.

Despite Lundman et al.'s demonstration of an acute, high fat meal inducing a 2-fold postprandial elevation in serum IL-6 concentrations 4 hr after feeding in both healthy and prediabetic humans with CHD, our HF treatment appeared to suppress IL-6, even after LPS administration (Lundman et al., 2007). This discrepancy in HF meal response between each study's respective control group could be attributed to differences in organism model or the earlier postprandial serum collection timepoint used in our study (Lundman, et al., 2007). Despite these differences in study design, the fidelity of our results demonstrating the suppression of the LPS response in HF-gavaged mice remain speculative as both treatments have been independently shown to stimulate inflammatory cytokine expression via TLR4 in several models (Erridge, Attina, Spickett, & Webb, 2007; Laugerette et al., 2011; Lundman, et al., 2007; Shi, et al., 2006). However, this response could be explained as a differential response to endotoxin in the presence or absence of circulating lipids as lipoproteins are well established to bind circulating LPS and facilitate its clearance, ultimately preventing TLR4 activation (Feingold et al., 1995; Levels, Abraham, van den Ende, & van Deventer, 2001; Mathison, Tobias, Wolfson, & Ulevitch, 1991; Munford, Hall, Lipton, & Dietschy, 1982; Ulevitch & Johnston, 1978).

TNFα is well recognized as an inflammatory cytokine secreted in response to LPS; however, our results demonstrated the predicted increase in TNFα expression with a HF meal and no literature-supported response to LPS (Ghanim et al., 2009; Lang, et al., 2003; Le Contel, Vinit, Parant, & Parant, 1990; Mannel, Northoff, Bauss, & Falk, 1987). As TNFα promotes lipid storage and impairs the insulin signaling cascade in skeletal muscle, it is not likely responsible for mitigating the metabolic adaptability of skeletal muscle to a high fat meal despite demonstrating similar trends to palmitate oxidation (Hotamisligil, et al., 1994; Steinberg et al., 2006). Therefore, it is possible that our singular collection timepoint missed the peaks of LPS-induced TNFα expression, leaving the actual response and timecourse of TNFα concentrations unobserved. The likelihood of our study design failing to observe an LPS-induced increase in TNFα concentration is supported by Le Contel et al.'s investigation of circulating cytokines after LPS injection (Le Contel, et al., 1990). Le Contel et al. observed TNFα concentrations to peak in mice after 1.5 hr after injection of a subseptic dose of LPS, much earlier than our measurement taken 5 hr post-injection (Le Contel, et al., 1990). While a delayed timepoint could explain this lack of TNFα response to LPS injection in mice, consistent with our lab's previous findings, it does not explain the interaction between LPS and the HF meal (Frisard, et al., 2010). It is possible that our acute induction of inflammation using LPS could have primed the mice for subsequent exposures to the inflammatory agonists associated with a HF meal. In their 2013 study, Borges et al. induced inflammation in Wistar rats using a 12 week HFD, subsequently measured their cytokine response to exogenous LPS injection, and compared these responses to those of rats on a control diet (Borges et al., 2013). Overall, they observed comparably blunted NF-κB activation from peritoneal macrophages in the HFD mice.

Though this study utilized a chronic model of inflammation induced by a HFD on Wistar rats, they demonstrated that prior inflammatory priming diminished the subsequent cytokine response to LPS injection (Borges, et al., 2013). It is important to bear in mind that their study was modeled conversely to our own; however, it does provide proof of concept for inflammatory priming via these overlapping pathways (Borges, et al., 2013).

Consistent with the literature, we observed an elevation in serum insulin concentrations in response to acute treatment with either HF or LPS (Cornell, 1985; Ghanim, et al., 2009; Kelleher, Bagby, & Spitzer, 1980; Manning et al., 2008). Considering this LPS-induced increase in insulin concentration, endotoxemia could be among the slew of environmental factors, including ROS, proposed to promote hyperinsulinemia, a condition characteristic of metabolically inflexible individuals with T2D (Corkey, 2012).

Inflammatory impairment of mitochondrial function in response to a HF meal was clearly demonstrated through our measures of mitochondrial respiration. The impairment of ADP-stimulated respiration in LPS treated mice after a HF meal suggests diminished ability to produce ATP in the face of ATP demand. As a similar interaction was observed in maximal respiration independent of ATP production, it is possible that the ETC itself is impaired under synergistic effect of our two inflammatory challenges. The unaffected function of CS under experimental conditions suggest that Kreb's cycle production of reducing agents is not responsible for the interactions observed in ADP-stimulated and maximal respiration. The meal-induced increase in state-4 respiration we observed was in concurrence with the effects observed by Clarke et al.; furthermore, LPS-induced impairment of this response suggests that inflammation interferes with the meal-induced uncoupling independent of ATP production (Clarke et al., 2012). As

uncoupling serves to reduce oxidative stress, it is possible that inflammation-induced impairments with ETC function in response to a HF meal are due to changes in ROS signaling (Flandin, Donati, Barazzone-Argiroffo, & Muzzin, 2005; Martinez, 2006; Papa & Skulachev, 1997). As part of a proposed anti-oxidant response, Jiang et al. has credited UCP3 as a mediator of impaired state-3 respiration during exercise, a state characterized by high ATP demand (Jiang et al., 2009). However, compromised oxidative efficiency via uncoupling only became evident after prolonged exercise, therefore we cannot, with any certainty credit the impaired state-3 respiration to uncoupling, particularly when our measurement of oligomycin-induced, state-4 respiration demonstrated a different trend in treatment interaction. In addition to ROS, lipid signaling is another proposed mediator of mitochondrial uncoupling, leaving a possible mechanism for the impairment of metabolic flexibility through various lipid species in circulation or within skeletal muscle (Keipert et al., 2013).

ROS are volatile oxidants capable of causing cellular damage, mitochondrial dysfunction, and acting as highly regulated signaling molecules directing cellular function in response to agonists including fatty acids and inflammatory markers (Barazzoni et al., 2012; Drabarek, Dymkowska, Szczepanowska, & Zablocki, 2012; Garcia-Ruiz, Colell, Morales, Kaplowitz, & Fernandez-Checa, 1995). Despite the literature's documentation of ROS as signals activated in response to inflammatory and metabolic stimuli, no changes in ROS production were observed in our study (Dandona, Ghanim, Chaudhuri, Dhindsa, & Kim, 2010; Lim et al., 2011). It is possible that changes in ROS production were part of the initial response to our treatments and were neutralized by our time of our indirect, *ex vivo* measurement. Despite the literature's emphasis on ROS as a critical mediator of metabolism and inflammation, no changes in

ROS production were observed in this study and possible ROS-focused mechanisms of inflammatory priming impairing the meal response extend beyond the reach of this study. However, our study does contain critical information regarding postprandial metabolism in an inflammatory environment.

The ineffectiveness of our treatments to modulate PDH, BHAD, CS, and MDH activity suggests that acute, low-grade inflammation does not impair skeletal muscle metabolic flexibility through these pathways. Doubt in the apparent non-response to our treatments could be enhanced through our comparatively small sample size. The uniformed impairment of PDH activity in the presence of mixed oxidative substrates with our treatments, would indicate a potential for increased statistical power to improve detection of physiologically significant treatment responses. In conjunction with doubts regarding the statistical reliability of this study's enzyme activity data, previous data from our lab indicate that increased duration and dosage of LPS injection in mice or cultured myocytes impairs both BHAD and CS (Frisard, et al., 2010). Furthermore, an increase in neutral lipid synthesis in these models suggests impairment of PDH activity based on ACC regulation. Indeed, continuous infusion of LPS increases transcription of PDK4 and decreases PDH activity in skeletal muscle within 24 hr of LPS infusion (Alamdari et al., 2008). Taken together, these results indicate that timing and dose of LPS treatment may be important.

CrAT, an enzyme responsible for the efflux of excess acetyl moieties from mitochondria, has been implicated as a regulator of metabolic flexibility via the glucose-fatty acid cycle proposed by Randle (Muoio, et al., 2012; Noland et al., 2009; Randle, et al., 1963). However, the unresponsiveness of CrAT to our treatments indicates that endotoxin-induced metabolic inflexibility does not appear acutely mediated through

CrAT. Other metabolite transporters shown to regulate skeletal muscle metabolic flexibility including CPT1 and fatty acid translocase/cluster of differentiation 36 (FAT/CD36) could be responsible for inflammation-induced metabolic impairment in skeletal muscle (Bruce et al., 2009; Smith et al., 2011).

Limitations

Though our study showed significant effects highlighting a unique interaction between acute inflammation and a high fat meal, limited sample size and sample quantity from each animal may have produced a profound effect on the results of this study. While increased sample size would likely bring about significance of trending data, increased sample quantity available from each mouse would have given us the ability to perform more measures investigating the mechanisms impairing metabolic flexibility. Increased N size would also facilitate the capacity to demonstrate a timecourse for concentrations of circulating hormones and cytokines. Further limitations contributing to study variability include the number of samples physically capable of being measured in one run using our equipment to measure radiolabeled substrate oxidation and mitochondrial respiration. However, methods minimizing day-to-day variability were utilized. Limitations in methodology could include our delayed, indirect, ex vivo measurement of ROS production. Though this method has demonstrated reliability for our past studies, it may be improved through the use of direct or *in vivo* methodology. The measurement of ROS production could also have been performed over a frequent timecourse to increase the opportunity for observing ROS signaling events.

Future Studies

As this study has supported the concept of inflammation regulating postprandial metabolism, further investigation is critical to an improved understanding of the significance of its role in inflammation. Beyond addressing the concerns expressed in the previous section on study limitations, future studies should investigate a wider degree of metabolic and inflammatory markers in circulation to understand the stimuli capable of regulating skeletal muscle under these treatments. In particular, it would be critical to observe how the resolution of inflammation through anti-inflammatory signals like IL-10 is affected by the HF meal challenge. Further mechanistic understanding of these pathways could be gained through the use of a microarray featuring critical genes found in various pathways associated to inflammation, oxidative metabolism, and oxidative stress. Such genes would include IL-1, IL-6, IL-10, TNFα, TLR4, IFNγ, AMPK, PPARα/δ, PGC1α/β, COX, and UCP3. Further investigation of metabolic function using the same treatments would include glycogen and neutral lipid synthesis. Investigation of therapeutic strategies would include various dietary and pharmaceutical interventions. Dietary interventions would include anti-oxidant rich beverages like orange juice or green tea and unsaturated fats like olive oil. Pharmaceutical intervention could include a low dose of aspirin to combat inflammation. Furthermore, application of these investigations to human subjects could provide findings of clinical significance

Conclusion

In conclusion, this study uniquely demonstrates that postprandial oxidative metabolism of a high fat meal is impaired in skeletal muscle of mice exposed to LPS. Though limited in power, we observed several significant interactions demonstrating an acute, low-dose of LPS to impair mitochondrial function after a high fat meal. As the

results currently stand, there is a lot of variability. Gaining statistical power through increasing N size should further support our hypothesis by bolstering our data for fatty acid oxidation and pyruvate decarboxylation while clarifying the functions of ROS signaling and enzyme activity in these models of inflammation induced metabolic flexibility. Overall, the data confirm the hypothesis that low grade inflammation can be a potent contributor to the metabolic inflexibility observed in obesity and T2D while highlighting a unique interaction in postprandial metabolism between acute, low grade inflammation and a HF meal.

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