The Role of High Saturated Fatty Acid Diets on Skeletal Muscle Metabolism and Inflammation

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Abstract

Heightened consumption of saturated fatty acids (SFA) is linked to obesity and insulin resistance. In vitro studies have shown that SFA-associated metabolic perturbations are mediated via induction of pro-inflammatory pathways. The purpose of this study was to examine the relationship between metabolic adaptive response to 5 days of high SFA feeding, independent of positive energy balance, and diet-induced agonism of pro-inflammatory pathways. A secondary aim was to determine if the metabolic adaptive response in skeletal muscle to a single, calorie dense, high fat meal was altered by 5 days of high saturated fat feeding. Twelve college-age, non-obese males were studied and skeletal muscle samples were obtained prior to and concluding the consumption of a high SFA diet. In a subset of volunteers (N=6), we fed participants a high fat meal after the initial skeletal muscle biopsy and measured changes in postprandial endotoxin concentrations for four hours following the meal challenge. A second biopsy was obtained four hours after the meal challenge. Skeletal muscle samples were used to measure fatty acid oxidation, glucose oxidation, oxidative enzyme activities, mRNA expression of metabolic targets, and phosphorylation and total content of inflammatory proteins. In response to five days of high SFA feeding, skeletal muscle glucose and complete palmitate oxidation were significantly reduced as was the ratio of complete to incomplete fatty acid oxidation, which we determined by the ratio of radio-labeled CO_2 to acid soluble metabolite production and the ratio of citrate synthase to β-HAD production. Considering that previous reports have linked the ratio of complete to incomplete fatty acid oxidation to skeletal muscle insulin sensitivity, our findings may suggest that the high SFA diet promoted the development of skeletal muscle insulin resistance. Five days of high SFA feeding also attenuated the meal challenge-induced up-regulation of oxidative genes while augmenting postprandial increases in plasma endotoxin concentrations. To assess the relationship between metabolic adaptability and diet-induced inflammatory response we categorized volunteers by the diet induced percent change in fatty acid oxidation. Volunteers who were the least capable to adapt to high SFA feeding displayed the most robust increases in phosphorylation of inflammatory proteins. Lastly, we measured the correlation between the meal challenge associated percent change in oxidative and inflammatory markers in samples obtained prior to and following five days of high SFA feeding. We observed positive associations between the percent change in oxidative and inflammatory markers in samples obtained prior to the high SFA diet that were not observed following five days of high SFA feeding. These results suggest that activation of pro-inflammatory proteins in response to a single high fat meal may be a normal adaptive response when habitual fat intake is less than 30%; however this response is dysregulated following only 5 days of high fat feeding These experiments highlight five important findings 1) five days of high SFA feeding reduced complete oxidation of glucose and fatty acids, 2) consumption of the 5-day high SFA diet attenuated increases in the mRNA expression of oxidative transcription factors which occurred with the consumption of a high SFA meal, 3) five days of high SFA feeding augmented postprandial endotoxin concentrations, 4) volunteers that were the least

capable to adapt to high SFA feeding displayed the most robust increases in plasma inflammatory markers in response to consumption of the high SFA diet, and 5) in response to five days of high SFA feeding associations between the high SFA meal challenge induced percent change in oxidation and inflammatory markers becomes dysregulated.

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Chapter 1: Introduction

In 2004 the US Surgeon General stated that obesity was the most important public health challenge of our time (1). This is an accurate description of a condition that is projected to replace smoking as the nation's leading cause of preventable death (3).

The American diet, which contains excessive amounts of dietary saturated fatty acids (SFA), plays a significant role in the increasing prevalence of obesity. Excessive SFA consumption is associated with reduced insulin sensitivity, excessive storage of fatty acids between skeletal muscle fibers, an inability to shift substrate metabolism in response to changes in substrate availability, and a reduced capacity to metabolize fatty acids (32-35, 61). Fifty-nine percent of the American population exceeds the recommended dietary allowance for SFA intake (61).

Data from our and other laboratories have shown that the toll-like receptor 4 (TLR4) pathway mediates SFA induced metabolic dysfunction (41, 42, 46, 51, 59). Toll-like receptor 4 is a transmembrane protein that that activates an intracellular, inflammatory signaling cascade in response to the presence of lipopolysaccharide (LPS), an endotoxin commonly found on the cell wall of gram negative bacteria which is released into circulation upon cellular death (49). The TLR4 pathway is linked to obesity in that skeletal muscle mRNA expression and protein content of TLR4 is elevated in obese individuals, type 2 diabetics, and individuals that preferentially store rather than oxidize dietary fats (41, 42). Furthermore; obesity is associated with metabolic

endotoxemia, a condition diagnosed by chronically elevated plasma LPS concentrations (63, 64).

Chronic activation of the TLR4 pathway is associated with the development of obesity-associated disruptions in insulin signaling and substrate metabolism. Mice that have been chronically infused with LPS have reduced insulin sensitivity and heightened weight gain compared to saline infused controls (54). Human primary myotubes cultured from non-obese humans display reduced oxidative capacity and a shift in substrate metabolism away from fatty acid oxidation to heightened glucose metabolism following exposure to to sub-septic concentrations of LPS (41).

A growing amount of evidence suggests that saturated fatty acids disrupt metabolic functioning through activation of the TLR4 pathway. Chronic consumption of a high fat diet increases plasma LPS concentrations to levels comparable to levels seen with metabolic endotoxemia (57). Furthermore; rodents whose TLR4 receptors are hypo-responsive to LPS agonsim have improved metabolic profiles and are protected against high fat diet induced metabolic dysfunction (46, 51, 59).

The goal of the proposed project was to assess the in vivo relationship between high SFA diet induced stimulation of pro-inflammatory pathways and metabolic adaptability to high SFA feeding as determined by insulin sensitivity, substrate metabolism, and transcriptional regulation of markers of oxidative phosphorylation.

Chapter 2: Review of the Literature

Obesity and its Associated Co-morbidities: Insulin Resistance and Metabolic Inflexibility

In 2004, the US Surgeon General stated that obesity was the most important public health challenge of our time (1). While one in three Americans are estimated to have a body mass index (BMI) in excess of 30kg/m^2 it may have been more appropriate to state that the metabolic syndrome, which encompasses obesity and its associated co-morbidities, is the nation's largest health care concern. The metabolic syndrome is diagnosed in individuals that are afflicted with two or more of the following conditions: obesity, insulin resistance, hypertension and hyperlipidemia. On average, an American's annual health care costs increases by 24% with the diagnoses of each additional comorbidity of the metabolic syndrome (69).

In addition to being preset in clusters, co-morbidities of the metabolic syndrome are intertwined in that they develop in response to skeletal muscle metabolic abnormalities that hinder the ability of the tissue to metabolize fatty acids and carbohydrates. For example, skeletal muscle insulin sensitivity is reduced by excessive intramyocellular lipid accumulation (IMCL), a characteristic of obesity (10, 14). Diacylglycerols (DAG) and ceramides, two lipid species that are intermediates of triacylglycerol metabolism, disrupt insulin signaling through stimulation of protein kinase C which inhibits insulin receptor substrate-1 (IRS-1) activity upon activation (10, 14). Despite these associations, not all lipid pools present within IMCL stores are disruptive to metabolism. Triacylglycerols (TAG) are thought to be inert and have no significant

influence on insulin signaling pathways (14). Although insulin resistance also develops in adipose tissue as a result of obesity, skeletal muscle insulin resistance is significant in that this tissue oxidizes approximately 80 percent of the glucose within the bloodstream in a postprandial state (9).

Insulin stimulates glucose uptake by initiating an intracellular signaling cascade that includes auto-phosphorylation of the insulin receptor, tyrosine phosphorylation of IRS-1, phosphorylation of phosphatidylinositol 3-kinase (PI3K) and phosphatidylinositol-dependent kinase (PDK), and activation of either akt/protein kinase B (akt/PKB) or atypical protein kinase C (aPKC) (7, 10). The cascade concludes with glucose transporter 4 (GLUT4) translocation from the golgi apparatus to the cellular membrane where the protein facilitates glucose entry into the skeletal muscle cell (7, 10).

The ability of insulin to initiate GLUT4 translocation to the cell membrane is inversely related to BMI, with the skeletal muscle of lean individuals clearing twice the amount of glucose from the blood stream than skeletal muscle of obese individuals (7, 8). Obese individuals display numerous disruptions in the insulin signaling cascade including heightened Serine307 phosphorylation of IRS-1, reduced insulin-stimulated insulin receptor activation, and suppressed phosphorylation of PI3K, all of which result in suppressed insulin stimulated glucose uptake and glycogen synthesis (7, 10).

Despite being insulin resistant, obese individuals have an increased dependence on glucose for ATP generation due, in part, to a reduced capacity to metabolize fatty

acids (8, 18, 20). There are two major classifications of skeletal muscle fibers that are identified according to their myosin ATPase activity; type I and type II skeletal muscle fibers (70, 71). In addition to containing higher activity of myosin, type I fibers are characterized by increased vascularization, heightened concentrations of skeletal muscle mitochondria, and a preferential use of fatty acids for ATP generation (70, 71). Type II fibers are subdivided into two categories; type IIa and type IIb fibers. While type IIa fibers are less oxidative than red, type I fibers, type IIa fibers are more dependent on fatty acids for ATP generation than glycolytic, white, type IIb fibers (70, 71). In a tissue such as the vastus lateralis, which contains a heterogeneous mix of red and white muscle fibers, the percentage type I fibers can vary from 13 to 98%. As BMI increases, the percentage of type I fibers present in this tissue is proportionally decreased (20).

Obese individuals also display higher ratios of glycolytic to oxidative enzyme activity than activity observed in the skeletal muscle of non-obese individuals. Studies have also shown that activity of β-hydroxylacyl CoA dehydrogenase (β-HAD), the enzyme that catalyzes the second step in β-oxidation, is inversely associated with BMI (8). Consequently, obese individuals contain elevated concentrations of type IIb skeletal muscle fibers and heightened activity of the glycolytic enzymes hexokinase (HK), phosphofructokinase (PFK) and glyceraldehyde phosphate dehydrogenase (GAPDH) (18, 20).

In line with these findings, obese individuals display depressed rates of complete fatty acid oxidation despite comparable amounts of fatty acid uptake into skeletal muscle

cells. Esterification of TAG is increased in the skeletal muscle of obese individuals by 69% compared to the TAG esterification observed in the skeletal muscle of non-obese individuals (16). In skeletal muscle samples obtained from morbidly obese patients, BMI greater than 50kg/m^2, complete palmitate oxidation is reduced by up to 80 percent (13).

Obese individuals are metabolically inflexible, meaning that their skeletal muscle is unable to facilitate shifts in substrate metabolism in response to changes in substrate availability and thus remains predominately glycolytic in conditions that stimulate fatty acid oxidation in non-obese individuals (8, 21-26). When presented with elevated concentrations of plasma free fatty acids, obese individuals preferentially store rather than oxidize fatty acids, which result in heightened lipid accumulation within peripheral tissues (13). Furthermore; following prolonged fasting, skeletal muscle respiratory quotient (RQ), which is an indicator of the ratio of glucose to fatty acid molecules that are being oxidized for ATP generation, is significantly higher in obese as compared to non-obese individuals suggesting that obese individuals preferentially metabolize glucose whereas fatty acids are the preferential substrate for ATP generation in non-obese individuals (8, 18).

It is important to note that the development of obesity associated metabolic abnormalities is thought to result from chronic agonsim of inflammatory pathways, another characteristic of the metabolic syndrome. Elevated transcription of inflammatory cytokines, which commonly occurs in the skeletal muscle of obese individuals, promotes the development of the metabolic syndrome due to the fact that cytokines are disruptive

to metabolism (42-44). The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) reduces insulin sensitivity while interleukin-6 (IL-6), which stimulates lipolysis and hepatic TAG secretion, is 2-fold higher in the skeletal muscle of obese compared to non-obese individuals (42, 43).

Transcription of IL-6 and TNF-α is regulated in part by nucleic factor κB (NFκB) (35, 45). When liberated from the IκBα complex, NFκB translocates to the cellular nucleus where it stimulates the transcription of inflammatory genes (45). Obese skeletal muscle has significantly depressed IκBα protein content, which suggests that NFκB translocation to the cellular nucleus is heightened as a result of the metabolic syndrome (42).

It has yet to be determined if obesity associated metabolic perturbations are present within skeletal muscle before the onset of obesity. Data from numerous studies suggests that some individuals inherently contain a reduced capacity to metabolize fatty acids which results in excessive weight gain (28-30). Studies measuring the relationship between RQ and weight gain have shown that individuals with heightened 24hr and non-sleeping RQ exhibit heightened weight gain and increased adiposity over a five year period, suggesting that this condition is present before the onset of obesity (28-30). Furthermore, volunteers with a reduced capacity to metabolize fatty acids, determined by skeletal muscle content of type 1, oxidative skeletal muscle fibers, are less able to lose weight when placed in weight loss intervention programs (65).

While there is sufficient data supporting the hypothesis that weight gain is associated with a genetic predisposition towards the development of obesity, these abnormalities are most apparent with high fat feeding. It's likely that both a predisposition towards the development of obesity and chronic intake of elevated amounts of fatty acids, namely SFA, jointly contribute to the development of metabolic syndrome.

Saturated Fatty Acids Disrupt Metabolic Functioning

To reduce the global burden of obesity, the World Health Organization has recommended replacing calories consumed from dietary SFA consumption with monounsaturated fatty acids (MUFA) (68). This recommendation stems from the understanding that SFA exert an obeso-genic influence on peripheral tissues. When exposed to elevated concentrations of palmitate, the most common SFA in circulation, skeletal muscle established from lean humans and rodents develop attributes of obesity including reduced mRNA expression of proteins involved in β-oxidation, heightened IMCL, DAG and ceramine concentrations, and skeletal muscle insulin resistance (14, 33, 37, 38, 67).

Insulin-stimulated glucose uptake is reduced by 30 percent, respectively, in skeletal muscle cells exposed to elevated concentrations of palmitate compared to cells treated with bovine serum albumin (BSA) (35). Studies have also shown that SFA-

induced suppression of insulin-stimulated glucose uptake occurs in conjunction with reductions in phosphorylation of protein kinase B (Akt), a component of the insulin signaling pathway, and heightened phosphorylation of IRS-1 on serine 307, which is commonly observed in the skeletal muscle of obese individuals following exposure to insulin, and reduced insulin-stimulated GLUT4 translocation to the cell membrane (33, 72). Saturated fatty acid-induced disruptions in insulin signaling have also been observed in vivo. Compared to chow fed controls and high MUFA diet (50% fat, 70% of fat calories from MUFA) fed rodents, mice fed high SFA diets (50% fat, 70% of fat calories from SFA) displayed significant reductions in insulin stimulated glucose uptake (66). The study also found that skeletal muscle mRNA expression of IRS-1 was significantly decreased in high SFA-diet fed animals compared to groups fed high MUFA and chow diets (66).

As with obesity, SFA-induced reductions in insulin sensitivity occur with disruptions in fatty acid oxidation. Whereas skeletal muscle cells incubated in elevated concentrations of the monounsaturated fatty acid (MUFA) oleate increase mRNA expression of oxidative transcription factors and proteins that facilitate β-oxidation such as carnitine palmitoyl transferase-1 (CPT-1) by up to seven-fold skeletal muscle CPT-1 mRNA expression is not increased in response to incubation in the SFA palmitate (33; 36). Furthermore, myotubes incubated in palmitate display a 50 percent decrease in mRNA expression of the oxidative transcription factor peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) (33; 36; 67).

In vivo comparisons between the influence of MUFA and SFA on fatty acid metabolism suggest that SFA promote heightened lipid storage and the development of metabolic inflexibility. Following 28 days of a high MUFA diet, non-obese subjects were able to compensate for elevated MUFA intake by increasing fatty acid oxidation (39, 40). On average, the group was in negative fat balance at the end of the high MUFA diet. When the same group of subjects were placed on a high SFA diet for 28 days, their ability to adapt to elevated fatty acid intake was severely compromised (39, 40). The group was in positive fat balance at the end of the diet period and less oxidative in the fed state compared to measures taken prior to the experimental diet (39; 40).

Saturated fatty acids are believed to be more detrimental to metabolism than MUFA because they stimulate pro-inflammatory pathways whereas MUFA do not (33; 35). Sinha et al., (35) proposed that insulin resistance can only be induced by fatty acids that activate NFκB. This hypothesis was supported with reports that palmitate-induced insulin resistance is attenuated when cells are co-incubated in salicylates, anti-inflammatory compounds that target inhibitor κB kinase β (IKKβ), an agonist of NFκB activation (35). Furthermore, inhibition of NFκB activity with the use of siRNA prevents palmitate-induced reductions in insulin stimulated GLUT4 translocation and glucose uptake (72).

Although NFκB inhibition is preventative against palmitate-induced metabolic dysfunction, recently published studies suggest that palmitate stimulates NFκB activation through agonism of the pro-inflammatory, transmembrane protein toll-like receptor 4

(TLR4). Studies have found that both rodents and skeletal muscle cells which lack the protein are protected against the development of high fat-diet induced obesity and SFA-associated metabolic derangements (41; 42; 46; 48; 51; 59).

Toll-Like Receptor 4 and SFA Induced Metabolic Dysfunction

The toll receptor family is a group of 13 inflammatory receptors that function to recognize pathogen-associated pattern molecules (PAMP). The receptors were initially discovered in *Drosophila,* but are present in numerous mammalian species including humans and mice. Toll-like receptor 4 was the first member of the family discovered in humans (47).

The primary function of TLR4 is to activate an intracellular inflammatory signaling cascade in response to gram negative bacteria (49). Therefore, lipopolysaccharide (LPS), an endotoxin present on the cell wall of gram-negative bacteria, serves as one of the primary ligands for the receptor (49). The TLR4 signaling cascade commences with ligand recognition by MD-2, a small extracellular glycoprotein that is attached to TLR4's extracellular membrane (49). Upon ligand association the TLR4/MD2 complex moves closer in proximity to CD-14, stimulating the translocation of myeloid differentiation factor 88 (MyD88), an adaptor protein, from the endoplasmic reticulum to the Toll/interleukin-1 receptor (TIR) domain of the TLR4 receptor (50-52). Auto-phosphorylation of IL-1R-associated kinase (IRAK) and the subsequent IRAK phosphorylation of tumor necrosis factor-associated factor 6 (TRAF6) and IKK, occurs as

a result of MyD88 joining to the TLR4 complex (52, 53). The cascade concludes with IKK phosphorylation of the IκBα complex, this action degrades the complex enabling NFκB to translocate to the cellular nucleus where it induces the transcription of pro-inflammatory cytokines (50-53).

Activation of NFκB by TLR4 also occurs through a MyD88 independent signaling cascade, which includes the adaptor protein Toll/IL-1R domain-containing adaptor inducing interferon-β (TRIF; TICAM-1), interferon regulatory factor 3 (IRF3), and interferon- β (IFN-β) (62). As with the MyD88 dependent pathway, the TRIF signaling cascade results in IKBα degradation (62). However; this pathway also has the capacity to activate NFκB within the nucleus resulting in late-phase stage of activation (62).

TLR4 Mediates Fatty Acid Induced Metabolic Dysfunction

The TLR4 pathway plays an integral role in the development of obesity and insulin resistance. This is evident in that the protein is more abundantly expressed in the skeletal muscle of individuals who are obese, insulin resistant, and who preferentially store rather than oxidize fatty acids (41; 42). The influence of TLR4 on the development of the metabolic syndrome is most apparent in skeletal muscle that has been exposed to

elevated concentrations of SFA which activates downstream targets of the TLR4 signaling pathway. Phosphorylated-IκBα protein levels are increased by seven-fold in human primary myotubes, which is indicative of heightened translocation of NFκB to the cellular nucleus (42). When placed on high fat diets skeletal muscle of C57/Bl6 mice displays heightened nuclear binding of the NFκB subunit p50, which is also indicative of heightened NFκB translocation to the cellular nucleus (46).

Inhibition of TLR4 signaling attenuates high fat diet-induced perturbations in insulin signaling and oxidative pathways, as evidenced in studies involving rodents that are hyporesponsive to ligand agonism of TLR4. Both C3H/HeJ and C57BL10/ScCr mice contain genetic mutations that inhibit ligand agonsim of TLR4 signaling, resulting in non-functional TLR4 proteins (41, 46, 48, 59). In the fasted state TLR4 deficient mice display lower fasting glucose levels and elevated mRNA expression of CPT-1 than controls (73). However, the beneficial influence of TLR4 deficiency on metabolism is most evident in high fat diet fed rodents.

Shi et al., (51) were the first group to report that C57BL/10ScCr mice do not exhibit reductions in insulin signaling subsequent to lipid infusion or chronic high fat feeding as seen in wild type controls. Tsukumo et al., (46) expounded upon these findings with reports that C3H/HeJ mice have significantly higher whole body insulin sensitivity and skeletal muscle mRNA expression of IRS-1 and AKT than genetically similar controls following chronic consumption of a high fat diet. To assess the in vitro influence of the mutation on skeletal muscle insulin sensitivity, isolated soleus muscle of C3H/HeJ

and control rodents was incubated in elevated concentrations of palmitate. The C3H/HeJ group utilized 30-40% more glucose subsequent to the palmitate treatment than soleus muscle isolated from the control animals (46). C3H/HeJ mice also appear to be more metabolically flexible in response to high SFA feeding, determined by a heightened respiratory exchange ratio (RER) in the fed state (46). The studies also validated that the protective effects observed in the C3H/HeJ and C57BL/10ScCr rodent models were mediated through TLR4 inactivation. When incubated in MTS510, a TLR4 antagonist antibody, palmitate-mediated insulin resistance was attenuated in isolated soleus muscle of wild type animals as observed in the soleus of C3H/HeJ mice (46).

While the previously discussed studies did not describe the fatty acid composition of the high fat diets, Davis et al have argued that the beneficial effects of the TLR4 non-functional mutations are only pronounced in response to the consumption of a high SFA diet (58). The study compared markers of weight gain and insulin sensitivity between C57BL/10ScN mice fed a high palmitate diet (60% of total fat intake from SFA) or a high fat control diet (10% of total fat intake from SFA). The high SFA group had significantly lower adipocyte sizes and epididymal fat pad weights compared to the high fat control group (58). In response to the high fat control diet, the C57BL/10ScN mice were not protected against diet-induced insulin resistance, increased transcription of inflammatory cytokines, or macrophage infiltration of adipose tissue as observed in animals fed a high palmitate diet (58).

Metabolic Endotoxemia

Toll-like receptor 4 is also linked with the development of diet-induced obesity in that obese individuals display chronic elevations in plasma endotoxin concentrations, a condition that has been termed metabolic endotoxemia (64, 63). Although the condition is predominately observed in obese and type 2 diabetic individuals (63, 64), postprandial plasma endotoxin concentrations have been reported to be elevated in non-obese subjects subsequent to the consumption of a high fat meal (62, 74). Furthermore, chronic high fat feeding results in elevations in plasma endotoxin concentrations in the fasted and fed state. Mice fed a 72% fat diet for four weeks displayed a 2.7 fold increased in plasma LPS levels (54). Interestingly, comparisons between mice fed high fat and high carbohydrate diets have shown that the development of endotoxemia is blunted in high carbohydrate fed animals (2).

Independent of high fat feeding, endotoxemia has the capacity to induce metabolic abnormalities in lean skeletal muscle similar to the abnormalities that occur in response to high fat feeding. Following four weeks of chronic LPS infusions, non-obese, chow fed mice display weight gain and fasting insulin levels that were comparable to mice placed on a 72% fat diet (54). Our laboratory has shown that injection of LPS into wild type animals attenuates fatty acid oxidation in the fasted state, thus increasing the production of neutral lipids (41). This study also showed significant decreases in β-HAD and citrate synthase enzyme activities which corresponded with elevations in serum FFA and TAG concentrations (41).

The TLR4 signaling pathway has emerged as an important mediator of SFA-induced metabolic dysfunction. While significant attention has been placed on the relationship between TLR4 activation and insulin sensitivity, there is a lack of information regarding TLR4's influence on skeletal muscle substrate selection. Considering that preferential metabolism of glucose over fatty acids is characteristically seen in individuals who are obese and who are predisposed towards excessive weight gain (18, 20); a more through understanding of TLR4's influence on metabolism can shed valuable insight into the pathogenesis of obesity and the metabolic syndrome.

Chapter Three: Specific Aims

Currently 30% of the American population is obese. This is a national health care concern due to the link between obesity, type II diabetes, and cardiovascular disease which are two of the nation's leading causes of morbidity. The increasing prevalence of obesity and obesity-associated disease is closely linked to heightened consumption of the "Westernized Diet" which characteristically contains excessive amounts of dietary fatty acids. The recommended daily allowance for fat and saturated fatty acid (SFA) intake is 30 and 10%, respectively. Approximately 60% of the American population exceeds both dietary guidelines. Epidemiological studies have shown that the type of fat consumed has more relevance in establishing disease risk than measures of total fat intake. Dietary SFA intake is closely linked to the development of the metabolic syndrome and its associated comorbidities. Following incubation in palmitate, the most common SFA present in circulation, myotubes display reduced insulin stimulated glucose uptake and glycogen synthesis. The adverse effects of palmitate on insulin-stimulated glucose metabolism are, in part, mediated through inflammatory pathways as co-incubation of myotubes with palmitate and the anti-inflammatory compound acetylsalicylate attenuates palmitate-induced insulin resistance. Furthermore, mice deficient in the inflammatory signaling protein toll-like receptor 4 (TLR4) do not display significant weight gain or insulin resistance following 8-weeks of a 60% fat diet. Preliminary data from the Hulver laboratory suggests that the TLR4 pathway is involved in obesity-associated skeletal muscle metabolic dysfunction. The TLR4 protein is more abundantly expressed in skeletal muscle of obese humans and is associated with reduced fatty acid oxidation and increased triacylglycerol synthesis. Additionally, in vitro (myotubes) and in vivo (mouse skeletal muscle) studies have shown that acute activation of the TLR4 pathway with LPS results in preferential storage of fatty acids within muscle as opposed to oxidation. Moreover, these TLR4-mediated effects are exacerbated in myotubes and mice that are predisposed to conditions of elevated palmitic acid content, which is suggestive of a

heightened sensitization of the TLR4 pathway by saturated fatty acids. The objective of the current proposal is to translate findings from cell cultures and animals to humans with the hypothesis that a pro-inflammatory response to consumption of a high saturated fat diet for five days in skeletal muscle somehow interferes with metabolic adaptation to elevations in fatty acid intake (i.e., improved oxidative capacity). Furthermore, this lack of metabolic adaptation will be associated with reduced whole-body insulin sensitivity. Our objective will be achieved through three specific aims.

Specific Aim 1: To test the hypothesis that a heightened activation of TLR4 signaling and subsequent pro-inflammatory response in skeletal muscle following high fat feeding will be associated with blunted metabolic adaptation, as determined by measures of skeletal muscle fatty acid oxidative capacity.

Specific Aim 2: To test the hypothesis that 5 days of high SFA feeding will augment plasma endotoxin levels.

Specific Aim 3: To test the hypothesis that plasma endotoxin in response to a high fat meal challenge will be altered following five days of high fat feeding.

Chapter Four: Research Design and Methods

Overview.

The Hulver laboratory has previously identified a negative correlation between TLR4 expression and fatty acid metabolism within skeletal muscle (41). Moreover, activation of the TLR4 pathway using LPS significantly reduces the capacity of skeletal muscle cells and rodent skeletal muscle tissue to oxidize fatty acids (41). Additionally, preliminary studies performed in the Hulver laboratory using primary human myotubes and C57Bl/6J mice have shown that the effects of TLR4 activation by LPS were more robust following exposure to media or a diet, respectively, enriched with saturated fatty acids (manuscript in preparation). These preliminary findings are the basis of the current proposal's hypothesis that saturated fatty acids disrupt skeletal muscle fatty acid metabolism through a mechanism involving a pro-inflammatory response.

Subject Recruitment.

Non-obese, (BMI <25) sedentary males between the ages of 18 and 40 were recruited for the study. This study is regarded as a proof of concept study to determine if high saturated fat feeding resulted in increased pro-inflammatory response, metabolic maladaptation (lack of an increase in fatty acid oxidation), and insulin resistance. As such, only males were studied with the hopes of expanding our findings to females in future studies. Participants were recruited from Blacksburg, Virginia and surrounding areas using print and electronic advertisements. Upon the expression of interest by a potential participant, a phone interview was conducted for initial screening. Subjects that met the inclusion criteria were invited to an orientation session explaining the procedures involved in the study, the commitments associated with study participation, and the risks and benefits of participation. The session was concluded following a question and answer period. Subjects that completed consent forms were further screened by: a) medical history; b) physical examination; and c) complete blood count, blood chemistry, and urinalysis. Thirty-four healthy, non-obese male humans were recruited under the inclusion/ exclusion criteria listed in the Table 1.

Inclusion/ Exclusion Criteria for Research Participants	
Inclusion	**Exclusion**
• Age, 18-40 years old • BMI < 30kg/m^2 • Blood Pressure < 140/90mmHG • Fasting TAG < 150 mg/dL • Fasting Glucose < 100 mg/dL • WHR < 0.9 • Habitual fat intake < 40% of total caloric intake • Sedentary < 2 days/week of extended activity • Weight stable for previous 6 months (± 1.5 kg)	• Past of current ischemic heart disease, respiratory disease, endocrine or metabolic disease, neurological disease, or hetatological-oncological disease • Smoking • Chronic medications • Alcoholism or other substance abuse • Psychological issues of behaviors that may indicate that dietary non-compliance is highly probable

Research Design.

Subjects were instructed to come to the laboratory after a ~12 hour fast for all visits. During the subjects' preliminary assessment, measures of height, body mass, and body composition as measured by Dual-energy x-ray absorptiometry (DEXA) were performed. A skeletal muscle biopsy and an intravenous glucose tolerance test (IVGTT) were performed to measure baseline insulin sensitivity and markers of inflammatory response. Subjects were asked to complete a physical activity questionnaire, an infection/inflammation questionnaire, and a medical history form. Blood was drawn from all subjects (approximately 3 tablespoons) and was used to measure metabolic and cardiovascular hormone levels. Upon inclusion to the study, subjects were given a 4-day (three weekdays and one weekend day) food record and instructed on proper completion of the forms by a registered dietician. Food records and the Harris-Benedict equation were used by a registered dietician to tailor the experimental diet to match the subject's habitual energy intake. . Energy balance was assessed by daily monitoring of body mass during the entire duration of the study. The diet was composed of 55% fat (45% of total fat intake from saturated fat), 15% protein, and 30% carbohydrate. Saturated fatty acids make up 24% of the total caloric intake. All food was prepared daily in a metabolic

kitchen. Subjects reported to the metabolic kitchen to eat breakfast and to have daily body weights recorded. Lunch and dinner were given to volunteers to consume at their leisure. During visits 2 and 3, a muscle biopsy was obtained from the vastus lateralis muscle. The sample was extracted from alternating legs on each visit. Approximately 300mg of tissue was extracted per biopsy and divided for skeletal muscle end point measures as detailed in Table 2. Within table 2, we have indicated the reason/relevance for each end point measure. Skeletal muscle samples to be used for gene expression and protein content analysis were snap frozen at the time of collection, transported in liquid nitrogen to Dr. Hulver's laboratory, and stored at -80°C until future analysis. All samples were analyzed at the completion of the study. The muscle samples used for measures of radiolabeled substrate metabolism were immediately placed in ice-cold assay buffer, stored on ice, and transported to Dr. Hulver's laboratory for analysis.

Research Design

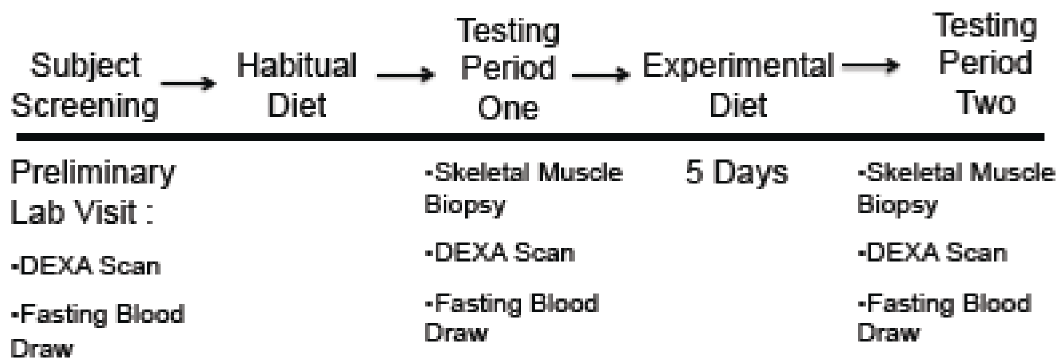


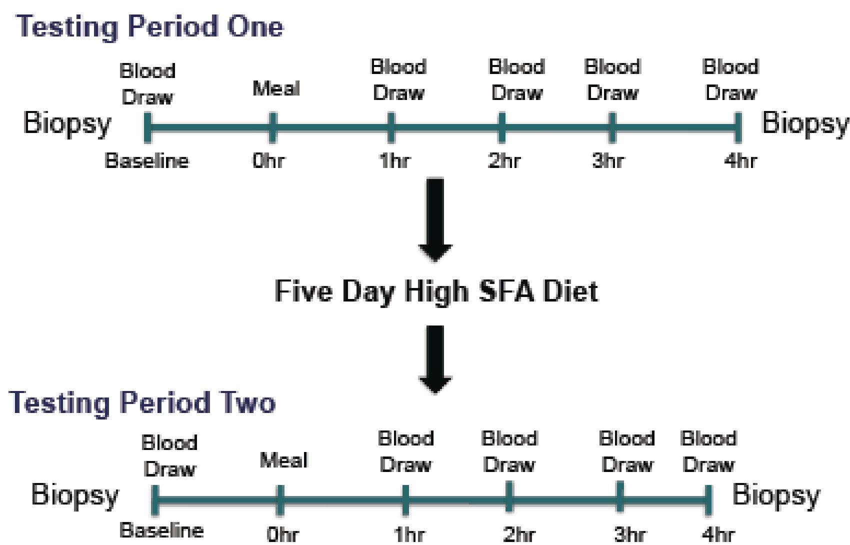
Table 2

Skeletal Muscle End Point Measures of Human Studies
Fatty Acid Oxidation
Glucose Oxidation
Mitochondrial Fatty Acid Oxidation
Bioplex Assay
- Phosphorylated: total JNK (pro-inflammatory protein)
- Phosphorylated: total p38 MAPK (pro-inflammatory protein)
- Phosphorylated: total AKT (insulin signaling protein)
- Phosphorylated: total ERK1/2 (pro-inflammatory protein)
- Phosphorylated: total IκBα (pro-inflammatory protein)
mRNA Expression
- PGC1α (oxidative transcription factor)
- PGC1β (oxidative transcription factor)
- PPARα (oxidative transcription factor)
- PPARΔ (oxidative transcription factor)
- PPARγ (oxidative transcription factor)
- NADH Dehydrogenase (oxidative protein)
- NFκB (inflammatory protein)
- MCP1 (inflammatory protein)
- TLR2 (inflammatory protein)
- TLR4 (inflammatory protein)
- Cyclophillin B (loading control)
Enzyme Activity
- Citrate Synthase (oxidative enzyme of the TCA cycle)
- β-HAD (oxidative enzyme of β-oxidation)

Cohort Two

Upon analysis of data obtained from cohort one we aspired to investigate the influence of elevations in plasma endotoxin concentrations on high fat diet associated shifts in skeletal muscle metabolism. To this end, a high fat meal challenge was included prior to and following high fat feeding to assess adaptive responses of oxidative metabolism, pro-inflammatory protein activation, and blood levels of endotoxin. As in cohort one, habitual dietary intake was used to assess high fat diet caloric content. Volunteers reported to the laboratory for testing period one after an overnight fast to obtain a skeletal muscle biopsy. Testing periods in cohort two differed from cohort one in that the frequently sampled IVGTT was replaced with a meal challenge. Immediately following the initial biopsy, volunteers were fed an 880kcal meal consisting of 24% carbohydrate,

13% protein, and 63% fat, 30% of which was composed to saturated fatty acids. The volunteers were placed in the supine position to obtain baseline blood samples, and remained in this position during the duration of the four-hour endotoxin challenge. Blood samples were collected at baseline and at one-hour intervals for a four-hour period and used to measure plasma endotoxin concentrations. After the 4hr blood draw, a second biopsy was performed. The previously described analyses were performed in samples obtained for the pre and post meal challenge biopsies.



Statistical Analysis.

Statistical analyses were performed using Prism GraphPad software (GraphPad software, La Jolla, CA). A Pearson's correlation analysis was used to determine R^2 and P values of correlation data. A repeated measures two-way ANOVA was used compare measures obtained in cohort 2 before and after the consumption of the high fat meal challenge at testing period one and two. All other measures were analyzed using a one-tailed student's or paired T test assuming unequal variance was used to determine P values (pre diet vs. post diet samples), which were considered significant at 0.05.

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Chapter 6. Manuscript

The role of a pro-inflammatory response in skeletal muscle's adaptation to high fat feeding in humans.

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Abstract

Diets that are rich in saturated fatty acids (SFA) promote the development of obesity and insulin resistance. In vitro studies have shown that SFA-associated metabolic perturbations are mediated via induction of pro-inflammatory pathways. The purpose of this study was to examine the relationship between diet-induced agonism of pro-inflammatory pathways and metabolic adaptive response to 5 days of high SFA feeding, which was determined by the volunteers' ability to increase radiolabeled substrate metabolism in response to the high SFA diet. A secondary aim was to determine if the metabolic adaptive response in skeletal muscle to a single, calorie dense, high fat meal was altered by 5 days of high saturated fat feeding. Twelve, college-age, non-obese males were studied and skeletal muscle samples were obtained prior to and directly after the consumption of a high SFA diet. Skeletal muscle samples were used to measure fatty acid oxidation, glucose oxidation, oxidative enzyme activities, mRNA expression of metabolic targets, and phosphorylated to total protein content of inflammatory proteins. We observed reductions in glucose oxidation and complete fatty acid oxidation, determined by the production of ^{14}C-labeled CO_2, in response to five days of high SFA feeding. When data were analyzed based on volunteers' response to high fat feeding, volunteers exhibiting decreased fatty acid oxidation in response to 5 days of high fat feeding also displayed heightened ratios of phosphorylated to total protein concentrations of pro-inflammatory proteins. We also report that postprandial elevations in endotoxin concentrations following the consumption of a high fat meal were augmented following five days of high SFA feeding. Interestingly, in skeletal muscle samples studied prior to

and 4 hours following a high fat meal challenge we observed a significant induction of mRNA levels of oxidative proteins; which was associated with increased phosphorylated to total protein ratios of pro-inflammatory targets. Meal challenge-induced stimulation of oxidative transcription factors was abrogated following 5 days of high fat feeding as were the correlations between oxidative and pro-inflammatory targets. This report illustrates two important findings: 1) a maladaptive oxidative response to 5 days of high fat feeding is associated with heightened activation of pro-inflammatory proteins; and 2) a single high fat meal challenge is met with increases in both pro-inflammatory response and induction of mRNA levels of oxidative proteins; a response that is abrogated following 5 days of high fat feeding. These results suggest that activation of pro-inflammatory proteins in response to a single high fat meal may be a normal adaptive response when habitual fat intake is less than 30%; however this response is dysregulated following only 5 days of high fat feeding.

Introduction:

To maintain a healthy body weight the World Health Organization has recommended that individuals substitute calories consumed from saturated fatty acids (SFA) with calories derived from monounsaturated fatty acids (MUFA) (Micha et al., 2010). This recommendation stems from the understanding that MUFA consumption is linked to improvements in metabolic profile while diets that are rich in SFA promote the development of obesity and its associated comorbidities including insulin resistance (Moon et al., 2010; Vessby et al., 2001) and dyslipidemia (Kein et al., 2005).

Corroborating evidence has shown that SFA induce these pathologies through a mechanism involving stimulation of pro-inflammatory pathways (Sinha et al., 2004; Coll et al., 2007). Saturated fatty acids are associated with heightened activation of toll-like receptor 4 (TLR4) and toll-like receptor 2 (TLR2), which induce transcription of pro-inflammatory markers through signaling cascades involving activation of nuclear factor κB (NFkB), p38 mitogen activated protein kinase (p38 MAPK), and c-jun N terminal kinase (JNK) (Youn et al., 2005). Inhibition of NFkB, TLR2 or TLR4 attenuates palmitate induced insulin resistance in cell culture and isolated soleus muscle (Tsukumo et al., 2007; Sinha et al., 2004). Furthermore, rodents lacking functional TLR4 display significantly hightened respiratory exchange ratios (RER) and have reduced visceral fat deposits when placed on high fat diets, which suggests that inhibition of immune signaling improves the rodents' ability to adapt to elevated fatty acid intake by increased fatty acid oxidation (Tsukumo et al., 2007).

Smith et al., (Smith et al., 2000) have previously reported that non-obese volunteers who display the most robust increases in fatty acid oxidation in response to five days of high fatty acid feeding are also the most insulin sensitive following the experimental diet. Considering that inhibition of NFκB and TLR4 appears to be protective against saturated fatty acid and high fat diet induced metabolic perturbations in rodents and cell culture; we aspired to investigate the relationship between high SFA diet induced insulin resistance, metabolic response to high SFA feeding, and stimulation of pro-inflammatory markers in skeletal muscle obtained from non-obese volunteers. We hypothesized that an increase in pro-inflammatory markers in skeletal muscle in response

to 5 days of a high saturated fat diet would be negatively associated with increased oxidative capacity, and positively associated with the development of insulin resistance.

Methods

Study Volunteers. Twelve, sedentary, college-age (21 +/- 1 years), non-obese (22.3 +/1 3.9 kg/m^2), Caucasian males were recruited and participated in the study under a protocol approved by the Institutional Review Board at Virginia Tech. Exclusion criteria included: smoking, taking medications that were antibiotic, anti-inflammatory, or known to alter carbohydrate or fatty acid metabolism, not weight-stable (+/- 5 pounds) for previous 6 months, habitual or planned exercise of more than 2 days per week, or habitual dietary consumption ≥ 30% fat.

Study Design. The primary objective of the current study was to investigate the relationship between pro-inflammatory response, insulin sensitivity, and metabolic adaptation in skeletal muscle in response to an acute (5 days), isocaloric (relative to habitual diet), high fat (50% of total caloric intake), high saturated fat (25% of total caloric intake) diet. The study was divided into two cohorts. During both cohorts, skeletal muscle measures of pro-inflammatory response, fatty acid oxidation in whole muscle homogenates and isolated mitochondria, oxidative enzyme function, and mRNA levels of metabolic targets were assessed prior to and following 5 days of high fat feeding. Subjects studied in cohort one underwent a frequently sampled intravenous glucose tolerance test (FSIVGTT) prior to and following five days of high fat feeding to assess changes in insulin sensitivity. Subjects studied in cohort two underwent a high fat meal

challenge with blood draws prior to and 1, 2, 3, and 4 hours post meal consumption. The primary measures in serum were lipids and endotoxin. Muscle biopsies were obtained pre and 4h post meal challenge to assess pro-inflammatory responses and metabolic adaptations.

Cohort 1. Volunteers in cohort 1 completed two testing periods. The first testing period occurred prior to experimental diet. Volunteers reported to the laboratory at ~7-9 AM following an overnight fast (~10-12h). A fasting blood sample was taken to measure insulin, glucose and lipid levels (total, high-density lipoprotein, low density lipoprotein, cholesterol, triglyceride and non-esterified fatty acid levels). Body composition was determined using a dual x-ray absorptiometry (DEXA) scanner and a skeletal muscle biopsy, as described below, was performed. Immediately following the biopsy, a FSIVGTT was performed. Volunteers began the 5-day high fat diet (dietary macronutrient content is described in detail below), on the morning following testing period one. Subjects reported to the metabolic kitchen daily to eat breakfast, receive meals for the day, and to have body mass measured and recorded. At the end of the 5-day high SFA diet subjects reported to the laboratory for testing period two, which was identical to testing period one as described above.

Cohort 2. We have previously reported that in the presence of "metabolic endotoxemia" concentrations of LPS (50pg/µL), fatty acid oxidation is suppressed while glucose metabolism is elevated in mice and in experiments using skeletal muscle cell cultures (Frisard et al., 2010). While circulating LPS concentrations in non-obese individuals are

usually well below 10pg/mL, plasma LPS concentrations are elevated in response to acute high fat feeding (Erridge et al., 2010; Ferrier et al., 2011). To this end, we aspired to (1) determine if circulating endotoxin was affected by a high fat meal challenge prior to and following 5 days of high SFA feeding; and (2) to determine if the adaptive response with regards to oxidative metabolism and pro-inflammatory markers to the meal challenge was altered by 5-days of high SFA feeding.

Volunteers in cohort 2 completed two testing periods. Volunteers reported to the laboratory at ~7-9 AM following an overnight fast (~10-12h). A fasting blood sample, DEXA scan and skeletal muscle biopsy were performed to assess the previously listed analyses. Research participants then consumed a high fat meal consisting of two Jimmy Dean sausage biscuits (880 kcal, 63% fat, 24%, 13% protein, 10% SFA). Blood samples were obtained prior to the meal challenge and at 1, 2, 3 and 4 hours post meal. A second biopsy was performed immediately concluding the 4hr blood collection. On the morning following the initial testing period volunteers began the 5-day experimental diet (described in detail below). Subjects reported to the metabolic kitchen daily to eat breakfast, receive meals for the day, and to have body mass measured and recorded. At the end of the 5-day experimental diet, subjects reported to the laboratory for testing period two which also consisted of muscle biopsies prior to and following a high fat meal challenge.

Dietary Assessment. Habitual caloric intake for each subject was determined using a self-reported 4-day food intake record, which was then compared to the Harris-Benedict

equation for each study participant. A registered dietician (RD) instructed volunteers to accurately report food intakes (e.g., portion sizes, food preparation methods, and brand names of products) with the use of two-dimensional food models. The RD reviewed all records with the participants upon completion for accuracy and sufficiency of detail. Food intake records were analyzed with the Food Intake Analysis System (FIAS 3.98 nutrient analysis program; University of Texas School of Public health, 1998). Volunteers were asked to provide food labels for products used to determine appropriate substitutions when the actual items consumed were not in the software database.

Upon determination of habitual caloric intake, the RD designed a 30% carbohydrate, 20% protein, and 50% fat experimental diet, which was isocaloric to the volunteers' habitual dietary intake. Saturated fatty acids accounted for 45% of the total fat content of the diet and 25% of total caloric intake. All meals were prepared daily by the RD. Subjects reported to the metabolic kitchen daily ~7-9AM to consume breakfast and to obtain body weight measures; lunch and dinner were provided to volunteers to consume at their convenience.

Anthropometric measures. Body weight was measured to the nearest 0.1kg with a balance scale while subjects were wearing only light indoor clothing (Detecto, Webb City, MO), and height (cm) was measured with a wall-mounted stadiometer while subjects were barefoot. Body weight was measured at each testing period and daily during the consumption of the 5-day high SFA diet. Body composition was determined

by a dual energy x-ray absorptiometry (GE Lunar Prodigy Advance Software version 8.10e).

Frequently Sampled Intravenous Glucose Tolerance Test. All volunteers were fasted and placed in the supine position prior to the FSIVGTT. An intravenous catheter was placed in each vein and blood samples were collected as previously described (Davy et al., 2002). Blood samples were immediately centrifuged at 4^0C for 20min at 2500g and analyzed for glucose concentrations with the glucose oxidase method by using a glucose autoanalyzer (Yellow Springs Instruments, Yellow Springs, OH). Insulin was measured using a Seimens Immulite 1000 Immunoassay System (Seimens Health Diagnostics, Deerfield, IL). Insulin and glucose values from the FSIVGTT were entered into the MINMOD program (version 3.0; R. Bergman, University of Southern California) for determination of insulin sensitivity (S_I), acute insulin response to glucose (AIR_G), and glucose effectiveness. This model uses measures of plasma glucose and insulin concentrations over a 3hr period to derive in vivo whole-body S_I as measured in the procedures described by Bergman et al., (Bergman et al., 1981).

Lipid and lipoprotein analysis. Blood plasma samples were obtained from volunteers in the fasted state at testing periods one and two. Blood samples were collected using an indwelling intravenous catheter into tubes containing heparin. Samples were inverted and centrifuged at 4^0C for 20min at 2500xg to obtain plasma, which was stored at -80^0C until analyzed. Plasma lipid and lipoprotein concentrations were measured in a commercial laboratory using conventional methods.

Skeletal muscle biopsies. Skeletal muscle biopsies were obtained from the vastus lateralis of participants under local anesthesia (1% lidocaine) using the Bergström needle suction technique (Bergström et al., 1962). Multiple passes were performed to obtain ~300mg of tissue. Muscle used for fatty acid oxidation, glucose oxidation, and enzyme activity was immediately placed in SET buffer (0.25M Sucrose, 1mM EDTA, 0.01M Tris-HCl and 2mM ATP) and stored on ice until homogenization (~25 min). Muscle used for mitochondrial isolation was placed in isolation buffer (67mM sucrose, 50mM Tris/HCl, 50mM KCl, 10mM EDTA/ Tris, and 10% bovine serum albumin; all from Sigma-Aldrich, St. Louis, MO) and stored on ice until homogenization. Muscle used for qRT-PCR was placed in Trizol (Invitrogen, Carlsbad, CA) and snap-frozen in liquid nitrogen. Muscle used for bioplex analysis was placed in phosphoprotein-lysis-buffer (Biorad, Hercules, CA) and snap-frozen in liquid nitrogen. Bioplex and RNA samples were stored at -80^{0}C for later analysis.

Skeletal Muscle Homogenate Preparation. Vastus lateralis muscle samples obtained from volunteers were weighed and placed in 200µL of SET buffer (0.25M Sucrose, 1mM EDTAQ, 0.01M Tris-HCl, and 2mM ATP). Each sample was minced 200 times with scissors and transferred to a glass homogenization tube and homogenized on ice using a Teflon pestle (12 passes at 3,000RPM). The sample was rested on ice for ~30 seconds and the homogenization step was repeated. The homogenate was transferred to an Eppendorf tube and fresh sample was used to measure glucose oxidation, fatty acid oxidation and enzyme activity assays. Homogenate protein concentrations were

determined spectrophotometrically using the bicinchoninic acid BCA assay (Thermo Scientific, catalog # 23225, Pittsburg, PA) and all substrate metabolism and enzymatic activities were expressed relative to mg protein used in the assays.

Mitochondrial Isolation. Vastus lateralis muscle obtained from volunteers was weighted and placed in 150mL of isolation buffer. A razor was used to mince tissue for 2min on a glass plate. Minced tissue was filtered and the supernatant was discarded. The homogenate was placed in trypsin and incubated at 4^0C for 30 minutes. Following the incubation period the sample was centrifuged at 200g for 3min. Trypsin was removed and the pellet was resuspended in 2.25mL of isolation buffer. The sample was homogenized using a motor operated Teflon Potter Elvehjem pestle (10 up and down pulses at ~85 RPM) and transferred to an Eppendorf tube where it was centrifuged at 700g for 10 minutes. The supernatant was removed and the pellet was resuspended in 5mL of isolation buffer. The sample was centrifuged at 8,000g for 10 minutes prior to resuspension in 100μL of isolation buffer. All steps were performed at 4^0C. Mitochondrial protein concentrations were determined spectrophotometrically using the BCA assay (Thermo Scientific, catalog # 23225, Pittsburg, PA). Isolated mitochondria were used fresh for fatty acid oxidation analysis.

Fatty acid oxidation. Fatty acid oxidation was assessed using whole muscle homogenates and isolated skeletal muscle mitochondria that were incubated in [1-^{14}C] palmitic acid as previously described (Hulver et al., 2005). Total fatty acid oxidation was

determined by measuring and summing the production of ^{14}C-labeled CO^2 and ^{14}C-labeled acid soluble metabolites.

Glucose oxidation. Glucose oxidation was assessed using whole muscle homogenates that were incubated in [U ^{14}C glucose] as previously described (Hulver et al., 2005).

Enzyme activity. Enzyme activities were assessed in muscle homogenates (20-fold dilution). Sample buffer consisted of 0.1 mol/l KH_2PO_4/Na_2PHO_4 and 2 mmol/l EDTA, pH 7.2. PFK, CS, and β-HAD activities were determined spectrophotometrically as previously described (Frisard et al., 2010).

Bioplex assay. Tissue samples were homogenized using a polytron homogenizer (30 seconds per sample). Homogenized samples were centrifuged at 4^0C at 30,000RPM for one hour. The supernatant was collected and the pellet discarded. Total and phosphorylated protein content of c-Jun N terminal kinase (JNK), p38 mitogen activated protein kinase (p38MAPK), inhibitor of nuclear factor of kappa light chain gene enhancer in B-cells alpha (IκBα), protein kinase B (AKT), and extracellular signal related kinase1/2 (ERK1/2) were measured using the Bio-Plex Pro Human Cytokine 8-plex assay (BioRad, Hercules, CA) according to the manufacturer's instructions. Fluorescence was measured by a Bio-Plex 200 system (BioRad, Hercules, CA) and protein concentrations were measured spectrophotometrically using the BCA assay (Thermo Scientific, catalog # 23225, Pittsburg, PA) and all results were expressed relative to mg protein used in the Bioplex assay.

RNA extraction and qRT-PCR. RNA was extracted using an RNeasy Mini Kit (Qiagen) and DNase I treatment (Qiagen, Valencia, CA), according to the manufacturer's instructions. qRT-PCR was performed using an ABI PRISM 7900HT Sequence Detection System instrument and TaqMan Universal PCR Master Mix used according to the manufacturer's specifications (Applied Biosystems, Foster City, CA). Target gene expression in human skeletal muscle tissue samples was normalized to cyclophilin B RNA levels. Primers and 5# FAM-labeled TaqMan probes were purchased as pre-validated assays (ABI) for the following gene targets; peroxisome proliferator activated receptor γ-coactivated 1-α and -β (PGC1α and PGC1β), tumor necrosis factor-α (TNFα), NADH Dehydrogenase, monocyte chemo-attractant protein 1 (MCP1), perosixome proliferator activated receptor-Δ, -α, and-γ (PPARΔ, PPARα and PPARγ), and nuclear factor κB 1 (NFκB). Relative quantification of target genes was calculated using the 2**Δ**CT method. Derivation of the 2 CT equation has been described in Applied Biosystems User Bulletin no. 2 (P/N 4303859).

Statistical Analysis. Statistical analyses were performed using Prism GraphPad software (GraphPad software, La Jolla, CA). Data were presented as means ± standard error (s.e.m.). A Pearson's correlation analysis was used to determine R^2 and P values of correlation data. A repeated measures two-way ANOVA was used compare measures obtained in cohort 2 before and after the consumption of the high fat meal challenge at testing period one and two. All other measures were analyzed using a one-tailed student's

or paired T test assuming unequal variance was used to determine P values (pre diet vs. post diet samples), which were considered significant at 0.05.

RESULTS

High fat diet composition and caloric intake. On average, the volunteers habitually consumed 2,458.5 kcal daily. Their mean dietary fat intake was 31.2% of total caloric intake while SFA consumption accounted for 10.8% of their total caloric intake. During the experimental diet volunteers consumed an average of 2,481 kcal daily. The mean fat content of the experimental diet was 54.9% of total caloric intake while SFA accounted for 24.8% of the total caloric intake.

Weight, body composition, or plasma glucose and lipid profiles were not influenced by 5 days of high SFA feeding. Weight, BMI, body fat percentage, fasting plasma glucose, fasting plasma TAG, fasting FFA, and cholesterol levels were measured in fasting conditions in cohorts one and two prior to and following five days of high SFA feeding. As the feeding paradigms were identical, data for both cohorts were combined for analysis. Five days of high fat feeding under energy balance conditions did not elicit any changes in body weight, BMI, body fat percentages, glucose, or plasma lipid profiles (Table 1).

Table 1

	Pre 5-Day HFD Levels	Post 5-day HFD Level
Weight	151.1 ± 6.5 lbs	150.9 ± 6.3 lbs
BMI	22 kg/m^2	22 kg/m^2
Body Fat Percentage	17 ± 2 %	17 ± 2 %
Plasma Glucose	88 ± 2 mg/dL	85 ± 3mg/dL
Plasma TAG	126.0 ± 18.4 mg/dL	110.1 ± 17.86 mg/dL
Plasma Cholesterol	153.5 ± 9.2 mg/dL	154 ± 8.9 mg/dL
LDL/HDL	1.98 ± 0.2 mg/dL	2.16 ± 0.2 mg/dL
LDL	88.3 ± 6.5 mg/dL	94.8 ± 6.2 mg/dL
HDL	47.5 ± 3.7 mg/dL	47.2 ± 5.4 mg/dL

Table 1. Five days of high SFA feeding did not significantly influence amphrometric measures as determined by a one-tailed paired T-test. Data was obtained from volunteers in cohorts one and two (pre-meal challenge samples only). Values are expressed as means ± SEM. No significant differences were observed.

Substrate metabolism, enzyme activity, and gene expression of oxidative and pro-inflammatory markers were not influenced by five days of high SFA feeding. In response to 5 days of high SFA feeding under energy balance conditions, we did not observe any changes in skeletal muscle fatty acid oxidation, acid soluble metabolite production, citrate synthase activity, β-HAD activity (Table 2), mRNA expression of metabolic (Table 3) or pro-inflammatory targets (Table 4). However, we did observe a significant decrease in glucose oxidation (Figure 1A). Complete fatty acid oxidation, determined by the production of ^{14}C-labeled CO_2 glucose oxidation, was reduced by 70% however; this reduction was not statistically significant (Figure 1B).

We also analyzed the ratio of complete vs. incomplete fatty acid oxidation determined by the production of ^{14}C-labeled CO_2 to the production of radio-labeled acid soluble metabolites (Figure 1C) and found that this ratio was slightly decreased with five days of high SFA feeding. However; we observed a significant decrease in the ratio of citrate synthase to β-HAD enzyme activity (Figure 1D), which is indicative of a decrease in the ratio of complete to incomplete fatty acid oxidation.

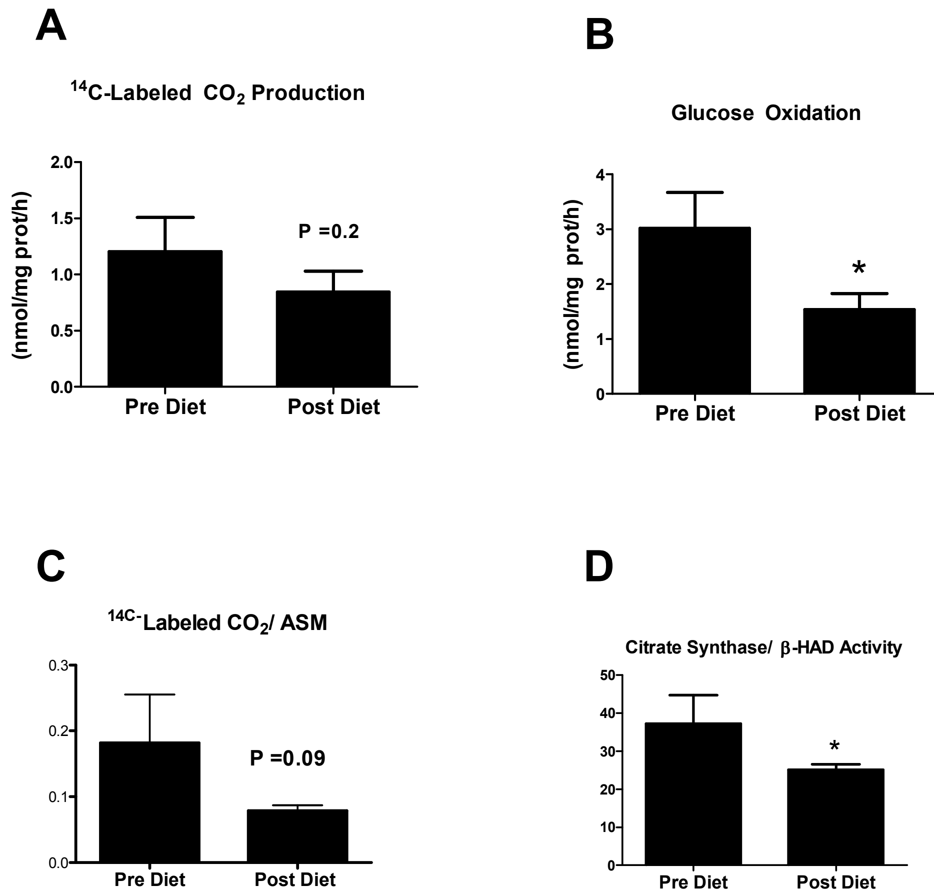

Figure 1. Radiolabeled substrates were used to determine fatty acid and glucose oxidation in skeletal muscle samples obtained prior to and following the consumption of a 5-day SFA diet. Data obtained from volunteers in cohort 1 and 2 (pre-meal challenge samples only) were combined. Glucose oxidation and CO_2 values were expressed as (nmol/mg protein/hr). A one-tailed paired T-test was used to determine significance. No significance differences were observed.

Table2.

	Pre 5-Day HFD	**Post 5-Day HFD**
Fatty Acid Oxidation (nmol/mg protein/hr)	13.2 ± 2.9	12.3 ± 2.4
Acid Soluble Metabolite Production (nmol/mg protein/hr)	10.9 ± 2.9	10.9 ± 2.2
CO_2 Production (nmol/mg protein/hr)	1.2 ± 0.3	0.9 ± 0.2

^{14}C-Labeled CO_2/ASM Production	0.2 ± 0.1	0.1 ± 0.0
Glucose Oxidation (nmol/mg protein/hr)	3.0 ± 0.6	1.5 ± 0.3
Citrate Synthase Activity (nmol/mg protein/min)	225.7 ± 47.6	219.2 ± 46.8
β-HAD Activity (nmol/mg protein/min)	14.8 ± 4.2	15.5 ± 4.6

Table 2. Radiolabeled substrates were used to determine fatty acid and glucose oxidation in skeletal muscle samples obtained prior to and following the consumption of a 5-day SFA diet. Data obtained from volunteers in cohort 1 and 2 (pre-meal challenge samples only) were combined. Fatty acid oxidation, ASM, CO_2, and glucose oxidation values are expressed as (nmol/mg protein/hr), citrate synthase and β-HAD values are expressed as (nmol/mg protein/min). A one-tailed paired T-test was used to determine significance. No significance differences were observed.

Table 3.

	Pre 5-day HDF	**Post 5-day HFD**
PGC1α mRNA	20.2 ± 4.3	26.6 ± 6.1
PGC1β	9.9 ± 2.9	11.2 ± 3.8
NADH Dehydrogenase	11.8 ± 1.6	10.8 ± 2.0
PPARα	24.4 ± 9.6	17.6 ± 6.8
PPARΔ	3.1 ± 0.8	2.6 ± 0.8
PPARγ	3.8 ± 1.2	4.6 ± 2.0

Table 3. mRNA expression of oxidative markers were measured in the skeletal muscle of volunteers prior to and following the consumption of a 5-day high SFA diet. Expression of PGC1α and NADH Dehydrogease was measured in cohort one and two (n=12) while expression of PGC1β, PPARα, PPARΔ, and PPARγ was exclusively measured in cohort 2 (n=6). Values are expressed relative to expression of cyclophilin B (arbitrary values). A one-tailed paired T-test was used to determine significance, values are presented as means ± SEM. No significance differences were observed.

Table 4.

	Pre 5-day HDF	**Post 5-day HFD**
TLR2	0.35 ± 0.07	0.31 ± 0.06
TLR4	6.65 ± 0.86	6.39 ± 0.86
MCP1	10.93 ± 1.91	9.97 ± 1.85
NFκB	0.07 ± 0.01	0.06 ± 0.01
TNFα	0.13 ± 0.02	0.08 ± 0.01

Table 4. mRNA expression of pro-inflammatory markers were measured in the skeletal muscle of volunteers prior to and following the consumption of a 5-day high SFA diet. Expression of TLR2, TLR4, MCP1, and NFκB was measured in cohort one and two (n=12) while expression of TNFα was exclusively measured in cohort 2 (n=6). Values are expressed relative to expression of cyclophilin B (arbitrary values). A one-tailed paired T-test was used to determine significance; values are presented as means ± SEM. No significance was observed.

The inability to increase fatty acid oxidation in response to high fat diet is associated with increases in inflammatory markers. Smith et al., (Smith et al., 2000) were one of the first groups to suggest that non-obese subjects varied significantly in their ability to compensate for elevations in fatty acid intake. To determine if diet-induced stimulation of pro-inflammatory markers varied by subject adaptability to high fat feeding volunteers in cohorts one and two were classified as responders (individuals who displayed a >50% increase in palmitate oxidation following the high SFA diet, N=4) and non-responders (individual who displayed a negative percent change in fatty acid oxidation following the consumption of the high SFA diet, N=4). We compared the percent change in the ratio of phosphorylated to total protein content of p38 MAPK, JNK, IκBα, AKT, ERK1/2. Whereas JNK and p38 MAPK concentrations were modestly decreased or unchanged in response to the diet in responders, the non-responders displayed a 100-200% increase, respectively, in the ratio of phosphorylated to total protein concentrations of the pro-inflammatory markers (Figure 3). These findings suggest that SFA-induced stimulation of pro-inflammatory markers is heightened in individuals with maladaptation in metabolic response to high SFA feeding.

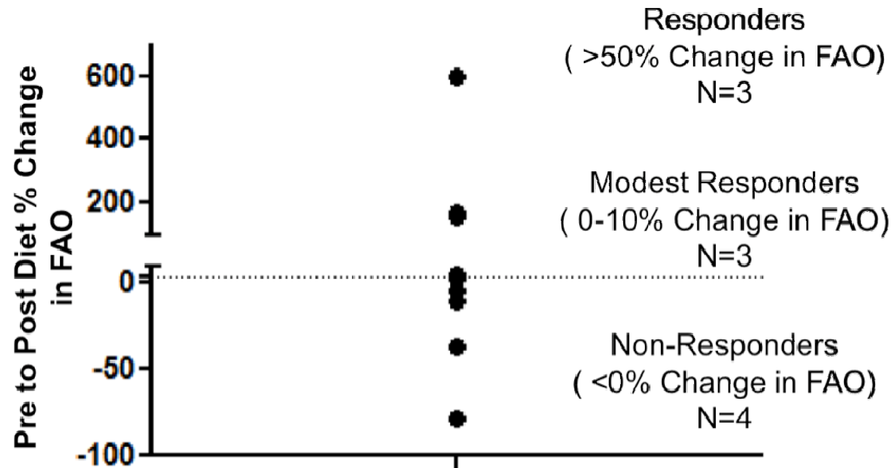

Figure 2. Volunteers were categorized by their percent change in fatty acid oxidation in response to five days of high SFA feeding. All volunteers with a >50% increase in fatty acid oxidation were labeled as responders. All volunteers with a 0-10% increase in fatty acid oxidation were labeled as modest responders. All volunteers with a <0% change in fatty acid oxidation were labeled as non-responders.

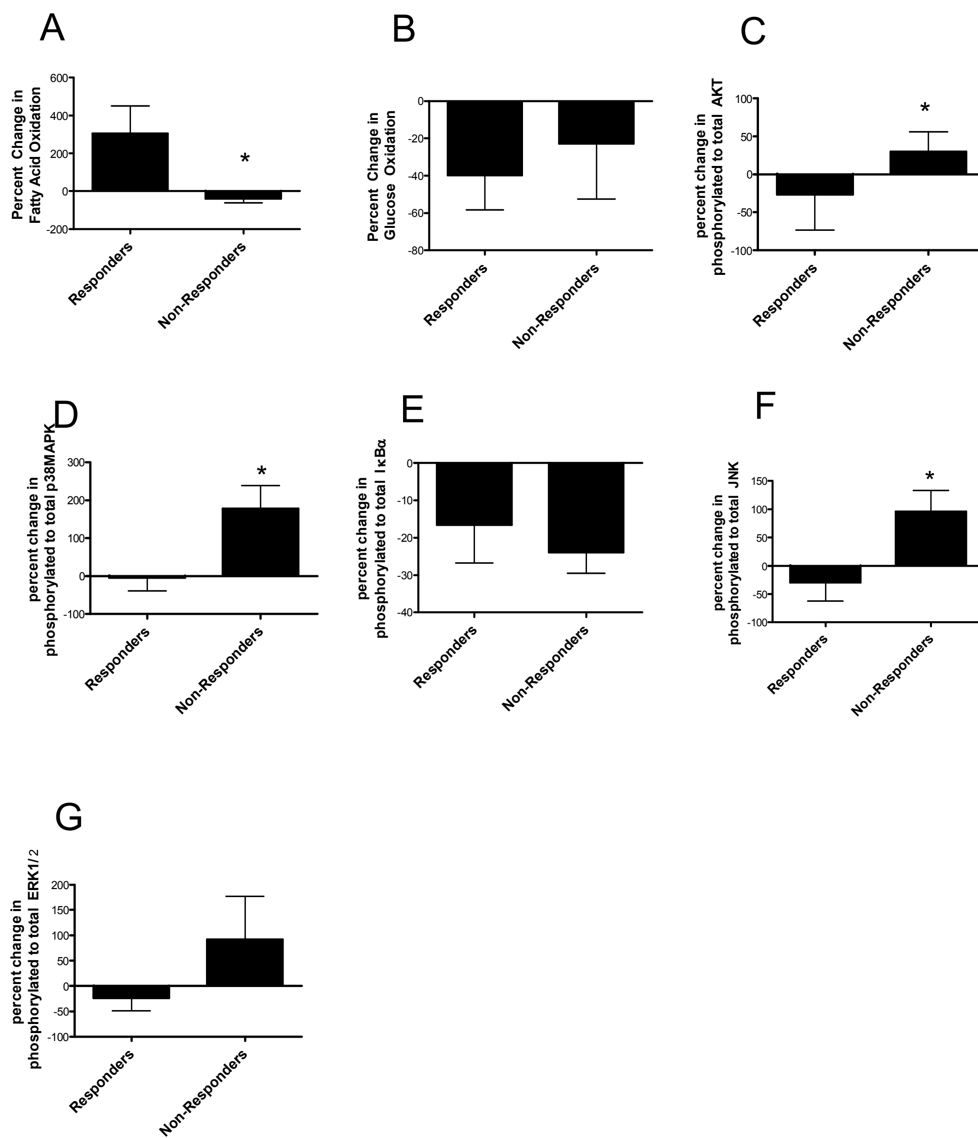

Figure 3. Volunteers from cohort one and two were classified as responders (>50% increase in fatty acid oxidation in response to consumption of high SFA diet) and non-responders (volunteers who did not display an increase in fatty acid oxidation in response to consumption of high SFA diet). The percent change in fatty acid oxidation (A), glucose oxidation (B), p:T AKT (C), p:t p38MAPK (D), p:T IκBα (E), p:t JNK (F), and p:t ERK1/2 (G) in responder and non-responder groups was summed and an one-tailed students t-test was used to measure statistical significance between the two groups. Values are expressed as means ± SEM, *P< 0.05.

Postprandial endotoxin levels are elevated in response to 5days of high SFA feeding. Plasma endotoxin concentrations have been reported to increase in response to chronic high fat diet consumption (Cani et al., 2007; Erridge et al., 2010). As such, we measured postprandial endotoxin concentrations at baseline and 1, 2, 3, and 4 hours following the meal challenge and compared those measures to samples obtained prior to and following 5 days of high SFA feeding. We observed a 2.5-fold increase in postprandial endotoxin concentrations at 1hr following the consumption of the high fat meal challenge in post-HFD vs. pre-HFD samples (Figure 4). Fasting endotoxin concentrations trended towards significance at baseline and were increased by 2-fold in response to the high SFA diet. We did not observe a significant elevation in post-diet endotoxin concentrations at baseline or 2-4 hours following the high fat meal challenge.

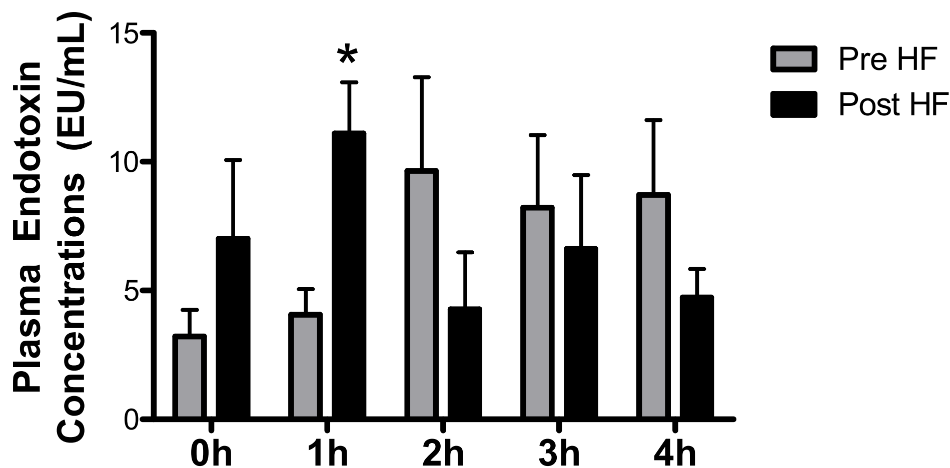

Figure 4. Plasma endotoxin concentrations were measured at baseline and 1-4 hours following the consumption of a high fat meal challenge. Endotoxin concentrations were compared between samples obtained prior to and following the consumption of a 5 day high SFA diet. In response to 5 days of high SFA feeding we observed a 2.5-fold increase in postprandial endotoxin concentrations one hour following the meal challenge. Significance was determined using a multi-level repeated measured ANOVA. Values are expressed as means ± SEM. A one-tailed, student's T-test was used to establish statistical significance, *P< 0.05.

Adaptive response to a high fat meal challenge is altered by 5 days of high SFA diet. We measured mRNA expression of oxidative and inflammatory proteins in response to high fat meal challenges conducted prior to and following 5 days of high fat feeding. In response to the meal challenge prior to the 5 days of high fat feeding, mRNA levels of PGC1β, PGC1α, PPARΔ, and NADH Dehydrogenase were significantly increased with no change in PPARγ (Figure 5 A-E). These observed increases in transcription of PGC1β, PGC1α, and NADH Dehydrogenase were not present in response to meal challenge after 5 days of high fat feeding. We did not observe changes in fatty acid oxidation, glucose oxidation, or enzyme activity of citrate synthase and β-HAD in response to the pre or post high SFA diet meal challenge (Table 5).

Table 5

	Pre Diet, Pre Meal Challenge	Pre Diet, Post Meal Challenge	Post Diet, Pre Meal Challenge	Post Diet, Post Meal Challenge
Fatty Acid Oxidation	17.6 ± 3.19	17.2 ± 4.10	19.2 ± 1.83	18.0 ± 1.12
CO2 Production	1.5 ± 0.43	1.4 ± 0.27	1.3 ± 0.20	1.7 ± 0.29
Acid Soluble Metabolite	14.5 ± 3.57	15.2 ± 3.75	16.9 ± 2.00	16.1 ± 1.26
CO2/ASM	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.01	0.1 ± 0.02
Mitochondrial Fatty Acid Oxidation	71.7 ±15.3	90.1 ± 26.7	103.2 ± 8.7	134.78 ± 26.8
Citrate Synthase	97.8 ± 8.97	101.2 ± 4.80	79.8 ± 5.34	84.9 ± 4.65
β-HAD	2.8 ± 0.29	3.7 ± 0.23	3.1 ± 0.14	3.9 ± 0.39

Table 5. Radiolabeled palmitate (A) and glucose oxidation (B) and enzyme activity of citrate synthase (C) and β-HAD was measured in cohort two volunteers during 4 conditions (1) the fasted state prior to the consumption of a 5-day high SFA diet (Pre Diet, Pre meal challenge), (2) 4hrs following the consumption of a high fat meal challenge and prior to the consumption of a 5-day high SFA diet (Pre Diet, Post Meal Challenge), (3) in the fasted state concluding the consumption of a 5 day high SFA diet (Post Diet, Pre meal Challenge), and (4) 4hours after the consumption of a high fat meal challenge concluding the consumption of a 5-day high SFA diet. Values are presented as means ± SEM. Significance was determined using a repeated measures two-way ANOVA. No significant differences were observed.

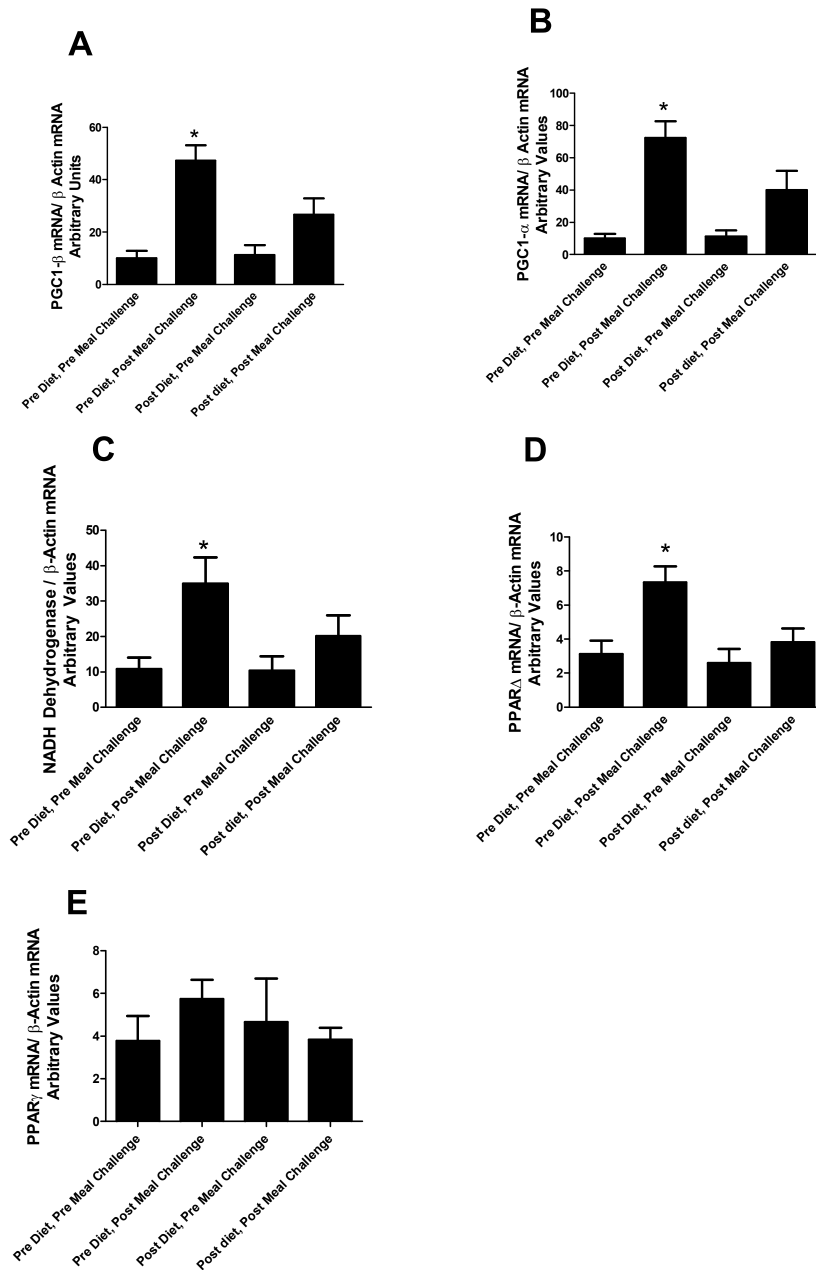

Figure 5. mRNA expression of markers of oxidative phosphorylation were measured in cohort 2 volunteers in 4 conditions (1) the fasted state prior to the consumption of a 5-day high SFA diet (Pre Diet, Pre meal challenge), (2) 4hrs following the consumption of a high fat meal challenge and prior to the consumption of a 5-day high SFA diet (Pre Diet, Post Meal Challenge), (3) in the fasted state concluding the consumption of a 5 day high SFA diet (Post Diet, Pre meal Challenge), and (4) 4hours after the consumption of a high fat meal challenge concluding the consumption of a 5-day high SFA diet. In response to the meal challenge mRNA expression of PGC1β, PGC1α, NADH Dehydrogenase and PPARΔ was significantly elevated. This increase was not observed in response to the meal challenge in post-diet samples. Significance was determined using a repeated measures two-way ANOVA, *$P<0.05$.

Consumption of a 5-day high SFA diet did not influence protein or mRNA expression of inflammatory markers. We did not observe a significant change in the ratio of phosphorylated: total protein content of JNK, ERK1/2, p38MAPK, or IκBα (table 6) in response to the high fat meal challenge or five days of high SFA feeding.

Table 6

	Pre Diet, Pre Meal Challenge	Pre Diet, Post Meal Challenge	Post Diet, Pre Meal Challenge	Post Diet, Post Meal Challenge
p:t AKT	0.08 ± 0.01	0.13 ± 0.03	0.12 ± 0.03	0.05 ± 0.01
p:t ERK1/2	0.96 ± 0.23	1.00 ± 0.22	1.20 ± 0.37	0.52 ± 0.17
p:t IκBα	0.04 ± 0.01	0.05 ±0.01	0.04 ± 0.00	0.03 ± 0.01
p:t JNK	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.004 ± 0.00
p:t p38MAPK	0.21 ± 0.04	0.45 ± 0.06	0.34 ± 0.07	0.41 ± 0.13
TLR2	0.5 ± 0.1	0.3 ± 0.5	0.39 ± 0.11	0.44 ± 0.03
TLR4	4.56 ± 1.09	7.04 ± 0.52	5.32 ± 1.64	5.10 ± 0.33
NFκB	0.08 ± 0.02	0.10 ± 0.02	0.06 ± 0.02	0.12 ± 0.03
MCP1	14.16 ± 2.87	11.16 ± 1.38	13.61 ± 3.02	11.06 ± 1.30

Table 6. The ratio of phosphorylated: total protein content of AKT, ERK1/2, IκBα, JNK, and p38MAPK was measured in samples obtained from cohort 2 volunteers in 4 conditions (1) the fasted state prior to the consumption of a 5-day high SFA diet (Pre Diet, Pre meal challenge), (2) 4hrs following the consumption of a high fat meal challenge and prior to the consumption of a 5-day high SFA diet (Pre Diet, Post Meal Challenge), (3) in the fasted state concluding the consumption of a 5 day high SFA diet (Post Diet, Pre meal Challenge), and (4) 4hours after the consumption of a high fat meal challenge concluding the consumption of a 5-day high SFA diet. Testing for statistical significance was performed using a repeated measures two-way ANOVA. No significant differences were observed.

Associations between inflammatory and oxidative markers in response to meal challenge are not observed with 5-days of high SFA feeding. The meal challenge-induced percent change in substrate metabolism, mRNA expression of oxidative and inflammatory genes, and the ratio of phosphorylated to total inflammatory protein content was measured in cohort two volunteers prior to and following the consumption a 5-day high SFA diet. In samples obtained prior to 5-days of high SFA feeding, we observed positive associations between phosphorylation of inflammatory proteins and substrate metabolism. The percent change in CO_2, ASM, and total fatty acid oxidation was associated with a ratio of phosphorylated:total protein content of IκBα and p38MAPK while total fatty acid oxidation was correlation to TLR2 mRNA expression. We also observed positive associations between glucose oxidation and phosphorylated:total protein content of IκBα, JNK and NFκB and associations between the percent change in mRNA expression of TLR2 and total fatty acid oxidation, NADH dehydrogenase, PPARΔ and PPARγ. Expression of PPARγ was associated with TLR4, and NFκB (Table 7). All correlations and P values are displayed in Table 8 (Appendix A).

Intriguingly, following 5 days of high SFA feeding, the relationships between meal challenge mRNA expression of oxidative and pro-inflammatory protein markers is ablated. These findings show that in response to a single high fat meal, when previously consuming habitual dietary fat content of <30%, there is an induction of mRNA levels of oxidative protein and that these induction occur in concert with activation of (phosphorylation) of pro-inflammatory protein. This not unlike what is observed in scenarios of pro-inflammatory response to muscle stress, such as muscle contraction

and/or exercise (Powers et al., 2009) which has been well characterized as a necessary response to invoke mitochondrial biogenesis. These data also show that as little as 5 days of high fat feeding abrogates these adaptive responses to a single high fat meal challenge, suggesting that chronic high fat feeding may be deleterious to the normal adaptive response to an influx of high levels of fatty acids following a high fat meal.

Table 7

	Pre 5-day HDF	**Post 5-day HFD**
NFkB mRNA and Glucose Oxidation	*R= 0.82, P= 0.04	R=-0.43, P= 0.39
p:t IκBα and Fatty Acid Oxidation	*R= 0.94, P< 0.01	R= 0.18, P= 0.74
p:t IκBα and CO_2 Production	*R= 0.89, P= 0.01	R=-0.01, P= 0.99
p:t IκBα and ASM Production	*R= 0.93, P< 0.01	R= 0.08, P= 0.89
p:t IκBα and Glucose Oxidation	*R= 0.82, P< 0.05	R= 0.29, P= 0.59
p:t ERK1/2 and CO_2 Production	*R= 0.99, P< 0.01	R=-0.35, P= 0.50
p:t p38 MAPK and CO_2 Production	*R= 0.89, P= 0.02	R=-0.78, P= 0.06
p:t p38MAPK and Fatty Acid Oxidation	*R= 0.86, P= 0.02	R= 0.18, P= 0.72
p:t JNK and Glucose Oxidation	*R= 0.86, P= 0.03	R=-0.26, P= 0.62
TLR2 and Fatty Acid Oxidation	*R= 0.83, P= 0.04	R= 0.15, P= 0.65
TLR2 and NADH Dehydrogenase	*R= 0.83, P= 0.04	R= 0.57, P= 0.23
TLR2 and PPARγ	*R= 0.92, P= 0.01	R= 0.60, P= 0.21
TLR2 and PPARΔ	*R= 0.86, P= 0.02	*R= 0.58, P< 0.05

A Pearson's Correlation Analysis was used to measure the association between the meal-challenge-induced percent change in skeletal muscle samples obtained before and after the consumption of a 5-day high SFA diet. Prior to five days of high SFA feeding, consumption of a high fat meal resulted in positive associations between numerous oxidative and pro-inflammatory markers which dissipated following five days of high SFA feeding. *P<0.05.

DISCUSSION:

Herein we present data suggesting that 1) five days of high SFA feeding reduces glucose oxidation and complete fatty acid oxidation in the fasted state, 2) adaptive response to high SFA feeding is associated with diet-induced stimulation of pro-inflammatory markers, 3) five days of high SFA feeding attenuates induction of oxidative markers that occurs in response to the consumption of a high fat meal and 4) induction of oxidative markers with a high fat meal challenge is associated with increased transcription and phosphorylation of pro-inflammatory proteins and that these associations are not evident following five days of high SFA diet intake.

Heightened SFA consumption is a well-established risk factor for the development of the metabolic syndrome and its associated co-morbidities (Hu et al., 1999; Salmeron et al., 2001; Romieu et al., 1988; Marshall et al., 1997). Following chronic consumption of a high SFA diet rodents develop skeletal muscle insulin resistance while non-obese volunteers display reduced fat oxidation in the fed state (Moon et al., 2010; Kein et al., 2005; Han et al., 2002; DeLany et al., 2000). Kein et al., (Kein et al., 2005) were one of the first groups to suggest that elevations in SFA intake prevent adaptive response to high fat feeding as seen following high MUFA intake. Concluding 30 days of high SFA feeding volunteers were less oxidative in the fed state compared to measures taken following the consumption of a 30 day high MUFA diet, and were in positive fat balance whereas volunteers were negative fat balance with high MUFA diet consumption. Our findings suggest that while skeletal muscle may be able to

adapt to acute high SFA feeding, such as the consumption of a meal rich in SFA, with five days of high SFA feeding, this adaptive response is no longer observed.

Unifying evidence within the last decade of research has implicated that SFA-induced metabolic perturbations are mediated through the immune system. Skeletal muscle cells exposed to elevated concentrations of palmitate, the most prevalent SFA in circulation, display concurrent elevations in transcriptional regulation of pro-inflammatory cytokines and depressed mRNA expression of markers of insulin signaling and oxidative phosphorylation (Coll et al., 2007; Coll et al., 2008). Significant attention has been placed on the prevention of SFA-induced metabolic derangements via inhibition of pro-inflammatory transcriptional regulators. Palmitate-induced insulin resistance is nearly ablated in myotubes that have been co-incubated in saturated fatty acids and anti-inflammatory compounds such as acetylsalicylate, which suppresses NFkB activation by inhibiting the activity of IKK, and antibodies that are inhibitory to TLR4 (Radin et al., 2008; Sinha et al., 2004; Tsukumo et al., 2007). Furthermore; in vivo studies have shown that genetic deletion of TLR4 and its associated co-receptors prevents the development of high fat diet induced insulin resistance and elevations in visceral and subcutaneous fat deposits while improving metabolic rates in rodents (Cani et al., 2007; Cani et al., 2008; Tsukumo et al., 2007, Davis et al., 2008; Radin et al., 2008).

While previous studies have established that pro-inflammatory pathways mediate the development of SFA-associated metabolic abnormalities we are one of the first groups to report that diet-induced stimulation of inflammatory markers is associated with

adaptive response to high SFA feeding prior to the onset of obesity. The individual percent change in pre- to post-diet fatty acid oxidation ranged from -78 to >200%. When volunteers were grouped by adaptive response, responders vs. non-responders, the percent change in the ratio of phosphorylated:total p38MAPK and ERK1/2 was increased by 100-200% in non-responders while responders did not display any significant changes in either of the inflammatory markers. While it is generally accepted that SFA inflate skeletal muscle transcription and content of pro-inflammatory proteins and transcription factors (Davis et al., 2008; Radin et al., 2008; Coll et al., 2008; Coll et al., 2007) there is a lack of understanding regarding the relationship between metabolic adaptability and diet induced stimulation of pro-inflammatory markers. Considering that skeletal muscle oxidative capacity is thought to be a predictor of weight gain over extended periods of time (Marr et al., 2004; Ellis et al., 2010), investigations into the mechanisms that govern adaptive response to high SFA feeding are critical to enhancing our understanding of the pathogenesis of the diet-induced obesity and genetic pre-disposition to the onset of this condition. Smith et al., (Smith et al., 2000) have previously reported that the failure to suppress RER in response to elevations dietary fat intake is linked to reductions in insulin resistance. We are the first group, to our knowledge; to assert that high SFA diet-induced phosphorylation of pro-inflammatory markers is heightened in non-obese volunteers with maladaptations in metabolic response to high fat feeding as determined by radio-labeled palmitate oxidation. This finding supports our hypothesis that metabolic adaptability is linked to diet induced stimulation of pro-inflammatory pathways.

Although inhibition of TLR4 and other pro-inflammatory proteins has consistently shown to be protective against the adverse influence of chronic SFA consumption on metabolism, we contend that in acute conditions of high SFA feeding SFA-associated stimulation of pro-inflammatory markers may, in fact, mediate skeletal muscle adaptive response. Following a single high fat meal challenge, prior to the commencement of the 5-day high SFA diet, we observed associations between the percent change in fatty acid oxidation, acid soluble metabolite production, and the production of ^{14}C-labeled CO_2 with the percent change in phosphorylated: total ERK1/2, p38MAPK, and IκBα as well as correlations between oxidative and inflammatory transcriptional regulators. Pro-inflammatory cytokines have previously been implicated in the regulation of substrate metabolism occurring with endurance exercise (Heldge et al., 2003; Wolsk et al., 2010). We are the first, to our knowledge, to preset data suggesting that (1) in response to a single high fat meal, the change in mRNA levels of oxidative targets is associated with activation of pro-inflammatory proteins, and (2) these associations are dissipated with 5 days of high SFA feeding. Therefore we also propose that five days of high SFA feeding dysregulates the immune system-mediated stimulation of adaptive response to high SFA feeding.

We also observed that chronic high SFA feeding augments high fat meal associated endotoxemia. We have previously reported that simulation of endotoxemia in rodents via intraperitoneal LPS injections (50pg/kg body weight) results in shifts in substrate metabolism that promote glucose over fatty acid oxidation thus enhancing neutral lipid production (Frisard et al., 2010). Considering that acute high fat feeding is

known to promote metabolic endotoxemia in non-obese volunteers (Ghamin et al., 2010, Erridge et al., 2010; Laugerette et al., 2009) our goal was to expound upon these and our findings to determine (1) if circulating endotoxin was influenced by a high fat meal challenge prior to and following five days of high fat feeding and to determine (2) if high SFA diet metabolic perturbations were more apparent following high fat meal induced endotoxemia. In the fasted state, plasma endotoxin concentrations were 2-fold higher, respectively, in post diet compared to pre-diet samples however; this increase was not statistically significant. One hour following the consumption of the high SFA meal challenge we observed a 2.5-fold increase in endotoxin concentrations in post-diet vs pre-diet samples. We also report that there were varying patterns of endotoxin presence in the blood with high fat feeding. Previous studies have shown that postprandial endotoxin concentrations are higher following the consumption of a high fat high carbohydrate meal compared to a low fat, high carbohydrate meal (Ghamin et al., 2009). In line with these findings we report that chronic high SFA feeding augments meal challenge-induced elevations in postprandial endotoxin concentrations. However; we observed a 2.5-fold increase in endotoxin concentrations at 1hr following consumption of the high SFA meal while at 4hr following consumption of the high SFA meal, when skeletal muscle samples were obtained, there was no significant difference in pre- and post-diet endotoxin concentrations. Therefore; we are unable to establish if the attenuation in meal challenge-induction of oxidative markers observed following five days of high SFA feeding occurred in response to high SFA diet induced endotoxemia.

In conclusion, this report illustrates two important findings: 1) a maladaptive oxidative response to 5 days of high fat feeding is associated with heightened activation of pro-inflammatory proteins; and 2) a single high fat meal challenge is met with increases in both pro-inflammatory response and induction of mRNA levels of oxidative proteins; a response that is abrogated following 5 days of high fat feeding. These results suggest that activation of pro-inflammatory proteins in response to a single high fat meal may be a normal adaptive response when habitual fat intake is less than 30%; however this response is dysregulated following only 5 days of high fat feeding

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Chapter Seven: Implications and future directions

The purpose of this study was to examine the relationship between metabolic adaptive response to 5 days of high SFA feeding, independent of positive energy balance, and diet-induced agonism of pro-inflammatory pathways. A secondary aim was to determine if the metabolic adaptive response in skeletal muscle to a single, calorie dense, high fat meal was altered by 5 days of high saturated fat feeding.

To determine the caloric content of the high SFA, which was designed to be isocaloric to habitual caloric intake, diet volunteers were given self-reported food records. While a registered dietician was employed to instruct volunteers on proper completion of the records, this method of dietary assessment can vary significantly in its accuracy and thus all reports were based off of the assumption that the habitual and high fat diets were isocaloric. To correct this discrepancy, future studies will replace the habitual diet period with a standardized, 30% fat diet administered to volunteers in the same fashion as the high fat diet.

The grouping of volunteers as responders and non-responders was solely based on the percent change in skeletal muscle substrate metabolism. To more accurately determine if a predisposition towards the development of metabolic syndrome is linked with diet-induced stimulation of inflammatory markers we will categorize volunteers

according to the presence of a family history of type 2 diabetes rather that by their metabolic response to high fat feeding.

In the current study, we were only able to the relationship between pro-inflammatory response and changes in fatty acid oxidation. To establish a direct link between pro-inflammatory response and the observed changes in fatty acid oxidation, future studies could be designed to include salicylates (inhibitors of NFκβ activity) with high saturated fat feeding. If the pro-inflammatory response (i.e., NFκβ activity) resulting from increased saturated fat intake directly causes reductions in fatty acid oxidation, then we would expect the reduction in fatty acid oxidation in skeletal muscle to be entirely or partially abrogated when salicylate is combined with an acute (5 days) high saturated fat diet.

Chapter Eight: Appendices

Appendix A

	CO_2	ASM	Total FAO	Glucose Ox	Citrate Synthase	β-HAD	PGC1α	PGC1β	NADH	PPARΔ	PPARγ	IκBα	ERK1/2	JNK	p38 MAPK	AKT	MCP1	NFκB	TLR4	TLR2
CO_2		0.322	0.171	0.482	-0.233	-0.226	0.367	0.597	0.292	0.299	0.121	-0.009	-0.351	0.028	-0.784	0.143	-0.079	-0.272	0.399	0.095
ASM	0.322		*0.978	-0.138	0.653	0.114	0.498	0.344	0.617	0.551	0.218	0.076	-0.158	0.243	0.092	0.673	-0.267	0.283	0.359	0.139
Total FAO	0.171	*0.978		-0.214	0.657	0.028	0.489	0.282	0.610	0.572	0.223	0.179	-0.025	0.378	0.184	0.637	-0.253	0.329	0.372	0.239
Glucose Ox	0.482	-0.138	-0.214		-0.299	-0.508	0.546	0.763	0.475	0.254	0.628	0.285	-0.454	-0.261	*-0.853	-0.113	-0.392	-0.431	0.558	0.174
Citrate Synthase	-0.233	0.653	0.657	-0.299		0.504	-0.072	-0.226	0.137	-0.125	-0.141	-0.449	0.103	-0.201	0.506	0.188	-0.615	-0.033	-0.138	-0.479
β-HAD	-0.226	0.114	0.028	-0.508	0.504		-0.450	-0.487	-0.319	-0.417	-0.389	-0.725	-0.268	-0.664	0.634	0.232	0.096	0.241	-0.403	-0.750
PGC1α	0.367	0.498	0.489	0.546	-0.072	-0.450		*0.935	*0.977	*0.936	*0.923	0.751	-0.585	0.197	-0.441	0.732	0.004	0.381	*0.980	0.690
PGC1β	0.597	0.344	0.282	0.763	-0.226	-0.487	*0.935		*0.876	0.799	*0.865	0.617	-0.653	0.046	-0.708	0.545	-0.065	0.130	*0.967	0.562
NADH	0.292	0.617	0.610	0.475	0.137	-0.319	*0.977	*0.876		*0.902	*0.899	0.651	-0.581	0.129	-0.312	0.790	-0.108	0.394	*0.946	0.578
PPARΔ	0.299	0.551	0.572	0.254	-0.125	-0.417	*0.936	0.799	*0.902		0.801	*0.821	-0.467	0.415	-0.259	0.790	0.260	0.602	*0.919	*0.816
PPARγ	0.121	0.218	0.223	0.628	-0.141	-0.389	*0.923	*0.865	*0.899	0.801		0.742	-0.660	-0.025	-0.365	0.781	0.020	0.378	*0.971	0.603
IκBα	-0.009	0.076	0.179	0.285	-0.449	-0.725	0.751	0.617	0.651	*0.821	0.742		-0.154	0.585	-0.268	0.419	0.341	0.469	*0.852	*0.971
ERK 1/2	-0.351	-0.158	-0.025	-0.454	0.103	-0.268	-0.585	-0.653	-0.581	-0.467	-0.660	-0.154		0.578	0.261	-0.738	-0.225	-0.402	-0.718	-0.043
JNK	0.028	0.243	0.378	-0.261	-0.201	-0.664	0.197	0.046	0.129	0.415	-0.025	0.585	0.578		-0.044	0.002	0.186	0.157	0.155	0.731
p38 MAPK	-0.784	0.092	0.184	*-0.853	0.506	0.634	-0.441	-0.708	-0.312	-0.259	-0.365	-0.268	0.261	-0.044		0.175	0.209	0.488	-0.422	-0.281
AKT	0.143	0.673	0.637	-0.113	0.188	0.232	0.732	0.545	0.790	0.790	0.781	0.419	-0.738	0.002	0.175		0.393	0.835	0.803	0.381
MCP1	-0.079	-0.267	-0.253	-0.392	-0.615	0.096	0.004	-0.065	-0.108	0.260	0.020	0.341	-0.225	0.186	0.209	0.393		0.716	0.135	0.400
NFκB	-0.272	0.283	0.329	-0.431	-0.033	0.241	0.381	0.130	0.394	0.602	0.378	0.469	-0.402	0.157	0.488	0.835	0.716		0.543	0.457
TLR4	0.399	0.359	0.372	0.558	-0.138	-0.403	*0.980	*0.967	*0.946	*0.919	*0.971	*0.852	-0.718	0.155	-0.422	0.803	0.135	0.543		0.740
TLR2	0.095	0.139	0.239	0.174	-0.479	-0.750	0.690	0.562	0.578	*0.816	0.603	*0.971	-0.043	0.731	-0.281	0.381	0.400	0.457	0.740	
TLR2 mRNA	0.390	0.309	0.039	0.150	0.538	0.656	0.101	0.102	0.039	0.027	0.008	0.105	0.451	0.266	0.372	0.231	0.566	0.041	0.000	

Table 8. Samples obtained following the 5-day high SFA diet were used to determine the association between the meal challenge induced percent change in oxidative and pro-inflammatory markers. The strength in the association of the measures was determined by the Pearson's correlation analysis. Values are expressed as R^2, *$P<0.05$.

	CO_2	ASM	Total FAO	Glucose Ox	Citrate Synthase	β-HAD	PGC1α	PGC1β	NADH	PPARΔ	PPARγ	IκBα	ERK1/2	JNK	p38 MAPK	AKT	MCP1	NFκB	TLR4	TLR2
CO_2		*0.990	0.749	0.795	0.051	0.580	-0.206	-0.175	-0.116	-0.043	0.124	*0.891	*0.998	0.772	*0.889	0.442	0.332	0.377	0.431	0.434
ASM	*0.990		*0.827	0.809	0.366	0.019	-0.139	-0.111	-0.038	0.034	0.196	*0.928	*0.987	0.736	*0.937	0.415	0.442	0.445	0.481	0.503
Total FAO	0.749	*0.827		0.770	0.400	0.093	0.322	0.328	0.453	0.502	0.615	0*.937	0.727	0.588	*0.862	0.434	0.659	0.738	0.793	*0.834
Glucose Ox	0.795	0.809	0.770		0.845	0.387	0.332	0.373	0.325	0.395	0.539	*0.815	0.768	*0.855	0.683	0.826	0.171	*0.817	0.799	0.664
Citrate Synthase	0.051	0.366	0.400	0.845		-0.103	0.664	0.694	0.506	0.534	0.553	0.168	-0.525	0.181	0.193	0.593	0.044	0.779	0.688	0.372
β-HAD	0.580	0.019	0.093	0.387	-0.103		0.268	0.253	0.249	0.222	0.232	0.481	0.463	*0.910	-0.248	*0.986	-0.190	0.359	0.328	0.274
PGC1α	-0.206	-0.139	0.322	0.332	0.664	0.268		*0.998	*0.969	*0.966	*0.932	0.111	-0.261	0.147	-0.175	0.569	-0.075	0.763	0.800	0.727
PGC1β	-0.175	-0.111	0.328	0.373	0.694	0.253	*0.998		*0.958	*0.960	*0.932	0.123	-0.230	0.176	-0.160	0.607	-0.100	0.781	0.825	0.726
NADH	-0.116	-0.038	0.453	0.325	0.506	0.249	*0.969	*0.958		*0.996	*0.968	0.241	-0.171	0.166	-0.056	0.517	0.063	0.749	0.823	*0.834
PPARΔ	-0.043	0.034	0.502	0.395	0.534	0.222	*0.966	*0.960	*0.996		*0.985	0.294	-0.100	0.218	0.002	0.567	0.065	0.784	0.864	*0.864
PPARγ	0.124	0.196	0.615	0.539	0.553	0.232	*0.932	*0.932	*0.968	*0.985		0.434	0.067	0.355	0.143	0.667	0.101	*0.852	*0.935	*0.924
IκBα	*0.891	*0.928	*0.937	*0.815	0.168	0.481	0.111	0.123	0.241	0.294	0.434		*0.876	0.783	*0.893	0.522	0.539	0.628	0.688	0.722
ERK 1/2	*0.998	*0.987	0.727	0.768	-0.525	0.463	-0.261	-0.230	-0.171	-0.100	0.067	*0.876		0.749	*0.897	0.395	0.351	0.335	0.375	0.385
JNK	0.772	0.736	0.588	*0.855	0.181	*0.910	0.147	0.176	0.166	0.218	0.355	0.783	0.749		0.531	0.876	-0.028	0.597	0.648	0.543
p38 MAPK	*0.889	*0.937	*0.862	0.683	0.193	-0.248	-0.175	-0.160	-0.056	0.002	0.143	*0.893	*0.897	0.531		0.164	0.719	0.418	0.357	0.448
AKT	0.442	0.415	0.434	0.826	0.593	*0.986	0.569	0.607	0.517	0.567	0.667	0.522	0.395	0.876	0.164		-0.306	0.766	0.949	0.654
MCP1	0.332	0.442	0.659	0.171	0.044	-0.190	-0.075	-0.100	0.063	0.065	0.101	0.539	0.351	-0.028	0.719	-0.306		0.261	0.040	0.298
NFκB	0.377	0.445	0.738	*0.817	0.779	0.359	0.763	0.781	0.749	0.784	*0.852	0.628	0.335	0.597	0.418	0.766	0.261		*0.915	*0.830
TLR4	0.431	0.481	0.793	0.799	0.688	0.328	0.800	0.825	0.823	0.864	*0.935	0.688	0.375	0.648	0.357	0.949	0.040	*0.915		*0.995
TLR2	0.434	0.503	*0.834	0.664	0.372	0.274	0.727	0.726	*0.834	*0.864	*0.924	0.722	0.385	0.543	0.448	0.654	0.298	*0.830	*0.995	

Table 7. Samples obtained prior to the 5-day high SFA diet were used to determine the association between the meal challenge induced percent change in oxidative and pro-inflammatory markers. The strength in the association of the measures was determined by the Pearson's correlation analysis. Values are expressed as R^2, *$P<0.05$.

Appendix B

Informed Consent for Participants of Investigative Projects

Department of Human Nutrition, Foods and Exercise

Virginia Tech

TITLE: **Effect of a High Fat Diet on Muscle Metabolism**

INVESTIGATORS: **Kevin P. Davy, Ph.D.**
Mathew W. Hulver, Ph.D.
Madlyn I. Frisard, Ph.D.
Brenda M. Davy, Ph.D., R.D.

MEDICAL DIRECTOR: **Jose Rivero, M.D.**

PURPOSE:
The amount of fat and glucose in the diet may influence the risk for diabetes and cardiovascular disease by altering metabolism (how the body gets energy from food in our diet). However, it is not clear how muscle tissue responds to different amounts of fat and glucose in the diet. Therefore, the purpose of this study is to determine how a high fat diet influences metabolism in muscle. Forty people will be included in this study.

METHODS:
You are being asked to participate in a study in which you will eat a high fat meal (for example, two sausage and egg biscuits) on three (3) occasions; before and after 5 days of eating your typical diet and again after 5 days of eating a high-fat diet. Blood samples will be taken before and for several hours following each meal. Muscle biopsies will also be taken before and after each meal for a total of six muscle biopsies.

You will not be able to participate if you have recently lost weight, exercise three or more times a week at a moderate to hard level (e.g., exercise that causes you to breathe hard and sweat), or if you use any medication or nutritional supplements that might influence the study variables. You will not be eligible to participate if you are allergic to lidocaine or bipivicaine.

The high fat diet will contain the same number of calories you normally eat so you should not gain or lose weight. You will be provided food (for example, McDonald's meals and other high fat foods) during the high fat portion of the study so that 50-60% of all the calories you eat come from fat. You will need to come to the laboratory each of the 5 days you eat a high fat diet to have your bodyweight measured as well as to pick up your food and return any uneaten foods from the previous day.

If you agree to participate in this study you will first be required to complete a personal health history questionnaire and the additional tests described below under Session 1. The results of your questionnaire and study tests may be discussed with the study medical director to determine your eligibility. Based on our evaluation, you may then be eligible to participate in the study. Eligible candidates will be non-smoking males or females between the ages of 18 and 40 years who do not have diabetes or high cholesterol as assessed by a medical history and blood tests. Your body mass index must be less than 35 kg/m^2 and your blood pressure also must be less than 140/90 mmHg.

There will be approximately 30 visits if you choose to participate in the study. The actual number and order of visits may depend on your schedule and the availability of the study staff. In addition, the order may differ from the order of appearance in this document. You will undergo Session 1 one time

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and session 2 three times (before and after 5 days of eating your typical diet and again following the high fat diet).

If you are female, we will ask you to schedule session 2 below close to the time of your monthly period because many of the variables we are measuring change during the menstrual cycle. You will be asked to notify us on the first day of your period. We will try to schedule your visits within 3-5 days of this time.

Session 1

- **Overnight Fast:** You will be asked to avoid eating for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process.

- **Pregnancy Test:** If you are female you will be required to have a pregnancy test. This will require you to collect 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study.

- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. Your height and weight will also be measured at this time. Your body weight will be measured on a standard digital scale. Your height will be measured with a standard stadiometer (ruler on the wall). Your waist, hip, and neck circumference will be measured using a measuring tape.

- **Blood Pressure:** You will be asked to sit quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.

- **Body Composition:** This test is to measure your body fat. You will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 5 minutes and there is no pain associated with the procedure. This procedure will be performed once at the beginning of the study and a second time at the end of the study. Your weight and height will also be measured at this time.

- **Physical Activity Questionnaire:** You will be asked a series of questions to estimate your usual physical activity level, which will require about 15 minutes to complete.

- **Catheter and Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure glucose, cholesterol, blood cells, liver and kidney enzymes and other factors to determine your eligibility.

Approximate time required: 1 hour

Session 2 (Your are being asked to complete this session three times; before and after 5 days of easting your typical diet and again after 5 days of eating a high fat diet)

- **Overnight Fast:** You will be asked to avoid eating or drinking for 12 hours and consuming caffeine-containing foods or beverages for 24 hours prior to this visit. This is to make sure that the test results will not be influenced by the food you eat or by the normal digestion process.

- **Pregnancy Test:** If you are female you will be required to have a pregnancy test. This will require collection of 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study.

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- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.

- **Muscle Biopsy:** You should not take aspirin, ibuprofen or other non-steroidal, anti-inflammatory medications (such as Advil, Motrin, Celebrex or Vioxx), or other medications or substances that may affect bleeding or bruising, for 72 hours prior and after this procedure. This procedure is used to sample a small amount of muscle (about 50-250 mg) from underneath the skin from the thigh. The actual biopsy site will be on the top of either the right or left leg half way between the knee and the hip.

  You will be asked to undergo this procedure 6 times, before and after each of two test meals and following the high fat diet and your typical diet. Neither a physician nor nurse may be present during the procedure. This procedure will be performed by a study investigator (Kevin P. Davy, Ph.D.) or co-investigator (Mathew Hulver, Ph.D.) who has been specifically trained to perform the biopsy. You will be lying down and your skin will be cleansed with iodine-type solution (Providine or Betadine). If you are allergic to iodine, we will use chlorhexadine which does not contain iodine. A sterile drape will be placed over the area and your skin and muscle tissue will be numbed by injecting numbing medication (lidocaine/bipivicaine) into the area with a small needle. If you allergic to lidocaine or bipivicaine, you cannot participate in this study. Then, a small incision (about 1/4 of an inch) will be made in the skin and a needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle. Some suction may be applied to the other end of the needle to help remove the muscle.

  After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleaned with saline and will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE bandage. You will be asked to keep the ACE bandage on for at least 10-15 minutes. You may take Tylenol for any discomfort you may experience following the biopsy. We will use the biopsy samples to measure factors which contribute to inflammation. The biopsy will take place at Dr. Jose Rivero's medical office in Christiansburg or the Human Integrative Physiology Laboratory (228 War Memorial Hall). Directions will be provided to you.

  You will be provided with instructions on how to care for the biopsy sites as well as what to look for if a problem were to occur.

- **Catheter and Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 3 tablespoons). We will measure various hormones that influence your metabolism (how your body burns calories and produces body heat) and cardiovascular system (the heart, blood vessel and lungs). Blood will be collected before the meal challenge and at 1, 2, 3, and 4 hours after the meal. The catheters will remain in your arms throughout the entire test.

- **Meal Challenge: On three occasions,** you will be asked to eat a test meal consisting of two breakfast sandwiches (e.g., egg and sausage) and a sugary drink. Blood will be collected before and 1, 2, 3, and 4 hours after the meal and a muscle biopsy will be performed before and approximately 4 hours after the meal.

Approximate time required: 5 hours.

Take-Home Tests

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- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day). You will also be asked to keep track of the food you eat for the 5 days you are in the habitual-diet phase of the study.

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all of the scheduled experiment.
- Follow all participant instructions for each session.
- Record any food you eat that has not been provided by the investigators.
- Return any uneaten food that has been provided by the investigators.
- Follow physical activity instructions provided by the investigators.
- Carefully read the instructions on consuming any food provided to you.
- Inform the study investigators if you are pregnant or intend to become pregnant during the study.

RISKS OF PARTICIPATION

- Catheter and Blood Draw: Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10 or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during or after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in catheter placement and blood draws will minimize these risks.
- HIV/AIDS: Your blood will be tested for the presence of HIV if one of the study investigators is exposed to your blood. There will not be any cost to you for this test. The results will be sent to your primary care physician or the study medical director, Dr. Jose Rivero, if you do not have a primary care physician. He/she will discuss them with you and provide you with the necessary referral for further evaluation and/or counseling if your results are positive. The results of your test will remain confidential.
- Muscle Biopsies: If you are allergic to lidocaine, you will not be allowed to participate in this study. There may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but you are not expected to experience discomfort during the biopsy procedure. Bruising in the area of the muscle biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately after the procedure to limit this potential effect and its accompanying tenderness. There is a slight risk of infection at the biopsy site. There is a small risk that you will become lightheaded, dizzy, or anxious before or during the procedures. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. These risks are minimized by having a trained individual perform the

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procedure. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

You will likely receive a scar from each of the biopsies performed but these are expected to be very small. These scars usually turn a purple color in the weeks to months following the biopsy and then fade considerably over time. The study staff will show you several pictures of examples of the scarring (greater than 1 year old) that can occur following similar biopsy procedures. It is important that you understand that these are just examples of the scarring that can occur. The actual scar you receive may be smaller or larger or differ in coloring. Individuals with darker skin (e.g., African Americans, Hispanics and Asians) tend to scar more than those with lighter skin. You should consider this before you agree to participate.

- DEXA Scan: The amount of radiation that you will receive in the DEXA exam (combined with the CT scan) is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks, however the exact increase in such risk is not known.

- Pregnancy: You should not become pregnant during this study because of the exposure to x-rays. If you are capable of having a child you must have a negative pregnancy test before each session that may pose a risk to an embryo or fetus (x-ray exposure or medication injection). You must agree to use an effective method of birth control, such as abstinence, condom use, oral contraceptives or use of an IUD to ensure that you will not get pregnant during the study. If you become pregnant during this study, you must notify your study investigator immediately. There may be unforeseen risks to the embryo or fetus in the event that you become pregnant during the study.

- Weight gain: It is unlikely that you will gain weight as a result of participating in this short term study. The investigators have designed the diet you will consume so that this does not happen. You should know that there is a small risk that small weight gain (1-2 pounds) will occur. You should also know that your weight can fluctuate 1-2 pounds over the course of 1-2 days even without changing your diet. However, if you do gain weight we will provide you with instructions on how to modify your diet and increase your physical activity to return to your original body weight.

- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.

- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION
Your participation will provide you with:

- Information on your body composition.
- Information on your blood pressure, cholesterol and glucose tolerance

COMPENSATION
You will be compensated $25 for completing each muscle biopsy. Muscle biopsies will be performed before and after each test meal and the test meal will be performed before and after your typical diet

and again after the high fat diet ($150 total). You can receive up to $150 for your participation in the entire study.

CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by subject numbers, without anything to identify you by name. In the event that your exercise test indicates you may have a heart problem, Dr. Rivero or investigators may want to share this information with your doctor but he will request your approval first.

FREEDOM TO WITHDRAW

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain muscle, body fat or other measurements that are necessary for the study. You may be able to participate in the study even if you to choose not to participate in the muscle and fat biopsies. All of the other sessions are required components.

INJURY DURING PARTICIPATION IN THIS STUDY

Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if injured as a result of your participation in this study. Any expenses that you incur including emergencies and long term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH

This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

SUBJECT PERMISSION

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:

- Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; After hours: 540-230-0486

- Chairman, Institutional Review Board for Research Involving Human Subjects: David Moore, Associate Vice President for Research (540) 231-4991

Name of Subject (please print)________________________________

Signature of Subject____ Date_________

Appendix C

INFECTION/INFLAMMATION QUESTIONNAIRE

Evaluator Script: I would like you to think if you had a cold, the flu, a dental infection or other infection during the past month. I am going to ask you about some symptoms that may have accompanied those types of conditions.

1) Did you have a cold, the flu, a dental infection or other infection in the past month?
   ( ) Yes ( ) No ( ) Refused ( ) Don't Know
   If yes, ( ) Within 1 week ( ) 2 weeks prior ( ) 3 weeks prior ( ) 4 weeks prior

In the prior month did you experience any of the following symptoms? [Note to examiner: If symptom was present, the timing of symptom onset and resolution (# days) prior to interview is recorded. If symptom is still present on the day of interview, place 0 in "Resolved___days ago".]

2) Did you feel feverish or have a fever? ( ) Yes ( ) No
   If Yes, Symptom Started ___days ago. Resolved_____days ago.
   Did you take your temperature? ( ) Yes ( ) No

3) Chills? ( ) Yes ( ) No
   If Yes, Started____days ago. Resolved____days ago.

4) Sore throat ? ( ) Yes ( ) No
   If Yes,.Started____days ago. Resolved____days ago.

5) Coughing? ( ) Yes ( ) No
   If Yes, Started____days ago. Resolved____days ago.

6) Sputum? ( ) Yes ( ) No
   If Yes,.Started____days ago. Resolved____days ago.

7) Sneezing? ( ) Yes ( ) No
   If Yes, Started____days ago. Resolved____days ago.

8) Runny nose or nasal congestion? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

If Yes to (5), (6), (7), or (8). Do you have seasonal allergies? ( ) Yes ( ) No
Do you have a chronic lung or sinus condition? ( ) Yes ( ) No
If Yes, are these symptoms typical for your chronic lung or sinus condition?
( ) Yes ( ) No

9) Ear pain or discharge? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

10) Run down feeling or achy muscles you feel may have been due to a cold or flu?
( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

11) Tooth/Gum pain? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.
If Yes, did you seek dental care?( ) Yes ( ) No
If Yes, did a Dentist find a cavity or other dental infection? ( ) Yes ( ) No

12) Mouth/gum (Y N), Skin (Y N), or Joint (Y N) redness or swelling?
If Yes, Started____days ago. Resolved____days ago.

13) Skin infection? ( ) Yes ( ) No
If Yes, Started____days ago. Resolved____days ago.

14) Nausea/Vomiting? ( )Yes( )No
If Yes, Started____days ago. Resolved____days ago.

15) Diarrhea? ( )Yes ( )No
If Yes, Started____days ago. Resolved____days ago.

16) Pain upon urination or urgency? ( )Yes ( )No
If Yes, Started____days ago. Resolved____days ago.

17) Cloudy discolored urine? ( )Yes ( )No

Urinalysis showing evidence of infection? ( )Yes ( )No
If Yes, Started____days ago. Resolved____days ago.

18) Did you seek medical care for any sort of cold, flu, or infection in the prior month?
( )Yes ( )No
If yes, diagnosis given____

19) Did you take any over the counter or prescription medications for a cold, flu, or any infection in the prior month?
( )Yes ( )No
If yes, names of medication____
____
____

Appendix D

Godin Leisure-Time Exercise Questionnaire

1. During a typical 7-Day period (a week), how many times on the average do you do the following kinds of exercise for more than 15 minutes during your free time (write on each line the appropriate number).

Times Per Week

a) STRENUOUS EXERCISE
(HEART BEATS RAPIDLY) ____________

(e.g., running, jogging, hockey, football, soccer,
squash, basketball, cross country skiing, judo,
roller skating, vigorous swimming,
vigorous long distance bicycling)

b) MODERATE EXERCISE ____________
(NOT EXHAUSTING)

(e.g., fast walking, baseball, tennis, easy bicycling,
volleyball, badminton, easy swimming, alpine skiing,
popular and folk dancing)

c) MILD EXERCISE ____________
(MINIMAL EFFORT)

(e.g., yoga, archery, fishing from river bank, bowling,
horseshoes, golf, snow-mobiling, easy walking

2. During a typical 7-Day period (a week), in your leisure time, how often do you engage in any regular activity long enough to work up a sweat (heart beats rapidly)?

1. Often _______ 2. Sometimes _______ 3. Rarely/Never _______

Appendix E

Activity Log

Participants in all groups are asked to keep a log of all exercise activity in order to monitor exercise behaviour for the duration of the study. In general, aerobic activities can be placed into three categories of exercise; below are the categories labelled: Group I activities, Group II activities, and Group III activities. Following is a list that provides examples of some of the activities that fit into each category. These are ONLY examples given to provide you a reference of how activities are categorized, to assist you in being as specific as possible when listing your activities.

Group I	Group II Activities	Group III Activities
Jogging (indoor/outdoor)	Cycling (outdoors)	Basketball
Running (indoor/outdoor)	Hiking	Country and western dancing
Walking (indoor/outdoor)	Aerobic dancing	Volleyball
Elliptical training (indoor)	In-line skating	Touch football
Cycling (indoors)	Swimming	Racquet sports
Stair Climbing	Rope skipping	Ultimate frisbee

Please note that if you are in either the Resistance or the Aerobic training groups, ONLY log the exercise activity that is OVER and ABOVE what you are currently prescribed within the study. For example, you may play touch football twice a week over and above the prescribed program that you received from Dr. Kell. If this were the case, only the participation in touch football would need to be indicated in the exercise activity log. This log is to be completed ONCE per MONTH.

Please answer the following questions:

1. During the last 4 weeks, how many minutes per week did you participate in exercise or physical activity that was not part of your prescribed exercise requirements for the Chronic Low Back Pain study? (Please check one)

< 30 minutes ________

21-40 minutes ________

41-60 minutes ________

61-90 minutes ________

91-120 minutes ________

> 121 minutes ________

2. During the last 4 weeks, what types of activities did you participate in (e.g., cycling outdoor) that were not part of your prescribed exercise requirements for the Chronic Low Back Pain study? (Please list them in the space provided and be as specific as possible)

____

____

____

REMINDER: **Please remember that if in the RESISTANCE group you are not supposed to be participating in any aerobic activity other than the prescribed warm-up and cool-down portion of the resistance program. **