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Defining the Role of Reactive Oxygen Species, Nitric Oxide, and Sphingolipid Signaling in Tumor Necrosis Factor - Induced Skeletal Muscle Weakness

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DEFINING THE ROLE OF REACTIVE OXYGEN SPECIES, NITRIC OXIDE, AND SPHINGOLIPID SIGNALING IN TUMOR NECROSIS FACTOR - INDUCED SKELETAL MUSCLE WEAKNESS

A dissertation has been submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

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2013

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ABSTRACT OF DISSERTATION

DEFINING THE ROLE OF REACTIVE OXYGEN SPECIES, NITRIC OXIDE, AND SPHINGOLIPID SIGNALING IN TUMOR NECROSIS FACTOR - INDUCED SKELETAL MUSCLE WEAKNESS

In many chronic inflammatory diseases, patients suffer from skeletal muscle weakness, exacerbating their symptoms. Serum levels of tumor necrosis factor-alpha (TNF) and sphingomyelinase are increased, suggesting their possible role in the progression of this weakness. This dissertation focuses on the role that reactive oxygen species (ROS) and nitric oxide (NO) play in mediating TNF-induced skeletal muscle weakness and to what extent sphingolipid signaling mediates cellular response to TNF.

The first aim of this work was to identify which endogenous oxidant species stimulated by TNF contributes to skeletal muscle weakness. In C57BL/6 mice (n=38), intraperitoneal injection of TNF elicited a 25% depression of diaphragm contractile function. In separate experiments, diaphragm fiber bundles harvested from mice (n=39) and treated with TNF ex vivo showed a 38% depression of contractile function compared to untreated controls. Using ROS and NO-sensitive fluorescence microscopy in parallel with a genetic knockout animal model, TNF-induced contractile dysfunction was found to be mediated by NO generated by a specific isoform of nitric oxide synthase (NOS), nNOS. Basal levels of ROS were necessary co-mediators, but were not sufficient to elicit TNF-induced diaphragm weakness.

The second aim of this dissertation was to investigate the extent to which sphingolipids could serve as a signaling cascade post-TNF stimulus leading to the generation of NO in skeletal muscle. The effects of TNF exposure in C2C12 skeletal muscle cells were studied in vitro using mass spectroscopy to measure sphingolipid metabolism and fluorescent microscopy to quantify oxidant production. TNF exposure was associated with significant mean increases in sphingosine (+52%), general oxidant activity (+33%), and NO production (+14%). These increases were due to specific modulation of nNOS as demonstrated by siRNA knockdown of neutral ceramidase and nNOS, and confirmed by pharmacologic inhibition using N-Oleylethanolamine and di-methylsphingosine.
In summary, these findings confirm NO as a major causative oxidant contributing to TNF’s deleterious phenotype in skeletal muscle. Moreover, the work suggests a new role for sphingosine in skeletal muscle and warrants further study of the enzymatic regulation of sphingosine to advance the discovery of new therapies for patients suffering from chronic inflammation.

KEYWORDS: Tumor Necrosis Factor-Alpha, Reactive Oxygen Species, Nitric Oxide, Sphingosine, Skeletal Muscle

Shawn A. Stasko

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CHAPTER ONE
INTRODUCTION

1.1. General Introduction

1.1.1. Skeletal Muscle Weakness. During conditions of chronic inflammation, patients show symptoms of muscular weakness independent of muscle atrophy (19, 125). This skeletal muscle weakness is associated with loss of function and is broadly referred to as contractile dysfunction. Skeletal muscle weakness is generally defined as ‘a loss of specific force’ which is the force generated by a muscle when normalized to cross-sectional area of the muscle fiber(s). This allows different muscles to be kinematically compared, regardless of size, angle of pennation, and anatomical orientation. The loss of muscle strength seen during states of chronic inflammation exacerbates an array of debilitating symptoms, such as breathlessness (79, 99) and exercise limitation (45, 65). Most importantly however, this weakness has been directly related to patient survival (65). How could inflammation in remote tissues weaken skeletal muscle? The mechanism appears to be multifaceted with systemic inflammation being a major contributor (68). Pro-inflammatory mediators like sphingomyelinase (SMase) and tumor necrosis factor – alpha (TNF) are both elevated in the serum of patients with chronic inflammation and correlate with muscle weakness (12, 23).

1.1.2. Tumor Necrosis Factor- Alpha. TNF is a pro-inflammatory cytokine produced by many different cell types, including skeletal muscle cells. In the clinic, patients with chronic inflammation have serum concentrations of TNF that are inversely related to their muscle strength (12, 132). TNF-induced weakness has long been
attributed to loss of muscle mass or cachexia and previously TNF was referred to as ‘cachectin’ in recognition of its catabolic action (71). In the laboratory environment, exposure to high concentrations of TNF depresses the force of excised muscle \textit{in vitro} (101, 135) as well as \textit{in vivo} (43). Similarly, cardiac overexpression of TNF in transgenic mice resulted in a 40% decrement in skeletal muscle functional capacity, despite no difference in muscle ultrastructure, phenotype, or bundle cross sectional area (69). Furthermore, TNF is known to directly stimulate an increase in ROS (69). Administration of an endogenous antioxidant mimic, Tempol, caused ROS levels to be similar to controls after TNF exposure, suggesting TNF-induction of oxidants (75). More recently, TNF was found to mediate its depression of specific force specifically through the TNFR1 complex (43). This effect of TNF appears to be regulated by cellular oxidants as antioxidant pretreatment abolished TNF-induced muscle weakness (43). Taken together, TNF stimulates an increase in oxidants and depresses the contractile function of skeletal muscle, however, the composition and source of the oxidants responsible for the weakness has yet to be firmly established.

\textbf{1.1.3. Sphingolipids in Skeletal Muscle.} Sphingolipids are a class of bioactive signaling lipids as well as integral components of the cellular membrane found ubiquitously throughout all higher-level organisms. Sphingolipid signaling molecules can be enzymatically synthesized \textit{de novo} from serine and palmitate utilizing a serine palmitoyl transferase and from the parent lipid sphingomyelin, using sphingomyelinase, to form ceramide, sphingosine, and sphingosine-1-phosphate. Ceramide and sphingosine are biologically active second messengers activated by the sphingomyelin signal transduction cascade in response to a variety of stimuli, in particular inflammatory
cytokines such as TNF (66), and IL-1B (64). In muscle and non-muscle cell types, ceramide is known to facilitate a number of cellular functions including apoptosis, inhibition of cell proliferation, and regulation of inflammatory processes (67, 70, 74). Although its neutral hydrophobic charge limits its potential as a diffusible intracellular sphingolipid messenger, ceramide may readily flip-flop across membranes (75). Sphingosine, on the other hand, is amphipathic in nature, and readily able to move through membranes as a signaling molecule (42). Both ceramide and sphingosine are thought to be responsible for negative effects in cellular function, while the third downstream signaling sphingolipid sphingosine-1-phosphate, is well documented to oppose their effects. Sphingosine-1-phosphate is generated via phosphorylation by a specific sphingosine kinase, and is known to induce its functions through both intracellular and extracellular mechanisms utilizing its five known receptor populations known as the Edg1-5 (115). The balance between the sphingosine and sphingosine-1-phosphate levels in a cell are thought to function as a ‘sphingolipid rheostat’, maintaining necessary levels of each species to preserve homeostatic function (115). Much of the literature on sphingolipid signaling and muscular cellular function revolves around the effects of ceramide and sphingosine-1-phosphate, while largely ignoring the intermediate sphingosine. But in fact, sphingosine in skeletal muscle has been shown to elicit profound effects on calcium handling which can potentially mediate the induction of fatigue and greatly affect the normal function of the tissue.

The major source of sphingosine in skeletal muscle cells is sphingomyelin, which is hydrolyzed by sphingomyelinase (SMase) to ceramide and then acted upon by ceramidase to produce sphingosine. Skeletal muscle T-tubule membranes contain the
largest amount of SMase protein and activity and in turn, also contain the highest sphingosine content (73). Resting sphingosine content is higher in the white glycolytic muscle fiber types and exercise to exhaustion increases this content three fold in excursionary limb muscles of both red and white fiber types (72). Sphingosine and its derivatives ceramide and sphingosine-1-phosphate, have two modes of action: (1) indirect effects on cellular responses through lysosphingolipid receptors, modulation of serine-threonine protein kinases, phospholipases, MAP kinases, and (2) by protein kinase-independent signal transduction wherein sphingosine or its derivatives act directly on intracellular calcium stores. In skeletal muscle, ceramide and its derivatives have largely been studied for their ability to regulate calcium release (106), modulate oxidative stress (30), and affect glucose uptake (60). Sphingosine, but not ceramide, blocks calcium release from isolated sarcoplasmic reticulum membranes containing the ryanodine receptor calcium release channel with significant inhibitory effects on [3H]ryanodine binding to the high affinity site of the sarcoplasmic reticulum / ryanodine receptor complex (51, 59, 99). Moreover, sphingosine inhibits the single channel activity of the ryanodine receptor, and only at high (>25 uM) levels does the channel paradoxically respond by gating in the open configuration (99). As a result, sphingosine may play a role in the development of muscle fatigue (59, 106) and play a secondary role in mediating cytokine-induced skeletal muscle weakness.

1.1.4. Free Radical Biology of Skeletal Muscle. Skeletal muscle continually generates two classes of diffusible, low molecular weight free radicals: reactive oxygen species (ROS) (144) and nitric oxide (NO) derivatives (92). Both ROS and NO are generated by muscle at low rates under resting conditions and at higher rates during
repetitive contraction. Both are detectable in the cytosol of skeletal muscle fibers and both are released into the extracellular environment. Both free radical cascades are derived from diatomic oxygen (119) and their biological activities involve reaction with common protein moieties, notably sulphydryl residues and metal centers. Despite their similarities, ROS and NO are: generated from different sources; regulated by unique networks of cellular mechanisms; and play specific roles in the facilitation of contractile and cellular function in skeletal muscle.

1.1.5. ROS in Skeletal Muscle. The potential sources of ROS in skeletal muscle include mitochondria, 5-lipoxygenase, sarcolemmal NAD(P)H oxidase, cyclooxygenase, and xanthine oxidase (86). Each source has been implicated as generation sites for superoxide anions (chemical formula O$_2^-$), the parent molecule of the ROS cascade. Resting skeletal muscle produces superoxide anion at a low rate, which dramatically accelerates during contractile activity. Superoxide anions are a by-product of mitochondrial electron transport, accounting for ~3% of total oxygen consumption by the organelle. Superoxide anions undergo spontaneous electron exchange reactions that give rise to a host of oxygen-based derivatives, among the most widely studied being hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^-$. H$_2$O$_2$ is less reactive than superoxide and is electrically neutral, favoring diffusion within and among cells. Superoxide and H$_2$O$_2$ function as signaling molecules, altering protein function via effects on regulatory sulphydryls or metal centers.

The biological activity of ROS is opposed by an array of endogenous antioxidant systems specifically localized in order to manage the oxidant demand of contracting muscle. As a first line of defense, circulating glutathione (GSH) reacts nonspecifically
with various oxidants to yield an oxidized glutathione dimer (GSSG). GSH is abundantly present in the cytosol at millimolar concentrations and utilizes a reaction facilitated by the enzyme glutathione peroxidase (GPx) to reduce several species of ROS. Glutathione is recycled from GSSG by a second enzyme, glutathione reductase, and preferentially kept in the reduced form to maintain proper redox potential and ability to buffer in the event of during a sudden change of ROS production (109). As a second line of defense, skeletal muscle expresses several ROS selective antioxidant enzymes. The most well known trio, superoxide dismutase (SOD), dismutes superoxide anions to hydrogen peroxide (4). Humans have three isoforms of SOD, specified by their cellular distribution (95). The most abundant and soluble form, SOD1, is located in the cytosol as a dimer utilizing copper-zinc as its catalytic site (CuZnSOD). The second, SOD2, is strictly localized to the mitochondria and is known as MnSOD because of its use of manganese as its reactive center. SOD3, the extracellular isoform, is found in tetramer formation, and is the least well known in skeletal muscle (3). After dismutation by SOD, H₂O₂ is enzymatically dehydrated to water and molecular oxygen by the endogenous antioxidant catalase, which is widely distributed throughout the cell.

The third line of ROS buffering capacity involves the action of nutrient antioxidants and free radical scavengers that include vitamin E, which compromises a family of lipid-soluble antioxidants, including alpha-tocopherol (86). These molecules are hydrophobic and primarily function to protect muscle membrane against oxidation. In contrast, vitamin C distributes to the aqueous phase where it directly scavenges ROS and facilitates redox cycling of vitamin E.
1.1.6. NO in Skeletal Muscle. Originally identified as endothelial derived relaxing factor (35), nitric oxide (NO) is synthesized from molecular oxygen and L-arginine by the enzyme NO synthase (56) with L-citrulline as the by-product (40). NO may exert antioxidant effects in biological systems by reacting directly with superoxide anions, by preventing the formation of longer-lived ROS, or by reversibly nitrosylating reactive thiol residues on regulatory proteins and thereby limiting thiol oxidation (138). In skeletal muscle, the primary isoform of NOS expressed is the neuronal-type (nNOS), but as an elongated, muscle-specific splice variant (nNOSµ) that associates with the dystrophin complex and is localized to the subsarcolemmal region (118). NO can also be produced by two other isoforms: endothelial (eNOS or NOS3) and inducible (iNOS or NOS2) isoforms. eNOS is expressed in low levels physically in skeletal muscle but is more present in the adjacent vascular endothelium (34). There is virtually no expression of iNOS in healthy skeletal muscle (81, 105), however, inflammatory stimuli have been shown to stimulate the expression of iNOS in muscle cells (44). Both nNOS and eNOS generate NO at relatively low rates to regulate intracellular and intercellular signaling events, while iNOS is thought to generate NO at a high rate under biological stimulus.

Both nNOS and eNOS are dimers; each monomer contains an amino-terminal oxygenase domain (N-terminal) and a carboxy-terminal reductase domain (C-terminal). The oxygenase domain binds the substrate L-arginine and contains the co-factor tetrahydrobiopterin (BH4) and a cytochrome P-450-type heme active site. The reductase domain contains binding sites for flavins (FMN, FAD) and the electron donor NADPH. The two monomers are dimerized and together produce NO following Ca2+ binding to calmodulin, which facilitates the electron transfer from the carboxyl-terminal reductase
domain to its heme-containing amino terminal domain.

Kobzik et al. (51) first described nNOS as the primary isoform of NOS in skeletal muscle, and found that it localized specifically in type II (fast) fibers and that NO-synthase activity increased as the proportion of type II fibers increased. Functionally, nNOS is initially regulated by the binding of calcium to calmodulin, while the specific activity can be activated by phosphorylation of the Ser1417 site and inhibited by phosphorylation of the Ser847 site (134). The effects of NO on skeletal muscle contractile function are well known. The work by Alloatti et al. addressed this directly and found that NO caused a direct depression of specific force (2). Pharmacologic blockade of constitutive NOS activity increases the force of submaximal contractions, shifting the force-frequency curve leftward, whereas NO donors decrease force (51). The mechanism behind how NO decreases force is still unclear, but two schools of thought prevail: (a) NO stimulates synthesis of cyclic guanosine monophosphate (cGMP) which functions as a second messenger to depress force (1); and (b) through a cGMP-independent mechanism, suggesting NO mediates contractile dysfunction by altering myofilament function (4).

1.2. Scope of Dissertation

1.2.1. Aims of Dissertation. This dissertation evaluates the effects of TNF on skeletal muscle function and oxidant production using respiratory (diaphragm) muscle isolated from C57BL/6 mice and in parallel using a cell culture model, C2C12 myotubes. This work explores the possibility that TNF induces skeletal muscle dysfunction through either or both ROS and NO, and this process is mediated by sphingolipid signaling (Figure 1.1).
1.2.2. Rationale. In skeletal muscle, exogenous ceramide, sphingomyelinase, and elevated oxidants are known to induce muscle weakness and accelerate fatigue (30, 86, 95, 123). TNF has been shown to increase oxidants and depress the contractile function of skeletal muscle (102). These effects of TNF have been shown to be transmitted through the TNFR1 complex and be redox sensitive, as pretreatment with antioxidants can attenuate this response (43). The role that sphingolipids play as mediators of TNF-induced oxidants and contractile dysfunction has yet to be elucidated. Sphingolipids are known to act as signaling proteins through an activated TNFR1 utilizing adaptor proteins (111, 143), but the molecular target of this cascade is also unknown. Identifying the downstream targets of this sphingolipid cascade acting as mediators of oxidant production and decreased contractile function will ultimately lead to new therapeutic targets for patients suffering from chronic inflammation and cytokine-induced muscle weakness. In total, we focused on deepening our understanding of the specific oxidant species and their source(s) generated in response to TNF and explored the role that skeletal muscle sphingolipids play in post TNFR1 signaling, specifically the species and their enzymatic regulation that promotes skeletal muscle oxidant production.

1.2.3. Model of TNF-stimulated contractile dysfunction. TNF is proposed to increase nNOS leading to skeletal muscle contractile dysfunction. Activated TNFR1 is proposed to stimulate sphingomyelinase-induced sphingosine which acts upon nNOS to stimulate the production of NO when in combination with basal ROS, mediates the process of contractile dysfunction.
1.2.4. Hypothesis and Specific Aims. My hypothesis is that the contractile dysfunction in skeletal muscle induced by TNF is dependent upon sphingolipid signaling events that result in an increase in nNOS-specific NO production. In the first aim, we tested whether the oxidant species ROS and NO are essential mediators of TNF-induced skeletal muscle contractile dysfunction. In the second aim, we investigated the effects of TNF on sphingolipid signaling and as mediators of oxidant production in skeletal muscle.
CHAPTER TWO

TNF SIGNALS VIA NEURONAL-TYPE NITRIC OXIDE SYNTHASE AND REACTIVE OXYGEN SPECIES TO DEPRESS SPECIFIC FORCE OF MURINE SKELETAL MUSCLE FIBER BUNDLES

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2.1. Abstract

Tumor necrosis factor (TNF) promotes skeletal muscle weakness, in part, by depressing specific force of muscle fibers. This is a rapid, receptor-mediated response in which TNF stimulates cellular oxidant production, causing myofilament dysfunction. The oxidants appear to include nitric oxide (NO); otherwise, the redox mechanisms that underlie this response remain undefined. The current study tested the hypotheses that: a) TNF signals via neuronal-type NO synthase (nNOS) to depress specific force, and b) muscle-derived reactive oxygen species (ROS) are essential co-mediators of this response. Mouse diaphragm fiber bundles were studied using live cell assays. TNF exposure increased general oxidant activity (P<0.05; DCFH oxidation assay) and NO activity (P<0.05; DAF assay), and depressed specific force across the full range of stimulus frequencies (1-300 Hz; P<0.05) by measuring contractile function in an ex vivo preparation. These responses were abolished by pretreatment with N(omega)-nitro-l-arginine methyl ester (L-NAME), a non-specific inhibitor of NO synthase activity, confirming NO involvement. Genetic nNOS deficiency replicated L-NAME effects on
TNF-treated muscle, diminishing NO activity (-80%; P<0.05) and preventing the decrement in specific force. Comparable protection was achieved by selective depletion of muscle-derived ROS. Pretreatment with either superoxide dismutase (SOD; degrades superoxide anion) or catalase (degrades H$_2$O$_2$) depressed oxidant activity in TNF-treated muscle and abolished the decrement in specific force. These findings indicate that TNF signals via nNOS to depress contractile function, a response that requires ROS and NO as obligate co-mediators.
2.2. Introduction

Tumor necrosis factor-α (TNF) is a pro-inflammatory cytokine that promotes muscle weakness in a variety of chronic diseases (139). TNF depresses muscle force via two general mechanisms. Muscle atrophy is the mechanism recognized most widely. At the cellular level, prolonged TNF exposure activates pro-catabolic signaling and upregulates the ubiquitin-proteasome pathway to cause protein loss and reduce cell size (60, 72-75). The resulting decline in muscle mass inexorably leads to weakness.

The second mechanism, termed contractile dysfunction, is less well understood. TNF has the capacity to depress specific force of skeletal muscle, (i.e., force per unit cross-sectional area), causing weakness independent of muscle mass. Contractile dysfunction occurs at circulating TNF levels that do not cause muscle atrophy (69), is stimulated via the TNF receptor subtype 1 (TNFR1) (43), and is associated with loss of myofibrillar function (43, 101). Muscle-derived oxidants play a central role in this response. TNF/TNFR1 signaling increases general oxidant activity in the cytosol within minutes; antioxidant pretreatment buffers the rise in oxidant activity and preserves specific force, arguing for causality (43, 69). The essential oxidants that mediate TNF-induced dysfunction appear to include nitric oxide (NO) derivatives. Alloatti and associates (2) showed that brief exposure to TNF increases NO production and depresses specific force in extensor digitorum longus (EDL) muscle of guinea pig. Both responses were abolished by pharmacologic blockade of NO synthase (NOS), suggesting a role for NO. While intriguing, these findings have not been confirmed independently.

The current study addressed the redox mechanism by which TNF stimulates contractile dysfunction. Initial experiments assessed the biological robustness of the NO
hypothesis using a respiratory skeletal muscle (diaphragm) from a different species (mouse). We then tested two original hypotheses:

2.2.2. Hypothesis 1. TNF signals via neuronal-type NOS (nNOS) to stimulate NO production and contractile dysfunction. At present, the NOS isoform that mediates TNF/NO signaling is undefined. Skeletal muscle constitutively expresses all three isoforms: inducible, endothelial, and neuronal NOS (51, 52, 91). These are independent gene products with distinct structures, intracellular locations, and mechanisms of regulation (117). The nNOS isoform transduces receptor-activated signaling cascades in other cell types (18, 107, 124) and localizes to the subsarcolemmal region of muscle fibers (9), placing nNOS in close physical proximity to the TNFR1 complex. We therefore assessed nNOS as a downstream component of TNF/TNFR1 signaling.

2.2.3. Hypothesis 2. Reactive oxygen species (ROS) contribute to contractile dysfunction stimulated by TNF. Compared to NO and its derivatives, the ROS cascade may be generated by separate intracellular sources, is derived from a chemically-distinct parent molecule, and can be regulated by independent mechanisms. Muscle-derived ROS are reported to function as second messengers in a variety of TNF-stimulated responses (57, 61, 75, 116). However, the role of ROS in contractile dysfunction has not been evaluated.

These two hypotheses were tested by either exposing C57BL/6 male mice to TNF in vivo via i.p. injection or their excised diaphragm fiber bundles to recombinant TNF in an ex vivo preparation. Live cell assays were used to measure changes in general oxidant activity, NO activity, and contractile function. The essentiality of muscle-derived NO and ROS to TNF-induced depression of contractile function were evaluated using selective
pharmacologic probes and genetic manipulations. Our findings identify nNOS as the source of TNF-stimulated NO and demonstrate that muscle-derived ROS are obligate co-mediators of the subsequent contractile dysfunction.

2.3. Materials and Methods

2.3.1. Animal care. Experiments were performed using 6-8 wk old male mice from two strains, wild-type (C57BL/6J; Jackson Labs, Bar Harbor, ME) and neuronal NO synthase or nNOS-deficient (nNOS<sup>−/−</sup>; B6.129S4-Nos1<sup>tm1Pbh</sup>/J, Jackson Labs, Bar Harbor, ME). Animals were maintained in a 12:12-h dark-light cycle and received water and food ad libitum. All procedures conformed to the guiding principles for use and care of laboratory animals of the American Physiological Society and were approved by the Institutional Animal Care and Use Committee of the University of Kentucky.

2.3.2. Muscle preparations. Each animal was deeply anesthetized by inhalation of isoflurane (Aerrane; Baxter Healthcare, Deerfield, IL) and killed by cervical dislocation followed by exsanguination. The diaphragm muscle was quickly excised and placed in Krebs-Ringer solution (in mM: 137 NaCl, 5 KCl, 1 MgSO<sub>4</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 24 NaHCO<sub>3</sub>, and 2 CaCl<sub>2</sub>) equilibrated with 95% O<sub>2</sub>-5% CO<sub>2</sub> (pH 7.3 at 37°C). For live-cell oxidant activity assays, the muscle was surgically separated into two hemi-diaphragms. For measurement of contractile function, bundles of muscle fibers were surgically isolated with portions of the rib and central tendon attached.

2.3.3. Live-cell oxidant activity assays: We used the fluorochrome probe 2′,7′-dichlorodihydrofluorescein diacetate (DCF; excitation/emission 492-495/517-527 nm; Molecular Probes, Eugene, OR) to measure total cytosolic oxidant activity (86) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM; 495/515 nm;
Molecular Probes) to measure cytosolic NO activity (55). Hemidiaphragms were pinned at near-optimal length ($L_0$) in a bath containing oxygenated Krebs-Ringer solution at 37°C. Each muscle preparation was loaded with fluorochrome by incubation in either DCF 20 μM or DAF-FM 5 μM for 20 mins and then was incubated for 30 min in buffer alone (control) or TNF 500 ng/mL. In a subset of experiments, muscles were pretreated with superoxide dismutase 1000 U/ml (SOD; Sigma-Aldrich), catalase 1 kU/mL; (Sigma-Aldrich), $N^G$-nitro-L-arginine methyl ester 10 mM (L-NAME; Sigma-Aldrich), or $N^G$-nitro-D-arginine methyl ester 10 mM (D-NAME; Sigma-Aldrich) to assess the contributions of muscle-derived ROS or NO derivatives. Fluorescence emissions of the oxidized fluorochromes were measured from a 0.27-mm$^2$ site on the muscle surface and background emissions were measured from a muscle-free area of the incubation media using an epifluorescence microscope (Nikon Eclipse TE2000; Nikon Instruments, Melville, NY) and a charge-coupled device camera (Cool Snap-ES, Photometrics, Tuscon, AZ). Final values for fluorescence intensity were corrected for background emissions.

2.3.4. Molecular probe chemistry: Experiments were performed in triplicate using a 24-well tissue culture plate with a reaction volume of one mL. The sensitivities of DCF and DAF-FM to ROS and NO derivatives were tested using a cell-free assay. DCF diacetate 50 μM and DAF-FM diacetate 20 μM were pre-incubated with esterase 4 U/mL in Krebs buffer for five minutes to produce DCF and DAF-FM, respectively. Each fluorochrome was mixed with a.) TNF 15 ng/mL, b.) hydrogen peroxide 1 mM, c.) hydrogen peroxide plus catalase 1 kU/mL, d.) the NO donor, 3-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine (NOC-7 100 μM ; EMD4Biosciences), e.)
NOC-7 plus hemoglobin 50 μM (NO scavenger; Sigma-Aldrich), or f.) Krebs-Ringer solution alone (control). After 10 mins, fluorescence was measured using the microscope system described above.

2.3.5. Contractile studies. Muscle fiber bundles were preconditioned by TNF exposure prior to contractile measurements. For in vitro preconditioning, fiber bundles were incubated at 37°C with TNF 500ng/mL, 3-[2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine 20 μM (NOC-5; EMD4Biosciences, San Diego, CA) as a source of exogenous NO derivative, or buffer alone (control) before contractile measurements. For in vivo preconditioning, mice received intraperitoneal (i.p.) injections of TNF 100 μg/kg (Pierce Biotechnology, Rockford, IL) or an equal volume of diluent prior to muscle excision. A subset of TNF-treated animals were pretreated with SOD 1 kU/μg, catalase 1 kU/μg, L-NAME 20 mg/kg, or D-NAME 20 mg/kg by i.p. injection prior to TNF administration.

Isometric contractile properties were measured in vitro. The fiber bundle was mounted by tying the rib to a glass rod and the central tendon to a force transducer (BG series 100 gm; Kulite, Leonia, NJ) using silk suture (4-0). The transducer was mounted on a micrometer used to adjust muscle length. The fiber bundle was positioned between platinum plate electrodes in a water-jacketed organ bath containing (+)-tubocurarine hydrochloride 0.025 mM (Sigma Aldrich) in Krebs-Ringer solution continuously gassed with 95% O2-5% CO2 at 37°C. Fiber bundle length was adjusted to maximize twitch force (optimal length, L0) stimulated using supramaximal voltage and 0.2 ms pulses (stimulator model S48; Grass Industries, Quincy, MA).

Force-frequency characteristics were determined using stimulus frequencies of 1,
15, 30, 50, 80, 120, 150, and 250 Hz (500 ms train duration) delivered every 2 minutes and interspersed by maximal tetanic contractions (P_0; 300 Hz). Transducer output was measured using a digital oscilloscope (model 546601B; Hewlett Packard, Palo Alto, CA). After the force-frequency protocol, muscle length was measured using an electronic caliper (CD-6” CS, Mitutoyo America, Aurora, IL). The fiber bundle was removed from the organ bath, excised from bone and connective tissue, blotted dry, and weighed. Fiber bundle weight and L_0 were used to calculate cross-sectional area (14). Specific force is expressed as N/cm².

2.3.6. Western blot. Tissue samples were homogenized in 2X protein loading buffer (120mM Tris pH 7.5, 200mM DTT, 20% glycerol, 4% SDS, 0.002% bromphenol blue). Proteins were loaded and separated by 4-15% SDS-PAGE gel electrophoresis (Criterion, BioRad) at 200V for 40 min. Total protein was assessed by staining using Simply Blue (Invitrogen). Proteins were wet transferred to PVDF membranes overnight at 100mA. Membranes were blocked (Odyssey blocking-buffer; LI-COR Biosciences, Lincoln, NE) for 1 h at room temperature and incubated with primary antibodies (anti-nNOS, ECM Biosciences, Versailles, KY; mouse phospho- JNK and rabbit total JNK, Cell Signaling, Danvers, MA) overnight in blocking buffer and an equal volume PBS plus 0.1% Tween (PBST), followed by four 5 min washes in PBST. Membranes were incubated with fluorescence-conjugated secondary antibodies (goat anti-mouse IRD 800, Rockland Immunochemicals, Gilbertsville, PA) in blocking buffer/PBST plus 0.01% SDS for 45 min, followed by four 5 min washes. Membranes were dried and blots were imaged using the Odyssey Infrared Scanner (Odyssey Infrared System, LI-COR) to quantify differences.
2.3.7. Statistical analysis. All comparisons were performed using Prism 5.0b software (Graphpad Software, La Jolla, CA). Paired comparisons between DCF and DAF-FM data were evaluated using Student’s paired t-tests. Differences between force-frequency curves were analyzed using two-way, repeated measures ANOVA, and a post-hoc Tukey Test. Differences were considered significant at the P<0.05 level. Results are reported as means ± SE.

2.4. Results

2.4.1. TNF effects on specific force and oxidant activity. As illustrated in Figure 2.1, TNF exposure increases general oxidant activity in the cytosolic compartment. This rise in oxidant activity is associated with decrements in specific force at all stimulus frequencies such that relative force, as a percentage of maximal force, is relatively unaffected. These responses are abolished by antioxidant pretreatment (43, 69), evidence that muscle-derived oxidants cause the fall in specific force stimulated by TNF.

2.4.2. Role of muscle-derived NO. General oxidant activity was monitored using DCF, a fluorescence probe that detects both NO derivatives and ROS (Fig. 2.2A). To assess the contribution of NO, we tested TNF effects on general oxidant activity in muscle fibers pretreated with L-NAME (NOS inhibitor) or D-NAME (inactive enantiomer). As shown in Figure 2.2B, TNF increased general oxidant activity in D-NAME-treated fibers by 42%, mimicking the response of muscle treated with TNF alone. L-NAME abolished this response, evidence that TNF increases general oxidant activity by stimulating NO production. NO involvement was further tested by use of DAF-FM, a fluorescence probe that preferentially detects NO derivatives (Fig. 2.2C); data from intact muscle fibers confirm that TNF increases cytosolic NO activity (Fig. 2.2D). The
increased NO was associated with a 25% depression of in vivo contractile function. Decrement in specific force (Fig. 2.3A) were abolished by pretreating muscle with L-NAME (Fig. 2.3B) but not D-NAME (Fig. 2.3C). These findings closely parallel results from guinea pig EDL muscle (2), suggesting that NO is an essential mediator of the contractile dysfunction stimulated by TNF.

2.4.3. TNF signaling via nNOS. The NOS isoform responsible for TNF-stimulated NO production has not been defined. Based on its role in receptor-mediated signaling and subsarcolemmal localization, we evaluated nNOS as the NO source. Skeletal muscle preparations from mice engineered for nNOS deficiency were compared with control animals of similar genetic background. Muscle preparations from the transgenic mice were confirmed to be nNOS-deficient by Western blot (Fig. 2.4A). The patency of TNF/TNFR1 signaling was assessed by measuring phosphorylation of Jun-N-terminal kinase (JNK), an established response to TNFR1 activation (25, 41, 72). TNF consistently increased JNK phosphorylation (Figs. 2.4B, 2.4C), evidence that TNFR1 signaling remains intact in nNOS-deficient muscle. Despite retaining TNFR1 function, NO signaling was profoundly disrupted by nNOS deficiency. The NO activity stimulated by TNF was diminished by 84% in nNOS-deficient muscle relative to muscle from genetically-intact wild-type muscle (Fig. 2.5A). Contractile function was also protected. The specific force-frequency relationship of nNOS-deficient muscle was not significantly diminished by TNF exposure (Fig. 2.5B). This finding cannot be attributed to NO insensitivity because further studies showed that direct exposure to NOC-5, an exogenous NO donor, depressed specific force of nNOS-deficient muscle (Fig. 2.5C). This mirrors the contractile depression caused by NO donors in wild-type muscle (51, 100) and
confirms that downstream targets of NO signaling are preserved in the nNOS transgenic mice. Overall, these data identify nNOS as the primary isoform by which TNF-stimulates NO synthesis and show that nNOS is an essential component of the signaling pathway that contributes to contractile dysfunction.

2.4.4. ROS as co-mediators. We tested the role of muscle-derived ROS in our system by using of exogenous catalase and SOD, anti-ROS enzymes that selectively deplete H$_2$O$_2$ and superoxide anions, respectively. In muscle exposed to TNF, catalase and SOD depressed general oxidant activity (Fig. 2.6A). This finding confirms that ROS are present in the cytosol of TNF-treated muscle and can be depleted experimentally by catalase and SOD. ROS depletion does not appear to disrupt TNF/NO signaling. NO activity is still stimulated by TNF despite pretreatment with catalase and SOD (Fig. 2.6B). This rise in NO activity is essential for contractile dysfunction but does not appear to be sufficient. Muscle-derived ROS are also required. ROS depletion by catalase and SOD abolishes TNF effects on specific force (Fig. 2.6C), identifying ROS and NO as obligatory co-mediators of contractile dysfunction.

2.5. Discussion

2.5.1. TNF and contractile dysfunction. TNF has a robust capacity to decrease the specific force of skeletal muscle. This response has been documented in respiratory muscle (43, 69, 135, 136) and limb muscle (2, 101, 145) of various mammalian species, although recent data suggest that individual muscles differ in their sensitivities to TNF (145). Contractile dysfunction can result from TNF overexpression in remote tissues (69, 145), systemic TNF administration in vivo (43, 136), or following TNF incubation in
vitro (2, 101, 135). Specific force is decreased within an hour (43, 69) and at TNF plasma levels that are too low to cause muscle atrophy (69).

Our laboratory has a long-standing interest in the cellular mechanisms of this response. Previous work using genetically-engineered mice showed that contractile dysfunction is stimulated by TNF binding to the TNFR1 receptor subtype (38, 43). The resulting decrement in specific force appears to be caused by myofilament dysfunction. In intact muscle fibers, TNF decreased specific force without depressing electrically-stimulated calcium transients, results that suggest myofilament involvement (101). This interpretation was confirmed by studying permeabilized muscle fibers from TNF-treated animals; myofibrillar force is depressed despite direct calcium activation (43).

The pathway that transduces the TNF/TNFR1 signal is less clear. Early reports implicated platelet activating factor (2) or prostaglandin synthesis (135), but more recent research has focused on muscle-derived oxidants. TNF increases biochemical and genetic markers of oxidative stress (8, 11, 62) and stimulates intracellular oxidant activity (43, 62, 69), changes that correlate with loss of force. More importantly, force can be preserved by pretreating muscle with nonspecific antioxidants (43, 69). These findings indicate that a rise in oxidant activity is required for contractile dysfunction, but do not identify the redox cascade that is required.

2.5.2. NO signaling via nNOS. A report by Alloatti and colleagues (2) indicated that TNF acts via muscle-derived NO to depress specific force. This finding is inconsistent with earlier reports that suggested TNF does not stimulate NO production by muscle cells (137) and that TNF opposes receptor-stimulated NO signaling (142). Therefore, we set out to independently evaluate NO as a second messenger for TNF. Our
data support those of Alloatti et al., confirming their conclusion that TNF signals via NO to depress specific force. This concurrence was reached despite qualitative differences between species (guinea pig vs. mouse) and muscle preparations (glycolytic limb muscle vs. aerobic respiratory muscle), which argues for biological robustness. The evidence that NO acts as a downstream messenger for TNF is consistent with the previous observation that direct NO exposure depresses specific force of skeletal muscle by altering myofilament function (4).

Our new results point to nNOS as the source of TNF-stimulated NO. Skeletal muscle fibers constitutively express nNOS, which binds alpha1-syntrophin to associate with the dystrophin complex (9), localizing nNOS to the subsarcolemmal region. nNOS has a well-established role in transducing activation signals from membrane receptors, including kappa opioid receptors (18), NMDA receptors (124), and muscarinic M2 and M4 acetylcholine receptors (107). For G-protein coupled receptors, recent data indicate that nNOS complexes with RGSZ2, a regulator of G-protein signaling, and physically associates with membrane receptors to initiate redox signaling (107). The mechanism by which TNFR1 interacts with nNOS is less clear. To our knowledge, the results reported here are the first to show that nNOS is required for TNFR1 signaling in any cell type.

2.5.3. ROS as co-mediators. We hypothesized that muscle-derived ROS contribute to TNF-induced dysfunction for several reasons. In our experience, ROS mimic the actions of TNF by increasing cytosolic oxidant activity (86) and by depressing specific force of unfatigued muscle via effects on myofilament function (3). Data from the current study demonstrate that muscle-derived ROS are required for TNF-induced dysfunction. However, our study provides no evidence that TNF increases general ROS
activity in the cytosol. It may be that ROS signaling occurs at low levels or in a compartmentalized process that protects overall redox state of the cell (47). Alternatively, basal ROS levels may play a permissive role in TNFR1 signaling, for example, by enabling signal transduction via redox-sensitive proteins (33) or reacting with TNF-stimulated NO to generate peroxynitrite (76).

2.5.4. Clinical relevance. Muscle weakness is a major complication of chronic inflammatory conditions including chronic obstructive pulmonary disease (COPD), chronic heart failure, rheumatoid arthritis, and aging. Circulating TNF levels correlate inversely with muscle strength in these conditions (6, 12, 68, 108), suggesting a TNF-mediated process. Consistent with this notion, chronic inflammatory conditions often increase biochemical markers of oxidative stress in muscle (5, 46, 77, 96, 131) and depress specific force (48, 63, 90, 125).

At present, there is no clinical standard of care nor drug or nutritional strategy to preserve muscle function in the face of chronic inflammation. Systemic TNF blockade could be beneficial but anti-TNF therapy has significant side-effects (21, 36, 128) and may increase mortality in some patient populations (16, 93). Alternatively, pharmacologic antioxidants could be used to target events downstream of TNF. For example, N-acetylcysteine is a reduced thiol donor that opposes contractile dysfunction caused by TNF in animals (69) and improves quadriceps endurance in individuals with COPD (53). However, at clinical doses, N-acetylcysteine causes adverse reactions that can limit its use (29, 103). NO- and ROS-specific interventions are new approaches made rational by our current findings. For example, several NOS inhibitors are available for experimental use in humans (17, 50, 110). Similarly, novel small peptide molecules that
inhibit mitochondrial ROS production are now being developed for clinical use (126). Our findings presented here suggest that drugs in these two categories have the potential to alleviate weakness caused by TNF in individuals with chronic inflammatory disease.

2.5.5. Conclusion. The signaling pathway by which TNF stimulates contractile dysfunction includes nNOS-derived NO and endogenous ROS as essential co-mediators. To our knowledge, obligate interdependence between these two redox cascades has not been demonstrated before in skeletal muscle. Most studies of redox signaling, including reports from our laboratory, have addressed one cascade or the other but not both. In the future, it will be useful to test for NO/ROS interdependence in other aspects of muscle cell physiology that are redox sensitive, such as glucose uptake, calcium regulation, and protein catabolism.
Figure 2.1. Direct TNF exposure increases cytosolic oxidant activity and depresses specific force in diaphragm fiber bundles. A. Representative fluorescence images depict hemi-diaphragms loaded with DCF and incubated in buffer alone (upper panel) or in TNF (lower panel, 500 ng/mL, 30 min). B. General oxidant activity quantified from
DCF fluorescence of hemi-diaphragms treated as in A.; TNF increased emissions in 8 of 8 paired comparisons; *P<0.05 (paired t-test), Buffer vs. TNF. C. The specific force-frequency relationship of murine diaphragm fiber bundles incubated with TNF (500 ng/mL, 30 min, open diamonds) was depressed across all experimental frequencies relative to control measured in buffer alone (closed circles); mean values shown ± SE; n = 3/group; *P<0.05 (Two-Way ANOVA) Buffer vs. TNF. D. Relative force as a percentage of maximal tetanic force (%Po) was unaffected by TNF at all stimulus frequencies; means are shown ± SE.
Figure 2.2. Tumor Necrosis Factor (TNF) induces both ROS and NO in diaphragm fibers. A: Reactivity of de-esterified DCF was measured by use of a cell-free system; relative to buffer alone (Krebs), DCF emissions were unaffected by TNF (15 ng/mL); hydrogen peroxide (H$_2$O$_2$, 1 mM) increased emissions, an effect abolished by catalase (1 kU/mL, H$_2$O$_2$+catalase); the NO donor NOC-7 (100μM) increased emissions, an effect abolished by reduced hemoglobin (50μM, NOC-7+HGB); n=3/group; means shown ±
SE; *P<0.05 Krebs vs. NOC-7 or H₂O₂; #P<0.05, H₂O₂ vs. H₂O₂+catalase, NOC-7 vs.
NOC-7+HGB. B. Muscles treated with TNF (500ng/mL) plus D-NAME (10uM; nNOS
inhibitor, inactive enantiomer) for how long increased DCF emissions; an effect that was
abolished by treatment with L-NAME (10uM, nNOS inhibitor, active enantiomer); mean
values shown + SE; n = 3/group; *P<0.05 (paired t-test), TNF + D-NAME vs. all other
groups. C. Reactivity of de-esterified DAF-FM exposed under cell-free conditions to
oxidants and redox interventions as in A; TNF, H₂O₂, and catalase had no effect on DAF-
FM emissions relative to buffer alone; NOC-7 increased emissions, an effect abolished
by reduced hemoglobin; n=3/group; means ± SE; *P<0.05, NOC-7 vs. NOC-7+HGB. D.
Diaphragm treated with 500ng/mL TNF (30 min) ex vivo increased cytosolic NO activity
as measured by DAF-FM assay relative to buffer-treated muscles; n=6/group; means ±
SE; *P<0.05 (paired t-test), Buffer vs. TNF.
Figure 2.3. Pharmacologic nitric oxide synthase inhibition preserves contractile function of TNF-treated diaphragm in vivo. A. Specific force of diaphragm fiber bundles from TNF-treated mice (i.p., 100μg/kg, 60 min, open diamonds) was depressed relative to data from buffer-treated control animals (closed circles); n = 3/group; means ± SE; *P<0.05, (Two-Way ANOVA) TNF vs. Control. B. Pharmacologic blockade of nNOS with L-NAME (i.p., 20mg/kg, 60 min) prevents the TNF-stimulated decrease in specific force compared to buffer-treated muscle (dotted line; reproduction of data in A for reference); n=6/group. C. The inactive enantiomer D-NAME (20mg/kg) did not alter the decrement of force in TNF-treated muscle (open diamonds) compared to buffer-treated muscle (dotted line; reproduction of data in A); n=6/group; means ± SE; *P<0.05 (Two-Way ANOVA) TNF + D-NAME vs. Buffer.
Figure 2.4. Tumor Necrosis Factor (TNF) signaling remains intact after neuronal nitric oxide synthase (nNOS) depletion in diaphragm muscle. A. Western blots illustrate constitutive expression of full-length nNOS (158 kDa) in diaphragm of wild-type mice and the absence of nNOS in diaphragm of nNOS−/− animals. B. Western blot illustrates the effects of TNF (in vitro, 500ng/mL, 30 min) on JNK signaling in nNOS−/− muscle. C. Quantification of B shows that TNF induces JNK phosphorylation in nNOS−/− muscle; data expressed as the ratio of phosphorylated:total JNK; means are shown ± SE; *P<0.05 (paired t-test) TNF + nNOS−/− vs. Buffer + nNOS−/−.
Figure 2.5. Neuronal NOS-derived NO is essential for TNF-induced contractile dysfunction.  
A. TNF-stimulated (in vitro, 500ng/mL, 30 min) nitric oxide activity as measured by DAF-FM is blunted in nNOS\(^{-/-}\) muscle as compared to muscle from strain-matched controls; n=3/group; means ± SE; *P<0.05 (paired t-test) C57BL/6 vs. nNOS\(^{-/-}\).  
B. TNF (i.p., 100 μg/kg, 60 min) administration to nNOS\(^{-/-}\) mice does not depress specific force of diaphragm fiber bundles; n = 3/group; means ± SE.  
C. Reintroduction of nitric oxide (NOC-5, 10 μM, 30 min) to TNF-treated nNOS\(^{-/-}\) muscle in vitro recovers phenotypic depression of specific force; n=3/group; means ± SE; *P<0.05 (Two-Way ANOVA) nNOS\(^{-/-}\) + TNF vs. nNOS\(^{-/-}\) + TNF + NOC-5.
Figure 2.6. Muscle-derived ROS co-mediates contractile dysfunction stimulated by TNF. A. Mice were treated with TNF (i.p., 100μg/kg, 60 min) following pretreatment with buffer, or superoxide dismutase (SOD) (i.p., 1000U/μg, 60 min) and catalase (CAT) (SOD/CAT, i.p., 1000U/μg, 60 min). Selective ROS depletion depressed general oxidant activity in fibers of TNF-treated animals as measured by DCF fluorescence; n=7/group; *P<0.05 (paired t-test) TNF vs. SOD/CAT + TNF. B. NO activity in fibers treated ex vivo with SOD/CAT (1000U/mL, 30 min) was increased by co-treatment ex vivo with TNF (500ng/mL, 30 min); n=3/group; means ± SE; *P<0.05 (paired t-test) SOD/CAT vs. SOD/CAT + TNF. C. Pretreatment of mice with SOD/CAT (i.p., 1000U/μg, 60 min, n=6) prevented the decrement in specific force caused by TNF administration (i.p., 100μg/kg, 60 min, open circles) relative to control values (n=3; closed circles); means ± SE.
CHAPTER THREE

THE ROLE OF SPHINGOSINE IN MEDIATING TNF-INDUCED NITRIC OXIDE PRODUCTION IN SKELETAL MUSCLE

3.1. Abstract

The inflammatory cytokine tumor necrosis factor α (TNF) stimulates the production of nitric oxide (NO) by neuronal-type nitric oxide synthase (nNOS) in skeletal muscle leading to muscle weakness; however the signaling pathways involved are not known. The current study investigates the hypothesis that sphingosine mediates TNF-induced nitric oxide production in C2C12 skeletal muscle cells. TNF stimulated the turnover of sphingomyelin as early as 30 seconds after exposure, based on mass spectrometry analysis and in vitro neutral sphingomyelinase activity assay. However, instead of increasing, the levels of ceramide, the product of sphingomyelinase, decreased by 11.8% (± 0.1, P<0.05). These changes were strongly correlated with an accumulation of the ceramide metabolite, sphingosine. Exogenous exposure of muscle cells to TNF, ceramide, or sphingosine increased general oxidant activity (+17.1% ± 6.4, +24.9% ± 4.7, +15.8% ± 6.2 respectively; P<0.05) and NO activity (+33.2% ± 6.8, +14.5% ± 1.0, +18.4% ± 0.8 respectively; P<0.05). TNF-induced NO activity dropped significantly following suppression of ceramide conversion to sphingosine by siRNA knockdown of neutral ceramidase 2 (ASAH2) and after pharmacologic inhibition with N-Oleoylethanolamine (-3.1% ± 1.0; P<0.05), indicating that sphingosine generation is both sufficient and required for TNF-induced nitric oxide production. Pharmacologic inhibition of sphingosine kinase by Di-methylsphingosine, did not affect sphingosine-stimulated production, thus excluding a role of the sphingosine metabolite sphingosine-1-
phosphate in the process. Finally, a gene silencing approach identified the neuronal form of NOS (nNOS), as the source of sphingosine induced nitric oxide production. Together these results identify sphingosine as key component in the pathway linking TNF with nNOS, NO production, and respectively muscle weakness.
3.2. Introduction

Skeletal muscle weakness is present in many patients suffering from diseases of chronic inflammation. Increased levels of circulating tumor necrosis factor-α (TNF) are characteristic in the inflammatory disease process suggesting a role for TNF in the progression of skeletal muscle weakness. Indeed TNF has been found to induce skeletal muscle weakness ex vivo (69), an effect that has been in part mediated by increased production of reactive nitrogen species (120).

Originally TNF had been named ‘cachectin’ in recognition of its ability to cause a loss of muscle mass, (and respectively weakness) known as ‘cachexia’. Recent studies however, revealed that TNF can cause skeletal muscle weakness independent of atrophy (43). When force measurements are normalized to muscle mass, specific force of muscle is depressed after in vivo or ex vivo exposure to TNF (43, 120). An increased generation of reactive nitrogen species seems to mediate this marked, atrophy-independent, loss of force-generating capacity (120). Several recent studies have focused on the underlying signaling mechanisms and have provided evidence that bioactive sphingolipids may play a role in TNF-induced muscle weakness. First, TNF treatment of skeletal muscle or C2C12 myotubes was shown to activate sphingomyelinase (SMase); the first enzymatic step in the generation of the bioactive sphingolipid metabolites. Second, ceramide, the direct product of SMase activation, has been shown to induce a rise in oxidant production (15, 30, 37, 140); Lastly, treatment of isolated diaphragm with recombinant SMase or with ceramide, has been shown to cause skeletal muscle weakness (30), an effect that can be blocked by the antioxidant N-acetylcysteine (30).
In spite of this evidence, the role of sphingolipids in regulation of muscle function is not well understood. Ceramide, sphingosine, and sphingosine-1-phosphate are the three main bioactive sphingolipids in muscle. Each can be derived from the plasma membrane sphingomyelin by the consecutive action of SMase, which generates ceramide, ceramidase, that hydrolyzes ceramide further to sphingosine, and lastly, sphingosine kinase, which phosphorylates sphingosine to sphingosine-1-phosphate. In non-muscle cells, these enzymes are activated by pro-inflammatory cytokines, including TNF (49, 104). The resulting generation of distinct sphingolipid products has been linked to various cellular responses, including changes in gene expression (20), cell adhesion, and migration (13). In skeletal muscle, ceramide has been shown to increase the production of oxidants and to stimulate apoptosis (30, 129), while its downstream metabolite, sphingosine, to alter calcium handling by modulating the normal function of the ryanodine receptor (106). While several lines of evidence suggest that bioactive sphingolipid metabolites indeed are necessary for induction of muscle weakness, in several scenarios and pathophysiological conditions, the exact mechanisms remain elusive.

Recently, our group reported that TNF-induced skeletal muscle weakness is mediated by reactive nitrogen species specifically generated by the neuronal nitric oxide synthase isoform (nNOS) (120). NO is synthesized from molecular oxygen and L-arginine by the enzyme NO synthase (NOS) with L-citrulline as the by-product (118). NO in healthy skeletal muscle was first described by Kobzik et al (51), and of the three known isoforms, skeletal muscle most highly expresses the neuronal NOS isoform as an elongated muscle specific splice variant (nNOSμ) localized to the subsarcolemmal region.
nNOS activity can be regulated by post-translational modification at specific residues, and requires calcium and translocation to the membrane for full activation. Exposure to NO can decrease the contractile function of skeletal muscle (2, 51) but the possibility that sphingolipids affect NO production in muscle, or for that matter in non-muscle cells, however has never been tested. The aim of this study is to understand the link between TNF, the activation of sphingomyelinase and nNOS in murine skeletal muscle-derived C2C12 cell line.

3.3. Materials and Methods

3.3.1. Cell Culture. Myoblasts from the murine skeletal muscle-derived C2C12 cell line (American Type Culture Collection, Rockville, MD) were cultured as in Smith, et al. (113). Cells were seeded at 10,000 cells/cm² in Dulbecco’s modified Eagle’s medium with 1.6g/L sodium bicarbonate, 10% fetal bovine serum, and 100U/mL PenStrep (Invitrogen, Carlsbad, CA) in 5% CO₂ at 37°C. After two days, myoblast cultures were serum restricted in FBS-free medium containing 2% horse serum. Mature myotubes were obtained 5 days after serum restriction. Cell culture medium was replaced every 48 hours until cells were used for experimentation.

3.3.2. Neutral sphingomyelinase (nSMase) activity. nSMase activity was assessed using the method as described by Nikolova-Karakashian, et al. (87) and Merrill, et al. (84). Sample (20 ug protein) was diluted into 30uL assay buffer [25 mM Tris pH 7.5,12 mM MgCl₂, 15 uM fluorescent C6-NBD-sphingomyelin (6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino) hexanoyl) sphingosyl phosphocholine); Invitrogen] and incubated at 37°C for 3 hours. All reaction buffers were supplemented with phosphatase and protease
inhibitor cocktails. Reactions were stopped by the addition of 500 uL methanol (HPLC grade, Mallinckrodt, St. Louis, MO). After further incubation at 37C for 30 min, the samples were centrifuged at 15,000 x g for 5 min and the generation of the fluorescent product, NBD-ceramide, was monitored by reverse phase HPLC using methanol:water:phosphoric acid (850:150:0.15, by volume) as a mobile phase.

3.3.3. Sphingolipid analyses. Sphingolipids were analyzed for content at two different facilities. TNF-treated myotubes were harvested in methanol and transferred to the University of Kentucky Center of Research in Obesity and Cardiovascular Disease Analytical Research Core for sphingolipid analysis (22). Lipids were extracted in methanol:cholorform:HCl. Ceramides and other sphingolipids were detected by HPLC tandem mass spectrometry using an ABI 4000 Q-Trap hybrid linear ion trap triple quadrupole mass spectrometer. The methods employed were minor adaptations of those published by Sullard, et al (122). Myotubes analyzed at the Lipidomics Core at the Medical University of South Carolina were harvested in methanol, dried under vacuum, and shipped on dry ice to the for further extraction and analysis. Sphingolipids were separated by high-performance liquid chromatography, introduced into the electrospray ionization source, and then analyzed by tandem mass spectrometry using a TSQ 7000 triple quadrupole mass spectrometer (Thermo-Fisher Scientific) as described previously (112).

3.3.4. Silencing RNA transfection. Neutral ceramidase (ASAH2) and neuronal-type nitric oxide synthase (NOS1) expression were silenced as described by Moylan, et al. (85) using siRNA (Silencer Select, Ambion – Invitrogen) technologies. Cultured cells were
transfected 3 days after serum restriction using 20 nM siRNA plus Oligofectamine (Invitrogen). Myotubes were tested 48 hours post-transfection.

3.3.5. Live-cell oxidant activity assays. Cytosolic oxidants and nitric oxide were measured as previously described by Stasko, et al. (120). Mature myotubes were loaded for 30 minutes with either the fluorochrome probe 2’,7’ – dichlorodihydrofluorescin diacetate (DCFH; 10 μM; excitation/emission 492-495/517-527 nm Molecular Probes, Eugene, OR) or 4-amino-5-methylamino- 2’,7’ difluorofluorescin diacetate (DAF-FM; 5 μM; 495/515 nm, Molecular Probes) prior to pharmacologic intervention (see below). Accumulation of the oxidized derivative DCF was measured using a fluorescence microscope (TE 2000S, Nikon, Melville, NY). Fluorescence images were captured using a CCD camera (CoolSNAP-ES, Roper Scientific Photometrics, Tuscon, AZ) controlled by a computer with image acquisition software (NIS Elements, Nikon). Accumulation of the fluorescent oxidized derivative DAF, was measured using a Synergy H1 Multi-Mode Microplate Reader (BioTek, Winooski, VT). Fluorescence was recorded and calculated by the Gen5 2.00 computer software (BioTek)

3.3.6. Pharmacologic solutions. After 30 minute loading with fluorochrome probe DCFH or DAF-FM, myotube cultures were treated with TNF 6 ng/mL, sphingosine 10μM (Avanti Polar Lipids, Alabaster, AL), or C6 ceramide 20 μM (Avanti Polar Lipids) for 15 minutes at 37°C. In a subset of myotube cultures, 30 minute co-pretreatment with the fluorochrome was done with pharmacologic inhibitors N-oleoylethanolamine (NOE; 10 μM; Sigma-Aldrich), N°-nitro-L-arginine methyl ester 10μM (L-Name; Sigma-Aldrich), N°-nitro-D-arginine methyl ester 10μM (D- Name; Sigma-Aldrich), or 2S-dimethylamino- 4E- octadecene- 1, 3R-diol 10μM (N,N-Dimethyl Sphingosine; DMS;
Cayman Chemical). All probes were introduced in their respective loading vehicles according to their published product information guidelines.

3.3.7. Statistical analysis. All comparisons were performed using Prism 5.0b (GraphPad Software, La Jolla, CA). Differences were considered significant at the P <0.05 level. Results are reported as means ± SE.

3.4. Results

3.4.1. Effect of TNF on sphingolipid metabolites. Activation of neutral sphingomyelinase is a hallmark of TNF action in many cell types. As illustrated in Figure 3.1, the exposure of C2C12 myotubes to TNF, similarly lead to a rapid increase in sphingomyelinase activity and to altered sphingolipid composition. As measured by mass spectrometry, exposure to TNF decreased total sphingomyelin content (Fig. 3.1A), which is consistent with an activation of sphingomyelinase. An in vitro assay for nSMase using NBD-SM as substrate also showed more active degradation of the NBD-SM substrate in lysates from TNF-treated cells as compared to lysates from control cells (data not shown). However, this increased SM hydrolysis was accompanied by a decrease, rather than an increase in ceramide content of the cells (Fig. 3.1B), as well as in the amount of NBD-Ceramide produced during the in vitro nSMase activity assay (Fig. 3.1C). Earlier studies on the role of sphingolipids in cytokine signaling have shown that activation of sphingomyelinase is sometimes coupled to activation of ceramidase (87), which might explain the lack of ceramide accumulation in our system. To test this, we measured the levels of sphingosine, the downstream metabolite of ceramide. Indeed, TNF treatments
lead an increase in sphingosine levels in the same time course (Fig. 3.1D). In contrast, the levels of sphingosine-1-phosphate showed no change (Fig. 3.1E). Together these data indicate that TNF indeed stimulated sphingomyelin turnover in skeletal muscle; however, instead of ceramide accumulation, a marked increase in the levels of ceramide metabolite sphingosine was observed, implicating the latter as a plausible mediator of TNF signaling.

3.4.2. TNF, ceramide, and sphingosine effects on oxidants and NO production. Direct exposure to TNF increased general oxidant activity (+14.5% ± 0.9; P<0.05) in the cytosolic compartment of skeletal muscle cells (Fig. 3.2A) as determined using DCFH, a fluorescent probe that is sensitive to both reactive oxygen (ROS) and nitrogen (RNS) species. In a similar fashion, exposure to C6 ceramide or sphingosine also increased the oxidant activity by +24.9% ± 4.6 and 15.8% ± 6.2 (P<0.05), respectively (Fig. 3.2A). To decipher the specific contribution of NO to this increased oxidant activity, we used DAF-FM, a fluorescent probe that preferentially detects RNS derivatives, and administered the same battery of stimulants. As expected, TNF increased DAF-FM fluorescence by 33.2% ± 6.8 (P<0.05). More notably, C6 ceramide and sphingosine treatment elicited similar effects by increasing DAF-FM fluorescence by 14.5% ± 1.0 and 18.3% ± 0.8 (P<0.05), respectively (Fig. 3.2B). Together, these data suggest these sphingolipid metabolites may act as potential mediators of TNF action upstream of NO production.

3.4.3. Sphingosine, a mediator of TNF post-receptor signaling. To distinguish whether ceramide or sphingosine mediate a rise in TNF-induced oxidant activity, C2C12
myotubes were transfected with siRNA against the neutral ceramidase (ASAH2), the main enzyme responsible for the conversion of ceramide to sphingosine, or siRNA against a scrambled control sequence (N1). Quantification of general oxidant activity indicated that the TNF-induced increase was eliminated with knockdown of neutral ceramidase ASAH2 (Fig. 3.3A), while the response was not affected in cells treated with the scrambled control. TNF-treated myotubes were then exposed to the general ceramidase inhibitor N-oleoylethanolamine (NOE). The pharmacological inhibition of ceramidase significantly blunted the induction of NO by TNF (P<0.05) (Fig. 3.3B). As follow up, siRNA targeted treatment of ASAH2 also completely eliminated the TNF-stimulated increase in NO activity measured using DAF-FM fluorescence (Fig. 3.3C).

Together these data suggested that sphingosine, rather than ceramide is involved in activation of NO generation. In several systems however, the biological effects of sphingosine have been attributed to its conversion to sphingosine-1-phosphate. To eliminate this possibility, additional experiments were done. Myotubes were pretreated with dimethylsphingosine (DMS) to inhibit the activity of sphingosine kinase, and sphingosine-induced NO generation was measured. Treatment with DMS however had no effect on the effects of sphingosine, suggesting that indeed sphingosine alone and not sphingosine-1-phosphate is sufficient to stimulate NO production (Fig. 3.4). In sum, these results indicated that sphingosine is both a sufficient and a required step in the pathway linking TNF and NO production.

3.4.4. nNOS, a new target of sphingosine. Recently, we identified the neuronal-type nitric oxide synthase (nNOS) isoform, as a source of NO generation during TNF-induced weakness in skeletal muscle (120). To test whether sphingosine acts up-stream of
nNOS in the TNF signaling cascade, myotubes were first treated with a general nitric oxide synthase (NOS) inhibitor, N\textsuperscript{\textomega} -nitro-L-arginine methyl ester (L-NAME) or its inactive enantiomer D-NAME, and NO production was quantified using DAF-FM fluorescence. Treatment with L-NAME abolished the sphingosine induced rise in NO production when compared to muscles cells treated with D-NAME indicating that indeed NOS is responsible for the elevation in NO production by sphingosine (Fig. 3.5A). An investigation of isoform specific NOS was performed using siRNA knockdown against the nNOS isoform. NO production increased when exposed to TNF (Fig. 3.5B) or sphingosine (Fig. 3.5C) in appropriate control-treated-groups, but was completely abolished upon depletion of nNOS. These results indicate nNOS as necessary for TNF-induced NO production, implicating that sphingosine-induced nNOS as an important new target for abrogating TNF-induced muscle weakness.

### 3.5. Discussion

This chapter delineates a novel component of the pathway by which TNF induces muscle weakness. Using a highly controlled cell culture system, we demonstrate that TNF-treated skeletal muscle cells rapidly increase the abundance of sphingosine in a sphingomyelinase and ceramidase dependent manner. The sphingosine accumulation is sufficient and required for TNF-induced NO production via the nNOS isoform.

Skeletal muscle weakness affects many patients suffering from diseases of chronic inflammation. Circulating TNF and SMase are elevated in the serum of these patients (12, 23) that correlate with their skeletal muscle weakness (23). TNF is known to depress muscle contractile function through a pathway that involves the TNFR1-activated
signaling cascade (43) and elevated oxidative stress (69), particularly reactive nitrogen species (120). The intermediate components of the pathway however remained unclear.

TNF and other cytokines have been known to stimulate sphingomyelinase activity and generate the bioactive metabolite ceramide in various cell types, including muscle (49, 88). In skeletal muscle, the TNF-induced stimulation of nSMase activity has been causatively linked to the induction of muscle weakness, since like TNF, exogenous sphingomyelinase can also depress contractile function of skeletal muscle ex vivo (30). As with TNF, the effects are dependent upon elevated oxidant stress, suggesting that nSMase and oxidative stress are components of the same pathway for TNF-induced muscle weakness (31). Based on these observations it was hypothesized that ceramide, the product of SMase activation may be a mediator of TNF-induced oxidative stress in the muscle. Our results support a role of sphingolipid signaling cascade in TNF-induced muscle weakness and NO generation, however they implicate sphingosine, the product of ceramide degradation, as the key mediator in the process. While indeed treatment of the cells with exogenous C6 ceramide mimicked well the effects of TNF on NOS, so did the addition of exogenous sphingosine. Numerous studies have shown that exogenous C6 ceramide can be rapidly hydrolyzed to sphingosine by neutral ceramidase in the cells, resulting in a net increase not only of ceramide content but also that of sphingosine. Although the opposite, i.e. the conversion of exogenously added sphingosine to ceramide, is also possible, this process is typically slower. More importantly however, instead of the anticipated rise in ceramide content after TNF stimulation, our results revealed a swift increase in sphingosine instead. In fact, TNF stimulated a decrease in ceramide species with no change in sphingosine-1-phosphate levels.
Increases in sphingosine in response to exogenous TNF have been previously reported for cardiomyocytes (58, 89), but not at such short time intervals as our observations. It should be noted that in contrast to ceramide, which is highly hydrophobic and remains at the site of production, sphingosine is amphipathic in nature and readily moves through the plasma membrane and cytosol (42). The ceramide generated at the plasma membrane upon nSMase activation might alter membrane properties to allow for calcium influx and other signaling events at the level of the plasma membrane (26), however only its product sphingosine could serve to transmit the signal intracellularly. In fact, sphingosine is found at high levels in the junctional T-tubules of skeletal muscle (88). These organelles are in close proximity to the cell surface sarcolemma in skeletal muscle (118), thus placing sphingosine near to nNOS and the primary site of calcium handling for cross-bridge activation, potentially affecting force generation (106).

With the successful identification of distinct sets of downstream targets for ceramide, sphingosine, and sphingosine-1-hosphate, the importance of sphingosine for cell signaling has been well established. Nevertheless, the specific enzymes responsible for its generation only recently began to immerge. The results from our present study indicate that elevated sphingosine levels are the results of apparently coordinated degradation of plasma membrane sphingomyelin to ceramide and then to sphingosine. Neutral sphingomyelinase 3 (smpd4) and neutral ceramidase (asah2) are likely the enzymes responsible for these conversions. A recent study by our group (submitted for publication) characterized smpd4 as the only form of neutral sphingomyelinase activated by TNF in skeletal muscle. This current study demonstrates that at least at very early time
points, the activation of smpd4 is coupled to the action of asah2 to generate sphingosine, rather than ceramide. Ceramidases are a family of 5 enzymes, which in addition to asah2, involves the acidic ceramidase, asah1, and the three forms of alkaline ceramidase, acer1-3. These 5 enzymes differ mainly in their subcellular localization and respectively, the pool of ceramide on which they act. Previous studies had shown that asah2 is important for the progression through cell cycle (141), neuronal differentiation (127), keratinocyte apoptosis (130) as well as during the intestinal inflammatory response (114). These and other studies help identify the ceramidases, including asah2, as attractive novel targets for drug development, especially anticancer treatments (7, 97, 121). In this study, the inhibition of neutral ceramidase specifically with siRNA or pharmacologically using a general inhibitor (NOE) prevented the TNF-stimulated oxidant activity and NO production. These results join relatively small number of published studies identifying asah2 as key ceramidase responsible for the conversion of ceramide to sphingosine during cell signaling.

An accumulation of sphingosine-1-phosphate, in addition to that of sphingosine was another possible explanation for the TNF-induced increase in NO activity. As shown by others, sphingosine-1-phosphate is known to cause an increase in NOS via activation of the endothelial NOS, NOS3, in endothelial cells (94) as well as elevation in ROS via extracellular receptor activation (98) in muscle. Our results using a pan-inhibitor of sphingosine kinase (di-methylsphingosine), exclude this possibility, as DMS had no effect on sphingosine-induced NOS NOS and/or ROS generation, suggesting that the effects of sphingosine on NOS system are not mediated via sphingosine-1-phosphate.
TNF-initiated oxidant signaling has important implications for skeletal muscle function. Previously our laboratory reported that TNF-initiated oxidant signaling in mouse diaphragm leads to muscle weakness (43) and that treatment of isolated mouse muscle fiber bundles \textit{ex vivo} with either C6-ceramide or bacterial sphingomyelinase stimulated oxidant production (30), thereby inhibiting force of contraction. Recently, we identified NO as the major TNF-induced oxidant, specifically from the nNOS isoform (120). The present study confirms TNF-induced NO production and suggests SMase-generated sphingosine as the mediator of these effects of TNF. A functional connection between nNOS and sphingosine however has never been documented. The molecular mechanisms by which sphingosine activates nNOS is currently unknown, but an effect on calcium homeostasis is one likely possibility. Our data suggest that general inhibition of NOS abolishes the effect of sphingosine on NO production in skeletal muscle cells. L-NAME is a well-known inhibitor of NOS that has been shown to block the effects of TNF on NO production (120). Furthermore, our cell culture model of siRNA-mediated ablation of nNOS was protective against TNF and sphingosine-induced NO production, indicating a functional connection between sphingosine and nNOS in skeletal muscle cells.
3.6. Figures

**Figure 3.1. Model of sphingolipid metabolism and sites of experimental inhibition.**

Sphingomyelin is hydrolyzed by sphingomyelinase into the ceramide. Ceramide is then converted into sphingosine by ceramidase. Sphingosine can be phosphorylated by sphingosine kinase into sphingosine-1-phosphate. ASAH2 siRNA knocks down ceramidase protein, limiting the ability of ceramide to be converted into sphingosine. N-olelethanolamine (NOE) is a pharmacologic inhibitor of ceramidase, effectively blocking the ability of ceramide to convert ceramide into sphingosine. Di-methylsphingosine (DMS) is a pharmacologic inhibitor of sphingosine kinase, blocking the conversion of sphingosine to sphingosine-1-phosphate.
Figure 3.2. Tumor Necrosis Factor (TNF) exposure increases general sphingomyelinase activity and alters sphingolipid composition in skeletal muscle cells. A. Total sphingomyelin content is decreased in C2C12 myotubes treated with TNF (6ng/mL, 15 mins) compared to PBS treated control; mean values shown ± SE; n = 3/group; *P<0.05 (paired t-test) Control vs TNF. B. Total ceramide species are decreased in C2C12 myotubes treated with TNF (6ng/mL, 15 mins) expressed as percent of untreated control; mean values shown ± SE; n = 10/group; *P<0.05 (paired t-test) Control vs. TNF. C. TNF (6ng/mL) decreases NBD-ceramide species at 30 seconds as the substrate in an in vitro neutral sphingomyelinase activity assay; mean values shown ± SE; n = 6/group; *P<0.05 (Two-Way ANOVA). D. Exogenous treatment of TNF (6ng/mL)
increases sphingosine as quantified by mass spectroscopy at 30 seconds when compared to baseline (time 0); mean values shown ± SE; n = 3/group; *P<0.05 (paired t-test) Time 0 vs. Time 30 seconds. E. Exogenous treatment of TNF (6ng/mL) does not alter sphingosine-1-phosphate levels as quantified by mass spectroscopy at any time point measured when compared to baseline (time 0); mean values shown ± SE; n = 3/group.
Figure 3.3. Tumor Necrosis Factor (TNF), C6 ceramide, and sphingosine increase general oxidant activity and nitric oxide in skeletal muscle cells. A. General oxidant activity quantified by fluorescent microscopy and use of 2’,7’-dichlorodihydrofluorescin diacetate was increased in C2C12 myotubes when treated with C6 ceramide (20µM, 30 mins), sphingosine (10µM, 30 mins), or TNF (6ng/mL, 30 mins), when compared to vehicle (PBS, 30 mins) control; mean values shown ± SE; n = 6/group; *P<0.05 (paired t-test) Vehicle vs. C6 Ceramide, Vehicle vs. Sphingosine, Vehicle vs. TNF. B. General oxidant activity quantified by fluorescent microscopy and
use of 4-amino-5-methylamino-2’,7’ difluorofluorescin diacetate was increased in C2C12 myotubes when treated with C6 ceramide (20µM, 30 mins), sphingosine (10µM, 30 mins), or TNF (6ng/mL, 30 mins), when compared to vehicle (PBS, 30 mins) control; mean values shown ± SE; n = 6/group; *P<0.05 (paired t-test) Vehicle vs. C6 Ceramide, Vehicle vs. Sphingosine, Vehicle vs. TNF.
Figure 3.4. Modulation of ceramidase protects against the rise in Tumor Necrosis Factor (TNF)-induced oxidants and nitric oxide. A. SiRNA targeted to neutral ceramidase (asah2) protects against TNF (6ng/mL, 30 mins) - induced increase in general oxidant activity as quantified from fluorescence of 2',7’ – dichlorodihydrofluorescein diacetate (DCF) in C2C12 myotubes transfected with siRNA targeting asah2 or control (n1); mean values shown ± SE; n = 7/group; *P<0.05 (paired t-test) N1 vs N1 + TNF; ## P<0.05 (Two-Way ANOVA) N1 + TNF vs. siRNA + TNF. B. SiRNA targeted to asah2 protects against TNF (6ng/mL, 30 mins) – induced increase in nitric oxide production. Nitric oxide production is quantified using 4-amino-5-methylamino- 2’,7’ difluorofluorescein diacetate, measured by microplate spectrofluoroscopy, and represented as fluorescence percent time 0; mean values shown ± SE; n = 6/group;
*P<0.05 (paired t-test) N1 vs. N1 + TNF; ## P<0.05 (Two-Way ANOVA) N1 + TNF vs. siRNA + TNF. C. General ceramidase inhibition protects against TNF (6ng/mL) – induced nitric oxide using N-oleoylethanolamine (NOE, 10µM, 30 mins) after 10 minutes. Nitric oxide is quantified as described in B and represented as fluorescence percent time 0; mean values shown ± SE; n = 6/group; *P<0.05 (paired t-test) NOE vs. TNF; ## P<0.05 (Two-Way ANOVA) TNF vs. NOE + TNF.
Figure 3.5. Sphingosine alone increases nitric oxide production. General sphingosine kinase inhibition protects against sphingosine (10µM) – induced nitric oxide using dimethylsphingosine (DMS, 10µM, 30 mins) after 10 minutes. Nitric oxide is quantified using 4-amino-5-methylamino-2’,7’ difluorofluorescin diacetate (DAF-FM), measured by microplate spectrofluoroscopy, and represented as fluorescence percent time 0; mean values shown ± SE; n = 6/group; # P<0.05 (paired t-test) Vehicle vs. Sphingosine; *P<0.05 (paired t-test) Vehicle vs. DMS vs ; ## P<0.05 (Two-Way ANOVA) DMS vs. DMS + Sphingosine.
Figure 3.6. Modulation of general nitric oxide synthase (NOS) and neuronal nitric oxide synthase (nNOS) protect against Tumor Necrosis Factor (TNF) and sphingosine (SPH)-induced nitric oxide. A. General NOS inhibition protects against sphingosine (10µM) – induced nitric oxide production in C2C12 myotubes. General NOS inhibition was done with N°-nitro-L-arginine methyl ester 10µM (L-Name) or the inactive enantiomer N°-nitro-D-arginine methyl ester 10µM (D-Name), nitric oxide production was quantified using 4-amino-5-methylamino- 2’,7’ difluorofluorescin diacetate (DAF-FM), emissions were measured by microplate spectrofluoroscopy, and values were represented as fluorescence percent time 0; mean values shown ± SE; n = 6/group; *P<0.05 (paired t-test) D-Name vs. D-Name + SPH ; ## P<0.05 (Two-Way
ANOVA) L-Name + SPH vs. D-Name + SPH. B. SiRNA targeted to NOS1 (nNOS) protects against TNF (6ng/mL, 30 mins) – induced increase in nitric oxide production when compared to scrambled N1 control in C2C12 myotubes. Nitric oxide production is quantified using 4-amino-5-methylamino- 2’,7’ difluorofluorescin diacetate, measured by microplate spectrofluoroscopy, and represented as fluorescence percent time 0; mean values shown ± SE; n = 6/group; *P<0.05 (paired t-test) N1 vs. N1 + TNF; ## P<0.05 (Two-Way ANOVA) siRNA + TNF vs. N1 + TNF. C. SiRNA targeted to NOS1 (nNOS) protects against sphingosine (10µM) – induced increase in nitric oxide production when compared to scrambled N1 control. Nitric oxide production is quantified as described in B and represented as raw fluorescence values after 10 minutes exposure; mean values shown ± SE; n = 7/group; # P<0.05 (paired t-test) N1 vs. siRNA; *P<0.05 (paired t-test) Vehicle vs. Sphingosine; ## P<0.05 (Two-Way ANOVA) Sphingosine vs. siRNA + Sphingosine.
CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

Patients suffering from diseases of chronic inflammation have skeletal muscle weakness that cannot be explained solely by disuse atrophy. This weakness amplifies a range of other symptoms, extending cost to the healthcare system. Our laboratory has had a long history of investigating the mechanisms of skeletal muscle weakness, particularly cytokine-induced muscle weakness. More recently, we have been interested in the role of the skeletal muscle redox environment and how oxidants mediate inflammatory response leading to contractile dysfunction. In experiments described here, we defined a critical role for ROS and NO in TNF-induced skeletal muscle weakness, and also implicated sphingolipids as important mediators of this signaling cascade. Using an in vivo model system, we injected C57BL/6 mice with TNF and measured the contractile function of intact diaphragm fiber bundles isolated from each animal after conditioning. To extend these findings, strain–matched fiber bundles were exposed ex vivo to TNF and contractile function was measured. In parallel, we used DCFH and DAF-FM live-cell microscopy as fluorescent probe assays to quantify diaphragm changes in general oxidant activity and NO production specifically. Further, by capitalizing on the power of genetic knockout animals, we showed that skeletal muscle exposed to TNF increases a neuronal nitric oxide synthase-specific NO production that, together with basal reactive oxygen species, is essential for TNF-induced contractile dysfunction.
It has been previously observed that patients with chronic inflammation have elevated levels of sphingomyelinase, the enzyme that converts sphingomyelin to ceramide, suggesting that sphingolipid metabolites may be critical mediators of TNF-induced muscle weakness. Using an in vitro system in which we could carefully measure TNF effects on sphingolipid metabolism, we showed the metabolite sphingosine was a major mediator of this response. Using siRNA knockdown and pharmacologic inhibitors of specific targets, we showed a potent physiologic interplay between sphingosine and cellular oxidant production. Taken together, our results suggest sphingosine induces nNOS-specific NO production, highlighting sphingosine as an important downstream mediator of TNF-induced NO production.

We believe the results of this work provide valuable guidance for investigators seeking to prevent or reverse TNF-induced contractile dysfunction in skeletal muscle. Continued investigative efforts at the intersection of the fields of sphingolipids and redox modulation might yield promising new targets for intervention. Our work provides strong rationale for pursuing three targets: (1) Ceramidase; (2) nNOSμ; and (3) Sphingosine Kinase.

- Ceramidase: Additional research on skeletal muscle function and how modulating the conversion of ceramide to sphingosine is needed. Exposure to TNF, sphingomyelinase, and short chain ceramide are known to depress contractile function, however yet to be elucidated is the effect of exogenous sphingosine. TNF-induced ceramidase activity and ceramidase isoform function has also been minimally explored in skeletal muscle. Experiments focused on defining isoform-
specific ceramidase function could be of particular interest as only pan-
ceramidase pharmacologic inhibitors are available. Our results suggest that sphingosine increases nNOS-specific NO production and that nNOS-specific NO is an essential mediator of TNF-induced contractile dysfunction. Identifying the ceramidase isoform in skeletal muscle responsible for conversion of ceramide to sphingosine could lead to development of specific inhibitors that could serve as effective therapeutic strategies against TNF-induced muscle weakness.

- **nNOSμ**: The effects of TNF on nNOSμ in skeletal muscle have yet to be functionally tested. nNOSμ is the skeletal muscle specific isoform of nNOS. Therefore, identifying the responsiveness of nNOSμ to a cytokine challenge is essential to characterizing its function in skeletal muscle homeostasis, as well as its role in cellular signaling in inflammation-induced skeletal muscle weakness. Besides its primary function to generate NO, little is known about this isoform as a player in other metabolic pathways. Utilizing nNOSμ siRNA in a cell culture system would provide a highly controlled environment in which to test its sensitivity to exogenous challenge. *In vivo* experiments using nNOSμ deficient knockout mice could test not only basal contractile function, but also function after challenge by exogenous stimuli.

- **Sphingosine kinase**: Our results set the stage for probing the mechanisms of enhanced conversion of sphingosine to sphingosine-1-phosphate. Skeletal muscle localization and quantification studies could be done, not only to further
characterize sphingosine kinase in muscle, but also to better understand methods of regulation and function based on cellular localization. If knockdown of sphingosine kinase using an siRNA in C2C12 cell culture models prevents an increase in TNF-induced NO production, then this would justify use of transgenic animals deficient in sphingosine kinase to measure contractile function under conditions of elevated TNF.
This section is included because of the valuable experience and training I gained from being involved in all aspects of this project. An opportunity such as this is rare to experience as a graduate trainee which included an introduction to working with human subjects, administering human physiologic testing, working with a third-party sponsor, and statistical analysis of a double-blind crossover study was directly related to the translational aspects of my training.

5.1. General Background

5.1.1. Study rationale. As reviewed elsewhere (32, 99), skeletal muscle fibers continually generate oxidants – reactive oxygen species and nitric oxide derivatives – that are detectable in both the cytosolic compartment and the extracellular space. Oxidant production is increased by strenuous exercise. As exercise intensity and duration increase, the rise in intracellular oxidant activity can depress myofilament function and alter calcium regulation, thereby disrupting contractile function of working myofibers. This process contributes importantly to the loss of force that occurs during fatiguing exercise.

Antioxidant pre-treatment can buffer the rise in muscle-derived oxidants during exercise, thereby delaying fatigue. Among antioxidants classes, reduced thiol-donors appear to be the most effective in opposing fatigue. These compounds increase the amount of reduced cysteine available for cellular resynthesis of glutathione (GSH), an
important non-enzymatic antioxidant that is abundant in muscle cells. Changes in the redox status of thiol markers – cysteine and GSH – reflect changes in oxidant activity during exercise and can function as biomarkers to predict performance. Pre-treatment with thiol-based antioxidants preserves thiol redox status during fatiguing exercise and limits the decline of muscle force (27, 28)

In human studies, the benefits of thiol antioxidants have largely been studied using N-acetylcysteine (NAC), considered as a drug and a nutritional supplement used to prevent hepatotoxicity in acetaminophen overdose. Pretreatment with NAC delays muscle fatigue in healthy subjects, an action documented by multiple laboratories using a wide variety of exercise protocols (80, 82, 83, 103). NAC also delays fatigue in patients with chronic obstructive pulmonary disease (54), suggesting thiol donors might be used to preserve mobility and facilitate physical activity in chronically ill patients. However, oral intake of NAC causes gastrointestinal side effects that can be unpleasant (29, 103), limiting its use in this setting.

DGB-01 is a cysteine-rich, non-heated whey protein isolate that supports glutathione synthesis, has antioxidant properties, and is commercially available as a nutritional supplement. DGB-01 has been shown to improve muscle performance during volitional tasks (38) but has not been tested for its capacity to inhibit muscle fatigue. The current study tested the hypothesis that DGB-01 supplementation would support thiol homeostasis, delay fatigue during strenuous exercise, and improve task performance on a 40km cycling time trial.

5.1.2. DGB-01 product information. DGB-01 is an instantized version of Immunocal®. Immunocal® is a nutritional supplement patented to raise glutathione
(PDR (Physician’s Desk Reference) 2008, Montvale, NJ: Thompson Healthcare Inc.). It has been available as a non-prescription supplement in the USA and Canada for more than 10 years. It is composed of specially prepared whey protein isolate. Specific proteins when in their undenatured form serve as potential glutathione precursors including lactoferrin, serum albumen, alpha lactalbumin, and immunoglobulins. These proteins are rich in cysteine and cystine residues that are bioavailable for cellular absorption and subsequent glutathione synthesis.

In clinical trials, Immunocal® significantly raised glutathione levels in conditions like obstructive lung disease (78), liver dysfunction in patients with chronic hepatitis B (133), cystic fibrosis (39), and in healthy athletes (38). To date, no serious side effects have been reported. Among the rare, adverse reactions are gastrointestinal bloating and cramps if the subject has not consumed enough water. Transient urticarial-like rashes may also occur in rare occasions.

5.1.3. Casein. Casein is a milk protein extract with an inclusive amino acid profile, slow digestion, and good bioavailability. It has effects on protein turnover and may promote accretion over time. Casein has little cysteine and was used as a placebo control in the current study.

5.2. Research Objectives

The purpose of this study was to evaluate DGB-01 effects on performance of a 40-km time trial in trained cyclists. The hypothesis was that DGB-01 will improve performance on the time trial as measured by a reduction in the amount of time required to complete the distance using a computerized cycle ergometer (CompuTrainer Lab; Racermate, Inc., Seattle, WA) Further, we hypothesize that DGB-01 will increase total
cysteine and the ratio of reduced-to-total cysteine as well as total GSH and the ratio of reduced-to-total GSH in the plasma of participants.

5.3. Investigational Plan

The study was a double-blind, placebo-controlled, randomized, crossover study that involved one clinical site. Each subject was tested for his capacity to perform the 40-kilometer time trial (cycling course of New York City Triathlon) after taking the study product DGB-01 or placebo (casein) as a dietary supplement. Subjects made a total of five visits to the test facility after the screening visit. Each subject was given a unique randomization number, corresponding to the treatment allocated. Each subject received either DGB-01 or casein during the initial period (Visit 2-3: daily intake of approximately 20g taken in (2) 10g dosages) for the product duration of 28 days. The 10g dosages were to be taken orally within 2 hours of waking and the second within 1-1.5 hours at the end of the primary workout for that day. On ‘rest days’ where there were no planned workouts, the second dose was taken within 2 hrs of lunch. After a washout period of at least 30 days, subjects received the alternative study product/supplement during the final period (Visit 4-5: daily intake of approximately 20g taken in (2) 10g dosages) and instructed to take as done prior in the first period (Visit 2-3) above. All subjects consumed the study product for at least 28 days between Visit 2 – 3 and between Visit 4 – 5. Every effort was made to schedule visits so that duration of supplementation was exactly 28 days.

5.4. Study Population

Twenty-six (26) healthy, male, trained cyclists, between the ages of 18-60 were screened and enrolled in the study. Of the 26, 23 finished the study in its entirety, and 19
complete profiles were utilized for data analysis. Two subjects were excluded for investigator discretion of poor performance well outside of what the subject demonstrated in prior visits, one subject was excluded due to a flat tire during a time trial run, and one subject left the study early not consuming all study packets to join a professional triathlon team located in San Diego, CA. All subjects enrolled: (1) had to be training or regularly competing in either triathlon or cycling competition; (2) have a VO₂max greater than or equal to 45 mL O₂/kg/min; and (3) be in the competitive phase of their annual training cycle. Subjects were recruited for the study by word-of-mouth, flier placement, and direct contact. Subjects were excluded if they had: (1) had a specific allergy to milk proteins; (2) currently on immunosuppressive medication; (3) had planned surgeries; (4) body weight greater than 285 pounds; (5) abnormal BUN, creatinine, hemoglobin, or hematocrit; or (6) were currently using dry whey protein supplements, N-acetylcysteine, or α-lipoic acid supplements.

5.5. Study Procedure

5.5.1. Screening. Visit 0: Informed consent for the protocol was obtained prior to any study-related test or procedure. The subject underwent a brief physical exam by Dr. William Black, MD focusing on the heart, lungs, legs, and back. Blood pressure, pulse/heart rate, respiratory rate, height, weight, medical history, and current prescriptions/supplements were recorded. Subjects also completed a cardiovascular questionnaire and had 5-10 mL of blood drawn for hemoglobin, hematocrit, BUN, and creatinine measurement.

5.5.2. Orientation. Visit 1: Subjects were introduced to the University of Kentucky Center for Clinical and Translational Science facility and program personnel.
Daily log books were given so each subject could record his daily exercise activities, injuries, illnesses, or other events that affected time trial performance. Baseline body composition [dual energy x-ray absorptiometry (DXA)] and maximal oxygen uptake/blood lactate concentration [maximal graded cycling exercise (Max GXT)] were performed. During the Max GXT, venous blood samples (7 mL) were drawn from the antecubital vein and heart function (via 12-lead ECG) were administered. After completing the Max GXT, the subjects completed a 40-km familiarization ride on the time trial course using their own cycling equipment and a computerized cycle ergometer (CompuTrainer Lab; Racermate, Inc. Seattle, WA) at a comfortable, self-selected pace.

5.5.3. Pre study product. Visit 2: Approximately one week after Visit 1, each subject returned to the lab prepared for a maximum effort on the same 40-km time trial test as ridden one week prior. Each subject was asked to complete two pre-exercise questionnaires: the Standard Overtraining Questionnaire of the French Society for Sports Medicine (SFMS) and the Adverse Effects Questionnaire. The subject then performed a 20-minute warm up followed by a maximal effort of the 40-km time trial on the ergometer. Venous blood samples (5 mL) were collected from the antecubital vein prior to exercise and during the final 30 seconds of the time trial. Plasma was isolated, transferred to the appropriate solutions for analysis by the Emory Clinical BioMarkers Laboratory (Emory, GA) (DP Jones, 200x) and stored at -80°C for post-hoc analyses of thiol metabolites (glutathione (GSH), oxidized glutathione (GSSG), cysteine (Cys), cystine (CySS), and cysteine-glutathione disulfide (CysGSH)). Plasma samples were stored for no longer than 10 days to prevent sample deterioration. After completeing the time trial, the subject received two post-exercise questionnaires: Foster’s Psychological
Complaint Index and Classification of Muscle Soreness. They also received two boxes, each including 28 pouches of study product – DGB-01 or casein-, mixing instructions, and a mixing container to standardize mixing procedure.

5.5.4. Study product start. The morning after Visit 2: Subjects awoke and completed the post-exercise questionnaires and began consuming one of two study products. The order of the study product administration was selected randomly. If subjects conducted normally scheduled exercise, they consumed the second of 2 packets 1-1.5 hours post exercise. This was repeated daily for 28 days.

5.5.5. Post study product. Visit 3: This visit occurred 28 days after taking the study product. The subjects were informed to save and bring back all opened study packet material and two post questionnaires. Again, each subject came prepared for a maximal effort on the 40-km time trial test. Each subject repeated the pre-exercise questionnaires, completed his 20 min warm up, and maximal effort 40-km time trial. Venous blood samples (5 mL) were drawn before exercise and during the final 30 seconds of exercise; plasma was isolated and stored for analysis as previously described. After the time trial, the subject was given the two post-exercise questionnaires to be filled out the following morning. The morning after Visit 3 the subject repeated both post exercise questionnaires and began a 28 day, study product free washout period. The subject continued using and filling out the daily Log book throughout the duration of the washout period.

5.5.6. Pre study products/supplement. Visit 4: (Pre Supplement B) at least 4 weeks after Visit 3, the subject returned to the lab and repeated the protocol used in Visits 2-3. The subject received 2 boxes of the opposite study product – DGB-01 or casein –
which contained approximately 10g of protein per packet. The next morning the subjects filled out the post exercise questionnaires and began taking their study product for the 28 days.

5.5.7. Post study product/supplement. Visit 5: the final visit occurred after 28 days of consuming the alternate supplement. The protocols from Visits 2-4 were repeated and the log book was collected. Subjects were given post exercise questionnaires and a self-addressed stamped envelope in which to be used to send back study questionnaires the following day. The subject brought back any study material, including unused product packets, in order for us to monitor compliance.

5.5.8. Statistical analysis. Based on prior data, we predicted that 20 subjects were required to achieve a minimum power of 0.8 and a type I error rate (p value) of 0.05. This sample size provided adequate statistical power to detect moderate differences in function between supplements. Data was managed electronically using commercial software and was analyzed using univariate analyses, analysis of variance, linear regression, and linear mixed models for repeated measures. The primary end point of the study was evaluated by the indices of exercise performance: time to complete the 40-km time trial (in seconds) of Group A with DGB-01 intervention in period 1 versus the completion of time trial of Group A with casein in period 2 and time trial of Group B with DGB-01 intervention in period 2 versus the time trial completion of Group B with casein in period 1. The secondary end points included changes in plasma thiol status (GSH, GSSG, CysGSH, CySS, Cys, total glutathione (TGSH), and total cysteine (TCys). TGSH was calculated as GSH + 2*GSSG + CysGSH. Total cysteine was calculated as Cys + 2*CySS + CysGSH. Results from questionnaires, SFMS Overtraining, Fosters
Psychological Complaint Index, and Classification of Muscle Soreness were also evaluated but results not reported.

5.6. End-point Results

5.6.1. Primary end-points. After analysis of 19 subjects, DGB-01 did not show a performance benefit in time to completion of the 40 kilometer time trial. The average time to completion (TTC) in all groups on Visit 2 was 4113.37 ± 252.83 seconds, Visit 3: 4080.68 ± 196.19 seconds, Visit 4: 4015.26 ± 223.64 seconds, and Visit 5: 4054.68 ± 240.83 seconds. When subjects consumed DGB-01, the average TTC pretreatment was 4090.95 ± 229.53 seconds and post treatment 4095.53 ± 229.27 seconds; a difference of +4.58 seconds (P = 0.894). When subjects consumed placebo casein, the average TTC pretreatment was 3921.3 ± 246.12 seconds and post treatment 3837.85 ± 206.51 seconds; a difference of -87.84 seconds (P = 0.097). Average power was also measured and compared between group treatments. The average power output (in watts) in all groups on Visit 2 was 264.58 ± 28.39, Visit 3: 267.58 ± 31.70, Visit 4: 267.58 ± 35.88, and Visit 5: 272.00 ± 39.72 watts. When subjects consumed DGB-01, the average power output difference between pre and post treatment was +0.053 watts (265.63 ± 30.98 vs. 265.68 ± 36.04, P = 0.991). When subjects consumed casein, the average power output difference between pre and post treatment was +9.05 watts (264.84 ± 33.68 vs. 273.89 ± 35.48, P = 0.013), yielding a significant improvement in power output. Overall, these results indicate no benefit in TTC performance or average power output with DGB-01. Instead, the findings suggest that casein may improve overall performance since TTC trended towards statistical significance and average power was significantly higher post supplementation.
5.6.2. Secondary end-points. Each subject had their plasma analyzed for changes in thiol status four times per study period: (1) pre exercise pre supplement; (2) post exercise pre supplement; (3) pre exercise post supplement; and (4) post exercise post supplement. When subjects consumed DGB-01, CySS and TCys increased significantly. DGB-01 did not increase reduced GSH, GSSG, or CysGSH; mean values of all markers reported in Table 5.2. When subjects consumed casein, no increases in any resting blood thiol markers prior to exercise were observed.

5.7. General conclusions

As a method of cycling performance testing, the computrainer was reliable during protocols requiring repeated measures when a strict calibration protocol was used. This observation is similar to other studies demonstrating the computrainer’s reproducibility and accuracy when compared with other laboratory methods collecting similar data (24).

Supplementing normal adult male cyclists for one month with a modified whey protein rich in cysteine increases resting cysteine level in the plasma but did not have any effect on performance of a 40 kilometer time trial. Similar changes in thiols were observed supplementing the same modified whey protein rich in cysteine, however using a different duration of supplementation and exercise protocol (38). Interestingly, by supplementing normal adult male cyclists for one month with casein (placebo), we found increases in average power output over a 40 kilometer time trial as compared to supplementing with whey protein. No studies have directly compared the effects of whey versus casein directly, but one study in soccer players demonstrated an increase in muscle mass in players consuming casein protein as compared to players consuming whey or hydrolyzed whey protein (11).
Finally, our results demonstrated a total plasma glutathione and glutathione-related increase in reducing capacity at the end of the 40K cycling TT. These results are countercurrent to what we hypothesized as the more oxidized glutathione redox potential has been discussed more prominently in the literature. Given the nature our exercise protocol, duration of exercise, timing of blood draw, and level of participant effort, we feel these results do not contradict, but add to the body of growing exercise literature measuring thiol changes in various tissues and compartments of blood.
Table A.1. Summary of Performance Data

<table>
<thead>
<tr>
<th></th>
<th>Time to Completion (seconds)</th>
<th>Average Power (watts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visit 2 Mean</td>
<td>4113.37 (252.83)</td>
<td>264.58 (28.39)</td>
</tr>
<tr>
<td>Visit 3 Mean</td>
<td>4080.68 (196.19)</td>
<td>267.58 (31.70)</td>
</tr>
<tr>
<td>Visit 4 Mean</td>
<td>4105.26 (223.64)</td>
<td>267.58 (35.88)</td>
</tr>
<tr>
<td>Visit 5 Mean</td>
<td>4054.68 (240.83)</td>
<td>272.00 (39.72)</td>
</tr>
<tr>
<td>Pre Treatment A</td>
<td>4090.95 (229.53)</td>
<td>265.631 (30.98)</td>
</tr>
<tr>
<td>Post Treatment A</td>
<td>4095.53 (229.271)</td>
<td>265.68 (36.04)</td>
</tr>
<tr>
<td>Difference A</td>
<td>+4.58</td>
<td>+0.053</td>
</tr>
<tr>
<td>P Value</td>
<td>0.894</td>
<td>0.991</td>
</tr>
<tr>
<td>Pre Treatment B</td>
<td>3921.3 (246.12)</td>
<td>264.84 (33.68)</td>
</tr>
<tr>
<td>Post Treatment B</td>
<td>3837.85 (206.51)</td>
<td>273.89 (35.48)</td>
</tr>
<tr>
<td>Difference B</td>
<td>-87.84</td>
<td>+9.05</td>
</tr>
<tr>
<td>P Value</td>
<td>0.097</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Summary of cyclist time to completion and average power generated during 40km time trial. Subjects consuming supplement A demonstrated no change in performance or average power compared to placebo B which trended towards a decrease in average time to completion and increased average power output; values are represented as means (SD); *P<0.05 (paired t-test).
<table>
<thead>
<tr>
<th>Visit</th>
<th>Mean</th>
<th>GSH (µM)</th>
<th>GSSG (µM)</th>
<th>CySGSH (µM)</th>
<th>Cys (µM)</th>
<th>CySS (µM)</th>
<th>Total Cys (µM)</th>
<th>Eh (GSH/GSSG)</th>
<th>Eh (Cys/CySS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.652 (1.481)</td>
<td>0.089 (0.152)</td>
<td>1.472 (0.800)</td>
<td>2.002 (0.891)</td>
<td>10.041 (3.166)</td>
<td>56.990 (8.708)</td>
<td>126.022 (19.600)</td>
<td>-128.586 (12.920)</td>
<td>-76.131 (8.468)</td>
</tr>
<tr>
<td>3</td>
<td>4.159 (2.109)</td>
<td>0.082 (0.827)</td>
<td>2.027 (1.273)</td>
<td>1.969 (1.273)</td>
<td>12.103 (6.832)</td>
<td>58.085 (17.796)</td>
<td>130.241 (38.584)</td>
<td>-128.923 (29.657)</td>
<td>-77.521 (18.859)</td>
</tr>
<tr>
<td>4</td>
<td>4.719 (3.022)</td>
<td>0.078 (0.092)</td>
<td>2.706 (2.058)</td>
<td>1.856 (1.074)</td>
<td>12.651 (5.759)</td>
<td>50.562 (13.465)</td>
<td>115.811 (29.073)</td>
<td>-142.290 (10.737)</td>
<td>-83.411 (10.662)</td>
</tr>
<tr>
<td>5</td>
<td>4.538 (2.303)</td>
<td>0.071 (0.074)</td>
<td>3.484 (4.316)</td>
<td>2.067 (0.971)</td>
<td>10.470 (2.750)</td>
<td>55.150 (11.149)</td>
<td>122.949 (23.871)</td>
<td>-140.731 (9.042)</td>
<td>-78.351 (5.781)</td>
</tr>
<tr>
<td>Pre Treatment A</td>
<td>4.137 (2.056)</td>
<td>0.095 (0.159)</td>
<td>2.083 (4.521)</td>
<td>1.865 (0.885)</td>
<td>11.746 (5.645)</td>
<td>52.875 (11.578)</td>
<td>119.361 (24.361)</td>
<td>-135.104 (14.594)</td>
<td>-80.659 (10.788)</td>
</tr>
<tr>
<td>Post Treatment A</td>
<td>4.336 (2.336)</td>
<td>0.081 (0.073)</td>
<td>2.186 (1.494)</td>
<td>3.026 (4.312)</td>
<td>12.927 (6.773)</td>
<td>59.605 (12.413)</td>
<td>135.164 (25.548)</td>
<td>-135.476 (12.166)</td>
<td>-81.449 (9.982)</td>
</tr>
<tr>
<td>Difference A</td>
<td>+0.198</td>
<td>-0.014</td>
<td>+0.103</td>
<td>+1.161</td>
<td>+1.181</td>
<td>6.730</td>
<td>15.803</td>
<td>0.372</td>
<td>-0.790</td>
</tr>
<tr>
<td>P Value</td>
<td>0.753</td>
<td>0.708</td>
<td>0.808</td>
<td>0.214</td>
<td>0.524</td>
<td>0.038</td>
<td>0.017</td>
<td>0.917</td>
<td>0.797</td>
</tr>
<tr>
<td>Pre Treatment B</td>
<td>3.793 (1.861)</td>
<td>0.058 (0.039)</td>
<td>1.765 (1.188)</td>
<td>1.912 (1.009)</td>
<td>10.745 (4.143)</td>
<td>53.055 (12.474)</td>
<td>118.768 (26.999)</td>
<td>-134.492 (13.933)</td>
<td>-78.519 (10.640)</td>
</tr>
<tr>
<td>Post Treatment B</td>
<td>4.271 (2.029)</td>
<td>0.071 (0.087)</td>
<td>2.108 (1.140)</td>
<td>2.018 (1.032)</td>
<td>10.553 (3.376)</td>
<td>56.009 (13.217)</td>
<td>124.707 (27.874)</td>
<td>-138.394 (11.270)</td>
<td>-78.157 (7.315)</td>
</tr>
<tr>
<td>Difference B</td>
<td>0.478</td>
<td>0.013</td>
<td>0.343</td>
<td>0.106</td>
<td>-0.192</td>
<td>2.954</td>
<td>5.939</td>
<td>-3.902</td>
<td>0.362</td>
</tr>
<tr>
<td>P Value</td>
<td>0.440</td>
<td>0.508</td>
<td>0.350</td>
<td>0.719</td>
<td>0.881</td>
<td>0.404</td>
<td>0.444</td>
<td>0.258</td>
<td>0.904</td>
</tr>
</tbody>
</table>

Summary of blood thiol markers when subjects were consuming supplement A or placebo B. Subjects consuming supplement A increased CySS and Total Cys as compared to placebo; values represented as means (SD) *P<0.05 (paired t-test).
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