Fiber Type-Specific Nitric Oxide Protects Oxidative Myofibers against Cachectic Stimuli

Zengli Yu1, Ping Li1, Mei Zhang3, Mark Hannink4, Jonathan S. Stamler1,2, Zhen Yan1,3*

Abstract

Oxidative skeletal muscles are more resistant than glycolytic muscles to cachexia caused by chronic heart failure and other chronic diseases. The molecular mechanism for the protection associated with oxidative phenotype remains elusive. We hypothesized that differences in reactive oxygen species (ROS) and nitric oxide (NO) determine the fiber type susceptibility. Here, we show that intraperitoneal injection of endotoxin (lipopolysaccharide, LPS) in mice resulted in higher level of ROS and greater expression of muscle-specific E3 ubiquitin ligases, muscle atrophy F-box (MAFbx)/atrogin-1 and muscle RING finger-1 (MuRF1), in glycolytic white vastus lateralis muscle than in oxidative soleus muscle. By contrast, NO production, inducible NO synthase (iNos) and antioxidant gene expression were greatly enhanced in oxidative, but not in glycolytic muscles, suggesting that NO mediates protection against muscle wasting. NO donors enhanced iNos and antioxidant gene expression and blocked cytokine/endotoxin-induced MAFbx/atrogin-1 expression in cultured myoblasts and in skeletal muscle in vivo. Our studies reveal a novel protective mechanism in oxidative myofibers mediated by enhanced iNos and antioxidant gene expression and suggest a significant value of enhanced NO signaling as a new therapeutic strategy for cachexia.

Introduction

Chronic diseases are often associated with and exacerbated by loss of lean body mass known as cachexia mainly due to skeletal muscle wasting. Recent research efforts have led to the current understanding of the molecular and signaling mechanisms responsible for skeletal muscle atrophy under various pathological conditions [1–7]. In particular, enhanced expression of muscle-specific E3 ligases, MAFbx/atrogin-1 and MuRF1, with function in protein ubiquitination have been shown to mediate proteosome-dependent protein degradation in muscle wasting. Therefore, expression of these genes is now served as an early marker of skeletal muscle atrophy. Skeletal muscles of different fiber type composition have different contractile and metabolic properties. Oxidative muscles (predominantly type I and/or Ila fibers) are generally rich, while glycolytic muscles (predominantly type IIId/x and/or IIb fibers) are poor, in mitochondria and capillaries. Interestingly, oxidative muscles are more resistant to atrophy than glycolytic muscles [8–13]; however, much remains unknown regarding the molecular mechanism(s) for the fiber type-specific muscle wasting. The “built-in” protection in oxidative myofibers provides an excellent experimental model to decipher the skeletal muscle wasting process and search for new drug target(s) to treat this detrimental symptom that affects the morbidity and mortality of many chronic diseases.

ROS are well recognized to have primarily deleterious effects in mammalian cells. High level, sustained increase in ROS leads to oxidative cellular injury that plays “cause-effect” roles in the pathogenesis of chronic diseases and syndromes including cachexia. The deleterious role of ROS in muscle wasting has been shown in previous studies. Firstly, increased production of ROS [14–17] and reduced antioxidant gene expression [18] are associated with muscle catabolism. Secondly, exogenous ROS activate proteosome-dependent protein degradation [6]. Lastly, antioxidant treatment attenuates skeletal muscle atrophy [19,20]. However, little is known about the fiber type differences of oxidative stress in skeletal muscle in cachexia.

The functional role of nitric oxide (NO) and related oxides of nitrogen (reactive nitrogen species, RNS) in skeletal muscle wasting is controversial and far less well understood except that it is well known that NO synthase (NOS) expression is often increased in muscle wasting disorders [11,21–25]. On the one hand, NO and RNS have been shown to acutely inhibit mitochondrial respiration and muscle contractile function [21,25–27]; thus a scavenger of ROS-RNS [23] or NOS inhibitor [25] prevents muscle dysfunction. On the other hand, iNOS expression has been shown to increase dramatically in disease-resistant oxidative muscles following pathological challenges, and iNOS inhibition increases disease susceptibility [11]. Furthermore, chronic inhibition of NOS resulted in muscle dysfunction and atrophy [28]. In light of these apparently conflicting findings, the functional role of NO and its derivatives in skeletal muscle wasting remain to be determined. In particular, there has not been an

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* E-mail: zhen.yan@duke.edu
attempt to define the functional relationship between ROS and NO in skeletal muscle under the catabolic condition.

We have recently obtained comprehensive physiological, morphological, biochemical and gene expression evidence in a mouse model of chronic heart failure to indicate that oxidative myofibers are more resistant than glycolytic myofibers to chronic heart failure [12]. Consistently, there are significant differences between these myofibers in their atrophic response to cachectic stimuli, such as LPS and TNF-α. To improve our understanding of the mechanism(s) underlying the protection associated with oxidative phenotype, we designed this study to focus on fiber type-specific differences in ROS and NO and to determine if NO plays a functional role of muscle atrophic process related to cachexia.

We obtained evidence that oxidative muscles had greater production of NO and expression of antioxidant genes, and endure lower oxidative burden than glycolytic muscles in response to cachectic stimuli. We then tested the hypothesis that up-regulation of antioxidant genes were dependent on NO, and thereby provided the protection from cachectic stimuli. This hypothesis was confirmed both in cultured muscle cells and in intact skeletal muscle in vivo. Results from this study provide potential mechanistic insights into the relatively greater resistance to cachexia associated with oxidative phenotype in skeletal muscle.

Results

Endotoxin induces less ROS production and muscle-specific E3 ligase expression in mouse oxidative soleus muscle than in glycolytic white vastus lateralis muscle

Oxidative myofibers are resistant to cachetic stimuli induced by chronic heart failure and inflammatory cytokines [12]. One possible explanation is that systemic inflammation induces less oxidative stress in oxidative than in glycolytic myofibers. To test this hypothesis, we injected mice with endotoxin and measured protein carbonylation as an index of oxidative stress. LPS injection (12 hours) did not result significant changes in carbonylated proteins in soleus muscles, but resulted in a significant (~50%) increase in white vastus lateralis muscles (Fig. 1A and 1B). LPS induced greater expression of muscle-specific E3 ligase MAFbx/atrogin-1 [1,3,4] in white vastus lateralis muscles than in soleus muscles (Fig. 1C and 1D). LPS also significantly increased MuRF1 expression in vastus lateralis muscles, but not in soleus muscles (Fig. 1C and 1E). These findings are completely consistent with our previous findings [12]. Thus, endotoxin challenge results in significantly less oxidative stress, hence less activation of the proteosome-dependant protein degradation, in oxidative than in glycolytic muscles. We have also observed similar changes in a mouse model of sepsis induced by S. aureas infection (unpublished results). Therefore, the fiber type-specific muscle atrophic response appears to be relevant to various cachetic conditions that can be recapitulated by endotoxin injection.

Endotoxin induces greater NO production and iNOS expression in mouse oxidative soleus muscle than in glycolytic white vastus lateralis muscle

To understand the possible functional role of NO-dependent signaling/gene regulation in fiber type specificity of muscle wasting, we assessed NO production in both soleus and white vastus lateralis muscles by measuring the stable NO product, nitrite. Nitrite concentration was not significantly different between these two muscles under basal conditions. However, LPS injection led to ~100% increase in nitrite concentration in soleus muscles, but not in white vastus lateralis muscles (Fig. 2A). The increase in nitrite in soleus muscle was associated with an enhanced iNOS expression (Fig. 2B, 2C and 2F) with majority of protein confined to the periphery of oxidative myofibers. In contrast, neuronal NO synthase (nNOS) expression was reduced in both soleus and white vastus lateralis muscles (Fig. 2D), whereas endothelial NO synthase (eNOS) expression was low in white vastus lateralis muscles under basal conditions and was induced to a level comparable to that in the soleus muscles (Fig. 2E) following LPS injection. These findings suggest that oxidative myofibers have greater inducibility in NO production, probably through induced expression of iNOS.

Endotoxin induces greater antioxidant gene expression in oxidative soleus muscle than in glycolytic white vastus lateralis muscle

Our finding of less oxidative stress in soleus muscles than white vastus lateralis muscles following endotoxin challenge prompted us to determine if enhanced antioxidant gene expression contributes to the protection of oxidative myofibers. We performed semi-quantitative RT-PCR analysis for mRNA of the antioxidant genes: superoxide dismutases (Sod1, Sod2 and Sod3) and catalase (Cat). LPS injection resulted in significantly increased expression for all four antioxidant genes in soleus muscles with no or less increases in white vastus lateralis muscles (Fig. 3A–E). We have, therefore, obtained evidence for differentially enhanced antioxidant gene expression in oxidative vs. glycolytic myofibers that are likely intrinsic to the muscles.

NO donor blocks TNF-α-induced muscle-specific E3 ligase expression and enhances iNOS and antioxidant gene expression in cultured muscle cells

Our findings that there are greater increases in NO production and antioxidant gene expression in oxidative than glycolytic muscles in response to cachectic stimuli suggest a functional link between NO production and the antioxidant defense system. To test this hypothesis, we cultured mouse C2C12 myoblasts with TNF-α in the presence and absence of NO donor, diethylenetriamine NO (DETA-NO). DETA-NO resulted in a moderate, but significant, reduction in the basal level expression of antioxidant genes in vastus lateralis muscles, but not in soleus muscles (Fig. 4A and 4B). DETA-NO increased TNF-α-induced MAFbx/atrogin-1 mRNA and completely blocked the induction of MAFbx/atrogin-1 induced by TNF-α (Fig. 4A and 4B). DETA-NO enhanced TNF-α-induced iNOS expression significantly (Fig. 4A and 4D). These findings indicate that NO is sufficient to reduce cytokine-induced activation of the proteosome-dependent catabolic pathway, which appears to be mediated by iNOS. To determine if NO induces antioxidant gene expression, we performed semi-quantitative RT-PCR analysis for Sod1, Sod2, Sod3 and Cat. DETA-NO increased the basal level and TNF-α-induced Sod3 and Cat expression, whereas Sod1 and Sod2 were not responsive (Fig. 4E–4I), suggesting that Sod3 and Cat play an important role in mitigating oxidative stress induced by cytokines in myocytes. To further confirm that these findings are due to NO-dependent transcriptional activation of the antioxidant genes, we transfected C2C12 myoblasts with the antioxidant response element (ARE) TATA-Ins luciferase reporter gene, pARE-Luc [29], and treated the myoblasts with or without DETA-NO for 24 hours [30]. The reporter gene expression was significantly stimulated by NO donor, suggesting that NO stimulates antioxidant genes through the cis-acting ARE DNA sequences.

S-nitrosoglutathione (GSNO) blocks endotoxin-induced muscle-specific E3 ligase expression and enhances iNOS and antioxidant gene expression in skeletal muscle in vivo

The findings in cultured muscle cells motivated an in vivo study to confirm the functional role of NO in intact skeletal muscle. We
injected the mice with the endogenous NO donor, GSNO (1 mg/kg, i.p.; 6 hours before and immediately before LPS injection) and performed semi-quantitative RT-PCR analysis in plantaris muscles (a muscle with a mixture of both oxidative type IIa and glycolytic type IId/x and IIb fibers). GSNO effectively blocked MAFbx/atrogin-1 mRNA expression induced by LPS (Fig. 5A and 5B) and promoted iNos mRNA expression both with and without LPS injection (Fig. 5C and 5D). Therefore, NO protects myofibers from endotoxin-induced oxidative stress and prevent activation of the proteosome-dependent catabolic pathway, possibly through induced expression of iNOS. RT-PCR analysis for Sod1, Sod2, Sod3 and Cat in plantaris muscles showed that GSNO resulted in increases in Sod3 and Cat, but not Sod1 and Sod2, expression with and without LPS injection (Fig. 5E–5I). These findings were entirely consistent with the findings in cultured myoblasts, suggesting the importance of SOD3 and CAT in NO-mediated protection against cachetic stimuli.

**Discussion**

The mechanisms underlying the protection of oxidative myofibers against cachexia have been unclear. The major findings of this study are: 1) Protein oxidation and degradative protein (muscle-specific E3 ubiquitin ligase) expression were lower in oxidative than in glycolytic muscles in response to cachectic stimuli; 2) iNOS expression, NO production and antioxidant gene expression were higher in oxidative muscles than in glycolytic muscles; and 3) NO donors enhanced iNOS and antioxidant gene expression and attenuated atrophic muscle responses both in vitro and in vivo. These findings together strongly suggest that NO-dependent up-regulation of the antioxidant genes, at least partly mediated by iNOS, protects oxidative myofibers against cachectic stimuli. The studies not only provide potential mechanistic insights into the functional protection in oxidative muscle fibers, but also
suggest the therapeutic value of enhanced NO signaling to the treatment of muscle wasting.

The fiber type-specific muscle wasting could be due to an intrinsic difference(s) in the oxidant/antioxidant system. We have recently shown that oxidative myofibers are protected from cachectic stimuli, such as LPS and TNF-α [12]. The finding in this study that endotoxin resulted in significantly less protein oxidation in oxidative muscle than in glycolytic muscle (Fig. 1A and 1B) is consistent with the notion that a more robust basal and/or inducible antioxidant system in oxidative myofibers prevents ROS from accumulating to a high level causing cellular damage. In fact, it has been shown that oxidative muscles have more robust expression and activity of antioxidant enzymes than glycolytic muscles [31,32], and there is less mitochondrial superoxide production in oxidative myofibers than glycolytic myofibers [33]. Our findings of enhanced antioxidant gene expression in oxidative muscles (Fig. 3A-3E) suggest that an inducible antioxidant system provides additional protection against muscle catabolism. Elucidation of the mechanism responsible for such an inducible defense system will likely lead to the discovery of new drug target(s) for cachexia.

We also found that oxidative muscles produce more NO than glycolytic muscle and exhibit attenuated atrophic responses following endotoxin challenge (Fig. 2A), raising the possibility of a cause-effect relationship between NO and muscle protection. Paradoxically, it has been shown that NO and RNS inhibit mitochondrial respiration and muscle contractile function [21,25–27], which could be prevented by ROS-RNS scavengers [23] or NOS inhibitors [25]. The interpretation was that iNOS mediated NO production was detrimental to muscle wasting. These previous findings were obtained in ex vivo experiments focusing on the acute effect of NO on muscle metabolic and contractile functions. Therefore, the mechanism(s) by which NO may protect against long-term muscle injury is currently not known. Our fiber type-specific analysis provides exciting new insight, suggesting induced NO production may play a protection against cachectic stimuli, rather than mediate muscle wasting.

NO production in skeletal muscle could involve nNOS (NOS1), iNOS (NOS2) and/or eNOS (NOS3) as they have been detected in skeletal muscles. A recent study showed that LPS injection resulted in increased NO production and changes in redox state in skeletal muscle, which were absent in iNos−/− mice [23],
demonstrating the relevance of iNOS in muscle wasting. Here, we observed that induced iNOS expression was associated with increased NO production in oxidative muscle that was protected from endotoxin challenge (Fig. 2A, 2B, 2C and 2F). In contrast, endotoxin resulted in decreased nNOS expression in both oxidative and glycolytic muscles (Fig. 2B and 2D). nNOS protein is specifically expressed in glycolytic myofibers as part of dystrophin glycoprotein complex [34] that maintains skeletal muscle contractile function [35,36]; reduced nNOS expression here is consistent with a general sarcolemmal injury [36]. On the other hand, induced eNOS expression in glycolytic myofibers (Fig. 2B and 2E) does not reconcile with the protection in oxidative myofibers. Taken together, cachexia-induced NO production in skeletal muscle appears to be mediated through enhanced iNOS expression. The apparent feed-forward regulation of NO production may prove to be critical in amplifying the signals necessary for the protection associated with oxidative phenotype.

Paradoxically, melatonin, a powerful scavenger of both ROS and RNS, abolished the increases in NO production and GSSG/GSH ratio in isolated mitochondria from skeletal muscles [23] and heart [37] in mice challenged with endotoxin; these increases were absent in iNos−/− mice. Our confined staining of oxidative myofibers of iNOS to the periphery is most consistent with mitochondrial localization of iNOS protein, which is in agreement with a recent finding [23]. The previous findings that cachetic responses were absent in iNos−/− mice were interpreted as evidence that iNOS was responsible for mitochondrial dysfunction and oxidative stress in muscles wasting. However, precaution should be taken regarding these findings with overall inhibition of iNOS since whole body genetic disruption of the iNos gene and application of iNOS inhibitor leads to loss of iNOS function not only in skeletal muscles, but also in macrophages, which is critical for the induction of sepsis [38]. On the other hand, a powerful protective function of iNOS in skeletal muscle are suggested in previous studies using a rat model of myasthenia gravis [11] caused by autoantibodies binding to and inhibiting the nicotinic acetylcholine receptors at the neuromuscular junctions. iNOS inhibitor converted disease-resistant soleus muscle to a disease-susceptible phenotype [11]. In this study, both in vitro and in vivo findings provide strong evidence support the protective function of NO in skeletal muscle in cachexia. Nevertheless, a formal

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**Figure 3. Endotoxin induces greater antioxidant gene expression in mouse oxidative than in glycolytic muscles.** Total RNA from SO and WV after LPS or saline (Con) injection were assayed for antioxidant gene expression by semi-quantitative RT-PCR. A) Gel images show levels of Sod1, Sod2, Sod3 and Cat mRNA expression with 18S ribosomal RNA as control; and B), C), D) and E) Quantification and comparison of Sod1, Sod2, Sod3 and Cat mRNA, respectively (n = 9; *, ** and *** P<0.05, 0.01 and 0.001, respectively).

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confirmation of iNOS function in skeletal muscle awaits the generation of an animal model of skeletal muscle-specific knockout of the \textit{iNos} gene.

We observed significant enhanced antioxidant genes in oxidative soleus muscles, but not in glycolytic white vastus lateralis muscles (Fig. 3A–3E), a first demonstration of fiber type-specific induction of antioxidant genes in a whole animal model of muscle wasting. We then addressed the question whether enhanced NO production/signaling renders protection against cachetic stimuli induced by endotoxin/cytokine. Both \textit{in vitro} and \textit{in vivo} findings suggest that NO reduces atrophic responses and enhances antioxidant gene expression in skeletal muscles (Fig. 4 and 5). To our knowledge, this is the first demonstration of simultaneous, fiber type-specific induction of \textit{Sod3} and \textit{Cat}. Based on these findings, we now conclude that oxidative myofibers possess an intact NO-dependent signaling and transcription system protecting the myofibers from oxidative stress by enhancing the antioxidant system. The finding of enhanced reporter gene activity

\begin{figure}
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\caption{NO enhances \textit{iNos} and antioxidant gene expression, and blocks TNF-\textalpha-induced \textit{MAFbx/atrogen-1} mRNA expression in cultured myoblasts. Total RNA from cultured C2C12 myoblasts following treatment with NO donor, DETA-NO (0.2 mM), for 24 hours in the presence or absence of TNF-\textalpha (10 ng/ml) were assayed for \textit{MAFbx/atrogen-1}, \textit{iNos} and antioxidant gene expression by semi-quantitative RT-PCR and total protein lysates from transfected C2C12 myoblasts were harvested and assayed for luciferase activities. A) Gel images show levels of \textit{MAFbx/atrogen-1} mRNA expression (in triplicates) with 18S ribosomal RNA as control; B) Quantification and comparison of \textit{MAFbx/atrogen-1} mRNA (n = 11; ** and *** \(P<0.01\) and 0.001, respectively); C) Gel images show levels of \textit{iNos} mRNA expression with 18S ribosomal RNA as control; D) Quantification and comparison of \textit{iNos} mRNA (n = 6; ** \(P<0.01\)); E) Gel images show levels of \textit{Sod1}, \textit{Sod2}, \textit{Sod3} and \textit{Cat} mRNA expression with 18S ribosomal RNA as control; F), G), H) and I) Quantification and comparison of \textit{Sod1}, \textit{Sod2}, \textit{Sod3} and \textit{Cat} mRNA, respectively (n = 6; * and ** \(P<0.05\) and 0.01, respectively); and J) pARE-Luc reporter gene activity in C2C12 myoblasts treated with or without DETA-NO for 24 hours (n = 6; * \(P<0.05\)).
\end{figure}
in cultured muscle cells transfected with the ARE reporter gene (Fig. 4J) further supports this conclusion and provides more direct evidence of transcriptional control. Further research should focus on the link between the NO signal and the transcriptional factors in skeletal muscle that is responsible for the inducible defense system.

It is worth noticing that NO donor was sufficient both in vitro and in vivo to induce iNOS expression (Fig. 4C and 4D and Figure 5. GSNO enhances \textit{iNos} and antioxidant gene expression, and blocks LPS-induced \textit{MAFbx/atrogen-1} mRNA expression in mouse plantaris muscles. Total RNA following NO donor GSNO injections (1 mg/kg, i.p. twice) in mice with LPS (1 mg/kg) or saline injection (Con) were assayed for \textit{MAFbx/atrogen-1}, \textit{iNos} and antioxidant enzyme mRNA expression by semi-quantitative RT-PCR. A) Gel images show levels of \textit{MAFbx/atrogen-1} mRNA expression (in triplicates) with 18S ribosomal RNA as control; B) Quantification and comparison of \textit{MAFbx/atrogen-1} mRNA (n = 11; ** and *** P < 0.01 and 0.001, respectively); C) Gel images show levels of \textit{iNos} mRNA expression with 18S ribosomal RNA as control; D) Quantification and comparison of \textit{iNos} mRNA (n = 6; ** and *** P < 0.01 and 0.001, respectively); E) Gel images show levels of \textit{Sod1}, \textit{Sod2}, \textit{Sod3} and \textit{Cat} mRNA expression with 18S ribosomal RNA as control; and F), G), H) and I) Quantification and comparison of \textit{Sod1}, \textit{Sod2}, \textit{Sod3} and \textit{Cat} mRNA, respectively (n = 6; * and ** P < 0.05, and 0.01, respectively).

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Fig. 5C and 5D), suggesting that both culture muscle cells and skeletal muscles have intact machineries to induce iNOS expression; the difference may be that oxidative muscle, but not glycolytic muscle, has the ability to trigger the initial increase in NO production. Future experiments should focus on elucidating the mechanism underlying this important regulatory event.

Accumulating evidence supports the view that NO and RNS play protective functions in various tissues/organs and disease models [39–41]. Perhaps, the most relevant findings are in the heart [42,43] through an iNOS-dependent mechanism [44] as cardiac muscle has metabolic and contractile functions that more closely resemble oxidative skeletal muscles. More recently, a calcineurin/NFAT (nuclear factor of activated T-cells) signaling cascade has been implicated in cardiomyocyte protection through the transcriptional control of the iNOS gene [45]. Ours is the first report that NO and RNS play a protective role against cachetic stimuli in skeletal muscle.

We believe that NO elicits signaling events leading to the transcriptional activation of the antioxidant genes though the exact signaling and molecular mechanisms responsible for the up-regulation of the antioxidant genes remain to be elucidated.

In summary, findings from this study are consistent with a hypothesis as following. Cachexia leads to increased oxidative stress and consequently activation of protein degradation pathways in glycolytic myofibers. Enhanced iNOS expression and NO production lead to enhanced antioxidant gene expression in oxidative myofibers, providing protection against oxidative stress (Fig. 6). These findings suggest a therapeutic value of enhancing NO signaling in skeletal muscle for cachexia. Future investigations should ascertain the functional role of enhanced NO signaling in protection against various types of muscle atrophy and elucidate the molecular mechanism(s) responsible for the fiber type-specific protection against various types of muscle atrophy.

**Materials and Methods**

**Experimental animals**

Male C57BL/6 mice (8–9 weeks old) from the Jackson Laboratory (Bar Harbor, ME) were maintained in light- (12:12 h light-dark cycle) and temperature-controlled quarter (21°C) provided with water and chow (Purina, Richmond, IN) ad libitum. They were intraperitoneally injected (i.p. 1 mg/kg) with *E. coli* LPS (Sigma, St. Louis, MO) or normal saline 12 hours before being sacrificed by isoflurane-induced anesthesia and cervical dislocation. LPS injection did not result in morbidity or mortality. Soleus and white vastus lateralis muscles were harvested. To determine the role of NO donor iNOS and NO-dependent up-regulation of the antioxidant genes, leads to less atrogyene expression and protein degradation. Thick and thin arrows represent great and little increase/stimulation, respectively.

**Nitrite assay**

Nitrite was assessed using the Griess Reagent System (Promega, Madison, WI). In brief, harvested skeletal muscles were homogenized immediately in ice-cold Protein Lysis Buffer (Cell Signaling Technology, Danvers, MA) supplemented with 50 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PMSF) and homogenized with an Ultra Turrax T25 Polytan™ homogenizer. The homogenates were centrifuged at 16,000 × g for 5 min at 4°C, and the supernatants were then transferred to 10K NMWNL-0.5 ml Ultralp Free Filter Unit (Millipore, Bedford, MA) and centrifuged at 16,000 × g at 4°C (about 30 min) to concentrate to a volume of about 60 μl. Protein concentration was then determined by using the RC DC protein assay (BioRad, Hercules, CA). Protein carbonylation was then determined in the whole muscle lysate (20 μg protein) by an immunoblot-based assay according to the instructions from the manufacturer. The intensities of all the bands in each of the lanes were quantified by using Scion Image (Scion Corporation, Frederick, Maryland) and normalized by α-tubulin.

**Cell culture**

C2C12 mouse myoblasts were grown and maintained as subconfluent monolayer in high-glucose Dulbecco modified Eagle ...
medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY). To examine the effect of NO donor and iNOS inhibitor, C2C12 cells seeded in 35 mm-wells at 1 x 10^4 and allowed to adhere for at least 12 hours prior to stimulation by TNF-α (R&D Systems, Minneapolis, MN) at 10 ng/ml with/without DETA-NO (Sigma, St. Louis, MO). The cells were harvested 20 hours later, and total RNA samples were isolated for RT-PCR analysis by using Trizol (Invitrogen, Carlsbad, CA). For transfection of pARE-Luc, C2C12 myoblasts were transfected with 0.5 μg with Lipofectamine 2000 according to the manufacturer’s instructions. After overnight transfection, the transfected cells were treated with/without DETA-NO (10 ng/ml) for 24 hours. Luciferase activity was assayed and is expressed as relative activity (fold change) in comparison to the value of transfected cells without DETA-NO. The transfection was done twice with triplicate samples for each condition.

**Semi-quantitative RT-PCR**

This analysis was performed as described [12] to measure MAFbx/atrogin-1, MuRF1, iNos, superoxide dismutases (Sod-1, Sod-2, Sod-3) and catalase (Cat) mRNA expression in skeletal muscles and in cultured C2C12 myoblasts. PCR primer pairs were designed using Primer3 search engine at www-genome.wi.mit.edu. The oligonucleotide primer pairs used in this study corresponded to the following nucleotides: MAFbx/atrogin-1: 1917–1936 and 2415–2396 (NM_026346); MuRF1: 50–69 and 452–433 (AF294790); iNos: 1813–1834 and 2301–2282 (NM_034328); α-NOS: 1515–1534 and 2002–1983 (NM_008713); β-NOS: 1261–1272 and 1735–1716 (NM_008712); Sod1: 168–187 and 503–564 (NM_011434); Sod2: 1218–1237 and 1647–1629 (BC060663); Sod3: 1347–1366 and 1909–2000 (NM_011453); and Cat: 237–256 and 643–626 (NM_034328). Results were normalized by 18s RNA and presented as fold change to soleus muscle in mice injected with normal saline.

**Statistics**

Data are presented as mean±standard error. For comparisons involving two factors, two-way ANOVA was performed followed by the Newman–Keuls test. For comparisons between two groups, the Student t-test was performed. *P*<0.05 was accepted as statistically significant for all the experiments in this study.

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**Author Contributions**

Conceived and designed the experiments: JS Z Yan Z Yu PL. Performed the experiments: Z Yan Yu PL. Analyzed the data: Z Yan Z Yu. Contributed reagents/materials/analysis tools: Z Yan MH. Wrote the paper: JS Z Yan.

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