Changes in Nitric Oxide and Inducible Nitric Oxide Synthase Following Stretch-Induced Injury to the Tibialis Anterior Muscle of Rabbit

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Abstract: This study investigated the changes in nitric oxide (NO) together with inducible nitric oxide synthase (iNOS) content and enzyme activity at 0, 4, 12, 24, and 48 h following acute muscle stretch injury. A single stretch injury was induced to the tibialis anterior muscle of 30 male New Zealand white rabbits (n = 6 at each time point). Injured and uninjured contralateral sham-operated muscles were harvested and analyzed for NO levels, iNOS content, and iNOS activity at each time point. Furthermore, three animals were used to estimate baseline NO levels and iNOS activity. There was a progressive reduction in NO content in the injured and the sham-operated muscles up to 24 h postoperation and stretch injury (p < 0.05). At 48 h postinjury, however, NO levels were 146% higher in injured muscles than in sham-operated muscles (p < 0.05). iNOS protein content was higher at 4 h and 48 h in injured versus sham-operated muscles (p < 0.05). Similarly, iNOS activity was higher at 4 h (p < 0.05) and at 48 h (p < 0.01) in injured versus sham-operated muscles. These results suggest that NO may play an active role during the postinjury recovery of skeletal muscle modulated by iNOS expression. [The Japanese Journal of Physiology 55: 101–107, 2005]

Key words: nitric oxide, inducible nitric oxide synthase, muscle stretch injury, rabbit.

Nitric oxide (NO) generation occurs through the conversion of L-arginine to NO and L-citrulline, which is controlled by NO synthases (NOS) [1]. There are three forms of NOS that catalyze NO production [2]. NO is a ubiquitous molecule involved in a variety of physiological functions, including vascular regulation, neurotransmission, immune regulation, and cell signaling [3–5]. NO is also known to have profound effects on skeletal muscle function, including contractility, blood vessel tone, blood flow, and satellite cell activation [6–9]. Resting skeletal muscle contains two constitutive types, neural NOS (nNOS) and, to a lesser extent, endothelial NOS (eNOS) [7, 10, 11]. The third or inducible form (iNOS) is expressed mainly in leukocytes and macrophages in response to inflammation. In contrast to eNOS and nNOS, iNOS can produce large amounts of NO over a prolonged period [12, 13], and skeletal muscle has been shown to induce iNOS mRNA and protein following endotoxin administration [14]. Because NO has both pro-inflammatory and anti-inflammatory properties, several possible roles for NO in muscle injury have been postulated. For example, leukocyte concentrations within muscle tissue following unloading and reloading can be decreased by NOS inhibitors [15]. Moreover, a recent study reported that rat skeletal muscle produced sustained increases in both iNOS activity and protein levels in response to crush injury [16]. In contrast to these effects of NO on inflammation, NO can also have direct effects on muscle function, growth, and repair [17]. However, the pathogenesis of acute muscle stretch injury and the factors that regulate inflammation and repair are not well understood. To clarify whether NO modulates either inflammation or regeneration/repair, or both, following...
In this communication, we used an anesthetized rabbit model to measure the time course for NO levels, iNOS protein content, and iNOS activity in stretch-injured skeletal muscles. We employed an isokinetic injury apparatus that quantifies tibialis anterior (TA) muscle-tendon shortening according to the method previously reported [18–20]. This in vivo model of acute mechanical muscle stretch injury would be different from the injury observed with exhaustive exercise and reported eccentric muscle contractions. Because injury was created by using a single stretch and stimulation of the muscle-tendon unit rather than repeated eccentric muscle contractions, this model can mimic the clinical injury pattern that occurs with a single stretch of the muscle-tendon unit, for example, the so-called “hamstring strain” [18]. However, we confirmed that this in vivo model of stretch injury showed a visible hematoma at the muscle-tendon junction, and that histological evaluation 48 h after injury revealed fiber tearing and inflammation at this junction [19].

METHODS

Animal care. Thirty-three male New Zealand white rabbits (2.7–3.0 kg) were housed individually and fed food and water ad libitum in a temperature-controlled room on a 12:12 h light–dark cycle. The University of Wisconsin-Madison Research Animals and Resource Center Review Committee approved the animal use protocol.

Injury model and protocol. Our model of a single stretch-induced injury to skeletal muscle has been described [18]. Briefly, all animals were anesthetized with an intramuscular preparation of ketamine (100 mg/kg), xylazine (2.5 mg/kg), and acepromazine (3 mg/kg). A 6 mm incision was made over the dorsum of the right foot just distal to the ankle joint to isolate the tendinous portion of the TA. The peroneal nerve was isolated through a 4 mm skin incision at the knee. The peroneal nerve was achieved, the ankle was plantar flexed through an arc at 450°/s. The torque-angular displacement time behavior was recorded. A sham operation of skin incisions was performed in the left foot and knee. The 4 h animals were maintained under anesthesia with 1 ml of i.m. Ketamine every 45 min until tissue harvest. For the remaining four groups (12, 24, and 48 h), all skin incisions were closed with 4-0 Ethilon suture, and the animals were returned to their cages after recovery from anesthesia with unrestricted activity. An evaluation 7 days after single stretch-induced injury in this protocol had demonstrated a functional deficit (percent ankle isometric torque; injured leg versus uninjured leg) of 47.5 ± 5.4% (mean ± SD) [20].

Tissue sampling. The entire TA muscle-tendon unit was surgically removed at its proximal attachment to the tibia and distal insertion into the foot. A one cm² block (approximately 2 g) of muscle tissue was removed from the myotendinous junction region of both the stretch-injured and the sham-operated controls. This sample always contained the area of visible hematoma and maximum injury. The tissue block was cut and one section (1.8 g) was snap-frozen in liquid nitrogen. The animals were sacrificed with Beuthanasia (0.4 ml/kg), i.v., placed into the marginal ear vein immediately following muscle harvest. In addition to the thirty animals evaluated following injury, three rabbits were used to estimate baseline NO levels and iNOS activity. Measurements were made in both legs of the three animals not exposed to stretch injury or skin incisions to ascertain the contribution of the surgical procedures to overall NO levels and iNOS activity.

NO content. The samples were homogenized at 0–4°C in ice-cold 0.1 M K₂HPO₄-KH₂PO₄ (pH 7.4) buffer (wt:vol of 1:10) with a motor-driven Potter-Elvehjem teflon glass homogenizer. The level of NO in homogenate was determined with the use of a commercially available kit (Nitric oxide colorimetric assay kit, Calbiochem, San Diego, USA). Because the Griess reagent measures only nitrite and biological systems contain both nitrite and nitrate, 0.01 U nitrate reductase (Sigma, St. Louis, MO) was added to each well, followed by the addition of NADH (0.02 mM). The samples were read at 540 nm by the use of a microplate reader (Molecular Devices, Sunnyvale, CA), and the results were expressed as pmol/mg protein [21]. Pilot assays established interassay/intra-assay variability of less than 3% (data not shown).

Inducible nitric oxide synthase (iNOS) protein content. SDS polyacrylamide gel electrophoresis (SDS-PAGE) in combination with an enhanced chemiluminescence plus (Amersham, Arlington Heights, IL) detection system was used to determine iNOS protein content in skeletal muscle. Tissue samples were homogenized in 50 mM Tris HCl, 1 mM EDTA, and 0.1% phenylmethylsulfonylfluoride (PMSF). We determined the relative amount of iNOS by using an anti-iNOS (IgGl) antibody (Transduction Laboratories, San Diego, CA). Autoradiographic signals were assessed by the use of a BioRad scanning densitometer (BioRad,
Hercules, CA). The results are expressed in arbitrary optical density units.

**Inducible nitric oxide synthase (iNOS) activity.** A measurement of iNOS activity was performed by an examination of the conversion of \(^{[3]H}\)-arginine to \(^{[3]H}\)-citrulline, using the method of Thompson [14]. The reaction buffer contained 3 mM \(n\)-2-hydroxyethylpiperazine-\(N'\)-protein-ethanesulfonic acid (HEPES), 4 mM NADPH, 20 mM tetrahydrobiopterin, 20 \(\mu\)M flavin adenine dinucleotide (FAD), 20 \(\mu\)M flavin mononucleotide (FMN), 1 mM dithiothreitol (DTT), 4 \(\mu\)M \(L\)-arginine, 4 \(\mu\)M ethylene glycol-bis (b-aminoethyl ether)-\(N,N',N'\)-tetraacetic acid (EGTA), and 2.0 mCi/ml \(L\)-[\(^{3}H\)]-arginine (Amersham, Arlington Heights, IL). The reaction was incubated at 37°C for 1 h, then terminated by an addition of buffer containing 40 mM HEPES, pH 5.2, 2 mM EDTA, and 2 mM EGTA. The samples were then applied to a 10 ml Dowex AG50WX-8 (Na\(^{+}\)) column (BioRad, Hercules, CA) and eluted with 40 mM HEPES. We collected the effluent in scintillation vials and quantified it by liquid scintillation spectroscopy, using a Wallac LKB 1211 Rack Beta counter (Wallac, Helsinki, Finland). One mM \(N\)-nitro-\(L\)-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, served as a negative control to assess background activity and the non-NOS-dependent conversion of \(L\)-[\(^{3}H\)] arginine to \(L\)-[\(^{3}H\)] citrulline. The results are expressed as picomoles per minute per microgram protein (pm/mln/mg protein). Pilot experiments demonstrated inter/intravariableity of less than 4%.

**Statistical analysis.** The means ± SEM values were calculated for all data sets. The data were analyzed with a 2-way analysis of variance with repeated measures to evaluate the two main treatment effects, injury and time, and their interaction (StatView). The Fisher’s Least Significant Difference post hoc test was used to find the differences between means when the observed F ratio was statistically significant (\(p < 0.05\)).

**RESULTS**

**NO content**

Baseline NO levels were approximately a third of the sham-operated or injured tissue values (Fig. 1). The NO levels at 0 h after surgery were significantly higher than those of the baseline (\(p < 0.05\)). The sham-operated muscles showed a decrease in NO content at 12, 24, and 48 h after surgery, compared to NO content at 0 h after surgery. Stretch-injured muscles showed decreased NO content at 24 h, compared to NO content at 0 h after injury. At 48 h, sham-operated muscle continued to have lower NO than the value at 0 h after surgery, whereas in stretch-injured muscles, NO was recovered to higher levels, compared to the value at 0 h after injury, and it was 146% higher than that of sham-operated muscles.

**iNOS content**

Figure 2A shows the immunoreactive levels of iNOS protein in sham-operated and injured muscles. Figure 2B shows densitometric quantification of iNOS pro-
The iNOS protein expression in injured muscles was not different from that in sham-operated muscles at 0, 12, and 24 h postinjury. However, iNOS proteins in injured muscles at 4 and 48 h were significantly higher than their sham-operated counterparts. Stretch injured muscles showed an increased expression of iNOS protein at 4 and 48 h, as compared to the value at 0 h after injury. On the other hand, sham-operated muscles showed a decreased expression of iNOS protein at 4 h, 12 h, and 24 h postsurgery.

**iNOS activity**

Similar to iNOS protein content, the iNOS enzyme activity was significantly higher in injured muscles at 4 h and 48 h after injury than the values of sham-operated muscles at each respective time point. The time course of iNOS enzyme activity in injured muscles displayed two peaks, with higher levels at 4 and 48 h after injury, compared to the value at 0 h after injury. At 24 h, injured and sham-operated muscles had decreased iNOS activity, compared to the value at 0 h after operation. iNOS activity was fully inhibited by the addition of L-NAME (results not shown).

**DISCUSSION**

The major finding in the current study was that stretch-injured muscles showed an increase in NO levels 48 h after injury in comparison to sham-operated tissues. During the initial 24 h after injury, similar changes in NO levels were found in sham-operated muscles and injured muscles. No significant increase in NO levels was observed in sham-operated muscles 48 h after surgery, whereas a significant increase was noted in injured muscles 48 h after injury. This finding may be of significance to muscle healing for several reasons. First, adequate levels of intracellular NO may be an important prerequisite for muscle healing after injury because it has been postulated that NO plays a central role in satellite cell activation and muscle regeneration.
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[8, 22]. Consistent with this role, various NO donors have been shown to stimulate myoblast proliferation in vitro [23]. Second, NO may provide some control on the extent of muscle inflammation and repair through its influence on leukocyte infiltration. Besides influencing myofiber regeneration, NO has been shown to affect collagen synthesis because NO generators can have proliferative and inhibitory effects on the mitotic activity of fibroblasts [24]. Finally, NO is a well-known vasodilative agent and conceivably increases blood flow to recovering myocytes following stretch injury [14]. Taken together, NO or intermediate(s), or both, may exert multiple biological effects to facilitate the healing of stretch-injured skeletal muscle in our model.

During the initial 24 h after injury, NO levels were higher compared to baseline levels in sham-operated and stretch-injured muscles. However, as noted in Figs. 2 and 3, the source of increased NO during the initial 4 h after injury or operation was different between injured muscles and sham-operated muscles. Although apparent expressions of iNOS proteins were not detectable in sham-operated muscles, injured muscles showed an enhanced expression of iNOS protein at 4 h and 48 h after injury, resulting in the enhanced activity of iNOS, which increased NO production and recovered NO levels. Thus an enhanced expression of iNOS protein appears to be the main mechanism. The observed iNOS-dependent increases in NO levels at 4 h and 48 h after injury may be specific phenomenon in stretch-injured muscles. On the other hand, in sham-operated muscles there was no increase in NO after the initial reduction. The initial sham procedure involving two skin incisions in sham-operated leg, therefore, may impose a systemic stress response to the affected region. Alternatively, the higher initial NO levels compared with the baseline animals may also have resulted from a general systemic bloodborne response from the surgery and injury to the opposite leg. Sustained elevations in blood NO levels have been noted for up to 48 h following abdominal surgery in a rat model [25]. However, we lack an adequate explanation for detectable iNOS enzyme activity for this injury.

The mechanism by which higher levels of iNOS protein were observed in injured muscles at 4 h and 48 h after injury compared to sham-operated muscles at respective time points after operation remains unclear at present. One possibility is that the enhanced expression of iNOS was caused by the infiltration and activation of neutrophils, which are known to release inflammatory cytokines [26]. Consistent with this hypothesis, our previous study showed an increase in myeloperoxidase activity, a biochemical marker for neutrophil presence, in injured muscles at 4 h and 48 h following injury [27]. Neutrophils undergo the respiratory burst to generate reactive oxygen species as signaling molecules to activate the nuclear factor (NFκB) and, in turn, iNOS activation [28].

Using a similar injury protocol, we previously reported an increase in the rate of oxidant production at 24 h after operation and injury in the sham-operated muscles.
and stretch-injured muscles [27]. This increase paralleled an increase in xanthine oxidase activity, suggesting that O$_2^-$ could be the underlying oxidant species. Therefore it is possible that in the injured muscle, the decline in NO levels during the initial 24 h postinjury may reflect increased NO consumption within muscle cells because of increased oxidant generation. It is well known that NO can function as a pro-oxidant [22, 29] and as an antioxidant [17, 22] molecule, depending on the physiological condition and the time following various forms of muscle damage. One interesting possibility is that the decreased total NO species (including nitrate and nitrite) observed in the first 24 h in both samples in the current study was due to a depletion of endogenous NO serving as an O$_2^-$ scavenger before de novo synthesis took place during the later phase of the postinjury time course. Further studies are needed to test this hypothesis.

Our results should be interpreted as resulting from the literature and previous studies on NO and muscle trauma. Rubinstein et al. [16] have shown with a rat muscle crush model that iNOS protein and mRNA are upregulated in response to injury. Their main finding was that iNOS mRNA was increased to its greatest extent at 6 h after injury and remained elevated to a lesser extent through 72 h postinjury. iNOS protein content was increased gradually beginning at 24 h and reaching a maximum at 72 h. These results are similar to some extent with our findings of increased iNOS protein content at 48 h. However, we found that increased iNOS protein and activity at 4 h postinjury was in contrast with the data of Rubinstein et al. [16]. This discrepancy may be related to differences in injury models and species. Others have shown a less sustained increase in iNOS mRNA and iNOS protein following endotoxin administration [22].

In conclusion, acute stretch injury to skeletal muscle can trigger a distinct time course for tissue NO levels along with parallel changes in iNOS protein and iNOS enzyme activity. NO production early following injury may be slower than its consumption, resulting in decreased tissue NO levels. However, by 48 h following injury, NO levels were elevated in stretch-injured skeletal muscles. The significance of the increased levels at 48 h is a topic of ongoing study.

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