IMMEDIATE FORCE LOSS AFTER ECCENTRIC CONTRACTIONS IS INCREASED WITH L-NAME ADMINISTRATION, A NITRIC OXIDE SYNTHASE INHIBITOR

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Abstract

Introduction—Nitric oxide (NO) signaling regulates many biological processes in skeletal muscle, wherein aberrant signaling contributes to myopathic conditions (e.g., Duchenne muscular dystrophy). NO has been shown to play a role in muscle regeneration after injury. However, less is known about its role during injury. In this study we aimed to determine whether NO synthase (NOS) inhibition exacerbates functional deficits immediately after the performance of eccentric contractions.

Methods—Wild-type mouse extensor digitorum longus (EDL) muscles underwent in vitro functional testing in the presence or absence of a non-specific NOS inhibitor (L-NAME, 10 mM) before and after performance of 10 eccentric contractions.

Results—After eccentric contractions, $P_0$ was reduced by ~25% for muscle in regular physiological solution but by ~50% with the addition of L-NAME ($P = 0.009$).

Conclusions—Non-specific blockade of NOS exacerbates functional deficits immediately after eccentric contractions, suggesting that NO signaling protects skeletal muscle from excessive injury in healthy muscle.

Keywords

eccentric contractions; force; L-NAME; muscle injury; nitric oxide synthase

Nitric oxide is a major regulator of many biological processes in skeletal muscle. Skeletal muscle primarily produces nitric oxide (NO) by expression of nNOS (neuronal) and less so by eNOS (endothelial). After injury and in diseased muscle, NO is also produced by iNOS (inducible, expressed by inflammatory cells). During muscle activity (including eccentric contractions) NO production increases to modulate myofiber force production and metabolism. NO signaling also plays an important role in mediating regeneration after muscle injury by regulating hepatocyte growth factor activity and satellite cell activation. In healthy rodents, disruption of NO signaling after injury diminishes regeneration and increases fibrosis. Moreover, in dystrophic mice, in which nNOS location and activity is altered, partial restoration of NO signaling prior to eccentric contractions attenuated muscle injury.
Immediate force loss after eccentric contractions is increased with L-NAME administration, a nitric oxide synthase inhibitor.

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Although the importance of NO in skeletal muscle regeneration has been demonstrated, its role during or immediately after eccentric contractions is not fully understood. Because NO can attenuate calpain activity\(^{10,11}\) and modulate excitation–contraction (E–C) coupling\(^1\) (these factors are thought to play a role in immediate functional deficits\(^{12–15}\)), NO may serve to protect skeletal muscle from excessive initial injury. We tested the hypothesis that pharmacological inhibition of NOS activity exacerbates functional deficits immediately after eccentric contractions performed \textit{in vitro}.

**METHODS**

All animal care and use procedures were approved by our institutional animal care and use committee. Extensor digitorum longus (EDL) muscles were isolated from adult (age 4–5 months of age), male, wild-type mice (C57BL/6) and were tested in an \textit{in vitro} organ bath system.\(^6\) Muscles underwent a battery of functional tests (Fig. 1A) in Krebs–Ringer bicarbonate buffer at 35°C with 95% O\(_2\)–CO\(_2\) balanced air perfused continuously. In some experiments, \(N\_o\text{/-nitro/-n-arginine methylester hydro-chloride (L-NAME), a non-specific NOS inhibitor, was added to the Krebs–Ringer buffer at a final concentration of 10 mM.}\) (Millimolar concentrations of L-NAME have been shown to modulate contractility of skeletal muscle\(^4\) and NO-mediated events in other organ systems.\(^17\)) Muscle resting tension was initially set to ~4.5 mN to correspond to anatomical muscle length (L\(_o\)).\(^8\) Muscles were stimulated directly by applying trains of 0.2-ms pulses with a supramaximal voltage. Isometric specific force was measured as a function of stimulation frequency (F–f: 10–300 Hz; 200-ms train),\(^6\) before and after L-NAME administration and after the performance of 10 eccentric or isometric (300 Hz) contractions, with a 3-minute rest between contractions. During the eccentric contractions, the muscle was shortened to 90% and then lengthened to 110% of L\(_o\) at 1.5 muscle lengths per second, while it was stimulated (300 Hz) for 133 ms.\(^8\) Muscle wet weight and L\(_o\) for all muscles was 11.6 ± 0.21 mg and 1.37 ± 0.01 cm, respectively. The stimulation frequency corresponding to half the amplitude of force (Freq\(_{50}\)) of the normalized F–f curve was determined using a four-parameter Hill equation.\(^6\) Statistical differences were assessed with one- and two-way analyses of variance (ANOVAs) and \(t\)-tests; \(a\) was set at 0.05.

**RESULTS**

Prior to injury, L-NAME administration depressed peak isometric force (P\(_o\)) by ~10% [Fig. 1C, E, G and I; L-NAME (n = 4), \(P = 0.035\); vs. <1% in Krebs (n = 4), \(P = 0.985\)] but elevated twitch force (P\(_t\)) by ~8% (\(P = 0.031\)). During the eccentric injury protocol, initial peak eccentric force was similar between L-NAME and Krebs muscles, but L-NAME promoted a greater loss of eccentric force (Fig. 1B; L-NAME vs. Krebs: ~38 vs. ~24%; \(P = 0.041\)). Immediately after eccentric contractions, isometric force was reduced across all frequencies by 44–52% in the presence of L-NAME (Fig. 1H) and to a lesser extent (24–37%) for Krebs (Fig. 1D). Control L-NAME muscles (n = 2) that performed 10 isometric instead of eccentric contractions demonstrated an ~8% reduction in isometric force during the protocol and a corresponding ~9% deficit in P\(_o\) from pre- to post-injury (\(P = 0.094\)), similar to findings we made previously with EDL muscle with Krebs only.\(^6\) Freq\(_{50}\) increased significantly from pre- to postinjury for Krebs (Fig. 1F; 92 ± 2 Hz vs. 106 ± 1 Hz, \(P = 0.008\), but not for L-NAME muscles (Fig. 1J; 87 ± 2 vs. 97 ± 4 Hz, \(P = 0.162\)).

**DISCUSSION**

We have demonstrated that L-NAME, an inhibitor of NOS, exacerbates functional deficits during and after eccentric contractions in healthy murine muscle, suggesting that NOS serves to partially protect skeletal muscle from injury. It is possible that the high
concentration of L-NAME used in this study may have non-specific biological effects,\(^\text{19}\) such as blocking of muscarinic acetylcholine receptor signaling.\(^\text{20}\) However, the effect of muscarinic acetylcholine receptor activity on \(P_o\) is incongruous with our findings with L-NAME.\(^\text{21}\) Further, the contractile phenotype mediated by millimolar concentrations of L-NAME\(^\text{4}\) (Fig. 1) is similar to that of nNOS\(^{-/-}\) muscle,\(^\text{4}\) suggesting that, in a whole-muscle preparation, millimolar L-NAME primarily inhibits NOS.

Because these experiments were performed \textit{in vitro} and were thus largely devoid of macrophages and hence iNOS activity, it is likely that nNOS\(_\mu\) serves to protect muscle from eccentric muscle injury. This is of interest when compared with findings showing that nNOS\(^{-/-}\) mice did not exhibit significant differences in regeneration from wild-type mice after a myotoxic injury,\(^\text{22}\) whereas non-specific pharmacological inhibition of NOS impeded inflammation\(^\text{23}\) and regeneration.\(^\text{7}\) The findings from those studies and this study support the supposition of Lynch and colleagues,\(^\text{22}\) who showed that nNOS\(_\mu\) plays a critical role in protecting skeletal muscle from injury during the injury bout, whereas iNOS primarily mediates regeneration thereafter.

A remaining question is how NO signaling protects healthy skeletal muscle from injury during eccentric contractions. Immediate functional deficits after eccentric contractions are primarily due to E-C uncoupling,\(^\text{12-14}\) likely the result of triadic protein damage caused by physical stress.\(^\text{16,24}\) However, we did not observe a rightward shift in the force–frequency curve (i.e., \(\text{Freq}_{50}\)) after injury with NOS inhibition, suggesting that the increased force deficits observed may have been due to increased damage of force-bearing proteins (e.g., titin, dystrophin, or desmin). Although the performance of eccentric contractions has been shown to immediately increase cytosolic \(\text{Ca}^{2+}\),\(^\text{12,13}\) which may increase proteolysis, our laboratory has demonstrated in otherwise healthy muscle using a similar injury protocol that immediate functional deficits are not improved via extracellular \(\text{Ca}^{2+}\) manipulation,\(^\text{25}\) blockade of stretch-activated \(\text{Ca}^{2+}\) channels,\(^\text{16}\) or inhibition of calpain.\(^\text{18}\) Collectively, these findings suggest that NO signaling in response to eccentric contractions protects healthy skeletal muscle from excessive damage, potentially by attenuating proteolytic activity. In myopathic conditions in which NO signaling is disrupted, this protective effect is likely diminished and results in a greater propensity for injury with high mechanical stress.

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**Abbreviations**

- **E-C**: excitation–contraction
- **EDL**: extensor digitorum longus
- **Freq\(_{50}\)**: stimulation frequency at half-amplitude of force–frequency curve
- **\(L_o\)**: optimal muscle length
- **L-NAME**: \(\text{N}^\\text{x}\)-nitro-L-arginine methylster hydrochloride
- **NO**: nitric oxide
- **NOS**: nitric oxide synthase
- **\(P_o\)**: peak isometric tetanic force
- **\(P_t\)**: isometric twitch force

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REFERENCES


FIGURE 1.
L-NAME increases functional deficits immediately after eccentric contractions. (A) EDL muscle testing was performed in vitro under physiological conditions using the experimental timeline depicted. (B) Muscles performed 10 eccentric contractions with or without L-NAME (10 mM) administration. A subset of muscles performed isometric contractions in the presence of L-NAME as an injury control. (C–J) Isometric force was assessed as a function of stimulation frequency (F–f) in the absence (C–F) or presence of L-NAME (G–J) and is expressed as specific force (C, D, G, H) or the ratio of peak isometric specific force ($P_0$) (E, F, I, J). *$P < 0.05$ where differences between values are noted. Values are listed as mean ± SE.