Nitric oxide impairs Ca\(^{2+}\) activation and slows cross-bridge cycling kinetics in skeletal muscle

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Heunks, Leo M. A., Mark J. Cody, Paige C. Geiger, P. N. Richard Dekhuijzen, and Gary C. Sieck. Nitric oxide impairs Ca\(^{2+}\) activation and slows cross-bridge cycling kinetics in skeletal muscle. J Appl Physiol 91: 2233–2239, 2001.—The effects of the nitric oxide (NO) donor spermine NONOate (Sp-NO, 1.0 mM) on cross-bridge recruitment and cross-bridge cycling kinetics were studied in permeabilized rabbit psoas muscle fibers. Fibers were activated at various Ca\(^{2+}\) concentrations (pCa, negative logarithm of Ca\(^{2+}\) concentration), and the pCa at which force was maximal (pCa 4.0) and ~50% of maximal (pCa\(_{50}\) 5.6) were determined. Fiber stiffness was determined using 1-kHz sinusoidal length perturbations, and the fraction of cross bridges in the force-generating state was estimated by the ratio of stiffness during maximal (pCa 4.0) and submaximal (pCa 5.6) Ca\(^{2+}\) activation to stiffness during rigor (at pCa 4.0). Cross-bridge cycling kinetics were evaluated by measuring the rate constant for force redevelopment after quick release (by 15% of optimal fiber length, \(L_o\)) and restretch of the fiber to \(L_o\). Exposing fibers to Sp-NO for 10 min reduced force and the fraction of cross bridges in the force-generating state at optimal fiber length, \(L_o\). Exposing fibers to Sp-NO for 10 min reduced force and the fraction of cross bridges in the force-generating state at optimal fiber length, \(L_o\). Exposing fibers to Sp-NO for 10 min reduced force and the fraction of cross bridges in the force-generating state at optimal fiber length, \(L_o\). Exposing fibers to Sp-NO for 10 min reduced force and the fraction of cross bridges in the force-generating state at optimal fiber length, \(L_o\). Exposing fibers to Sp-NO for 10 min reduced force and the fraction of cross bridges in the force-generating state at optimal fiber length, \(L_o\).

IN SKELETAL MUSCLES, mechanical action results from the cyclic interaction between actin and myosin, which is regulated primarily by Ca\(^{2+}\) release from the sarcoplasmic reticulum and subsequent binding to troponin C (TnC). In the Huxley-Simmons model of muscle contraction, cross bridges cycle between two functional states: a force-generating state, in which cross bridges are strongly bound to actin, and a non-force-generating state, in which cross bridges are detached from or weakly attached to actin (16, 17). Two apparent rate constants, one for cross-bridge attachment (\(f_{app}\)) and another for cross-bridge detachment (\(g_{app}\)), describe the transitions between the two primary functional states of cross bridges.

Skeletal muscles express nitric oxide (NO) synthase (NOS), which is the primary source for NO in vivo (19, 20). It has been demonstrated that skeletal muscles generate NO at rest and that NO generation is enhanced by contractile activity (2, 19). Exposing skeletal muscle fibers to NO has been shown to impair contractile activity in vitro (19, 26). The effect of NO on cross-bridge cycling kinetics remains more controversial. Some studies have reported that exposing fibers to an NO donor causes a slowing of maximum shortening velocity (13, 26), but in the study by Andrade et al. (1), exposure of intact fibers to an NO donor did not affect maximum shortening velocity. The effect of NO on cross-bridge cycling kinetics has also been evaluated by measuring the rate of force redevelopment (\(k_{tr}\)) (1, 13). Andrade et al. found that exposure to an NO donor had no effect on \(k_{tr}\) in intact fibers, whereas Galler and colleagues (13) reported that exposure to an NO donor caused a slowing of \(k_{tr}\). In both of these studies, \(k_{tr}\) was measured only during maximum activation, and it is possible that the effect of NO on cross-bridge cycling kinetics is more pronounced during submaximal Ca\(^{2+}\) activation. Accordingly, in the present study, we hypothesize that the depressant effects of NO on muscle fiber force and

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Permeabilized Rabbit Psoas Single-Fiber Preparation

Permeabilized single fibers were prepared from glycerinized rabbit psoas muscle (Ward's Natural Scientific Establishment). A 1-cm strip of muscle was excised and placed in a relaxing solution of the following composition: 7.0 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 1 mM free Mg 2+; 4.42 mM MgATP 2−; 20 mM imidazole, 15 mM creatine phosphate, 1 mg/ml creatine phosphokinase, and sufficient KC1 to adjust the ionic strength to 150 mM. The pH was adjusted to 7.0 with KOH. The pCa of the relaxing solution was 9.0. Single fibers were then dissected using fine forceps under a microscope. These fibers were kept for 30 min in a skinning solution that was of the same composition as the pCa 9.0 solution, except 1% Triton X-100 was added. The temperature of the skinning solution was 15°C to thermoequilibrated the fibers before measurements.

The composition of the activating solutions was the same as that of the relaxing solution, except the pCa was adjusted to range between 7.0 and 4.0 with the following steps: 7.0, 6.5, 6.2, 6.1, 6.0, 5.9, 5.8, 5.7, 5.6, 5.5, 5.4, 5.3, 5.2, 5.1, 5.0, 4.5, and 4.0. The composition of the solution for maximum rigor activation was the same as that of the pCa 4.0 solution, except MgATP was omitted. The final concentrations of free rigor activation was the same as that of the pCa 4.0 solution, except MgATP was omitted. The final concentrations of free MgATP were 15°C to thermoequilibrated the fibers before measurements.

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Measurement of Fiber Mechanical Properties

Fibers were mounted in a temperature-controlled flow-through acrylic chamber (120-μl volume) with a glass cover-slip bottom. Fixation of fiber ends with glutaraldehyde reduced compliance at the attachment points, and two stainless steel hooks were used to mount the fiber horizontally in the chamber with aluminum foil T clips. One end of the fiber was attached to a force transducer (model AE-801, Aksjeselskapsforlaget) with a resonant frequency of 5 kHz; the other end was attached to a servomotor (model G120DT, General Scanning) with a step of 800 μs. First-order laser (He-Ne laser; model 58, sp-NO omitted from the relaxing solution. These experiments revealed that 1,000 μM Sp-NO reduced submaximal force to 81 ± 7% of baseline value. No reduction in force generation was observed at lower concentration of Sp-NO.

Experimental Protocols

Determination of Ca 2+ concentration generating 50% of maximal force. After measurement of dose-response relationship of Sp-NO (see Permeabilized Rabbit Psoas Single-Fiber Preparation). Each time a clear plateau was reached, the next pCa solution was perfused through the fiber chamber. From the resulting force-pCa curve, the Ca 2+ concentration generating 50% of maximal force (pCa 50) was determined using sigmoid regression analysis (SigmaPlot; Fig. 1). The calculated pCa 50 was 5.6 (see results) and was used in subsequent experiments for submaximal Ca 2+ activation.

Measurement of dose-response relationship of Sp-NO. In preliminary studies, the dose-response relationship of Sp-NO on force generation was tested. This relationship was only assessed on submaximal force. Testing of the dose-response relationship on the other experimental measurements (maximal force, ktr, and stiffness) as well would require numerous additional experiments, since repeated determination of all these measurements results in significant rundown in force in control fibers. Accordingly, after measurement of force at pCa 4.0 and pCa 5.6, the fibers were successively exposed for 10 min to a range of Sp-NO concentrations (100, 500, and 1,000 μM) dissolved in relaxing solution. Between the different concentrations, force generation at pCa 5.6 was determined. A similar protocol was used for time controls, with Sp-NO omitted from the relaxing solution. These experiments revealed that 1,000 μM Sp-NO reduced submaximal force to 81 ± 7% of baseline value. No reduction in force generation was observed at lower concentration of Sp-NO.
Therefore, in all subsequent experiments using Sp-NO, the final concentration was 1.0 mM. Although this concentration seemed to be high, all experiments had to be performed at 15°C. This low temperature reduced the rate of release of NO from Sp-NO.

Effect of Sp-NO on cross-bridge recruitment and cycling kinetics. To determine baseline properties, the fiber chamber was successively perfused with pCa 9.0, pCa 4.0 rigor, pCa 9.0, pCa 5.6, pCa 4.0, and pCa 9.0. Subsequently, the fiber was exposed to pCa 9.0 containing 1 mM Sp-NO for 10 min. Then Sp-NO was washed out and the force measurements repeated, although the order of introducing the solutions was different (pCa 9.0, 5.6, 4.0, 9.0, 4.0 rigor, and 9.0, respectively). The order of activation after exposure to Sp-NO was changed, since pilot experiments revealed that pCa 4.0 rigor activation significantly reduced force at pCa 5.6 in time controls. Consequently, rigor force was measured after measurement of submaximal force.

Fiber stiffness was measured at pCa 9.0, before submaximal and rigor activation, and during all subsequent activations (pCa 5.6, 4.0, and 4.0 rigor). The $k_n$ was determined at pCa 5.6 and pCa 4.0. A similar protocol was used in time controls, but Sp-NO was omitted.

A potential issue with use of NO donors is whether the observed effects are mediated via NO or via the parent compound and degradation products. To verify that NO was the mediator of changes in force production, 100 mM oxyhemoglobin (HbO2) was used to scavenge NO in the presence of Sp-NO. HbO2 was prepared by mixing 330 mg of bovine hemoglobin (Sigma Chemical) in 5 ml of phosphate buffer solution. Hemoglobin was reduced to HbO2 by the addition of 35 mg of sodium hydrosulfite (Sigma Chemical) and passed through a 250-ml Sephadex G50 column. A CO-oximeter (Instrumentation Laboratory, Lexington, MA) was used to determine the concentration of the HbO2. In addition, experiments were conducted using degraded Sp-NO, which was obtained by maintaining the Sp-NO stock solution for 6 h at room temperature. Degraded Sp-NO was used in similar experimental protocols as described above.

Statistics

Data were analyzed with SPSS/PC+ (version 9.0, SPSS, Chicago, IL). Between-group data were compared using the independent variable t-test (e.g., difference in baseline force between time control and Sp-NO-treated fibers). Within-group data were compared using two-tailed dependent variables t-test (e.g., difference in force due to Sp-NO treatment within group). Statistical significance was accepted at $P < 0.05$. Values are means ± SE.

RESULTS

Measurement of pCa50

Force-pCa2+ characteristics of psoas fibers were determined to identify the pCa50 (Fig. 1). At pCa 4.0, force reached a clear plateau. Therefore, in subsequent experiments, pCa 4.0 was used for measuring maximal force. Furthermore, calculated pCa50 was 5.62 (coefficient of variation = 1.7%). Accordingly, in subsequent experiments, pCa 5.6 was used for measuring submaximal force.

Effects of Sp-NO on Maximal and Submaximal Force

Exposure to Sp-NO significantly reduced force generated at pCa 4.0 and pCa 5.6 (Table 1). The ratio of force generated by pCa 5.6 to maximal force was significantly reduced in Sp-NO-treated fibers (44 ± 2 preexposure vs. 36 ± 2% postexposure, $P < 0.05$) but not in time controls (39 ± 4 vs. 40 ± 4%, $P > 0.05$), indicating that Sp-NO reduced Ca2+ sensitivity of force generation. Preexposure force tended to be lower in the Sp-NO group than in time controls. In addition, significant rundown in force generation was observed at pCa 4.0 and pCa 4.0 rigor. Therefore, forces were also expressed as percentage of preexposure value (Fig. 2). Exposure to Sp-NO did not affect force generated at pCa 4.0 rigor compared with time control. Force generation at pCa 5.6 was significantly reduced after exposure to Sp-NO compared with time controls (75 ± 4 vs. 99 ± 4%, $P < 0.05$). Similarly, exposure to Sp-NO reduced maximal force compared with time controls (91 ± 1 vs. 97 ± 1%, $P < 0.05$). Exposure to degraded Sp-NO did not affect force generation at any level of activation ($n = 7$; Fig. 2). Furthermore, the effect of Sp-NO on submaximal force generation was abolished in the presence of HbO2 (results not shown).

Table 1. Effect of Sp-NO on force generation in permeabilized rabbit psoas single fibers

<table>
<thead>
<tr>
<th>Time Control ($n = 9$)</th>
<th>Sp-NO ($n = 8$)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Preexposure</td>
</tr>
<tr>
<td>pCa 5.6</td>
<td>2.87 ± 0.46</td>
</tr>
<tr>
<td>pCa 4.0</td>
<td>5.84 ± 0.76</td>
</tr>
<tr>
<td>pCa 4 rigor</td>
<td>6.05 ± 0.83</td>
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</tbody>
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Values are means ± SE expressed in N/cm². Sp-NO, spermine NONOate; pCa, negative logarithm of Ca²⁺ concentration. †Significantly different from preexposure; ‡significantly different from time control ($P < 0.05$).
Effect of Sp-NO on $\alpha_{fs}$

The $\alpha_{fs}$ was measured to determine actomyosin filament overlap. At pCa 4.0, ~75% of the available cross bridges were in the strongly attached state, whereas this percent was lower during submaximal activation (Fig. 3). At pCa 4.0 and pCa 5.6, $\alpha_{fs}$ was significantly lower in Sp-NO-treated fibers than in time controls. Degraded Sp-NO did not affect $\alpha_{fs}$ at (sub)maximal activation ($n = 7$, data not shown).

Effect of Sp-NO on $k_{tr}$

The $k_{tr}$ was measured to study cross-bridge cycling kinetics. A representative trace is shown in Fig. 4A, and grouped data are shown in Fig. 4B. Figure 4B clearly shows the Ca$^{2+}$ dependency of the $k_{tr}$. Exposure to Sp-NO significantly reduced $k_{tr}$ during submaximal, but not maximal, Ca$^{2+}$ activation. Degraded Sp-NO did not significantly affect $k_{tr}$ at (sub)maximal activation ($n = 7$).

DISCUSSION

The present study demonstrates that, in permeabilized rabbit psoas fibers, Sp-NO reduces force generation at submaximal Ca$^{2+}$ activation, reduces $\alpha_{fs}$, and slows cross-bridge cycling kinetics (reflected by a slower $k_{tr}$ during submaximal activation). The effect of Sp-NO on force generation is consistent with a number of previous studies using different preparations and different NO donors (13, 19, 26, 29). Similarly, the more pronounced effects of Sp-NO on force generation at submaximal Ca$^{2+}$ activation are in general agreement with previous studies (1, 26). Together, these results indicate an NO-induced reduction in the Ca$^{2+}$ sensitivity of cross-bridge recruitment. The effect of NO on cross-bridge cycling kinetics is more controversial, with one study indicating no effect (1) and another reporting a significant slowing (13). The results of the present study indicate that the effect of Sp-NO on $k_{tr}$ is relatively modest and is limited to submaximal Ca$^{2+}$ activation. Thus these observations may partially reconcile the discrepancies in these previous observations.
Sp-NO, a nucleophilic type of NO donor with the general structure \([XN(O)NO]^-\), spontaneously releases NO (21). The rate of NO release is only dependent on pH, temperature, and the nucleophilic residue (23), and it is therefore easy to control. The rate of release of NO from Sp-NO is \(\sim 30\) times lower than release from the frequently used NO donor diethylamine NONOate (21). Therefore, a higher concentration of Sp-NO is required to obtain similar biological effects (15). It is important to consider that the effects of NO donors can be independent of the release of NO. In the present study, these potential independent effects were controlled by demonstrating that degraded Sp-NO did not affect the mechanical properties of psoas fibers. The absence of an effect of degraded Sp-NO is consistent with a previous report demonstrating no effect of degraded Sp-NO on smooth muscle mechanical properties (15). This previous study also showed that HbO\(_2\) abolished the depressant effects of Sp-NO on smooth muscle force generation, indicating that NO release mediates the effects of Sp-NO (15). Similarly, the results of the present study in skeletal muscle supported these findings, inasmuch as HbO\(_2\) abolished the effects of Sp-NO on submaximal force generation.

In the present study, chemiluminescence measurements of NO, were used to approximate changes in NO concentration induced by Sp-NO at various concentrations (100, 500, and 1,000 \(\mu M\)). As noted above, the chemiluminescence method is not specific for free NO, but it detects other oxidative products of NO as well (e.g., NO\(_2\) and NO\(_3\), NO (22). However, these chemiluminescence measurements did verify that NO concentration increased as Sp-NO concentration increased, strongly indicating that NO concentration also increased. In addition, it was shown that the reduction in force generation at submaximal Ca\(^{2+}\) activation (pCa 5.6) was dependent on Sp-NO concentration. At 1,000 \(\mu M\) Sp-NO, the concentration inducing the greatest reduction in force, NO concentration was 892 \(\mu M\), and it is likely that the NO concentration was even less. However, it is difficult to determine the physiological relevance of the Sp-NO concentration used in the present study, since the local concentration of NO in skeletal muscle fibers remains unknown. Although NO release has been measured from intact skeletal muscle tissue in vitro (2), no data are available concerning the NO concentrations within subcellular compartments. This is important, since within muscle fibers, NOS expression is not uniform (19, 20), suggesting that localized areas of higher NO concentration may exist.

In the present study, we found that Sp-NO reduced the ratio of stiffness during Ca\(^{2+}\) activation to maximal rigor activation \((\alpha_{fs})\), indicating an effect on the number of cross bridges in the strongly bound state (12, 17, 18). The parallel changes in \(\alpha_{fs}\) and force induced by Sp-NO indicate that NO influences cross-bridge recruitment, rather than the force generated per cross bridge.

Brenner and colleagues (4, 5, 7) presented an analytical framework based on the Huxley two-state model of cross-bridge cycling (with \(f_{app}\) and \(g_{app}\)) that showed the relationship between the number of strongly bound cross bridges \((\alpha_{fs})\) and cross-bridge cycling kinetics at any given level of Ca\(^{2+}\) activation

\[
\alpha_{fs} = f_{app} / (f_{app} + g_{app})
\]

Brenner and colleagues utilized measurements of \(k_{tr}\) to derive the components of cross-bridge cycling kinetics

\[
k_{tr} = f_{app} + g_{app}
\]

The results of the present study demonstrated that Sp-NO reduces \(k_{tr}\) at submaximal (pCa 5.6) but not maximal (pCa 4.0) Ca\(^{2+}\) activation. These results are consistent with the effects of Sp-NO on force and \(\alpha_{fs}\) and suggest an influence on cross-bridge recruitment \((f_{app})\) at submaximal Ca\(^{2+}\) activation. The effects of NO donors on \(k_{tr}\) in single skeletal muscle fibers have been previously studied with various outcomes (1, 13). Gallier et al. (13) found that \(S\)-nitroso-N-acetylpenicillamine slowed \(k_{tr}\), whereas Andrade et al. (1) found no
effect of S-nitroso-N-acetylcysteine on $k_{tr}$. Both studies evaluated $k_{tr}$ during maximal activation. Moreover, the methods used to determine $k_{tr}$ in both studies did not include controlling sarcomere length and restretching fibers to original length, both of which might affect $k_{tr}$ (7). In addition, Andrade et al. evaluated the effect of S-nitroso-N-acetylcysteine on only a very small number of intact fibers ($n = 3$).

A key issue is the potential site of action of NO. The results of the present study are not consistent with a direct effect of NO on the force generated per cross bridge, since Sp-NO did not influence fiber mechanics at maximum Ca$^{2+}$ activation (pCa 4.0). However, the Sp-NO-induced reductions in force $\alpha_{st}$ and $k_{tr}$ during submaximal Ca$^{2+}$ activation indicate a decrease in the Ca$^{2+}$ sensitivity of cross-bridge recruitment, which could result from a number of underlying mechanisms including altered Ca$^{2+}$ binding to TnC. Binding of Ca$^{2+}$ to TnC induces a conformational change in the troponin complex, resulting in an increase in the availability of myosin-binding sites on actin (30). However, there is no direct evidence that NO affects Ca$^{2+}$ binding to TnC. TnC (and troponin I) contain cysteine residues, which are known to be susceptible to reactive oxidants (10, 27). Biochemical studies showed that disulfide cross-linking of these residues decreased Ca$^{2+}$ sensitivity of TnC (25). In fact, after disulfide cross-linking of cysteine residues, the troponin complex was no longer able to regulate actomyosin ATPase activity in a Ca$^{2+}$-dependent matter. Although no direct biochemical evidence exists that NO is able to alter troponin function, a change is likely to occur when NO modifies the cysteine residues. Such a modification would be consistent with the results of the present study. First, the dependence of force and $k_{tr}$ on Ca$^{2+}$ is mediated through TnC and thin-filament regulation (9, 28). Second, force and $k_{tr}$ can be dissociated by modifying TnC characteristics (9, 28). Alternatively, the depressant effect of NO on force and $k_{tr}$ could be mediated through modification of the hyperreactive thiol SH-1 and SH-2 on the myosin head (26). However, the absence of an effect of Sp-NO during maximum Ca$^{2+}$ would be inconsistent with such a mechanism.

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