Nitric oxide contributes to vascular smooth muscle relaxation in contracting fast-twitch muscles

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Grange, Robert W., Eiji Isotani, Kim S. Lau, Kristine E. Kamm, Paul L. Huang, and James T. Stull. Nitric oxide contributes to vascular smooth muscle relaxation in contracting fast-twitch muscles. Physiol Genomics 5: 35–44, 2001.—During skeletal muscle contraction, NO derived from neuronal nitric oxide synthase (nNOS) in skeletal muscle fibers or from endothelial cells (eNOS) may relax vascular smooth muscle contributing to functional hyperemia. To examine the relative importance of these pathways, smooth muscle myosin regulatory light chain (smRLC) phosphorylation was assessed as an index of vascular tone in isolated extensor digitorum longus (EDL) muscles from C57, nNOS−/−, and eNOS−/− mice. The smRLC phosphorylation (in mol phosphate per mol smRLC) in C57 resting muscles (0.12 ± 0.04) was increased 3.7-fold (0.44 ± 0.03) by phenylephrine (PE). Reversal of this increase with electrical stimulation (to 0.19 ± 0.03; P < 0.05) was partially blocked by Nω-nitro-L-arginine (NLA). In nNOS−/− EDL, the PE-induced increase in smRLC phosphorylation (0.10 ± 0.02 to 0.49 ± 0.04) was partially decreased by stimulation (0.25 ± 0.04). In eNOS−/− EDL, the control value for smRLC was increased (0.24 ± 0.04), and PE-induced smRLC phosphorylation (0.36 ± 0.06) was decreased by stimulation even in the presence of NLA (to 0.20 ± 0.02; P < 0.05). These results suggest that in addition to NO-independent mechanisms, NO derived from both nNOS and eNOS plays a role in the integrative vascular response of contracting skeletal muscle.

endothelial nitric oxide synthase; neuronal nitric oxide synthase; exercise hyperemia; skeletal muscle

THE NITRIC OXIDE SYNTHASES (NOSs) constitute a family of three distinct isoforms including neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) enzymes (23). NOSs all function to convert L-arginine to L-citrulline and NO in the presence of molecular oxygen and appropriate cofactors (22). nNOS and eNOS are constitutively expressed and require Ca2+/calmodulin for activation. Among its many biological functions, NO generated in endothelial cells initiates a relaxation cascade in smooth muscle via a cGMP pathway that activates cGMP-dependent protein kinase (PKG). This kinase acts through a variety of cellular mechanisms to reduce intracellular calcium concentration ([Ca2+]i), decrease Ca2+-dependent phosphorylation of smooth muscle myosin regulatory light chain (smRLC), and thereby bring about smooth muscle relaxation. The decreased tone leads to vasodilation and increased blood flow in the affected vascular bed (21).

In contracting skeletal muscle, increased blood flow to match energy demand is known as active/functional hyperemia and is due in part to a decrease in vascular resistance (1). Vascular resistance can be decreased by relaxation of smooth muscle cells in the arterioles and precapillary sphincters that control access to capillary beds within the active muscles. Arterioles can dilate in a very localized manner (8) in response to many of the metabolites that are released in exercise (e.g., K+, adenosine, ATP, H+, etc.), as well as to changes in tissue Po2 (14, 29). Despite the obvious advantage of matching blood flow to metabolic demand, the specific connection between these exercise-induced signals and local arteriolar dilation remains elusive (9).

Recently, it was proposed that NO produced by eNOS and nNOS (13) may also regulate functional hyperemia, because NO is produced in contracting skeletal muscle (2). NOS activity has also been implicated in functional hyperemia at the microcirculatory level (17, 26). For example, in isolated rat arterioles, increased sensitivity following exercise training has been attributed to NOS activity (17), and in the hamster cremaster muscle individual arterioles demonstrate decreased dilation in the presence of nitro-L-arginine methyl ester (l-NNAME) during field stimulation (10). However, other studies have reported no changes in the hyperemic response to skeletal muscle contraction in the presence of NOS inhibitors (rabbit, Ref. 28; human, Ref. 42; dog, Ref. 3). Conversely, in humans, the NOS inhibitor Nω-monomethyl-L-arginine (l-NMMA) administered during exercise attenuated forearm blood flow 20–30% compared with exercise alone (7). It has also been suggested for humans that NO is an important vascular regulator at rest and during recovery from exercise rather than at the onset or during the activity itself (30). The conflicting results may arise from the experimental model tested, administration of the NOS inhibitor before or during contractions, or differ-
ences in the intensity of the exercise (24). In studies where a positive relation between NO and exercise hyperemia is observed, it is not clear whether NO is derived from endothelium, skeletal muscle fibers, or both.

NO is released from contracting skeletal muscle (2), indicating that repetitive skeletal muscle contractions lead to activation of a Ca\(^{2+}\)/calmodulin-dependent NOS; therefore, both nNOS and eNOS may be activated (15, 16). However, NO produced in muscle fibers during contractile activity may be predominantly nNOS derived (11). The \(\mu\)-form of nNOS (34) expressed in fast-twitch skeletal muscle fibers is localized to the sarcolemma with dystrophin and is enriched in neuromuscular junctions, costameres, and myotendinous junctions (4, 5, 25). The localization of nNOS at the sarcolemma suggests that NO could readily diffuse out to smooth muscle cells of adjacent vasculature, bind to soluble guanylyl cyclase (sGC), and initiate the cGMP relaxation cascade. In support of this possibility, a 1.7-fold increase in cGMP content induced by electrical stimulation in control mouse extensor digitorum longus (EDL) muscles in vitro was blocked by N\(^{\omega}\)-nitro-L-arginine (NLA), an NOS inhibitor (19). Most importantly, cGMP content was not increased in electrically stimulated nNOS-deficient EDL (19). Furthermore, the increases in cGMP appear related to the content of nNOS in the muscle fibers. Soleus muscles from control mice (19) and EDL muscles from \(mdx\) mice (18), both of which have less nNOS, do not increase cGMP formation during electrical stimulation in vitro. As well, reduction in sympathetic \(\alpha\)-adrenergic vasoconstriction is attenuated during muscle contraction in \(mdx\) compared with C57 mice (40) and is more prominent in fast- compared with slow-twitch oxidative muscles (39). Collectively, these results suggest that nNOS activity is necessary for increased cGMP formation in contracting fast-twitch skeletal muscle. cGMP formation in eNOS\(^{-/-}\) mice in response to stimulation is attenuated despite the presence of nNOS protein, but the increase can be blocked by NLA treatment (19). These results suggest the presence of nNOS-derived NO, and also decreased vascular sensitivity to NO compensates for the absence of eNOS (19). To be considered an effective contributor to functional hyperemia, however, nNOS-derived NO, like eNOS-derived NO, in control muscle must not only yield increases in cGMP but also modulate vascular tone. Because decreases in \([Ca^{2+}]_{i}\), associated with cGMP formation result in a decreased extent of smRLC phosphate content and smooth muscle force development (20, 37), smRLC phosphate content can be used as a biochemical index of vascular tone and indirectly as an index of blood flow during muscle activation.

In this study, we test the hypothesis that NO derived from nNOS and/or eNOS contributes to decreases in smRLC phosphate content in contracting EDL. To separate the contribution of NO derived from nNOS or eNOS, we have examined EDL muscles from control mice as well as mice lacking nNOS or eNOS. We demonstrate that the decrease in smRLC phosphate associated with contracting C57 EDL in vitro can be partially blocked by the NOS inhibitor, NLA, suggesting the presence of an NLA-sensitive component of relaxation. Our data also suggest that the NLA-sensitive relaxation component requires both nNOS and eNOS.

**METHODS**

**Mice**

C57, nNOS knockout (nNOS\(^{-/-}\)), and eNOS knockout (eNOS\(^{-/-}\)) mice were obtained as reported previously (19). All mice were maintained on a 12:12-h light-dark cycle and were given food and water ad libitum. The methods employed in this study were approved by the Institutional and Animal Care Advisory Committee at the University of Texas Southwestern Medical Center at Dallas.

**Skeletal Muscle Preparation**

Fast-twitch EDL muscles (mass, 12–15 mg; length, 10–14 mm) were isolated from 12- to 16-wk-old C57, nNOS\(^{-/-}\), and eNOS\(^{-/-}\) male euthanized with a lethal dose of pentobarbital sodium (250 mg/kg body wt ip). Individual muscles were suspended with 4-0 suture from a Grass model FT03C isometric force transducer (Astro-Med) and clamped at the base of the organ bath. Muscles were incubated in an oxygenated (95% O\(_2\)-5% CO\(_2\)) physiological salt solution (PSS; pH 7.6, 30°C) containing (in mM) 120.5 NaCl, 4.8 KCl, 1.2 MgSO\(_4\), 20.4 NaHCO\(_3\), 1.6 CaCl\(_2\), 1.2 NaH\(_2\)PO\(_4\), 10 glucose, and 1.0 pyruvate. Muscles were stimulated by square pulses of 0.2-ms duration at a voltage and at a muscle length \((L_0)\) to elicit maximal isometric twitch force (model S48 stimulator, Grass Instruments) via closely flanking platinum electrodes. Following an equilibration period of ~30 min to establish \(L_0\) and low levels of smRLC phosphate content, stimulation alone, drug(s) alone, or stimulation plus drug(s) experiments were initiated.

**Experimental Treatments**

The overall objective of this study was to determine whether an NO-dependent pathway was activated during contraction of fast-twitch skeletal muscle that could lead to decreases in smRLC phosphate content and thereby contribute to functional hyperemia. To address this question, we used three mouse genotypes and a variety of physiological and pharmacological interventions. The experimental treatments comprising our approach, along with the purpose for each, and the predicted smRLC phosphorylation response for each treatment with each mouse genotype are briefly described in Table 1.

All muscles were pretreated for 60-s with 10 \(\mu\)M phenylephrine (PE) to first increase smRLC phosphorylation, followed by two 10-min washes with PSS to decrease phosphorylation. During baseline experiments, the extent of smRLC phosphate content was determined under the following treatments: 1) control (C); 2) electrical stimulation at 30 Hz for 15 s (ST); 3) incubation with 10 \(\mu\)M PE for 60 s (PE); 4) incubation with 10 \(\mu\)M sodium nitroprusside for 30 s (SNP); and 5) incubation with 1 mM NLA, an NOS inhibitor, for 30 min (NLA). The primary purpose of the baseline treatments was to establish the control smRLC phosphate content and changes resulting from each of the other treatments alone in each of the mouse genotypes. For example, we expected that smRLC phosphate content would be significantly elevated above control with PE treatment to stimulate smooth muscle contraction and that we could use this to determine de-

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creases, if any, in smRLC phosphate content when PE was combined with a treatment such as electrical stimulation. The combined treatments included 1) 1 mM NLA for 30 min with 30 Hz stimulation the final 30 s (ST-NLA); 2) 10 μM PE for 60 s with 30 Hz stimulation the final 30 s (ST-PE); 3) 60 s of 10 μM PE with 10 μM SNP (SNP-PE); 4) 1 mM NLA for 30 min with addition of 10 μM PE the final 60 s (PE-NLA); and 5) 1 mM NLA for 30 min with addition of 10 μM PE the final 60 s and 30 Hz stimulation the final 30 s (ST-PE-NLA). The primary purposes of these treatments were to 1) determine whether an NO-dependent pathway contributes to decreases in PE-induced smRLC phosphate content and 2) to analyze contributions from nNOS and/or eNOS. At the conclusion of each treatment, muscles were frozen with tongs prechilled in liquid nitrogen and stored at −80°C for subsequent analysis.

### Sample Preparation

smRLC phosphate content was determined in samples employing modifications to the methods of Persechini et al. (27) and Walker et al. (41). Frozen muscles were weighed in a reach-in cold box (−30°C) on a Cahn electrobalance and then thawed in a 10% trichloroacetic acid (TCA)/acetone slurry containing 1 mM dithiothreitol (DTT). Thawed samples were homogenized in 500 μl ice-cold 10% TCA/1 mM DTT/water with a ground glass mortar and pestle for 20 s at 70 rpm. After centrifugation at 3,000 rpm for 2 min in a tabletop centrifuge, sample pellets were washed three times with 500 μl ethyl ether (Fisher), air-dried in a fume hood for 20 min, and resuspended in 300 μl sample buffer (in mM: 18.5 Tris, 20.4 glycine, 9.2 DTT, and 4.6 EDTA; pH 8.6) containing 8 M urea. To ensure complete protein solubilization, myosin RLC (18). Following incubation, membranes were washed briefly (15 s) in 100% methanol, placed on Whatman no. 1 filter paper, and dried 30 min at 80°C. Membranes were then transferred to Immobilon-P transfer membrane (polyvinylidene fluoride (PVDF), Millipore) at 25 V for 60 min in ice-cold transfer buffer.

Following Western transfer, membranes were washed 20% TCA/2 mM DTT/water was added to the supernatant fraction and, the samples incubated on ice for 20 min. Following centrifugation for 7 min at 7,000 rpm, the supernatant fraction was aspirated and the pellet resuspended in 8 M urea sample buffer as described above with the addition of saturated sucrose and 0.004% bromophenol blue. Samples were solubilized by saturation with urea crystals and the pH was maintained by adding 2.5 M Tris base (pH 11.0) and mixed vigorously for 60 min at room temperature. Samples were stored at −80°C.

### Quantification of smRLC Phosphate Content

Twenty microliters of each sample were subjected to polyacrylamide gel electrophoresis using a mini-gel system (Bio-Rad). The cathode buffer was supplemented with 2 mM thioglycolate and 2 mM DTT. After pre-electrophoresis at 400 V for 60 min, samples were loaded, and electrophoresis was performed at 400 V for 100 min. Following electrophoresis, the gels were rinsed twice, for 5 min each time, in Western transfer buffer at room temperature (25 mM Tris, 192 mM glycine, 0.05% SDS, and 20% methanol, pH 7.2). Proteins were then transferred to Immobilon-P transfer membrane (polyvinylidene fluoride (PVDF), Millipore) at 25 V for 60 min in ice-cold transfer buffer.

Following Western transfer, membranes were washed briefly (15 s) in 100% methanol, placed on Whatman no. 1 filter paper, and dried 30 min at 37°C. Membranes were then fixed in 0.4% glutaraldehyde (8% stock EM grade; Polysciences) for 30 min, washed in PBS (pH 7.4) three times, for 8 min each time, and blocked in 5% Amersham Liquid Block (Amersham, UK)/PBS for 60 min (all steps room temperature). Membranes were incubated overnight at 4°C with a primary monoclonal antibody (ascites diluted 1:15,000; a generous gift of Kathy Trybus) in 0.5% Amersham Block/PBS, which recognizes smooth muscle, but not skeletal muscle, myosin RLC (18). Following incubation, membranes were washed (five 5-min washes) in PBS and then incubated 60 min at room temperature with goat anti-mouse IgG(H+L) conjugated with alkaline phosphatase (1:10,000; Southern Biosciences, La Jolla, CA). The membranes were washed (five 5-min washes) in PBS and then incubated 60 min at room temperature with goat anti-mouse IgG(H+L) conjugated with alkaline phosphatase (1:10,000; Southern Biosciences, La Jolla, CA). Eighteen microliters of 1 mg/ml nitro blue tetrazolium (NBT)/5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were added to each membrane. The reactions were allowed to proceed for 30 min, and the membranes were then washed in tap water to stop the reactions.

### Table 1. Predicted smRLC phosphate content for each mouse genotype with experimental treatments addressing the role of NO in vascular regulation of fast-twitch skeletal muscle

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Purpose</th>
<th>Proposed smRLC Phosphate Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Control</td>
<td>Basal smRLC phosphorylation</td>
<td>low/lownOS+/−/eNOS+/−</td>
</tr>
<tr>
<td>2) ST</td>
<td>Effect of muscle contraction on basal smRLC phosphorylation</td>
<td>low/lownOS+/−/eNOS+/−</td>
</tr>
<tr>
<td>3) PE</td>
<td>Alpha-adrenergic stimulation of smooth muscle contraction</td>
<td>high/high/nOS+/−/eNOS+/−</td>
</tr>
<tr>
<td>4) SNP</td>
<td>Effect of NO donor on basal smRLC phosphorylation</td>
<td>low/lownOS+/−/eNOS+/−</td>
</tr>
<tr>
<td>5) NLA</td>
<td>Effect of NOS inhibitor on basal smRLC phosphorylation</td>
<td>modest/modest/nOS+/−/eNOS+/−</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Combined</th>
<th>Purpose</th>
<th>Proposed smRLC Phosphate Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) ST-NLA</td>
<td>Effect of NOS inhibitor on smRLC phosphorylation during muscle contraction (ST)</td>
<td>low/lownOS+/−/eNOS+/−</td>
</tr>
<tr>
<td>2) ST-PE</td>
<td>Can contracted smooth muscle (PE) be relaxed (smRLC dephosphorylation) with skeletal muscle contraction (ST)?</td>
<td>decreased/unchanged/decreased</td>
</tr>
<tr>
<td>3) SNP-PE</td>
<td>Can contracted smooth muscle be relaxed by a NO donor?</td>
<td>decreased/decreased/decreased</td>
</tr>
<tr>
<td>4) PE-NLA</td>
<td>Does smRLC phosphorylation increase with PE treatment if NOSs are inhibited?</td>
<td>increased/increased/increased</td>
</tr>
<tr>
<td>5) ST-PE-NLA</td>
<td>Does elevated smRLC phosphorylation decrease with stimulation if NOSs are inhibited?</td>
<td>stay high/stay high/stay high</td>
</tr>
</tbody>
</table>

smRLC, smooth muscle myosin regulatory light chain; ST, stimulation; PE, phenylephrine; SNP, sodium nitroprusside; NLA, N•-nitro-l-arginine; NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS.
Biotechnology Associates). After washing in PBS (three 5-min washes), the membranes were incubated in alkaline phosphatase assay buffer (50 mM Tris and 1 mM MgCl₂, pH 9.5; 3 times for 2 min each time). The phosphorylated and nonphosphorylated RLC bands were detected by chemiluminescence (CSPD substrate and Sapphire Enhancer, Tropix), according to the manufacturer's instructions; CSPD substrate is disodium 3-(4-methoxyspiro-[1,2-dioxetane-3,2',5'-chloro]tricyclo[3.3.1.1^{3,7}]decan)-4-yl)phenyl phosphate. The ratio of phosphorylated RLC to total RLC was determined by densitometry (Molecular Analyst; Bio-Rad) and reported as moles of phosphate per mole RLC (18, 27).

Statistics

Differences in smooth muscle RLC phosphate content between the mouse genotypes and across treatments were analyzed by a two-way ANOVA; effects of treatments within a given mouse genotype or effects of mouse genotype within a given treatment were examined with a one-way ANOVA. Duncan’s multiple range post hoc test was applied to all significant main effects to determine differences between means. Differences were considered significant for \( P < 0.05 \). Data are means ± SE.

RESULTS

In this study, we developed a sensitive assay to measure vascular smRLC phosphate content in isolated EDL muscles from C57, eNOS\(^{-/-}\), and nNOS\(^{-/-}\) mice under a variety of conditions to dissect the contribution of nNOS- and eNOS-derived NO to smRLC dephosphorylation in contracting skeletal muscle. Figure 1 is a representative blot that illustrates the differences in smRLC phosphate content among the three genotypes for selected treatments. In all cases smRLC phosphorylation by this mobility shift assay was easily measured. Importantly, the similarity in staining intensities indicated no significant adaptive changes in the relative amounts of smRLC phosphorylation in the different animal groups.

Treatment Responses for Each Genotype

C57 EDL. To examine the contribution of NOSs to functional hyperemia, we evaluated the responses of EDL muscles obtained from C57 mice in which both eNOS and nNOS were present. A pharmacological approach was used to address the following primary question: is there an NO-dependent pathway induced by skeletal muscle contraction that leads to decreases in smRLC phosphorylation? C57 EDL muscles were subjected to a series of treatments to assess their impact on smRLC phosphorylation, first for each treatment alone (baseline) and then in combination (Table 1). smRLC phosphate contents for selected treatments applied to C57 EDL muscles are reported in Fig. 2. Basal smRLC phosphorylation for the control condition was 0.12 ± 0.04 mol phosphate/mol RLC, and this value was not significantly changed by either electrical stimulation (0.07 ± 0.03), 10 μM SNP, an NO donor (0.11 ± 0.02), or 1 mM NLA, an NOS inhibitor (0.19 ± 0.06). However, as predicted (Table 1), treatment with 10 μM PE, an α-adrenergic agonist, induced a significant increase in smRLC phosphorylation (0.44 ± 0.03; \( P < 0.05 \)). This result indicated smRLC phosphate content in EDL muscles could be readily elevated above basal levels with PE to mimic a vasoconstritor response.

To test for an association between skeletal muscle contraction, NO, and functional hyperemia, treatment combinations of PE and either electrical stimulation (i.e., muscle contraction) or SNP (NO donor) were used to test for decreases in smRLC phosphate content (Fig. 2). Electrical stimulation at 30 Hz (ST-PE; 0.19 ± 0.03) or treatment with 10 μM SNP (SNP-PE; 0.14 ± 0.03) during the final 30 s of a 60-s treatment with 10 μM PE returned smRLC phosphate content to control levels. To determine whether the dephosphorylation was NO dependent, EDL muscles were pretreated with NLA and then were subjected to PE treatment with and without electrical stimulation. We first determined there were no changes in basal smRLC phosphate content when stimulation and NLA were combined...
ing that all or most of the endogenous NOS activity was inhibited by NLA, muscle activation itself had a potent dephosphorylation effect by NO-independent mechanisms. However, stimulation did not return the smRLC phosphate content to control values in the ST-PE-NLA condition, whereas it did in the absence of NLA (i.e., ST-PE). This result suggests there is also an NLA-sensitive component that contributes to dephosphorylation. That is, the additional decrease required to return smRLC phosphate content to control values from the ST-PE-NLA value could be attributed to NLA-sensitive mechanisms. However, the source of the NO cannot be determined on the basis of these data. To discriminate the source of NO, we applied the same treatments described above to EDL muscles from nNOS−/− and eNOS−/− mice.

**nNOS−/− EDL.** In the absence of NOS, will skeletal muscle contraction still result in decreased smRLC phosphorylation similar to that observed for the C57 EDL? The smRLC phosphate contents for selected treatments applied to nNOS−/− EDL muscles are reported in Fig. 3 (see also Table 1). Basal smRLC phosphorylation was 0.10 ± 0.02 mol phosphate/mol RLC, and this was not significantly changed by either ST (0.08 ± 0.02), SNP (0.11 ± 0.01), or NLA (0.20 ± 0.06). There was no change

Fig. 2. Decreases in the extent of PE-induced smRLC phosphate content in C57 EDL involve NLA-sensitive and NO-independent relaxation pathways. Extent of and changes in PE-induced smRLC phosphorylation were assessed under various treatments. *smRLC phosphorylation values were greater than control (P < 0.05). †PE in the absence and presence of NLA (PE-NLA) increased smRLC phosphorylation to levels greater than all other treatments (P < 0.05). Both stimulation and SNP in the presence of PE reduced smRLC phosphorylation to control levels, indicating the presence of both stimulation-dependent (ST-PE) and NO-dependent (SNP-PE) dephosphorylation pathways. ‡Different from all treatments except ST-PE (P < 0.05). Thus stimulation in the presence of NLA decreased PE-induced smRLC phosphorylation (ST-PE-NLA) but not back to control levels (P > 0.05), suggesting the presence of both NO-independent and NLA-sensitive (including NO-dependent; see DISCUSSION) dephosphorylation pathways. Definitions are as for Fig. 1. Data are means ± SE; number of muscles for each treatment = 4–19. High n values are associated with the control and PE treatments included as internal controls for basal and maximal smRLC phosphate content, respectively, within many of the experiments.

(ST-NLA; 0.17 ± 0.04). However, in the PE-NLA condition, smRLC phosphate content was ~50% greater than PE alone (0.65 ± 0.04; P < 0.05). This potentiated response suggested that the endogenous production of NO that normally opposes smRLC phosphorylation via the cGMP-dependent relaxation pathway (21) and potentially via cGMP-independent pathways (35) was attenuated by NLA. This conclusion appears reasonable, as incubation with a lower concentration of a more specific NOS inhibitor, 100 μM 7-nitroindazole, also significantly attenuated cGMP production in stimulated EDL muscles (data not shown). However, NLA may also inhibit NO-independent pathways to affect smRLC phosphorylation; we therefore describe this outcome as NLA sensitive.

Because the decrease in smRLC phosphorylation in the ST-PE condition was potentially NO dependent, EDL muscles were stimulated in the presence of PE and NLA (ST-PE-NLA) to determine whether the decrease in RLC phosphorylation could be prevented when NOSs were inhibited. In the ST-PE-NLA condition, the mean smRLC phosphate content (0.31 ± 0.02) was decreased 64% relative to the mean PE-NLA peak value [(PE-NLA − ST-PE-NLA)/(ST-PE-NLA − control); (0.65 − 0.31)/(0.65 − 0.12) × 100 = 64%]. Assum-
from basal levels in the ST-NLA condition (0.13 ± 0.06). As with the C57 EDL, treatment with PE induced a significant increase in smRLC phosphorylation (0.49 ± 0.04, P < 0.05). smRLC phosphorylation was decreased from the PE alone values in the SNP-PE condition (0.29 ± 0.02, P < 0.05), indicating the presence of a viable NO-dependent pathway. However, smRLC phosphate content in the ST-PE condition was also decreased (0.25 ± 0.04, P < 0.05). In contrast to results obtained with EDL muscles from C57 mice, both treatments were insufficient to return the elevated smRLC phosphate content to control levels (Fig. 3).

As with the C57 EDL response, smRLC phosphorylation was potentiated in the PE-NLA condition (0.68 ± 0.03). Electrical stimulation resulted in a significant 33% decrease in smRLC phosphate content in the ST-PE-NLA condition (to 0.49 ± 0.06, P < 0.05) compared with that of the PE-NLA condition [(0.68 – 0.49)/(0.68 – 0.10) × 100 = 62%]. This decrease was likely due to an NO-independent mechanism. This response is about half the relative decrease in mean smRLC phosphate content between the PE and the ST-PE conditions [(0.49 – 0.25)/(0.49 – 0.10) × 100 = 62%]. Note that the extent of smRLC phosphorylation with ST-PE-NLA treatment is not different from that of the PE treatment but is greater than either the SNP-PE or the ST-PE treatments (P < 0.05; Fig. 3).

eNOS−/− EDL. In the absence of eNOS but presence of nNOS, will skeletal muscle contraction result in decreased smRLC phosphorylation similar to that observed for the C57 EDL? The smRLC phosphate contents for selected treatments applied to eNOS−/− EDL muscles are reported in Fig. 4 (see also Table 1). Basal smRLC phosphorylation was significantly elevated to 0.24 ± 0.04 mol phosphate/mol RLC and was decreased similarly by both ST (0.10 ± 0.01, P < 0.05) and SNP (0.06 ± 0.01, P < 0.05) but not by NLA (0.36 ± 0.06; Fig. 4). An unexpected outcome was the blunted response to PE alone (0.36 ± 0.04) that was not different from control values. smRLC phosphate content in the ST-PE condition was decreased (0.18 ± 0.05, P < 0.05) compared with PE alone. However, smRLC phosphate content was also decreased from basal levels in the ST-NLA condition (0.12 ± 0.02, P < 0.05), suggesting a stimulation-dependent but NO-independent relaxation pathway. smRLC phosphorylation was similar to control values in the SNP-PE condition (0.27 ± 0.06), which contrasted with the significant decrease from control values in the SNP alone condition. Taken together, these two results suggest that smooth muscle in eNOS−/− EDL may be less sensitive to SNP in the presence compared with the absence of PE.

As with both the C57 and the nNOS−/− responses, PE-NLA treatment yielded a potentiated smRLC phosphate content compared with PE alone (0.63 ± 0.05, P < 0.05). Again unexpectedly, smRLC phosphate content in the ST-PE-NLA condition (0.20 ± 0.02, P < 0.05) was significantly decreased from the PE-NLA values. This outcome together with the ST-NLA result suggested the presence of stimulation-dependent but NO-independent relaxation mechanisms in eNOS−/− EDL.

Summary of treatment effects between each mouse genotype. The pattern of responses for smRLC phosphate content for the three genotypes across treatments is depicted in Fig. 5. Among the three genotypes, the pattern was essentially similar for three of the baseline treatments (ST, SNP, NLA) and three of the combined treatments (ST-PE, ST-NLA, PE-NLA). The most consistent responses across the three genotypes were to the ST, SNP, and PE-NLA treatments. smRLC phosphorylation values for each genotype under the ST and SNP conditions were all ~0.10 mol phosphate/mol RLC, while in response to PE-NLA, smRLC phosphate content was dramatically increased over PE alone (P < 0.05; Fig. 5). In contrast, differences between the genotypes were noted in the control, PE, SNP-PE, and ST-PE-NLA treatments. The eNOS−/− control value (0.24 ± 0.04) was about twofold greater than either the C57 (0.12 ± 0.03) or nNOS−/− (0.10 ± 0.02) control levels (P < 0.05), indicating a role for eNOS in maintaining a low extent of smRLC phosphate content under basal conditions. The eNOS−/− PE response (0.36 ± 0.04) was significantly blunted compared with the nNOS−/− response (0.49 ± 0.04, P < 0.05). This suggests, as with the depressed resting cGMP level and smaller absolute response to the NO donor, SNP, in
Fig. 5. The extent of EDL smRLC phosphate content in EDL muscles obtained from C57, eNOS−/−, and nNOS−/− mice differ for specific treatments. Asterisks and symbols denote significant differences between the genotypes within a given treatment. *eNOS−/− EDL have a greater basal level of smRLC phosphorylation than either C57 or nNOS−/− EDL (P < 0.05). †eNOS−/− compared with nNOS−/− EDL are less responsive to PE (P < 0.05). ‡C57 EDL are more sensitive to SNP, an NO donor, for reducing PE-induced smRLC phosphorylation than either nNOS−/− or eNOS−/− EDL (SNP-PE; P < 0.05). §nNOS−/− EDL smRLC phosphorylation was greater than either C57 or eNOS−/− EDL (P < 0.05), indicating decreased sensitivity to stimulation (ST) in the presence of NLA, an inhibitor of NOS activity. Data are means ± SE; number of muscles for each treatment within genotype = 4–22. High n values are explained in legend to Fig. 2.

EDL in vitro (19), that the signal cascade leading to smRLC phosphorylation may be compromised in some way in eNOS−/− EDL muscles. SNP reduced PE-induced smRLC phosphorylation in C57 mice (0.14 ± 0.03) to a greater extent than either the nNOS−/− (0.29 ± 0.02) or eNOS−/− (0.27 ± 0.06) muscles (P < 0.05). This result suggests that the presence of both nNOS and eNOS may be necessary for the muscle to respond completely to SNP. Both the C57 (0.31 ± 0.02) and eNOS−/− (0.20 ± 0.02) dephosphorylation responses to the ST-PE-NLA treatment were greater than those for nNOS−/− (0.49 ± 0.06; P < 0.05).

DISCUSSION

Major Finding

In this study, we evaluated the potential contribution of NO to reduction of PE-induced smRLC phosphate content in contracting muscles in vitro and attempted to discriminate the contributions of NO derived from nNOS and eNOS. Overall, the patterns of response to many of the treatments in each of the C57, nNOS−/−, and eNOS−/− genotypes were similar, with significant differential responses between the genotypes limited to only four of the treatments: control, PE, SNP-PE, and ST-PE-NLA. Comparisons of the responses to these treatments between and within the three genotypes suggest that NO derived from nNOS in C57 EDL contributes to vascular smooth muscle cell relaxation during skeletal muscle contraction, as assessed by smRLC phosphate content, but that vascular regulation overall also requires eNOS.

NO Contributes to Vascular Modulation in C57 EDL

In C57 EDL muscles, stimulation potently reduced PE-induced smRLC phosphate content to a level similar to control values (i.e., ST-PE). The presence of an NO-dependent mechanism is supported by the decrease in PE-induced smRLC phosphorylation to control values by SNP (PE-SNP), an NO donor. Furthermore, a stimulation-dependent decrease in PE-induced smRLC phosphate content occurs coincident with increases in cGMP (18). However, the complete decrease in PE-induced smRLC phosphate content with stimulation alone is only partially blocked by NLA (ST-PE-NLA), which indicates a functioning NO-independent pathway. In agreement with our previous report (19), therefore, total smooth muscle cell relaxation induced by stimulation can be divided into NO-independent (e.g., metabolites) and NLA-sensitive (including NO-dependent and non-NO-dependent relaxation mechanisms inhibited by NLA) mechanisms. The source of the NO cannot be determined from results obtained from C57 muscles; therefore, we evaluated the changes in PE-induced smRLC phosphate content in nNOS−/− and eNOS−/− EDL.

NO-Independent Relaxation Occurs in nNOS−/− EDL

In nNOS−/− EDL, stimulation reduced PE-induced smRLC phosphorylation but not to control values. This response likely represents an NO-independent relaxation mechanism but could also include NLA-sensitive mechanisms, including NO derived from eNOS. Both eNOS and nNOS are considered contributors to basal NOS activity in skeletal muscle (31). In the present study, eNOS and not nNOS appears to represent the major contributor to basal tone, because smRLC phosphate content was approximately twofold greater in the eNOS−/− compared with the C57 and nNOS−/− EDL control values. During contractions of nNOS−/− EDL, however, NO produced from eNOS may be minimal because increased cGMP formation was absent (19). This limited cGMP formation was not due to the absence of an NO-responsive pathway in nNOS−/− EDL, because SNP still increased cGMP formation (19) and partially reduced PE-induced smRLC phosphate content (herein). Additionally, cGMP formation was not increased in contracting soleus muscles, which have more eNOS but less nNOS than EDL muscles (19). The blunted dephosphorylation in nNOS−/− EDL in the presence of NLA (e.g., ST-PE-NLA) along with the low contents of cGMP indicate that a pathway independent of NO derived from eNOS may be inhibited. Additionally, the sensitivity of the stimulation-dependent, NO-independent pathway may be attenuated, much like the eNOS−/− EDL appears less sensitive to PE and SNP (19). Collectively, these data suggest that in contracting nNOS−/− EDL, decreases in smRLC phosphate content are probably not due to an NO-dependent pathway. Thus in the absence of...
nNOS, NO-independent relaxation pathways become dominant, whereas in C57 muscles nNOS-derived NO could contribute to vascular relaxation during contraction.

**Apparent Need for eNOS-Derived NO as Modulator of Vascular Sensitivity**

In eNOS$^{-/-}$ EDL, an underlying role for eNOS-derived NO as a modulator of basal tone and possibly as an indirect modulator of both smooth muscle contraction and relaxation signaling pathways is revealed. An intriguing finding in the eNOS$^{-/-}$ EDL is the blunted response to PE compared with those of C57 and nNOS$^{-/-}$ EDL, which is not different from the eNOS$^{-/-}$ control values. This suggests, as with the diminished cGMP response to SNP in eNOS$^{-/-}$ EDL (19), the signal cascade leading to smRLC phosphorylation may also be compromised. Nevertheless, the blunting effect is removed in the PE-NLA condition. The effect of NLA on smRLC phosphate content appears to be amplified in the presence of PE; yet under basal conditions, the NLA-sensitive pathways, which may include that mediated by nNOS-derived NO, only weakly compensate for the absence of eNOS.

The stimulation-induced decrease in PE-induced smRLC phosphate content in eNOS$^{-/-}$ EDL (ST-PE) could be due to both NO-independent and NLA-sensitive mechanisms. Surprisingly, however, SNP did not attenuate smRLC phosphorylation in the presence of PE. These data conflict with the decrease in basal smRLC phosphorylation with SNP treatment, which indicates NO-dependent relaxation mechanism(s) are intact. However, cGMP formation in response to SNP is attenuated in eNOS$^{-/-}$ compared with nNOS$^{-/-}$ or C57 EDL (19), suggesting the pathway is compromised. Thus the cGMP-dependent pathway may cope with reduction of smRLC phosphate content under basal but not under elevated smRLC phosphate conditions (e.g., PE induced).

**Apparent Emphasis on NO-Independent Relaxation Pathways in eNOS$^{-/-}$ and nNOS$^{-/-}$ EDL**

In the absence of eNOS or nNOS, vascular regulation may adapt by favoring NO-independent pathways as demonstrated by the dramatic decrease in smRLC phosphorylation when eNOS$^{-/-}$ EDL are stimulated in the presence of PE and NLA (e.g., nNOS blocked). Although not yet reported for nNOS$^{-/-}$ mice, vascular adaptations similar to those reported for eNOS$^{-/-}$ mice might also be invoked. Such adaptations might include intrinsic changes in the endothelial and/or smooth muscle cells. For example, an enhanced release of prostaglandins from endothelial cells is thought to maintain flow-induced skeletal muscle arteriolar dilation in eNOS$^{-/-}$ mice in situ (36). Alternatively, endothelium-derived hyperpolarizing factor (EDHF) is thought to shift from a minor to a major role in mediating acetylcholine-induced smooth muscle relaxation in eNOS$^{-/-}$ skeletal muscle arterioles via Ca$^{2+}$-dependent K$^+$ channels (12). One or both of these pathways may be activated during muscle contractions in vitro. Thus smooth muscle cells might adapt to the decrease in basal NO from eNOS and a decrease in activity-stimulated NO from nNOS to initiate cellular modifications to increase sensitivity to some signaling molecules (e.g., prostaglandins, EDHF) and decrease sensitivity to others (e.g., NO). This form of adaptation might explain the apparent decrease in sGC sensitivity (i.e., decrease in absolute cGMP production) to NO derived from SNP in the eNOS$^{-/-}$ EDL (19).

**Role of NO Derived from nNOS and eNOS in Functional Hyperemia**

The mechanism responsible for the rapid increase of exercise-related vasodilation within the first 0–7 s of contractile activity is considered obscure (32). Muscle vasodilation has been observed with only a single contraction, suggesting that the muscle pump and/or a vasoactive substance or substances are responsible (32). The idea of an NO-dependent relaxation cascade mediated through cGMP appears reasonable, because both the increase in cGMP formation and the decrease in PE-induced smRLC phosphorylation associated with muscle contraction can be blocked with NLA in C57 EDL (Ref. 19, and present study). The use of knockout animals could have identified the source of the NO. Instead, experiments with eNOS and nNOS knockout animals revealed intriguing interactions between eNOS and nNOS and the sensitivity of the vasculature to both vasorelaxation and vasoconstriction. The presence of eNOS appears necessary to maintain low vascular basal tone, to establish sensitivity of the cGMP-dependent relaxation cascade to NO during muscle contraction, and may also influence adrenergic pathways that lead to vasoconstriction. The presence of nNOS also appears to influence the relative contribution of NO-dependent and NO-independent relaxation pathways during muscle activation. From these interactions we conclude that to effect NO-dependent relaxation during muscle contraction requires the presence of both NOS isoforms. This outcome illustrates one of the limitations in employing knockout animals to dissect the function of a specific protein but also illustrates the need to explore a number of different experimental approaches to interpret function.

During muscle contraction, eNOS in endothelial cells could be activated by a variety of agonists (e.g., acetylcholine, bradykinin, substance P; Ref. 6) and/or shear stress (38). For example, in response to the action of the muscle pump, eNOS might be activated by muscle compression. However, in soleus muscles that have an eNOS content greater than that of EDL, there is no increase in cGMP with stimulation, and cGMP formation in contracting nNOS$^{-/-}$ EDL (i.e., eNOS present) is also attenuated (19). On the basis of these observations, NO derived from eNOS does not appear responsive to muscle contraction and, therefore, may be more prominent in maintaining vascular tone and modulating vascular sensitivity to NO. The latter effect on vascular sensitivity is clearly demonstrated in the...
eNOS<sup>−/−</sup> EDL subjected to the ST-PE-NLA treatment in which PE-induced smRLC phosphorylation was dramatically reduced in an NO-independent manner. If eNOS is not the principal source of NO during muscle contraction, then we suggest that nNOS may contribute NO to a greater extent, at least during the initial stages of functional hyperemia, because of 1) its localization at the sarcolemma; 2) the observation that it is apparently preferentially activated coincident with the skeletal muscle contractile apparatus (11); and 3) cGMP formation is not increased in contracting nNOS<sup>−/−</sup> EDL (19). One reason for the difficulty in establishing NO as an initial vasoactive substance modulating functional hyperemia in many studies is the possibility that its effects could be masked because of the apparent redundancies in regulating blood flow during exercise (e.g., by other metabolites; Ref. 33). This overlap may be particularly overt during the onset of exercise. However, on the basis of the data from our isolated muscle model, one possible role of nNOS-derived NO may be to initiate the rapid onset of functional hyperemia coincident with muscle contraction, because of the apparent functional hyperemia that results from the overlap of NO derived from eNOS and nNOS. Alterations in sensitivity in the vascular smooth muscle in contracting fast-twitch skeletal muscle appear complicated because of their mitochondrial relationships. In eNOS knockout mice skeletal muscle arteriolar dilation to acetylcholine is mediated by EDHF. Am J Physiol Heart Circ Physiol 278: H762–H768, 2000.


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