Skeletal muscle contractions stimulate cGMP formation and attenuate vascular smooth muscle myosin phosphorylation via nitric oxide

Kim S. Lau[a,*], Robert W. Grange[a], Wen-Jinn Chang[1,a], Kristine E. Kamm[a], Ingrid Sarelius[b], James T. Stull[a]

[a] *Department of Physiology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235-9040, USA*
[b] *Department of Pharmacology and Physiology, School of Medicine and Dentistry, University of Rochester Medical Center, 601 Elmwood Avenue, Box 711, Rochester, NY 14642-8711, USA*

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Abstract Nitric oxide generated by neuronal nitric oxide synthase in contracting skeletal muscle fibers may regulate vascular relaxation via a cGMP-mediated pathway. Neuronal nitric oxide synthase content is greatly reduced in skeletal muscles from *mdx* mice. cGMP formation increased in contracting extensor digitorum longus muscles in vitro from C57 control, but not *mdx* mice. The increase in cGMP content was abolished with N^G-nitro-L-arginine. Sodium nitroprusside treatment increased cGMP levels in muscles from both C57 and *mdx* mice. Skeletal muscle contractions also inhibited phenylephrine-induced phosphorylation of smooth muscle myosin regulatory light chain. Arteriolar dilation was attenuated in contracting muscles from *mdx* but not C57 mice. NO generated in contracting skeletal muscle may contribute to vasodilation in response to exercise.

Key words: Nitric oxide synthase; cGMP; Skeletal muscle; Vascular smooth muscle

1. Introduction

The Ca^{2+}/calmodulin-dependent neuronal isoform of nitric oxide synthase (nNOS) is expressed in normal skeletal muscle where it is co-localized with dystrophin to the sarcolemma. However, in dystrophin-deficient skeletal muscle from DMD patients and *mdx* mice, both nNOS expression and activity are greatly reduced [1,2]. Ca^{2+}/calmodulin-activated nNOS catalyzes the conversion of L-arginine to L-citrulline and the vasodilatory signaling molecule NO [3]. NO is synthesized in contracting skeletal muscle [4], suggesting that increases in $[Ca^{2+}]_i$ associated with contraction are sufficient to activate the Ca^{2+}/calmodulin-dependent nNOS.

It is well established that NO derived from endothelial cells produces vasodilation of arteries due to the subsequent activation of sGC in smooth muscle cells [5,6]. In exercising skeletal muscle, marked vasodilation occurs in blood vessels to accommodate increased metabolic demands of the muscle (functional or exercise hyperemia). Hyperemic responses in exercising rats and humans were attenuated by NOS inhibitors [7–10]. Thus, pharmacological studies in vivo and in vitro show NO may contribute to the hyperemic response in skeletal muscle. However, the origin of NO, from endothelial cells or muscle fibers, has not been determined. Therefore, we considered the possibility that NO produced from Ca^{2+}/calmodulin-dependent nNOS in contracting skeletal muscle fibers diffuses to adjacent vascular smooth muscle cells to elicit localized relaxation via a cGMP-mediated pathway. NO in vascular smooth muscle activates sGC to increase cGMP formation, thereby leading to a decrease in $[Ca^{2+}]_i$ with subsequent inhibition of myosin RLC phosphorylation and contraction [5,11,12]. We found that contraction in EDL muscles from C57 mice elicited increases in cGMP content and reduced smooth muscle RLC phosphorylation. Rather than rely solely on a pharmacological approach to explore this signaling pathway, we took advantage of attenuation of nNOS content in *mdx* mouse skeletal muscle. The contraction-induced increase in cGMP and arteriolar dilation was diminished in muscles from *mdx* mice. These results suggest that NO derived from nNOS in contracting skeletal muscle contributes to the vasodilatory response to exercise. Additionally, this regulatory pathway may be compromised in dystrophin-deficient skeletal muscles from *mdx* mice and humans with DMD.

2. Materials and methods

C57BL and *mdx* mice were purchased from Jackson Laboratories. Chemicals and biochemicals were purchased from SIGMA. Immunochemical reagents were obtained from Amersham, UK. ^{125}I-cGMP was a gift from Dr. David Garbers.

2.1. Isolated muscles

EDL muscles (approximately 12 mm in length; 1.5 mm diameter, and 12 mg wet weight) were isolated from fully anesthetized mice, mounted on Grass FTO3.C force transducers and continuously gassed with 95% O_2/5% CO_2 while immersed in PSS containing (in mM) 120.5 NaCl, 4.8 KCl, 1.2 $MgSO_4$, 1.5 $CaCl_2$, 1.2 Na_2PO_4, 20.4 $NaHCO_3$, 10.0 dextrose and 1.0 pyruvate at 30°C and pH 7.6. EDL muscles were subjected to electrical stimulation at 30 Hz (0.2 ms square pulse width) for 15 s, with 1 mM N^G-nitro-L-arginine (NLA) for 30 min and 10 μM sodium nitroprusside (SNP) or 10 μM phenylephrine as described below. At 1 mM NLA, the contractile properties of the EDL muscle were not changed. Mounted muscles were quick-frozen by tongs pre-chilled in liquid nitrogen after specific treatments.

*Corresponding author. Fax: +1 (214) 648-8685.
E-mail: Lau01@utsw.swmed.edu

[1] Current address: B-Cell Molecular Immunology Section, Laboratory of Immunoregulation, NIAID/NIH, Bldg. 10, Rm. 11B10, 10 Center Dr., MSC 1876, Bethesda, MD 20892, USA.

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; sGC, soluble guanylyl cyclase; RLC, regulatory light chain; L-NMMA, N^G-monomethyl-L-arginine; PSS, physiological salt solution; NLA, N^G-nitro-L-arginine; $[Ca^{2+}]_i$, intracellular calcium; PVDF, polyvinylidene fluoride; SNP, sodium nitroprusside; HRP, horse radish peroxidase; EDTA, ethylenediaminetetraacetic acid; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; CHAPS, 3-[(3-chlolamido propyl)dimethyl-ammonia]-1-propane sulfonate; Hz, hertz; DMD, Duchenne's muscular dystrophy

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2.2. Biochemical measurements

Frozen muscles were homogenized in 10% trichloroacetic acid followed by centrifugation. The cGMP content was measured in the soluble fraction by a radioimmunoassay [13].

Myosin RLC phosphorylation was measured by immunoblot analysis after urea-glycerol PAGE to resolve phosphorylated and non-phosphorylated forms of smooth muscle myosin RLC [14]. Briefly, protein precipitated by trichloroacetic acid was solubilized in urea and subjected to electrophoresis in a polyacrylamide gel containing glycerol. Resolved proteins were transferred onto PVDF membrane and blocked with blocking reagent for 60 min. The membranes were then probed for smooth muscle myosin RLC using a monoclonal antibody that was specific for smooth muscle myosin RLC. Bound antibody was detected with HRP-conjugated goat anti-mouse IgG antibody followed by development with ECL chemiluminescence detection reagents. The fraction of phosphorylated RLC relative to the total RLC was quantified by scanning laser densitometry.

2.3. Vascular response measurements in mouse cremaster muscle

Adult male mice were anesthetized with pentobarbital sodium (70 mg/kg, i.p.), tracheotomized, maintained with supplemental anesthetic as needed via a jugular venous catheter, and kept at body temperature of 37°C by radiant heating. The right cremaster muscle was prepared and the microvasculature viewed by intravital microscopy as described elsewhere for hamsters [15] except that the vascular connections between the muscle and the epididymis were left intact. The prepared cremaster muscles were superfused with PSS containing (in mM) 131.9 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, and 20 $NaHCO_3$. The buffer was equilibrated with 0–5% O_2/5% CO_2, balanced with N_2 to give pH 7.37 at 34 ± 0.5°C. Electrical field stimulation via silver foil electrodes was used to contract the muscle for 2 min each at 0, 2, 4, and 8 Hz (1 ms duration, 4–10 V). Each 2 min stimulation was followed by 3 min recovery and each was duplicated before moving to the next higher frequency. Arteriolar diameters were measured off-line from videotapes using an electronic calliper calibrated against a videotaped stage micrometer. The average diameter during the 10 s immediately following stimulation was used to represent the steady-state diameter achieved during the 2 min stimulation [16].

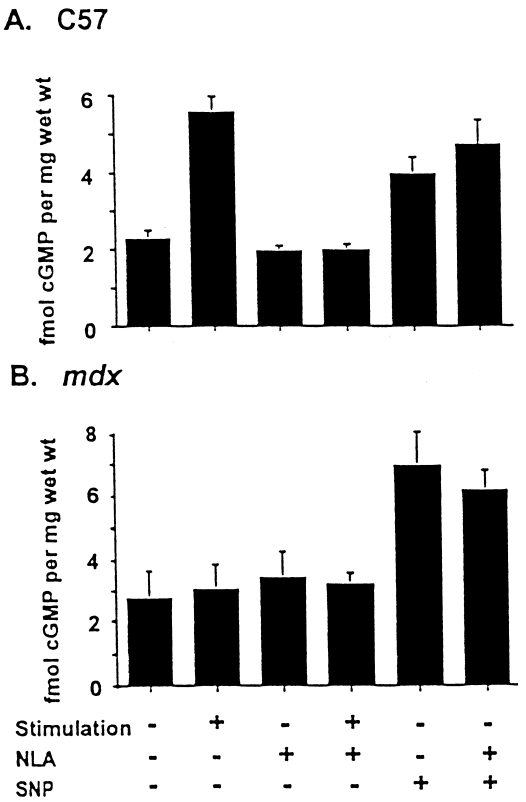

Fig. 1. Nitric oxide generated in contracting EDL muscle stimulates cGMP formation. Isolated EDL muscles from C57 mice (A) or *mdx* mice (B) were either electrically stimulated at 30 Hz for 15 s, incubated with 1 mM NLA (30 min), or 10 μM SNP (30 s), or subjected to combinations as indicated. Values are means ± S.E.M. for at least four muscles for each group.

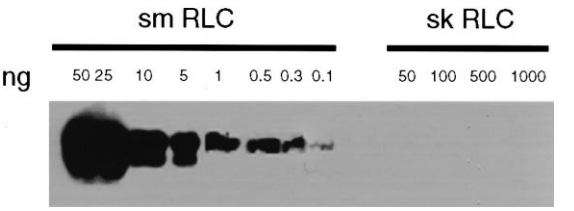

Fig. 2. A monoclonal antibody reacts specifically with smooth muscle RLC. Increasing amounts of purified smooth and skeletal muscle RLC were subjected to SDS-PAGE and Western blotting. A monoclonal antibody raised against smooth muscle RLC was used to detect smooth muscle and skeletal muscle myosin RLCs.

3. Results

3.1. cGMP formation in mouse EDL skeletal muscle

Under control, resting conditions, EDL muscles from C57 mice contained 2 fmol cGMP per mg wet wt tissue (Fig. 1A). cGMP content was increased 246% with electrical stimulation. Treatment of the muscles with 1 mM of the NOS inhibitor NLA did not affect the resting level of cGMP but completely inhibited the increase due to electrical stimulation. Addition of SNP increased cGMP content to 4 fmol cGMP per mg wet wt tissue which was not affected by 30 min pretreatment with NLA (Fig. 1A).

cGMP formation in EDL muscles from *mdx* mice was also measured (Fig. 1B). In contrast to results with muscles from C57 mice, electrical stimulation of *mdx* muscles did not increase cGMP formation (Fig. 1B). However, the muscles were responsive to the NO donor SNP. Thus, contraction of *mdx* muscles with attenuated nNOS expression does not lead to increased cGMP formation. However, an NO-responsive guanylyl cyclase is apparently still present.

3.2. Smooth muscle myosin regulatory light chain phosphorylation in isolated skeletal muscle

The extent of phosphorylation of vascular smooth muscle RLC was measured with a monoclonal antibody specific for

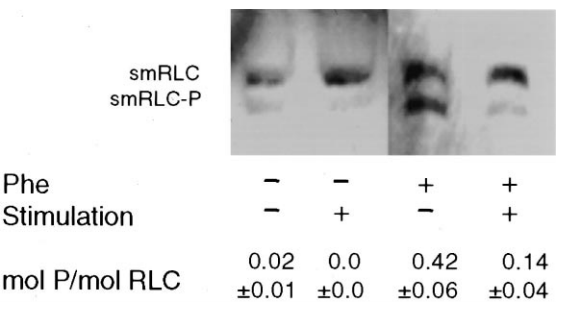

Fig. 3. Skeletal muscle contraction inhibits smooth muscle RLC phosphorylation. Non-phosphorylated (smRLC) and phosphorylated (smRLC-P) smooth muscle myosin RLCs were resolved by urea/glycerol PAGE and detected with a monoclonal antibody raised against the smooth muscle RLC. Mean values ± S.E.M. of smooth muscle RLC phosphorylation in EDL muscles treated for 60 s with 10 μM phenylephrine (Phe), electrical stimulation for 15 s, or Phe for 60 s plus electrical stimulation during the last 30 s ($n \geq 6$).

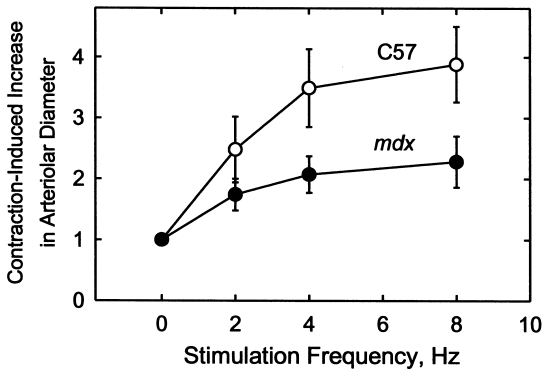

Fig. 4. Contraction-induced arteriolar dilation in nNOS-deficient *mdx* mice is attenuated $P < 0.01$. Diameters of second and third order arterioles in mouse cremaster muscle were measured immediately after contraction at different frequencies. For protocol see Section 2. Results are expressed as a ratio of the measured diameters after stimulation to that at rest (0 Hz). Values are the means ± S.E.M. for arterioles from C57 (○) and *mdx* (●) mice.

smooth muscle RLC. This antibody binds to smooth muscle RLC and shows no immunoreactivity to skeletal muscle RLC (Fig. 2). The monoclonal antibody detected smooth muscle RLC directly in Western blots of skeletal muscle homogenates (Fig. 3) where the smooth muscle RLC is a much less abundant protein relative to the skeletal muscle RLC. The extent of smooth muscle RLC phosphorylation was low under resting conditions (< 0.05 mol phosphate/mol RLC) or with electrical stimulation (Fig. 3). However, with the addition of the α-adrenergic agonist, phenylephrine, the extent of smooth muscle RLC phosphorylation increased to over 0.4 mol phosphate/mol RLC. More importantly, the extent of phosphorylation in the presence of phenylephrine decreased significantly in response to electrical stimulation of the skeletal muscle.

3.3. Vascular response to skeletal muscle contraction

Contraction-induced increases in arteriolar diameters determined in ten vessels in seven *mdx* mice and nine vessels in nine C57 mice showed a significantly diminished vascular dilation in the *mdx* vessels (Fig. 4). From an initial similar resting diameter (9.5 ± 2.0 μm in *mdx* vs. 6.7 ± 0.8 μm in C57), the mean response of the *mdx* mice was less than that of controls at each stimulation frequency ($P < 0.01$). All vessels studied were second and third order arterioles with similar mean maximal diameters of 27.8 ± 2.1 μm and 26.5 ± 3.0 μm for control and *mdx* muscles, respectively.

4. Discussion

We examined the hypothesis that NO produced by nNOS in skeletal muscle fibers may contribute to vascular relaxation during muscle contraction via the cGMP-myosin RLC cascade. cGMP content increased 246% in contracting mouse skeletal muscles in close agreement with the 230% increase in cGMP content reported for contracting frog gastrocnemii muscles subjected to the same stimulation protocol [17]. At the time this latter report was published twenty years ago, NO and NOSs were unknown so that the mechanism accounting for the increased cGMP formation could not be explored. We show the contraction-dependent increase in cGMP formation was totally abolished with the NOS inhibitor NLA. However, a similar increase in cGMP production by the NO donor SNP was not affected by the NOS inhibitor. Thus, increased cGMP formation in contracting skeletal muscle was due to activation of a NOS. In *mdx* mice, the amount of nNOS in skeletal muscle is greatly reduced [2]. This observation may explain why contraction of *mdx* muscles did not lead to increased cGMP formation. However, the cGMP response to SNP in *mdx* muscle was unaffected, indicating the cGMP signaling cascade is probably intact. These results support the hypothesis that nNOS stimulates cGMP formation in response to contractions in skeletal muscle.

While the mechanisms by which cGMP acts to decrease cytosolic Ca^{2+} concentrations are not fully defined [5,12], cGMP is recognized as an important second messenger modulating smooth muscle contractility. Increases in $[Ca^{2+}]_i$ activate Ca^{2+}/calmodulin-dependent myosin light chain kinase, resulting in phosphorylation of smooth muscle myosin RLC and contraction [18,19]. This cascade of cellular responses is inhibited by increases in cGMP formation which act to decrease $[Ca^{2+}]_i$. In stimulated C57 EDL muscle, smooth muscle myosin RLC phosphorylation was inhibited, presumably due to the cGMP-mediated decrease in smooth muscle $[Ca^{2+}]_i$. However, it is not known if the cGMP response to electrical stimulation or SNP is specifically localized to vascular smooth muscle. cGMP-dependent vasodilation of arterioles has been reported in rat cremaster muscle [20] and other tissues [21,22]. We also find that the arteriolar response to contraction in cremaster muscles from *mdx* mice was attenuated, providing physiological evidence consistent with our hypothesis.

In summary, these findings are consistent with the hypothesis that Ca^{2+}-dependent nNOS is activated in contracting skeletal muscle, and the NO produced results in increased amounts of cGMP with subsequent inhibition of smooth muscle myosin RLC phosphorylation. This NO-dependent cascade provides a cellular mechanism supporting functional hyperemia in contracting skeletal muscle. This hyperemic response may be compromised in dystrophin-deficient muscles in patients with DMD.

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