ORIGINAL PAPER

Nitric oxide regulates stretch-induced proliferation in C2C12 myoblasts

Quinlyn A. Soltow · Vitor A. Lira · Jenna L. Betters · Jodi H.D. Long · Jeff E. Sellman · Elizabeth H. Zeanah · David S. Criswell

Abstract Mechanical stretch of skeletal muscle activates nitric oxide (NO) production and is an important stimulator of satellite cell proliferation. Further, cyclooxygenase (COX) activity has been shown to promote satellite cell proliferation in response to stretch. Since COX-2 expression in skeletal muscle can be regulated by NO we sought to determine if NO is required for stretch-induced myoblast proliferation and whether supplemental NO can counter the effects of COX-2 and NF-κB inhibitors. C2C12 myoblasts were cultured for 24 h, then switched to medium containing either the NOS inhibitor, L-NAME (200 μM), the COX-2 specific inhibitor NS-398 (100 μM), the NF-κB inhibiting antioxidant, PDTC (5 mM), the nitric oxide donor, DETA-NONOate (10–100 μM) or no supplement (control) for 24 h. Subgroups of each treatment were exposed to 1 h of 15% cyclic stretch (1 Hz), and were then allowed to proliferate for 24 h before fixing. Proliferation was measured by BrdU incorporation during the last hour before fixing, and DAPI stain. Stretch induced a twofold increase in nuclear number compared to control, and this effect was completely inhibited by L-NAME, NS-398 or PDTC ($P < 0.05$). Although DETA-NONOate (10 μM) did not affect basal proliferation, the NO-donor augmented the stretch-induced increase in proliferation and rescued stretch-induced proliferation in NS-398-treated cells, but not in PDTC-treated cells. In conclusion, NO, COX-2, and NF-κB are necessary for stretch-induced proliferation of myoblasts. Although COX-2 and NF-κB are both involved in basal proliferation, NO does not affect basal growth. Thus, NO requires the synergistic effect of stretch in order to induce muscle cell proliferation.

Keywords C2C12 myoblasts · Skeletal muscle · NF-κB · COX-2 · Flexcell · DETA-NO

Abbreviations

NOS	Nitric oxide synthase
COX	Cyclooxygenase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
L-NAME	L-N^G-nitroarginine methyl ester
D-NAME	D-N^G-nitroarginine-methyl ester
PDTC	Pyrrolidine dithiocarbamate
DETA-NO	Diethylenetriamine NONOate
BrdU	5-Bromo-2-deoxyuridine
DAPI	4′6-Diamidino-2-phenylindole
VEGF	Vascular endothelial growth factor
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor 1

Q. A. Soltow · V. A. Lira · J. L. Betters · J. H. D. Long · J. E. Sellman · E. H. Zeanah · D. S. Criswell (✉)
Center for Exercise Science, Department of Applied Physiology & Kinesiology, University of Florida, Gainesville, FL 32611, P.O. Box 118206, USA
e-mail: dcriswell@hhp.ufl.edu

Introduction

Skeletal muscle fibers are terminally differentiated and incapable of undergoing myogenesis. Therefore, mature myofibers are reliant on a population of quiescent muscle precursor cells, termed satellite cells, that reside beneath the surface of the basal lamina. Satellite cells provide a

source of myonuclei necessary for skeletal muscle maintenance, growth, and repair of injured fibers.

In order for satellite cells to contribute to muscle size regulation, they must be activated and stimulated to proliferate. Proliferating muscle precursor cells, which at this stage can be referred to as myoblasts, immigrate to areas of the muscle fiber that have encountered injury or stretch and fuse with the existing fiber. Thus, stimulation of activated satellite cells to induce myoblast proliferation is a critical step for the growth and maintenance of skeletal muscle.

Basal proliferation of myoblasts can be induced by growth factors such as VEGF (Germani et al. 2003), HGF (Tatsumi et al. 2002), IGF-1 (Chakravarthy et al. 2001), and also by prostaglandin $F_{2\alpha}$ (Zalin 1987). More importantly, myoblasts proliferate in response to various mechanical stimuli including shear stress, contractile activity, and passive stretch. Enhanced proliferation of myoblasts following a mechanical stimulus will increase the likelihood that myoblasts will reach the area of injury or stress with a population of myogenic cells sufficient to support muscle growth or regeneration.

Deformation of the cytoskeleton that occurs during muscle contractions can be mimicked in vitro. Stretching provides stress to the sarcolemma and has been shown to activate satellite cells through a nitric oxide-dependent mechanism (Anderson 2000; Betters et al. 2008). Nitric oxide is a mechanical transducer and effector molecule in skeletal muscle cells leading to metabolic changes (Lira et al. 2007; Drenning et al. 2008, 2009), skeletal muscle hypertrophy (Smith et al. 2002; Soltow et al. 2006) and enhanced mytoube fusion (Lee et al. 1994; Long et al. 2006; Pisconti et al. 2006). C2C12 muscle cells subjected to cyclic or static loading show an increase in nNOS expression and NO production (Tidball et al. 1998; Zhang et al. 2004), and following mechanical loading of primary satellite cells, NO activates release of HGF leading to increased satellite cell activation/proliferation (Tatsumi et al. 2002). However, nitric oxide's specific effects on proliferation in activated myoblasts, and its potential interaction with known regulators of myoblast proliferation has yet to be elucidated.

Mechanical stretch of skeletal muscle stimulates the phospholipase A2-mediated liberation of arachidonic acid from the cell membrane (Vandenburgh et al. 1993). Arachidonic acid is then converted to prostaglandins by cyclooxygenase (COX) enzymes. Intermittent repetitive mechanical stimulation increases progaglandin E_2 and $F_{2\alpha}$ release from muscle cells (Vandenburgh et al. 1990) and these prostaglandins are necessary for stretched-induced proliferation (Otis et al. 2005). Inhibition of COX-2 decreases the total number of myoblasts in regenerating skeletal muscle suggesting a potential role for COX-2 in regulating myoblast proliferation (Bondesen et al. 2004).

We have previously demonstrated that NOS activity regulates COX-2 expression during skeletal muscle overload in vivo (Soltow et al. 2006), and this implies a potential link between NO and COX-2 control of mechanically-stimulated myoblast proliferation.

Both NOS and COX-2 have NF-κB binding domains in their promoter regions, making them susceptible to NF-κB regulation. NF-κB activity is necessary for NOS expression and NO production in chick myoblasts after 2 days of culture (Lee et al. 1997), and COX-2 activity is enhanced by NF-κB activation (Lim et al. 2001). Cyclic mechanical stretch induces NF-κB transcriptional activity, which promotes myoblast proliferation and inhibits myotube formation in C2C12s (Kumar et al. 2004a). Thus, NF-κB inhibits myogenic differentiation by delaying exit from the cell cycle thereby enhancing proliferative effects during stretch.

Therefore, given the mechanistic links between mechanical changes in muscle, NOS/COX-2/NF-κB activity, and satellite cell proliferation, we speculated that the proliferation of myoblasts following mechanical stretch is controlled by stress-mediated NF-κB activation leading to upregulation of NOS and COX-2 signaling. In this report we hypothesized that NOS activity is necessary for stretch-induced myoblast proliferation and that an NO donor can augment the stretch effect on myoblast expansion.

Materials and methods

Materials

The non-specific nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester hydrochloride (L-NAME), COX-2-specific inhibitor NS-398, and the nitric oxide donor diethylenetriamine NONOate (DETA-NO) were obtained from Cayman Chemical (Ann Arbor, MI). The inactive isomer of L-NAME, D-N^G-nitroarginine-methyl ester hydrochloride (D-NAME), was obtained from Alexis Biochemicals (Plymouth Meeting, PA). The NF-κB inhibitor ammonium pyrrolidinedithiocarbamate (PDTC) was obtained from Sigma (St. Louis, MO). 4′,6′-diamidino-2-phenylindole hydrochloride (DAPI) mounting medium and Texas Red antibody were obtained from Vector Laboratories, Inc. (Burlingame, CA).

Cell culture

Early passages of C_2C_{12} cells, ranging from passages 1–4 (ATCC, Manassas, VA) were plated at equal density (30,000 cells per well) in Dulbecoo's Modified Eagle's Medium (DMEM) (Mediatech, Herndon, VA) supplemented with insulin-transferrin-selenium (ITS) (Invitrogen, Grand Island, NY) and 1% penicillin/streptomycin. The

cultures were maintained in ITS (containing no serum) for 24 h to reduce the exposure of growth factors prior to mechanical stimulation. Visual inspection at the conclusion of all experiments demonstrated little cell fusion and low confluency (40–50%). For basal proliferation experiments, cells were switched to DMEM supplemented with 2% horse serum with 1% penicillin/streptomycin with the following treatments added to the medium: no treatment (control), L-NAME (200 μM), DETA-NO (10 μM), NS-398 (100 μM), NS-398 + DETA-NO, PDTC (5 mM), and PDTC + DETA-NO.

Mechanical stimulation using cyclic strain

The C_2C_{12} cells were plated on type I collagen-coated flexible-bottom plates (Bioflex plates, Flexcell International, McKeesport, PA) and incubated at 37°C in an incubator maintained at 5% CO_2 for 24 h before applying mechanical strain. Immediately prior to the initiation of stretch, the medium was aspirated and changed to DMEM supplemented with 2% horse serum and 1% penicillin/streptomycin with the following treatments added to the medium: no treatment (stretch), L-NAME (200 μM), DETA-NO (10, 50, 100 μM), NS-398 (50, 100, 200 μM), NS-398 (100 μM) + DETA-NO (10 μM), PDTC (5 mM), and PDTC + DETA-NO (10 μM). The cells were subjected to cyclic strain at 1 Hz (1 s of 15% stretch alternating with 1 s of relaxation) for 1 h using a computer-controlled vacuum stretch apparatus (FX-4000T Tension Plus System, FlexCell International) with a vacuum pressure that is sufficient to generate 15% mechanical strain. Replicate control samples were maintained under static conditions with no applied cyclic strain.

Nitric oxide production

To confirm the presence of functional NOS activity in C2C12 myoblasts and its response to the stretching protocol, separate cultures were grown on Bioflex plates as described above. Before beginning the stretch procedure, myotubes were incubated at 37°C for 30 min in serum-free DMEM containing 10 μM DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; Invitrogen, Carslbad, CA). After loading was completed, cells were rinsed three times with phenol red-free, serum-free DMEM, followed by addition of phenol red-free DMEM with no further additions (controls) or containing L-NAME (200 μM). Half of these cultures were subjected to the 1 h stretching protocol described above and the other half served as non-stretched controls. One plate (six cultures) of non-stretched cells was treated for 1 h with DETA-NO (100 μM) as a positive control for NO production. Immediately after the 1 h stretch/treatment period, the media was

aspirated and the cells harvested by scraping in 100 μl of dH_2O. Cell lysates (75 μl) were quickly transferred to 96-well plates and analyzed for fluorescence in a Spectra-Max M5 multi-detection reader (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 485 and 536 nm, respectively. All procedures using DAF-FM were performed in a darkened room. After fluorescence measurement, protein concentrations were assessed using the DC Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA).

Myoblast proliferation and cell number assays

For proliferation assays, control and stretched myoblasts were incubated with 25 μM 5-bromo-2′-deoxyuridine (BrdU) for 1 h beginning 23 h after the cessation of stretch. All subsequent steps were performed at room temperature unless otherwise noted. After 24 h, cells were fixed with 2% paraformaldehyde in PBS for 10 min. For BrdU labeling, fixed cells were denatured with 1 N HCl in PBS for 30 min at 45°C and neutralized with 0.1 M borate buffer (5 μM borax, 20 mM boric acid; pH 8.3) for 10 min. After 1 h incubation with blocking buffer (5% goat serum, 0.25% Triton X-100, 5 μg/ml BSA in PBS) to reduce non-specific binding, cells were incubated with a rabbit monoclonal antibody against BrdU (1:100 in blocking buffer, Megabase Research) for 2 h. Cultures were washed with PBS containing 0.1% Tween (PBS-T) and then incubated with a Texas Red goat anti-rabbit IgG (1:100 in PBS-T, Vector Laboratories) for 2 h followed by further washes with PBS-T. For MyoD staining, fixed cells were permeabilized for 15 min with 0.5% TritonX-100, blocked for 30 min in 5% goat serum in PBS and incubated for 1.5 h with a mouse monoclonal antibody for MyoD (1:20 in 0.5% BSA). Cultures were washed with PBS and incubated for 1.5 h with Alexa Fluor 488 goat anti-mouse secondary antibody (1:66 in TBS). After staining, the Flexcell membranes were cut and mounted to slides using Vectashield mounting medium with DAPI (Vector Laboratories) to stain nuclei. Because the BrdU + nuclei and DAPI staining were highly correlated during the initial stretching experiments, the subsequent data were analyzed using DAPI staining as a marker of proliferation. The myoblasts were visualized on a Zeiss microscope with Rhodamine and DAPI filters. BrdU + and DAPI-stained myoblasts were counted at several fields on the membrane using ImageJ version 1.32 J software (NIH).

To confirm cell viability following each of the pharmacological treatments, separate cultures were trypsinized and treated with 0.4% trypan blue (Sigma Chemical) in PBS. The proportion of viable cells (excluding the dye) determined by cell counting in a hemacytometer was consistently greater than 90% for all treatments.

RNA isolation and quantitative real-time PCR

Twenty-four hours after cessation of stretch, total RNA was extracted from cultured cells by harvesting in 1 ml of TRizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Concentration and purity of the extracted RNA was measured spectrophotometrically at A_{260} and A_{280} in 1X Tris–EDTA buffer (Promega, Madison, WI). Reverse transcription (RT) was performed using the SuperScript III First-Strand Synthesis System for RT–PCR according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). Reactions were carried out using 3.25 µg of total RNA and 2.5 µM oligo(dT)$_{20}$ primers. First-strand cDNA was treated with two units of RNase H and stored at –80°C. Primers and probes were obtained from ABI Assays-On-Demand service [COX-2 (GenBank NM_011198.3, assay Mm01307329_m1) and GAPDH (GenBank NM_008084.2, assay Mm999999 15_g1)] and consisted of Taqman 5′ labeled FAM reporters and 3′ nonfluorescent quenchers. Primer and probe sequences from this service are proprietary and therefore are not reported. Quantitative real-time PCR for the target genes was performed in the ABI Prism 7700 Sequence Detection System (ABI, Foster City, CA). Each 25 µl PCR reaction, performed in duplicate, contained 1 µl of cDNA reaction mixture. In this technique, amplification of the fluorescently labeled probe sequence located between the PCR primers is monitored in real-time during the PCR program. The number of PCR cycles required to reach a pre-determined threshold of fluorescence (called the CT) is determined for each sample. The procedure is referred to as the comparative CT method (Bustin 2002). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the appropriate normalizing gene since the expression in C2C12 cells was not significantly altered during any of the treatments ($P > 0.05$).

Statistical analysis

Data are presented as means ± SEM. Data were analyzed by independent samples t-tests for basal proliferation assays and two-way ANOVAs (stretch vs. treatment) for stretching experiments using SPSS (version 12.0.1). Significance was established at $P < 0.05$.

Results

Cyclic mechanical stretch stimulates myoblast proliferation and nitric oxide production

The stretch protocol consistently increased proliferation of C2C12 myoblasts by twofold (+104%; Fig. 1), as measured by whole cell count with DAPI staining. For the initial experiments, we also measured BrdU incorporation as a second estimate of cell proliferation. The number of BrdU$^+$ cells increased by 149% after stretch (Fig. 1).

The stretch protocol increased NO concentration in C2C12 myoblast by approximately threefold, compared to non-stretched control cells. Specifically, mean (±SEM) DAF-FM fluorescence (fluorescence – background per µg protein) was 12.2 ± 1.9 for myoblasts exposed to the stretch protocol, compared to 3.9 ± 0.8 for control, non-stretched myoblasts ($P < 0.05$, $n = 5$ per group). Addition of 200 µM L-NAME to the treatment media tended to lower DAF-FM fluorescence (L-NAME without stretch = 2.7 ± 1.1) compared to control, non-stretched cells ($P = 0.22$) and completely prevented the stretch-induced increase (L-NAME + stretch = 3.2 ± 1.0). Non-stretched myoblasts treated with 100 µM DETA-NO as a positive control for NO production exhibited DAF-FM fluorescence (per µg protein) of 16.3 ± 1.0.

Nitric oxide stimulates C2C12 myoblast proliferation

To determine if NOS activity is necessary for myoblast proliferation, cells were treated with the non-specific NOS inhibitor, L-NAME (200 µM). Treatment with L-NAME attenuated the stretch-effect by 35% but did not completely eliminate the increase in proliferation with stretch (Fig. 2a). Administration of L-NAME to unstretched cells had no effect on proliferation (Fig. 2b). To test the specificity of the NOS inhibitor, L-NAME, cells were treated with the inactive enantiomere D-NAME as a control. As determined by DAPI stain, D-NAME had no effect on either basal or stretch-induced proliferation when compared to untreated cells (results not shown). To determine the effect of NO on C2C12 myoblast proliferation during stretch, we supplemented the medium with 10, 50, 100 µM of the NO-donor, DETA-NO. The higher doses (50 and 100 µM) of DETA-NO had no additional effect on stretch-induced proliferation, but 10 µM DETA-NO stimulated proliferation 61% over stretch alone (Fig. 3a). Addition of 10 µM DETA-NO to basally growing cells had no impact on proliferation (Fig. 3b).

Nitric oxide rescues proliferation deficits caused by COX-2 inhibition

Myoblast proliferation decreased linearly after stretch with increasing concentrations of NS-298 (Fig. 4a): 25% decrease with 50 µM, 47% decrease with 100 µM, and 80% decrease with 200 µM NS-398. Further, addition of 100 µM NS-398 to the medium of unstretched cells significantly reduced basal proliferation by 35% compared to controls (Fig. 4b).

Fig. 1 Stretch stimulates myoblast proliferation. (**A**) Quantification of myoblasts staining positive for BrdU incorporation or DAPI. Cells were fixed 24 h after the cessation of mechanical stretch. Representative images of control (unstretched) myoblasts (**B**) and stretched myoblasts (**C**) stained for BrdU at 10× magnification. Representative images of stretched cells showing that >95% of DAPI-stained nuclei (**D**) are also myogenic by staining MyoD-positive (**E**). Values represent mean ± SEM. $n = 6$ cultures/treatment; * Significantly different from control group
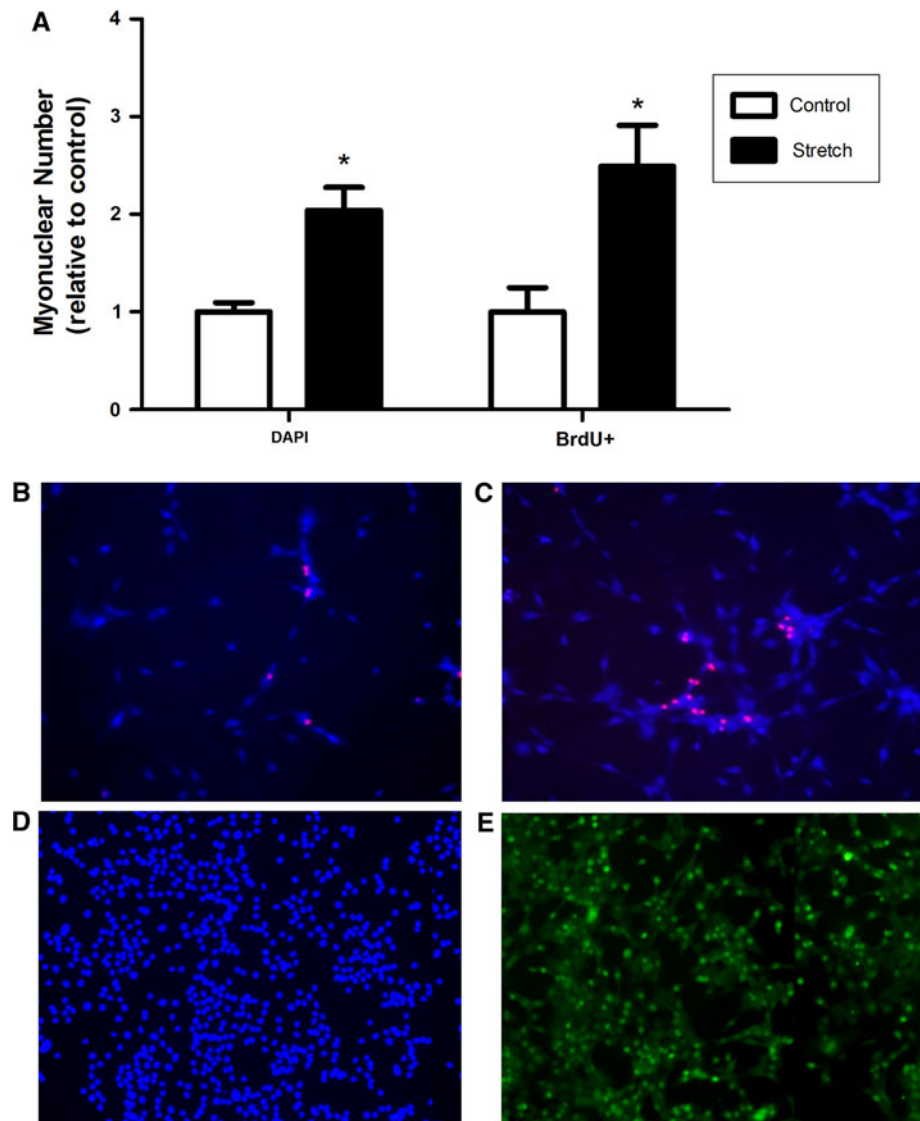
Our lab has demonstrated that NOS can regulate COX-2 expression involved in skeletal muscle growth (Soltow et al. 2006). Therefore, we tested the impact of exogenous NO on myoblasts stretched in the presence of the COX-2-specific inhibitor. The addition of 10 μM DETA-NO completely rescued stretch-induced myoblast proliferation in cells treated with the COX-2 inhibitor (Fig. 5a). However, supplementation with DETA-NO could not restore basal proliferation of myoblasts treated with NS-398 (Fig. 5b).

NF-κB activity is essential for myoblast proliferation

We sought to test if NF-κB activity is necessary for stretch-induced myoblast proliferation and the impact of supplementation with NO. To inhibit NF-κB activity, the drug, PDTC, was used, which has previously been shown to inhibit both NF-κB and NOS expression and activity in myoblasts (Lee et al. 1997) and COX-2 expression in rat heart, lung, and liver (Liu et al. 1999). Administration of 5 mM PDTC completely prevented stretch-induced proliferation (Fig. 6a) and significantly decreased basal proliferation (Fig. 6b). Addition of DETA-NO did not rescue the proliferation decrement caused by PDTC treatment in unstretched or stretched myoblasts (Figs. 7a, b).

Stretch of myoblasts induces COX-2 mRNA expression

Cyclic stretch of C2C12 myoblasts induced a fivefold increase in COX-2 mRNA, which was unaffected by co-treatment with the NF-κB inhibitor, PDTC (Fig. 8).

Discussion

In the present study, we demonstrate that NO is essential for stretch-induced proliferation and that administration of
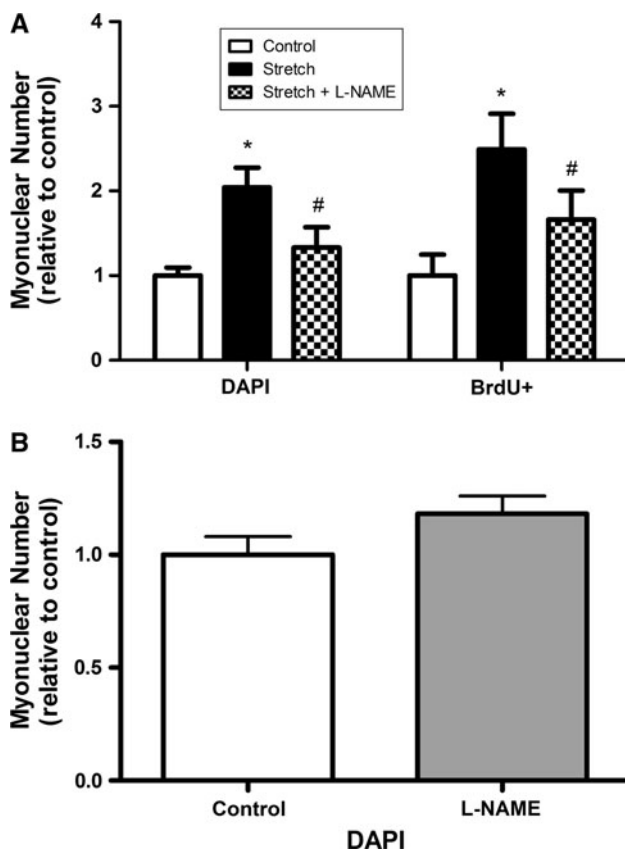
Fig. 2 NOS activity is necessary for stretch-induced proliferation. **a** Quantification of myoblasts staining positive for BrdU incorporation or DAPI stain 24 h following a 1 h stretch protocol. L-NAME (200 μM) was administered immediately prior to mechanical strain. **b** NOS activity is not necessary for basal proliferation of myoblasts. Quantification of myoblast number following a 24 h treatment of L-NAME (200 μM). Values represent mean $\pm$ SEM. $n = 6$ cultures/treatment; * Significantly different from control group. # Significantly different from stretch group

exogenous NO can potentiate the proliferative effects of cyclic stretch on myoblasts in a dose-dependent manner. It has previously been documented that the COX-2 pathway is necessary for myoblast proliferation in response to stretch (Otis et al. 2005) and that NF-κB plays a role in inhibition of myogenic differentiation following stretch (Kumar et al. 2004a). Therefore, we also tested the potential for an exogenous NO-donor to rescue stretch-induced myoblast proliferation during pharmacological inhibition of COX-2 and NF-κB signaling. Our data confirm that both COX-2 and NF-κB are critical for both basal and stretch-induced proliferation, and we are the first to show that NO supplementation can attenuate the proliferation decrement seen with COX-2 inhibition during stretch. Interestingly, NO-donor administration at the concentrations studied did not affect basal proliferation of C2C12 myoblasts. Thus, we demonstrate the following: (1) Stretch-induced myoblast proliferation is NO-dependent;

(2) supplementation with an NO-donor can overcome the negative effects of COX-2 inhibition on stretch-induced proliferation; and (3) myoblast expansion is stress sensitive since the antioxidant/NF-κB inhibitor, PDTC, drastically reduced nuclear numbers independent of stretch.

Cyclical mechanical stretch of skeletal muscle in vitro activates multiple pathways that are stimulated during in vivo muscle deformation, including NOS production of NO (Tidball et al. 1998), activation of satellite cells (Wozniak and Anderson 2007), and proliferation of myoblasts (Summers et al. 1985; Kumar et al. 2004a). Application of stretch to differentiated skeletal muscle myotubes leads to myotube hypertrophy (Vandenburgh and Kaufman 1979), an increase in cytoskeletal proteins, increased elastic modulus and cell integrity (Zhang et al. 2004). Therefore, cyclic stretch in culture can mimic certain physiological responses that occur when skeletal muscle is stressed in vivo.

Because skeletal muscle fibers are terminally differentiated, satellite cells must be activated to enter the cell cycle, divide, and immigrate to the stressed area for repair, growth, and maintenance. The proliferation of these muscle precursor cells is critical to supply sufficient numbers of myonuclei to the muscle fiber at the area of need. A primary activator of satellite cells is NO (Anderson 2000; Wozniak and Anderson 2007). NO release is triggered by mechanical stimuli, such as stretch, in cultured myoblasts, single myofibers, whole-muscle cultures, and in vivo (Tidball et al. 1998; Anderson and Pilipowicz 2002; Tatsumi and Allen 2004; Tatsumi et al. 2006; Yamada et al. 2006, 2008) making NO an important molecule for transforming mechanical changes into cellular signals. Using the NO-detecting fluorophore, DAF-FM, we demonstrate that cyclic stretch of C2C12 myoblasts induces production of NO via activation of NOS activity. The non-isoform-specific inhibitor of NOS (L-NAME) applied during stretch, prevented NO production and caused a significant reduction, but not complete inhibition, of myoblast proliferation. This suggests that although NO plays a significant role in stimulating myoblasts to proliferate, NO does not account for the entire stretch response.

Another proliferative factor secreted by stretched myoblasts is a product of the COX-2 pathway. Intermittent repetitive mechanical stimulation increases prostaglandin (PG) E$_2$ and F$_{2\alpha}$ release from muscle cells (Vandenburgh et al. 1990) and these prostaglandins induce proliferation (Otis et al. 2005; Mendias et al. 2004). Genetic and pharmacological inhibition of the COX-2 pathway prevents the release of PGE$_2$ and PGF$_{2a}$ and completely abrogates the stretch–response in primary cultured myoblasts (Otis et al. 2005). Using the COX-2 specific inhibitor NS-398, we also show inhibition of myoblast proliferation after stretch. Moreover, we were able to reverse this effect when cells were concurrently treated with the NO-donor DETA-NO.

Fig. 3 Nitric oxide promotes stretch-induced proliferation. (**a**) Quantification of a dose–response of the NO-donor DETA-NONOate administered immediately prior to initiation of stretch and representative images of (**b**) control; (**c**) stretch; (**d**) stretch + 10 μM DETA-NO; (**e**) stretch + 50 μM DETA-NO; and (**f**) stretch + 100 μM DETA-NO. (**g**) Nitric oxide is not sufficient to promote basal proliferation of myoblasts. Quantification of myoblast number following a 24 h incubation of 10 μM DETA-NONOate to the cell culture medium. Values represent mean ± SEM. $n = 6$ cultures/treatment; * Significantly different from control group. # Significantly different from stretch group
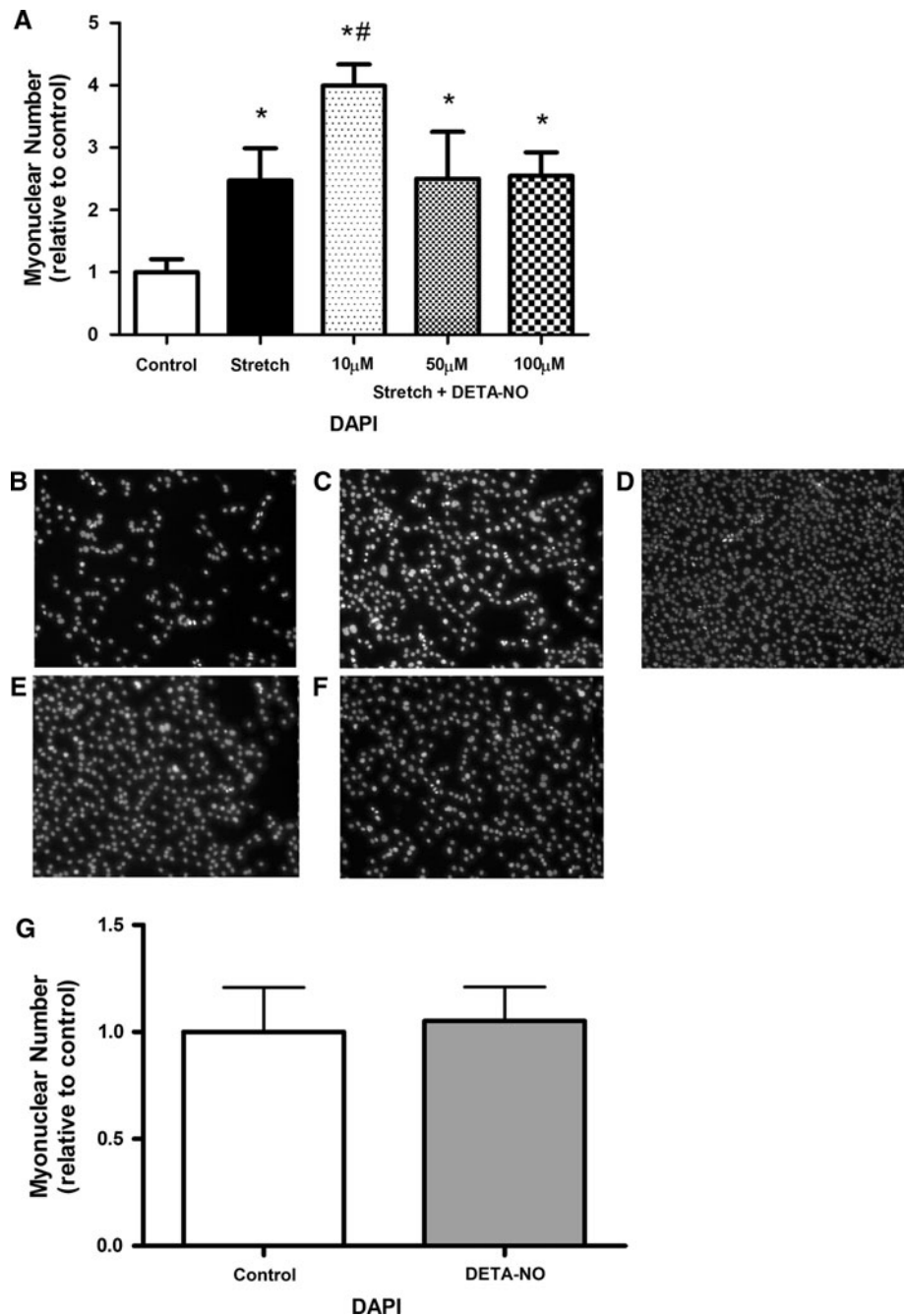
This suggests that NO production lies downstream of COX-2 activation in the stretch-proliferation signaling pathway. Perhaps via prostaglandin $F_{2\alpha}$ induction of iNOS expression and activity, as has recently been reported in luteal endothelial cells (Lee et al. 2009). On the other hand, our previous in vivo work in an overload model of skeletal muscle hypertrophy, showed that NO regulates the expression of COX-2 in the plantaris, and the activities of both NOS and COX-2 are necessary for skeletal muscle hypertrophy (Soltow et al. 2006). Considering these data with the present myoblast study, we suspect that treatment

with the long-acting NO-donor DETA-NO (half-life of 20 h) likely upregulated COX-2 expression, augmenting the more than fivefold increase in COX-2 mRNA observed with stretch (Fig. 8), potentially exceeding the inhibitory capacity of the 100 μM NS-398 treatment. This increase in COX-2 expression would allow increased prostaglandin production and lead to rescued proliferation. Future studies should measure the effect of NO donors on myoblast PGE_2 and $PGF_{2\alpha}$ content to confirm this hypothesis.

Another study performed in C2C12 myoblasts also demonstrated a >3.5-fold increase in COX-2 expression
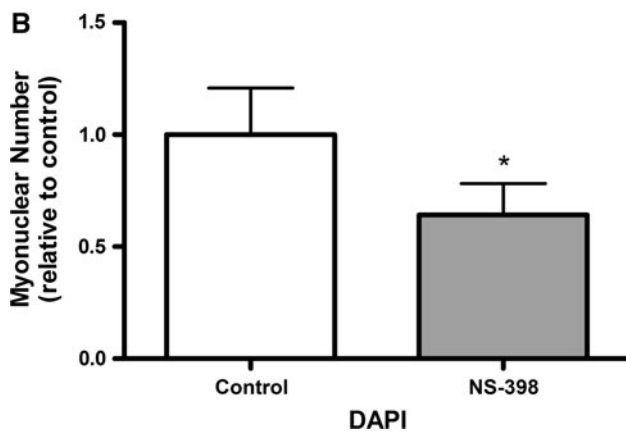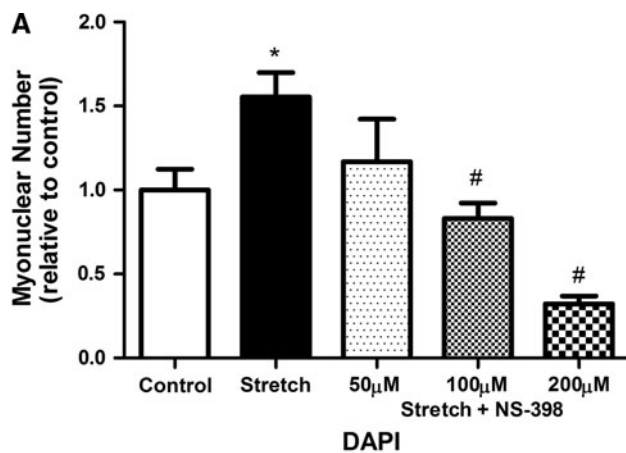
Fig. 4 COX-2 activity is necessary for stretch-induced and basal proliferation of myoblasts. **a** Quantification of a dose–response of the COX-2-specific inhibitor NS-398 administered immediately prior to initiation of stretch. **b** Quantification of myoblast number following a 24 h incubation of 100 μM NS-398 to the cell culture medium. Values represent mean ± SEM. $n = 6$ cultures/treatment; * Significantly different from control group. # Significantly different from Stretch group

Fig. 5 Nitric oxide rescues proliferation deficits in stretched myoblasts treated with a COX-2 inhibitor. **a** Quantification of myoblast number following stretch and concurrent treatments of the COX-2-specific inhibitor NS-398 (100 μM) and the nitric oxide donor DETA-NONOate (10 μM). Treatments were administered immediately prior to initiation of stretch. **b** Nitric oxide supplementation does not rescue proliferation deficits causes by COX-2 inhibition during basal growth. Quantification of myoblast number following a 24 h incubation of 100 μM NS-398 and 10 μM DETA-NONOate to the cell culture medium. Values represent mean ± SEM. $n = 6$ cultures/treatment; * Significantly different from control group. # Significantly different from stretch group

after stretch (Otis et al. 2005), and they speculate that NF-κB may provide the signaling link between mechanical stretch and increased COX-2 mRNA expression since NF-κB binds multiple sites within the COX-2 promoter region. We show here that PDTC treatment does not affect COX-2 mRNA during stretch (Fig. 8), which suggests that COX-2 expression is not dependent on NF-κB. Alternatively, COX-2 expression may be regulated by other mechanosensitive proteins, such as caveolin, which has been shown in other cell types (Rodriguez et al. 2009; Kwak et al. 2006; Spisni et al. 2003). Although we cannot determine the exact nature of the interaction between COX-2 and NO, it is clear that NO plays a critical role in the COX-2-dependent stretch-induced augmentation of myoblast proliferation that is not dependent on NF-κB.

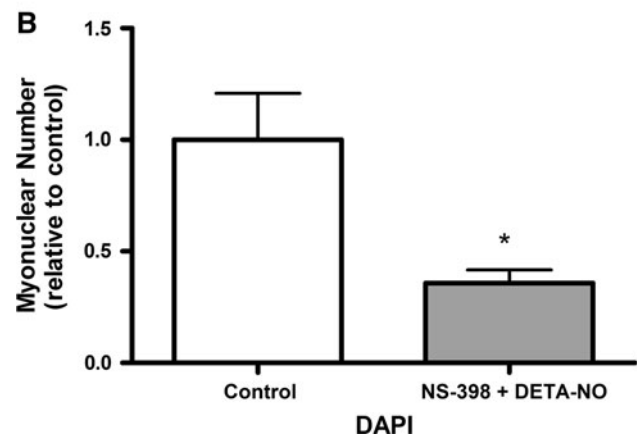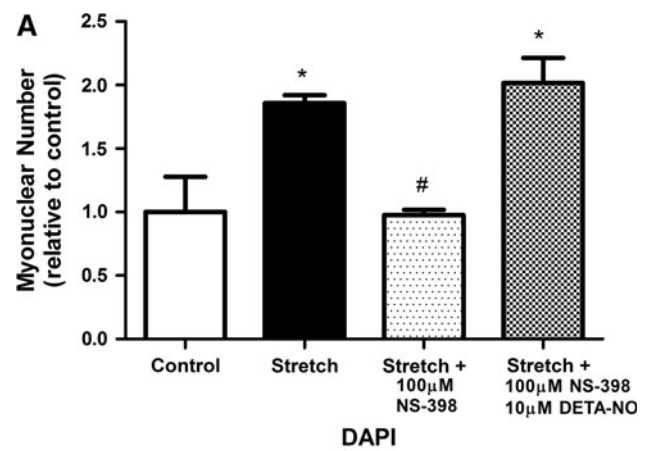Regulation of myogenesis is believed to be a redox-mediated event, and the oxidation state that induces entry

into, and exit from the cell cycle is tissue specific. For example, reducing conditions induce a proliferative effect and oxidative states lead to differentiation in adipocytes and smooth muscle cells (Csete et al. 2001; Su et al. 2001). However, under oxidizing conditions, mesenchymal stem cells and myoblasts (Csete et al. 2001; Hansen et al. 2007) are stimulated to proliferate and differentiation is down-regulated. Maintenance of a low redox potential and a reducing redox environment correlate with an increase in C2C12 muscle differentiation. Perturbations that result in more positive redox potentials (more oxidative) of C2C12 cells significantly slows their differentiation, and these morphologic observations are supported by changes in expression of the myogenic bHLH transcription factors (Hansen et al. 2007).
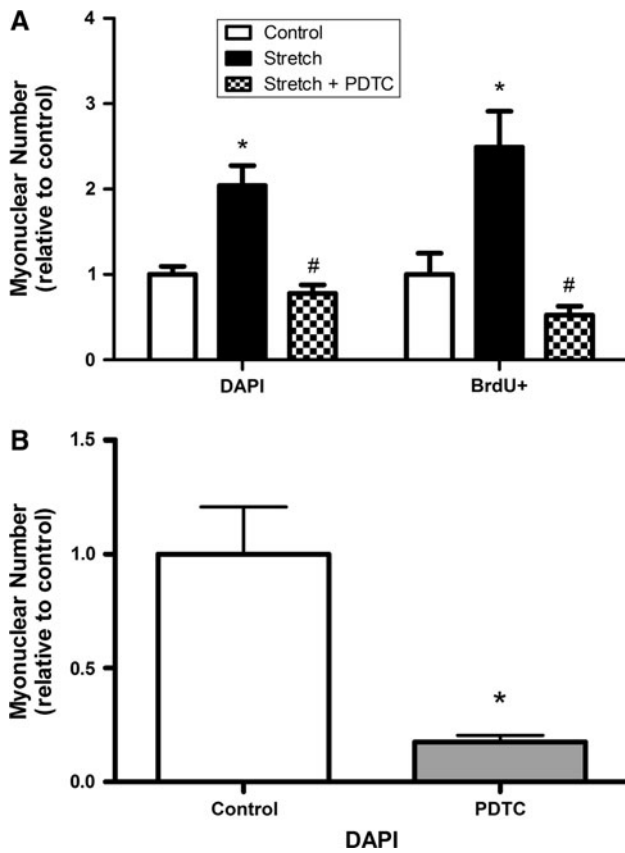
Fig. 6 NF-κB activity is necessary for stretch-induced and basal proliferation of myoblasts. **a** Quantification of myoblasts staining positive for BrdU incorporation or DAPI stain 24 h following a 1 h stretch protocol. The NF-κB inhibitor PDTC (5 mM) was administered immediately prior to mechanical strain. **b** Quantification of myoblast number following a 24 h incubation of 5 mM PDTC to the cell culture medium. Values represent mean ± SEM. $n = 6$ cultures/treatment; * Significantly different from control group. # Significantly different from stretch group

Fig. 7 Supplemental nitric oxide does not rescue proliferation deficits in stretched myoblasts treated with an NF-κB inhibitor. **a** Quantification of myoblast number following stretch and concurrent treatments of the NF-κB inhibitor PDTC (5 mM) and the nitric oxide donor DETA-NONOate (10 μM). Treatments were administered immediately prior to initiation of stretch. **b** Nitric oxide supplementation does not rescue proliferation deficits causes by NF-κB inhibition during basal growth. Quantification of myoblast number following a 24 h incubation of 5 mM PDTC and 10 μM DETA-NONOate to the cell culture medium. Values represent mean ± SEM. $n = 6$ cultures/treatment; * Significantly different from control group. # Significantly different from stretch group

Both COX-2 and iNOS have NF-κB binding domains in their promoter regions, and NF-κB has been shown to induce genes important for proliferation and differentiation of myogenic cells (Kumar et al. 2004b; Newton et al. 1997). To study the role of NF-κB in stretch-mediated proliferation, we utilized the drug PDTC, which has previously been used as a potent NF-κB inhibitor (Liu et al. 1999). Administration of PDTC to C2C12 myoblasts significantly blunted cell proliferation both during stretch and in basal conditions. This is in agreement with another study of C2C12 myogenesis (Kumar et al. 2004a) that demonstrated an increase in NF-κB DNA binding and promoter activity during 17% cyclic stretch, and overexpression of the NF-κB inhibitory protein IκB$\alpha\Delta$N completely abrogated the proliferative effects of stretch on C2C12 myoblasts. Altogether, it appears that NF-κB activity is necessary for C2C12 myoblast proliferation.
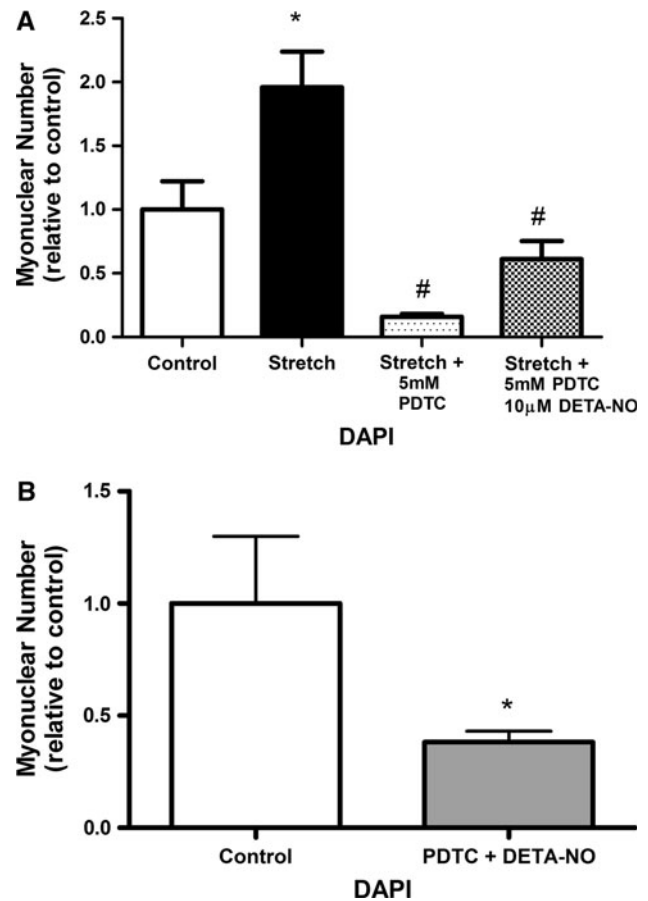
In addition, it is possible that the method of action for PDTC's inhibition of NF-κB is through its antioxidant capacity and not by direct inhibition of the protein. PDTC can effectively scavenge both hydroxyl and superoxide radicals (Shi et al. 2000) that may otherwise activate NF-κB. Therefore, addition of PDTC to culture media would supply a reducing environment that would thereby inhibit proliferation and promote differentiation of C2C12 cells as shown by Hansen and colleagues (Hansen et al. 2007). This redox and NF-κB-dependent regulation of myoblast proliferation seems to be independent of NO signaling effects since our NO donor did not influence PDTC effects.

Lastly, the NO-donor did not affect basal growth in any of the experimental conditions. The explanation for this
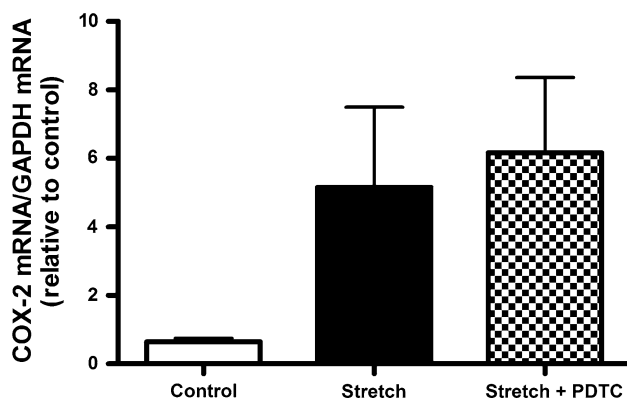
Fig. 8 Relative quantification (mean ± SEM) of mRNA transcripts for cyclooxygenase-2 (COX-2) in C2C12 myoblasts 24 h post-stretch, relative to unstretched control cells. The NF-κB inhibitor PDTC (5 mM) was administered immediately prior to mechanical strain. $n = 5$ cultures/treatment

could be that the concentration of the DETA-NO was not sufficient to induce proliferation without the stretch stimulus. We did not perform a dose response in non-stretched cells and only used the dose sufficient to induce the most proliferation during cyclic stretch. Thus, while larger doses of DETA-NO may have affected basal proliferation, the synergistic effects of stretch are necessary for the 10 μM dose of DETA-NO to induce proliferative effects on C2C12 myoblasts.

In conclusion, NO is an important signaling molecule for the regulation of skeletal muscle myogenesis during cyclic mechanical stretch. Supplemental NO can potentiate proliferative effects during mechanical activity and can provide a larger pool of skeletal muscle myoblasts for repair and growth. The restoration of myoblast proliferation seen with NO supplementation during treatment with the COX-2 inhibitor also suggests potential therapeutic benefits for individuals using COX-2-selective anti-inflammatory drugs or NSAIDS.

References

Anderson JE (2000) A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. Mol Biol Cell 11(5):1859–1874

Anderson J, Pilipowicz O (2002) Activation of muscle satellite cells in single-fiber cultures. Nitric Oxide 7(1):36–41

Betters JL, Lira VA, Drenning JA, Soltow QA, Criswell DS (2008) Supplemental nitric oxide augments satellite cell activity on cultured myofibers from aged mice. Exp Gerontol 43(12):1094–1101

Bondesen BA, Mills ST, Kegley KM, Pavlath GK (2004) The COX-2 pathway is essential during early stages of skeletal muscle regeneration. Am J Physiol Cell Physiol 287(2):C475–C483

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29(1):23–39

Chakravarthy MV, Booth FW, Spangenburg EE (2001) The molecular responses of skeletal muscle satellite cells to continuous expression of IGF-1: implications for the rescue of induced muscular atrophy in aged rats. Int J Sport Nutr Exerc Metab 11(Suppl):S44–S48

Csete M, Walikonis J, Slawny N, Wei Y, Korsnes S, Doyle JC, Wold B (2001) Oxygen-mediated regulation of skeletal muscle satellite cell proliferation and adipogenesis in culture. J Cell Physiol 189(2):189–196

Drenning JA, Lira VA, Simmons CG, Soltow QA, Sellman JE, Criswell DS (2008) Nitric oxide facilitates NFAT-dependent transcription in mouse myotubes. Am J Physiol Cell Physiol 294:C1088–C1095

Drenning JA, Lira VA, Soltow QA, Canon CN, Valera LM, Brown DL, Criswell DS (2009) Endothelial nitric oxide synthase is involved in calcium-induced Akt signaling in mouse skeletal muscle. Nitric Oxide 21(3–4):192–200

Germani A, Di Carlo A, Mangoni A, Straino S, Giacinti C, Turrini P, Biglioli P, Capogrossi MC (2003) Vascular endothelial growth factor modulates skeletal myoblast function. Am J Pathol 163(4):1417–1428

Hansen JM, Klass M, Harris C, Csete M (2007) A reducing redox environment promotes C2C12 myogenesis: implications for regeneration in aged muscle. Cell Biol Int 31(6):546–553

Kumar A, Murphy R, Robinson P, Wei L, Boriek AM (2004a) Cyclic mechanical strain inhibits skeletal myogenesis through activation of focal adhesion kinase, Rac-1 GTPase, and NF-kappaB transcription factor. Faseb J 18(13):1524–1535

Kumar A, Takada Y, Boriek AM, Aggarwal BB (2004b) Nuclear factor-kappaB: its role in health and disease. J Mol Med 82(7):434–448

Kwak JO, Lee WK, Kim HW, Jung SM, Oh KJ, Jung SY, Huh YH, Cha SH (2006) Evidence for cyclooxygenase-2 association with caveolin-3 in primary cultured rat chondrocytes. J Korean Med Sci 21:100–106

Lee KH, Baek MY, Moon KY, Song WK, Chung CH, Ha DB, Kang MS (1994) Nitric oxide as a messenger molecule for myoblast fusion. J Biol Chem 269(20):14371–14374

Lee KH, Kim DG, Shin NY, Song WK, Kwon H, Chung CH, Kang MS (1997) NF-kappaB-dependent expression of nitric oxide synthase is required for membrane fusion of chick embryonic myoblasts. Biochem J 324(Pt 1):237–242

Lee SH, Acosta TJ, Yoshioka S, Okuda K (2009) Prostaglandin F(2alpha) regulates the nitric oxide generating system in bovine luteal endothelial cells. J Reprod Dev 55(4):418–424

Lim JW, Kim H, Kim KH (2001) Nuclear factor-kappaB regulates cyclooxygenase-2 expression and cell proliferation in human gastric cancer cells. Lab Invest 81(3):349–360

Lira VA, Soltow QA, Long JH, Betters JL, JSellman JE, Criswell DS (2007) Nitric oxide increases GLUT4 expression and regulates AMPK signaling in skeletal muscle. Am J Physiol Endocrinol Metab 293(4):E1062–E1068

Liu SF, Ye X, Malik AB (1999) Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents in vivo expression of proinflammatory genes. Circulation 100(12):1330–1337

Long JH, Lira VA, Soltow QA, Betters JL, Sellman JE, Criswell DS (2006) Arginine supplementation induces myoblast fusion via augmentation of nitric oxide production. J Muscle Res Cell Motil 27(8):577–584

Mendias CL, Tatsumi R, Allen RE (2004) Role of cyclooxygenase-1 and -2 in satellite cell proliferation, differentiation, and fusion. Muscle Nerve 30(4):497–500

Newton R, Kuitert LM, Bergmann M, Adcock IM, Barnes PJ (1997) Evidence for involvement of NF-kappaB in the transcriptional

control of COX-2 gene expression by IL-1beta. Biochem Biophys Res Commun 237(1):28–32

Otis JS, Burkholder TJ, Pavlath GK (2005) Stretch-induced myoblast proliferation is dependent on the COX2 pathway. Exp Cell Res 310(2):417–425

Pisconti A, Brunelli S, Di Padova M, De Palma C, Deponti D, Baesso S, Sartorelli V, Cossu G, Clementi E (2006) Follistatin induction by nitric oxide through cyclic GMP: a tightly regulated signaling pathway that controls myoblast fusion. J Cell Biol 172(2):233–244

Rodriguez DA, Tapia JC, Fernandez JG, Torres VA, Muñoz N, Galleguillos D, Leyton L, Quest AF (2009) Caveolin-1-mediated suppression of cyclooxygenase-2 via a beta-catenin-Tcf/Lef-dependent transcriptional mechanism reduced prostaglandin E2 production and survivin expression. Mol Biol Cell 20:2297–2310

Shi X, Leonard SS, Wang S, Ding M (2000) Antioxidant properties of pyrrolidine dithiocarbamate and its protection against Cr(VI)-induced DNA strand breakage. Ann Clin Lab Sci 30(2):209–216

Smith LW, Smith JD, Criswell DS (2002) Involvement of nitric oxide synthase in skeletal muscle adaptation to chronic overload. J Appl Physiol 92(5):2005–2011

Soltow QA, Betters JL, Sellman JE, Lira VA, Long JH, Criswell DS (2006) Ibuprofen inhibits skeletal muscle hypertrophy in rats. Med Sci Sports Exerc 38(5):840–846

Spisni E, Bianco MC, Griffoni C, Toni M, D'Angelo R, Santi S, Riccio M, Tomasi V (2003) Mechanosensing role of caveolae and caveolar constituents in human endothelial cells. J Cell Physiol 197:198–204

Su B, Mitra S, Gregg H, Flavahan S, Chotani MA, Clark KR, Goldschmidt-Clermont PJ, Flavahan NA (2001) Redox regulation of vascular smooth muscle cell differentiation. Circ Res 89(1):39–46

Summers PJ, Ashmore CR, Lee YB, Ellis S (1985) Stretch-induced growth in chicken wing muscles: role of soluble growth-promoting factors. J Cell Physiol 125(2):288–294

Tatsumi R, Allen RE (2004) Active hepatocyte growth factor is present in skeletal muscle extracellular matrix. Muscle Nerve 30(5):654–658

Tatsumi R, Hattori A, Ikeuchi Y, Anderson JE, Allen RE (2002) Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. Mol Biol Cell 13(8):2909–2918

Tatsumi R, Liu X, Pulido A, Morales M, Sakata T, Dial S, Hattori A, Ikeuchi Y, Allen RE (2006) Satellite cell activation in stretched skeletal muscle and the role of nitric oxide and hepatocyte growth factor. Am J Physiol Cell Physiol 290(6):C1487–C1494

Tidball JG, Lavergne E, Lau KS, Spencer MJ, Stull JT, Wehling M (1998) Mechanical loading regulates NOS expression and activity in developing and adult skeletal muscle. Am J Physiol 275(1 Pt 1):C260–C266

Vandenburgh H, Kaufman S (1979) In vitro model for stretch-induced hypertrophy of skeletal muscle. Science 203(4377):265–268

Vandenburgh HH, Hatfaludy S, Sohar I, Shansky J (1990) Stretch-induced prostaglandins and protein turnover in cultured skeletal muscle. Am J Physiol 259(2 Pt 1):C232–C240

Vandenburgh HH, Shansky J, Karlisch P, Solerssi RL (1993) Mechanical stimulation of skeletal muscle generates lipid-related second messengers by phospholipase activation. J Cell Physiol 155(1):63–71

Wozniak AC, Anderson JE (2007) Nitric oxide-dependence of satellite stem cell activation and quiescence on normal skeletal muscle fibers. Dev Dyn 236(1):240–250

Yamada M, Tatsumi R, Kikuiri T, Okamoto S, Nonoshita S, Mizunoya W, Ikeuchi Y, Shimokawa H, Sunagawa K, Allen RE (2006) Matrix metalloproteinases are involved in mechanical stretch-induced activation of skeletal muscle satellite cells. Muscle Nerve 34(3):313–319

Yamada M, Sankoda Y, Tatsumi R, Mizunoya W, Ikeuchi Y, Sunagawa K, Allen RE (2008) Matrix metalloproteinase-2 mediates stretch-induced activation of skeletal muscle satellite cells in a nitric oxide-dependent manner. Int J Biochem Cell Biol 40(10):2183–2191

Zalin RJ (1987) The role of hormones and prostanoids in the in vitro proliferation and differentiation of human myoblasts. Exp Cell Res 172(2):265–281

Zhang JS, Kraus WE, Truskey GA (2004) Stretch-induced nitric oxide modulates mechanical properties of skeletal muscle cells. Am J Physiol Cell Physiol 287(2):C292–C299