Nitric-oxide Synthase Is a Mechanical Signal Transducer That Modulates Talin and Vinculin Expression*

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Mechanical stimuli can cause changes in muscle mass and structure which indicate that mechanisms exist for transducing mechanical stimuli into signals that influence gene expression. Myotendinous junctions show adaptations to modified muscle loading which suggest that these are transcriptionally distinct domains in muscle fibers that may experience local regulation of expression of structural proteins that are concentrated at these sites. Vinculin and talin are cytoskeletal proteins that are highly enriched at myotendinous junctions that we hypothesize to be subject to local transcriptional regulation. Our findings show that mechanical stimulation of muscle cells in vivo and in vitro causes an increase in the expression of vinculin and talin that is mediated by nitric oxide. Furthermore, nitric oxide-stimulated increases in vinculin and talin expression occur through a protein kinase G-dependent pathway and therefore differ from other mechanisms through which nitric oxide has been shown previously to modulate transcription. Analysis of vinculin mRNA distribution in mechanically stimulated muscle fibers shows that the mRNA is highly concentrated at myotendinous junctions, which supports the hypothesis that myotendinous junctions are distinct domains in which the expression of cytoskeletal proteins is modulated by mechanical stimuli through a nitric oxide and protein kinase G-dependent pathway.

Application of mechanical loads to myotubes in vitro yields similar cellular responses (10–13). Although little is known of the mechanisms of mechanical signal transduction in muscle, previous investigations have provided evidence that soluble factors released by muscle cells experiencing loads in vitro may contribute to signaling an increase in protein synthesis (12). In addition, stretch-activated ion channels (14) could provide increases in the concentration of specific cytosolic ions that are capable of activating signaling pathways that can influence gene expression. However, the extent to which these systems of mechanical signal transduction may contribute to modifying the expression of specific proteins in muscle is unknown.

Myotendinous junctions (MTJs),1 which are highly specialized sites of force transmission across the muscle cell membrane (15), are responsive to changes in their mechanical environment (16). Increased mechanical stimulation in vivo or in vitro causes an increased expression of structural proteins that are concentrated at these sites (13). The increased concentration at MTJs of the mRNA encoding proteins whose expression is increased at MTJs suggests that muscle cell nuclei near the MTJ preferentially transcribe those mRNAs.

The local regulation of mRNA and protein synthesis at MTJs that are experiencing modified loading suggests that mechanical signal transduction that regulates the expression of these molecules occurs at MTJs. Recently, neuronal nitric-oxide synthase (nNOS) was shown to be concentrated at MTJs (17), and both its activity and expression are positively regulated by mechanical loading of muscle in vivo and in vitro (18–20). Because nitric oxide (NO) has been shown to modify gene expression in other systems (21–25), it is feasible, but untested, that increased [NO] that is generated during increased muscle loading could affect the expression of structural proteins. Furthermore, nitric oxide that is generated during increased muscle use has a short half-life (26), so its effects in muscle would be local and consistent with the expectation that gene expression in nuclei located near the MTJ would be most affected.

In this investigation, we test the hypothesis that NOS is a mechanical signal transducer in muscle that positively regulates the expression of structural proteins enriched at MTJs. We assay the effects of mechanical stimulation that are mediated by the NOS signaling pathway on the expression of talin and vinculin, which are cytoskeletal proteins that are highly enriched at MTJs and are links in a chain of proteins that couple thin filaments to integrin. Talin was selected for study because its expression is up-regulated by mechanical stimulation of muscle (13). Vinculin was selected because previous

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‡ This abbreviation used are: NO, nitric oxide; NOS, neuronal nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; MTJ, myotendinous junction; DMEM, Dulbecco's modified Eagle's medium; PRG, cGMP-dependent protein kinase; L-NAME, (N' nitro-l-arginine methyl ester); SNAP, 5-nitroso-N-acetylpenicillamine; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction.
investigations have shown that it plays an important role in mechanical coupling in transmembrane assemblies of structural proteins (27).

**EXPERIMENTAL PROCEDURES**

**In Vitro Mechanical Loading Protocol—**C2C12 muscle cells were subjected to cyclic strains using a mechanical cell stimulator (Cell Kinetics, Pennsylvania, PA), which consists of a stainless steel plate containing wells in which the floor is a silastic membrane. Culture conditions were identical to those used previously (20). Myotubes were cyclically strained using a 6.7% mean deformation of the membrane and adherent cells. Five cycles of strain during a 20-s period followed by 10 s of no strain were applied to the cells, and then the strain cycle was repeated two more times, followed by a 30-min period of no strain. Mean strain rate was 3.4% per min with KT5823 only, followed by 2 h with SNAP and KT5823. NO concentration in culture media at the end of the experimental treatments was measured using previously described procedures (20).

**Modulation of [NO] and cGMP-dependent Protein Kinase Activity in Vitro**—Concentration in **vivo** was modulated by the addition of NO donors or NOS inhibitors to cultures. In addition, potential NO-mediated events in target cells were blocked downstream of NO stimulation by inhibition of cGMP-dependent protein kinase (PKG) with KT5823. Cultures in which NO activity was inhibited received 100 μM Nω-nitro-l-arginine methyl ester (L-NAME) in 10% FBS in DMEM containing no phenol red immediately prior to the beginning of experimental loading protocols. Cultures subjected to experimental inhibition of NOS activity were subjected to a media exchange at the same time as cultures receiving L-NAME.

Cultures that were stimulated with exogenous NO donor received 100 μM S-nitroso-N-acetylpenicillamine (SNAP) in DMEM containing 10% FBS for 2 h prior to collection for analysis. PKG was inhibited in cultured myotubes by incubation in 2 μM KT5823 for 2 h before analysis. Cells treated with both SNAP and KT5823 were incubated for 30 min with KT5823 only, followed by 2 h in SNAP and KT5823. NO concentration in culture media at the end of the experimental treatments was measured using previously described procedures (20).

**In Vivo Mechanical Loading Protocol—**Modified loading of rat hindlimb musculature in **vivo** was achieved using previously described procedures in which the hindlimb musculature experienced 10 days of unloading followed by 2 days of reloading by normal body weight (13, 28). At the end of the period of unloading or reloading, animals were euthanized, and plantaris muscles were collected for analysis.

**Modulation of [NO] in **vivo**—**Animals experiencing NO inhibition during muscle reloading followed unloading received water containing the NO inhibitor L-NAME at 0.5 mg/ml that was provided ad libitum starting 1 day prior to reloading. Control animals received untreated drinking water.

**Northern Blots—**RNA was isolated from whole plantaris muscles according to the technique of Chomczynski and Sacchi (29). Electrophoresis and hybridizations were performed as described previously (20). Transfer efficiency and uniformity of loading were checked by staining the membrane with methylene blue. Blots were hybridized with 32P-labeled probes generated by random priming (Amersham Pharmacia Biotech). Following hybridization at 65 °C, blots were washed with 0.05 m sodium phosphate, 0.75 m sodium chloride, 5 mM EDTA, and 0.1% SDS for 1 h and exposed to autoradiographic film.

**Probes for Northern Hybridization—**The following probes were used to hybridize Northern blots: 1) mouse talin cDNA (generous gift from Dr. L. C. Coll, EMBL accession number L18880); 2) 590-base pair product. The sequence of the primers was derived from the mouse cDNA sequence.2 Briefly, RNA was isolated (21), and 1 μl of the reaction mixture was used to make cDNA using random primers (10 μg/ml) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) in 20 μl at 42 °C for 30 min. Following cDNA synthesis, 2 μl of the reaction was used to perform PCR using Taq polymerase (Promega), 2.5 mM MgCl2, 4% MeSO4, and 0.2 μM dNTP (Amersham Pharmacia Biotech). Cycling conditions were 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C for 25 cycles.

**RESULTS**

**Mechanical Signal Stimulation Increases Talin and Vinex Expression in Myotubes via an NO-dependent Mechanism—**C2C12 myotubes subjected to mechanical stimulation by periodic cyclic loading in **vivo** for 24 h showed large increases in the concentration of both talin and vinculin mRNA by approximately 3- and 2.5-fold, respectively, compared with control myotubes not subjected to stimulation (Fig. 1). The presence of the NOS inhibitor L-NAME prevented the increases in talin and vinculin mRNA concentrations caused by mechanical stimulation of myotubes in **vivo** (Fig. 1). Measurement of NO concentration in the culture media of mechanically stimulated myotubes and unstimulated controls showed that stimulation nearly doubled NO production by myotubes, from 0.5 to 0.9 pmol NO/mg/min (±0.04; n = 4).
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FIG. 1. Northern analysis of mRNA collected from C2C12 myotubes grown on gelatin-coated, silastic membrane and assayed for vinculin mRNA (upper panel), talin mRNA (middle), or 28 S ribosomal subunit (lower). A, myotubes not subjected to mechanical stimulation; B, myotubes subjected to mechanical stimulation prior to collection for RNA isolation; C, myotubes mechanically stimulated while in culture medium; and D, myotubes mechanically stimulated while in culture medium containing l-NAME.

Exogenous NO Stimulates Vinculin and Talin Expression via PKG-dependent Mechanism—Addition of 100 μM SNAP produced 30 ng NO/mg/min (± 0.01; n = 3) over the 2-h incubation period. The increased NO concentration was accompanied by an increase in the concentration of vinculin mRNA to 170% of control values and an increase in the concentration of talin mRNA to approximately 260% of control values (Table I). Inhibition of cGMP-dependent protein kinase with KT5823 prevented the increase in vinculin or talin mRNA stimulated by SNAP (Fig. 2). PKG inhibition by KT5823 in the presence of SNAP reduced vinculin mRNA concentration below that of control cultures that were not stimulated by SNAP. Application of KT5823 alone did not significantly decrease vinculin or talin mRNA concentrations (Fig. 2) which indicates that the KT5823 inhibition of the SNAP-stimulated increase of talin and vinculin mRNA does not occur through inhibition of a NO-independent positive regulator of their expression. However, KT5823 application to myotubes in the absence of SNAP resulted in a significant increase in vinculin mRNA concentration, which indicates that there must be other NO-independent, PKG-modulated controls on vinculin expression that remain unidentified.

Talin and Vinculin mRNA Half-life Measurements—Stability of talin and vinculin mRNAs were measured by assaying for changes in their concentration over time in the presence of actinomycin D, used to inhibit transcription. These assays showed that the half-life for talin mRNA stability in C2C12 myotubes is approximately 12.1 h, and the half-life for vinculin mRNA is approximately 27.3 h (Figs. 3–5).

Increased Talin and Vinculin Expression during Muscle Loading Is Inhibited by NOS Inhibition—Increased loading of plantaris muscles by 2 days of normal weight-bearing following a 10-day period of unloading produced an increase in vinculin mRNA concentration to 2.6 times control and in talin mRNA to 1.8 times control concentrations (Fig. 6; Table II). The increased concentrations of both talin and vinculin mRNA during increased muscle loading was greatly attenuated in animals that received l-NAME in their drinking water during the reloading period (Fig. 6). GAPDH mRNA concentration as a fraction of total mRNA decreased during muscle reloading, but l-NAME had no detectable influence on the effect of reloading on GAPDH mRNA concentration. Thus, NOS inhibition does not affect the changes in concentration of all mRNAs that are caused by muscle reloading.

Vinculin mRNA was primarily concentrated at MTJs in muscles experiencing increased loading (Fig. 7), as has been shown previously for talin mRNA (13). Negative control fibers showed no labeling at the MTJ following the in situ RT-PCR procedure. Positive control fibers showed strong labeling of nuclei following the in situ RT-PCR procedure (Fig. 7). In situ RT-PCR of β-actin mRNA showed no detectable differences in the concentration of that mRNA at MTJ and non-MTJ regions of the fibers on reloaded muscles (Fig. 8). Thus, the relatively high concentration of vinculin and talin mRNA at MTJs does not occur for all mRNA. RT-PCR showed that the primers and RT-PCR conditions used for in situ RT-PCR yielded products only of the predicted size for vinculin or β-actin mRNA, indicating that the reaction products seen by in situ RT-PCR were specific for vinculin or β-actin mRNA.

DISCUSSION

Our findings show that the increases in talin and vinculin mRNAs that result from mechanical stimulation of muscle fibers are mediated by NO. Thus, NOS acts as a mechanical signal transducer in muscle cells and may couple modifications in the mechanical environment to changes in gene expression. Several previous investigations have reported that increased expression of structural proteins in skeletal muscle occurs when muscle is subjected to increased mechanical stimulation (9, 13, 34, 35), but little is known of the mechanisms by which those stimuli from the mechanical environment are transduced into chemical information that can influence gene expression. Although NO synthesis has been shown to be positively regulated by mechanical stimulation of cells in bone (36), endothe-
Northern analysis for vinculin mRNA collected from myotubes cultured in media containing actinomycin D. The Northern blot used for densitometry is shown in Fig. 2, which is representative of the results from three experiments. Mean value obtained by densitometry for untreated cells in 10% FBS in DMEM was set at 100 and used to normalize other samples in each data set.

Relative density of signals in Northern analysis of select mRNAs in plantaris muscles of animals experiencing modified muscle loading

The Northern blot used for densitometry is shown in Fig. 6 and is representative of the results from three experiments. Mean value obtained by densitometry for unloaded muscle samples was set at 100 and used to normalize other samples in each data set.

| Table I |
|-----------------|-----------------|-----------------|-----------------|
| [Vinculin mRNA] | 100 (S.E. = 23; n = 3) | 168 (S.E. = 9; n = 3)* | 60 (S.E. = 15; n = 3)* |
| [Talin mRNA]    | 100 (S.E. = 16; n = 3) | 258 (S.E. = 42; n = 3)* | 104 (S.E. = 16; n = 3) |

* Indicates significant difference from untreated cells at \( p = 0.05 \).

Fig. 3. Northern analysis of mRNA collected from C2C12 myotubes and assayed for vinculin mRNA (upper panel) or talin mRNA (lower panel). A, myotubes in culture media only; B, myotubes in culture media containing actinomycin D for 2 h; C, myotubes in culture media containing actinomycin D for 8 h; D, myotubes in culture media containing actinomycin D for 24 h.

Fig. 4. Linear regression of densitometry of autoradiograph of Northern analysis for vinculin mRNA collected from myotubes cultured in media containing actinomycin D. \( r = -0.73; p = 0.007 \).

Fig. 5. Linear regression of densitometry of autoradiograph of Northern analysis for talin mRNA collected from myotubes cultured in media containing actinomycin D. \( r = -0.96; p < 0.0001 \).

nNOS at an optimal site for sensing changes in the mechanical environment. Furthermore, increased skeletal muscle loading causes an increase both in nNOS expression (20) and activity (18, 20), and NO has been shown to function as a signaling molecule in several cell types (39). Finally, the short half-life of NO (26) suggests that it would locally regulate changes in transcriptional activity. This local regulatory effect may have particular functional significance in skeletal muscle where the transcriptional activities of individual nuclei located in a single muscle fiber can function independently. This independent regulation of transcriptional activity of individual myonuclei in a single cell has been best demonstrated for myonuclei located near the neuromuscular junction in which the increased expression of acetylcholine receptor subunits can be regulated...
independently of non-neuromuscular junction nuclei in the same cell (40, 41). The finding reported here that NO transduces mechanical signals to influence the expression of talin and vinculin and that NOS, NO, and the mRNAs of talin and vinculin are most concentrated at MTJs indicates that MTJs may also be a distinct nuclear domain in muscle cells in which NO plays a role in the local modulation of transcription. Other evidence indicating that MTJs may be distinct nuclear domains in muscle includes the findings that the mRNA for myosin heavy chain (34) and talin (13) increase in concentration at the MTJ during modified muscle use.

The observations reported here indicate that NO-mediated modulation of talin and vinculin mRNA concentrations occurs primarily by influencing the rate of transcription, although the possibility that there is some NO-mediated influence on talin and vinculin mRNA stability cannot be excluded entirely. This conclusion is supported by the finding that NO stimulation of myotubes for 24 h caused an approximately 170% increase in vinculin mRNA and 260% increase in talin mRNA, although the half-lives of those mRNAs were approximately 27 and 12 h, respectively. If NO were causing the observed increases in mRNA concentrations reported here solely through RNA stabilization, the maximum increase that would be observed in 24 h stimulation would be approximately 44% for vinculin and 93% for talin, which would be insufficient to explain the majority of the NO-mediated increase in mRNA concentrations.

Although there are several mechanisms through which NO can function as a signaling molecule, to our knowledge the only previously identified mechanism through which it has been shown to influence transcription is through structural modification of transcription factors (23–25). For example, c-Fos, c-Jun, and NF-κB binding affinities for target DNA sequences are all influenced by NO (23–25). Current evidence indicates that NO modifies transcription factors by S-nitrosylation of cysteines located in or near DNA binding domains (23–25) and that the NO-mediated effects are independent of guanylate cyclase activation (21). The present findings show that NO modulation of talin and vinculin expression is blocked by inhibition of PKG and therefore NO is not influencing transcription of talin and vinculin through direct interaction with transcription factors but rather through a cGMP- and PKG-dependent pathway. NO modulation of talin and vinculin expression also differs from the mechanisms through which it modulates the expression of genes regulated by AP-1 or NF-κB (21–25) in that NO is a positive modulator of talin and vinculin but negatively regulates genes whose expression is influenced by AP-1 or NF-κB binding.

Other mechanisms may also exist at MTJs for mechanical signal transduction that may influence the expression of genes for cytoskeletal proteins. The integrin-associated complex of proteins is highly concentrated at MTJs (15) and provides a
prominent candidate system for this function because integrins have also been shown to function in signal transduction. Mechanical loads placed on integrins and associated proteins influence the assembly of complexes of structural proteins at the loading site (42–44) and activate kinases concentrated at those sites (42). Although integrin loading per se has not been shown to regulate the expression of genes encoding cytoskeletal proteins, integrin binding of extracellular matrix molecules can dramatically influence gene expression (e.g., Refs. 32, 45, and 46). The co-distribution of nNOS and the integrin complex and the role of both proteins in influencing the response of cells to changes in their mechanical environment support the possibility that future investigations will show synergistic interactions between the two systems when muscle cells respond to changes in their mechanical environment.

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