Cellular Responses to Mechanical Stress
Selected Contribution: Mechanical strain increases force production and calcium sensitivity in cultured airway smooth muscle cells

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Smith, Paul G., Chaity Roy, Steven Fisher, Qi-Quan Huang, and Frank Brozovich. Selected Contribution: Mechanical strain increases force production and calcium sensitivity in cultured airway smooth muscle cells. J Appl Physiol 89: 2092–2098, 2000.—Cultured airway smooth muscle cells subjected to cyclic deformational strain have increased cell content of myosin light chain kinase (MLCK) and myosin and increased formation of actin filaments. To determine how these changes may increase cell contractility, we measured isometric force production with changes in cytosolic calcium in individual permeabilized cells. The pCa for 50% maximal force production was 6.6 ± 0.4 in the strain cells compared with 5.9 ± 0.3 in control cells, signifying increased calcium sensitivity in strain cells. Maximal force production was also greater in strain cells (8.6 ± 2.9 vs. 5.7 ± 3.1 μN). The increased maximal force production in strain cells persisted after irreversible thiophosphorylation of myosin light chain, signifying that increased force could not be explained by differences in myosin light chain phosphorylation. Cells strained for brief periods sufficient to increase cytoskeletal organization but insufficient to increase contractile protein content also produced more force, suggesting that strain-induced cytoskeletal reorganization also increases force production.

contractility; myosin light chain kinase; myosin; cytoskeleton

Prolonged exposure of visceral organs to abnormal mechanical stress increases both the deposition and the contractility of smooth muscle. For example, lungs of premature infants treated with mechanical ventilation develop increased smooth muscle deposition and reactivity, leading to chronic airway disease. It is difficult to assign increases in airway smooth muscle (ASM) quantity or reactivity specifically to mechanical stress in this and similar diseases because supportive structures are damaged and contractile agonists are released from surrounding tissue as a result of injury. To eliminate confounding variables while studying the effects of mechanical stress on ASM, we developed a system whereby cultured ASM cells are subjected to cyclic deformational strain approximating the degree seen in vivo (28). By use of this system, we reported that strain-induced increases in cell myosin light chain kinase (MLCK) are accompanied by increased phosphorylation of the 20-kDa regulatory light chain of myosin (LC20), increased maximal velocity of shortening, and increased extent of shortening of individual cells (30). In this way, strain-induced increases in MLCK increase cell contractility in ways analogous to changes seen in other models of hyperresponsive ASM (14, 17).

MLCK activation by the calcium-calmodulin pathway is essential for activation of smooth muscle contraction. Accordingly, increased expression of MLCK is a potential mechanism of calcium sensitization (6). To determine whether calcium sensitivity of force production accompanies strain-induced increases in MLCK, we studied the force response of individual permeabilized cells to incremental increases in intracellular calcium and found both increased calcium sensitivity and increases in the maximal force production in strain cells. Increased maximal force production was not entirely explained by differences in LC20 phosphorylation because similar results were noted when strained cells...
were subjected to irreversible thiophosphorylation. Short-term strain also increased force production independent of increases in contractile protein production, although actin filament formation was increased, suggesting that increased cell organization may also contribute to force generation independent of contractile protein synthesis.

**METHODS**

**Cell Culture and Strain Apparatus**

Trachealis muscle was harvested and digested in collagenase and elastase with soy trypsin inhibitor as previously described (28). Freshly dissociated cells were seeded into flasks at a density of $5 \times 10^4$ cells/cm² in Ham's F-12-DMEM with 10% fetal bovine serum, penicillin, streptomycin, and amphotericin. Cells were passaged to collagen type I-coated Silastic membranes (Flexcell, McKeesport, PA) when 80–90% confluent. First- and second-passage cells were used for these studies. To subject cells to mechanical stress, the plates were positioned over a manifold connected to a vacuum source. The vacuum was programmed by computer software (Flexcell) to cause a 10% increase in surface area of the membranes for 2 s, followed by 2 s of relaxation.

In most studies, cells were subjected to strain as described above for 10–12 days to study the effects of long-term strain. In other experiments, cells were subjected to strain for only 48 h to study the more acute effects of strain. This shorter duration was chosen because we have previously noted orientation, elongation, and actin filament organization in this short period (27). In all experiments, medium was replaced with low-serum medium for 48 h before force generation measurements to eliminate serum factors that may influence contractility.

**Contraction Measurements**

*Force production sensitivity to calcium.* To study single cell contractility, cells of similar length (100–150 μm) and morphology (elongated, spindle-shaped cells) were chosen to minimize differences in cell phenotype previously noted for cultured ASM cells (12, 19, 27). To determine the calcium sensitivity of force production in isolated cells, cells were permeabilized with *Staphylococcus aureus* α-toxin (250 hemolytic units/ml; GIBCO BRL Life Technologies, Grand Island, NY) in relaxing solution before contraction measurements (2). Cells were then rinsed and transferred to the movable stage of a Nikon inverted microscope. Force production was measured as previously described (25). The ends of the cells were attached to pulled glass capillary tubes with glue. One capillary tube was attached to a force transducer (Cambridge 406A, Cambridge Technology, Watertown, MA; resolution 0.01 μN, compliance 0.001 nm/μN). The transducer was calibrated by attaching known masses, and drift was <0.1 μN over 5 min. To record force, the cell was lifted from the membrane and then activated by changing the fluid bathing the cells, from pCa 9 to pCa 4, in a stepwise manner using a continuous flow-through system to minimize artifact from fluid level changes. Force production was displayed and recorded for later analysis. Force was noted to rise over 1–2 min and was allowed to plateau before the experiment proceeded to the next highest Ca²⁺ concentration (Fig. 1).

*Maximal force production after thiophosphorylation.* In separate studies, the maximal force production of individual cells was measured after LC₀⁻ was irreversibly thiophosphorylated. In this way, neither activity of MLCK nor phosphatase was directly involved in the initiation or maintenance of contraction or relaxation (24). Cells were permeabilized as above but were then treated with three changes, 5 min each, of a buffer in which the ATP was replaced by ATPγS. Greater than 95% of LC₀⁻ is thiophosphorylated with this protocol (23). The cells were then transferred to the microscope stage for force production measurements during activation with solution containing ATP.

**Solutions**

The calcium buffers used in this experiment were mixed according to an iterative computer program that calculates the amount of stock solutions to mix for a given set of free ion concentrations (2). Buffers contained 5 mM EGTA, 5 mM MgATP, 1 mM free Mg²⁺, 25 mM creatine phosphate, 25 mM $N,N$-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid, and methanesulfonic acid buffer pH 7.0. Mixtures of solutions at pCa 9 (relaxing solution) and pCa 4 were used to provide solutions of intermediate pCa. Rigor solution contained neither MgATP nor creatinine phosphate.

**Determination of Myosin Heavy and Light Chain Isoform Expression**

Myosin isoforms may be responsible for variations in the contractile function of smooth muscle cells (11, 13). To determine whether strain-induced changes in single-cell contractility might be due in part to differences in isoform expression of either myosin heavy chain (MHC) or the 17-kDa essential myosin light chain (LC₁₇), cells were subjected to RT-PCR. Oligonucleotide primers that bracketed the alternative exons of the MHC head region and C-terminus were used in the RT-PCR reaction as previously described (8). Total RNA was isolated from control and strain cells as well as for native canine trachealis muscle and chicken gizzard for comparison, and 2.5 μg of RNA was reverse transcribed. One-tenth of the RT products were subjected to PCR with melting at 95°C for 1 min and annealing and extension steps at 72°C elongation for 1 min, for a total of 35 cycles. PCR products were separated on 2% agarose gels, visualized with ethidium bromide, and directly quantified by using Biorad Multi-Analyist software.

**Evaluation of MLCK by Western Blotting**

To compare contractile protein content of strain and control cells, cells were maintained in serum-free media for 48 h after confluency growth and were harvested by scraping in lysis buffer (1 mM EDTA, 3 mM Na₂PO₄, 20 mM 3-(N-morpholino)propanesulfonic acid, 1% Triton X-100, 1 mM MgCl₂, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml
leupeptin] as previously described (29). Samples were normalized for total protein, and proteins were separated in 10% SDS-polyacrylamide gels before being electrophoretically transferred to nitrocellulose membranes. Non-specific sites were blocked with 5% dry milk in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.3% Tween 20. Primary antibodies against the proteins of interest were applied for 1 h at room temperature. Horseradish peroxidase-conjugated secondary antibodies were applied, and bands were detected by chemiluminescence (Super Signal, Pierce, Rockford, IL).

Statistics

The force-vs.-pCa relationship was analyzed by expressing force production as percentage of maximum (pCa = 4). The data were then fit to the Hill equation

$$\text{Relative force} = 100\% \times \frac{1}{1 + 10^{(p\text{Ca} - p\text{Ca}_{50})/h}}$$

where pCa is the negative log10 of Ca2+ concentration, pCa50 is the pCa at half-maximal force level of the Hill fit, and h is the Hill coefficient. Maximum force production (measured at pCa 4) and force production after thiophosphorylation or acute strain were compared by unpaired t-test. Significance was assigned to P values < 0.05.

RESULTS

Strain Increases Force Production Sensitivity to Calcium

To investigate strain-induced changes in the calcium sensitivity of force, cells were permeabilized, and individual cells were attached to a force transducer. Force production was then recorded during exposure of the cells to increasing concentrations of calcium. At least 10 cells were tested from each of four populations in each condition (control or strain), and force was expressed as a percent of maximum (pCa = 4). Strain cells demonstrated increased sensitivity to calcium with a pCa50 of 6.6 ± 0.4 compared with 5.9 ± 0.3 in the control cells. When absolute values of force production were compared, the maximum force produced was also increased (8.6 ± 2.9 μN vs. 5.7 ± 3.1 μN, strain vs. control, respectively, mean ± SD; Fig. 2, Table 1).

![Graph showing force-pCa relationship for strain and control cells.](image)

Table 1. Data comparing force production of strain and control cells from separate experiments

<table>
<thead>
<tr>
<th>Condition</th>
<th>pCa50</th>
<th>Max. Force</th>
<th>Thiophosphorylation force</th>
<th>Acute strain force</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>6.6 ± 0.4*</td>
<td>8.6 ± 2.9*</td>
<td>10.8 ± 3.7*</td>
<td>6.1 ± 2.6*</td>
</tr>
<tr>
<td>Control</td>
<td>5.9 ± 0.3</td>
<td>5.7 ± 3.1</td>
<td>8.0 ± 2.8</td>
<td>3.9 ± 1.5</td>
</tr>
</tbody>
</table>

Values (in μN) are means ± SD for 7–10 cells per experiment from four experiments for each study. *Strain is significantly greater than control at P < 0.05.

Maximal Force Production Measurements After Thiophosphorylation

To determine whether the increased maximal force production noted in strain cells might be explained solely by increased cell content of MLCK resulting in differences in LC20 phosphorylation, in separate experiments, cells were permeabilized and treated with ATPγS to irreversibly phosphorylate LC20. After attachment to the force transducer, cells were then activated by solution containing ATP. For both the control and strain cells, thiophosphorylation increased the maximal force compared with the calcium sensitivity studies. However, maximal force production of thiophosphorylated strain cells was again greater than the maximal force produced by thiophosphorylated control cells (Table 1). This indicates that increased maximal force was due, in part, to changes other than the quantitative changes in LC20 phosphorylation that resulted from increased MLCK.

Effects of Short-Term Strain on Contractility

To determine whether increases in cytoskeletal organization might lead to increased maximal force production in strain cell independent of increased contractile protein content, we subjected cells to strain for only 48 h. This was sufficient time to increase cytoskeletal organization (Fig. 3) but did not increase cell content of MLCK (Fig. 4). In these studies, intact cells were depolarized with 80 μM KCl. Comparison of maximal force production between cells subjected to strain for 48 h and control cells revealed an increase in maximal force production from 3.9 ± 1.5 μN (n = 37) in the control cells to 6.1 ± 2.5 μN (n = 45) in the strain cells (Table 1).

Effects of Strain on Contractile Protein Expression

We have shown, in several studies, that strain increases total myosin and MLCK content of cultured ASM cells (29–31). To determine the effects of strain on expression of isoforms A and B of MHC and isoforms of LC17, RT-PCR was performed on strain (10–12 days) and control cells. There were no differences in the profiles of the MHC isoforms expressed on any of the three experiments performed (Fig. 5). Both strain and control cells expressed only the exon-excluded splice-out variant of MHC head, identical to the native trachealis muscle. The expression of the exon-excluded splice variant of the LC17a isoform was increased in the
strain cells (90%) compared with the control cells (60%). In this way, the expression of LC\textsubscript{17} isoforms in the strain cells was more closely matched to the native trachealis muscle expression, in which the expression was exclusively LC\textsubscript{17a}.

DISCUSSION

In this study, we demonstrate strain-induced increases in the calcium sensitivity of force production of cultured ASM cells. This finding is consistent with increased cell content of MLCK. The additional finding of increased maximal force production cannot be entirely explained by increased LC\textsubscript{20} phosphorylation but may be due to several factors, including organizational changes and maintenance of more differentiated expression of light-chain isoforms. Smooth muscle hypertrophy and/or hyperplasia are frequent consequences of chronically abnormal mechanical stress on visceral tissue. The cellular mechanisms responsible for increased airway reactivity are unclear, although changes intrinsic to the smooth muscle seem likely. Increased calcium sensitization of force production is one characteristic of hyperresponsive ASM. There are several potential mechanisms responsible for calcium sensitization. One of the leading candidate mechanisms is inhibition of myosin phosphatase through Rho kinase phosphorylation of the myosin-binding subunit of the phosphatase (32). As discussed below, this may be an active process in our system because many of the effects of strain on cultured smooth muscle cells are identical to effects noted with pharmacological activation of RhoA, the activating enzyme of Rho kinase. Such effects of strain include filament organization and focal adhesions (27) and inhibition of phosphatase activity (31). Increased cellular content of MLCK could also contribute to increased calcium sensitization because MLCK is activated by calcium-calcmodulin and is essential for activation of actinomyosin ATPase (5). Increases in MLCK have been correlated with increased maximal velocity of shortening in some disease models of hyperresponsive smooth muscle (14) and in cultured cells (19, 30). The present study demonstrates an increase in calcium sensitivity of force production consistent with the increased cell content of MLCK that we have shown in previous studies (29–31). Another potential mechanism for calcium sensitization of ASM is decreased phosphatase activity, which would also result in an increase of LC\textsubscript{20} phosphorylation. We did not investigate the potential for decreased phosphatase activity in the present study, although we have previously demonstrated a decrease in phosphatase activity in ASM cells subjected to strain (31). Because the total amount of LC\textsubscript{20} phosphorylated reflects both phosphorylation by MLCK and dephosphorylation by myosin light chain phosphatase, it is possible that both processes contributed to the calcium sensitization in our system. However, alterations in kinase and phosphatase activity cannot entirely explain the increased maximal force production seen in the strain cells, because thiophosphorylation of LC\textsubscript{20} is not subject to degradation by phosphatases (24) and, yet, maximal force was still greater in strain cells after thiophosphorylation. Maximal force generation was greater in the thiophosphorylation studies than the maximal force at pCa 4 in the calcium-sensitivity studies. Because pCa 4 was used in both studies, this general increase is most likely due to more complete LC\textsubscript{20} phosphorylation after thiophosphorylation (23). Thus increased maximal force in strain cells cannot be explained on the basis of differences in activation (LC\textsubscript{20})

Fig. 3. Mechanical strain increases actin filament formation and organization within 48 h. Cells were subjected to strain for 48 h, fixed and stained with rhodamine-conjugated phalloidin (A), and compared with control (B).

Fig. 4. Mechanical strain for 48 h does not induce increased cell content of myosin light chain kinase (MLCK). Cells were subjected to mechanical strain for 48 hrs and compared by Western blotting to identical cells for content of MLCK. Examples shown are representative of strain (S1–S3) and control (C1–C3) cells from 3 experiments and showed no appreciable difference between the 2 conditions: strain 328 ± 52 vs. control 341 ± 88 arbitrary units, as measured by densitometry. MW, positions of molecular weight, in kDa.
phosphorylation), and the results point to differences at the level of the contractile filaments.

The cellular mechanisms of increased maximal force production in the cells subjected to strain in our study appear to be multifactorial. The rapid changes in organization of cytoskeletal and contractile elements noted with strain could increase cell contractility by increasing the number of the contractile units, i.e., cross-bridge numbers and filament length (9), or by enhancing their efficiency through focusing force along a unified vector. Regarding the latter of these possibilities, if it were assumed that the contractile elements in control cells were randomly oriented and that strain only increased the orientation of contractile elements without an increase in number of force-generating units, analyzing the directional angle of force generation could theoretically predict the magnitude of differences between strain and control cells attributable to this effect. Random orientation of contractile elements relative to the long axis of the cells would have a mean vector of force of 45° relative to filaments lying parallel to the long axis of the cell. Force production from randomly oriented filaments would be produced at a level equal to cos 45°, or 0.7. Because mean force values generated by control cells varied from the mean values of the strain cells to a greater extent than predicted by this theoretical model (60%), orientation of contractile elements alone does not account for differences. In fact, differences in force generation were probably not caused by differences in filament direction, because the control cells studied were elongated and positioned in their long axes between the glass probes so that filaments were not randomly oriented. We would suggest instead that increased numbers of filaments formed, increased amounts of myosin, or increased numbers of cross bridges or other effects from strain, as outlined below, account for the increased force generation of strained cells. Of secondary interest was the fact that the mean values of force generation in the studies after acute strain were, in general, less than the force generated with thiophosphorylation or high concentrations of calcium. This finding is consistent with other studies that noted relatively less force generation with KCl depolarization than with other methods of activation.

Certainly, the increased content of myosin that we noted previously (29, 31) could increase force-generating units through increased numbers of cycling cross bridges. Various isoforms of MHC (15) and essential light chain (13, 21) may increase cell contractility, but we did not detect any strain-induced changes in MHC isoform expression. The COOH-terminus isoforms of MHC, previously designated SM1 (204 kDa) and SM2 (200 kDa), were not examined but have not been shown to directly influence contractile parameters (22). Previously, we reported increased cell content of SM1 isoforms with strain (29), and this could contribute to increased filament assembly (3). We did not find differences in the SM-MHC head insert isoforms expressed in the cultured cells (either strain or control) compared with native trachealis muscle; all samples expressed the exon-excluded splice variant (SM-A) (15). However, we found increased expression of the exon-included variant of the 17-kDa regulatory light chain (LC17n) in cultured cells not exposed to strain. This isoform was not found in the native trachealis and was only found in low amounts in the strain cells, suggesting that increased expression in the control cells represents dedifferentiation not seen in strain cells. Increasing the expression of LC17n has been shown to decrease the rate of force activation in gizzard smooth muscle cells (13). It is possible that differences in the expression of LC17n/LC17s could change stiffness of the lever arm or kinetics of the cross-bridge cycle to alter force per cross bridge and could also account for some of the differences in the force generation seen between strain and control cells. Although some studies (20) have shown that increasing the amounts of LC17s decreases the velocity of shortening, which is in agreement with our study, others have shown a dependence of smooth muscle shortening kinetics and corresponding ATPase activities on MHC isoform differences (18). This latter study differed from ours in that kinetics were measured by using a laser trap and an in vitro motility
mixture assay rather than whole cells so that the results are not equivalent.

Another strain-induced cell change not addressed by these experiments is activation of integrin- or membrane-associated proteins, which have identified roles in regulation of cell contractility. We have previously noted strain-induced tyrosine phosphorylation of focal adhesion proteins such as p125 focal adhesion kinase and paxillin (26). Similar protein tyrosine phosphorylation has been noted in intact smooth muscle (33) and may be responsible for either organization of contractile elements or contractile function and calcium regulation of smooth muscle (7). It is interesting to note that the strain-induced morphological changes and myosin phosphatase inhibition we have reported are identical to cell changes induced by activation of the small GTPase RhoA in other studies (4, 16). Furthermore, there is evidence that mechanical strain influences RhoA activation (34), and RhoA is known to have a role in smooth muscle contraction. Although we have reported inhibition of strain-induced organization during exposure to tyrosine kinase inhibitors, the present study does not exclude the possibility that strain-induced RhoA activation or protein tyrosine phosphorylation contributes to increased cell contractility.

Smooth muscle contractile function is influenced by either short-duration (10) or more chronically induced mechanical stress. The cellular effects of mechanical stress are numerous and both have immediate effects on the biochemical makeup of the cell and ultimately affect cell organization. The strain-induced alterations in cell phenotype revealed by this cell culture system reflect the contractile changes noted in studies of intact airways (whole animal or human) and smooth muscle strips (1, 17). Specifically, the mechanically induced events produced in this cell culture model include an increase in calcium sensitivity that correlates with increased LC30 phosphorylation and velocity of shortening. Increased force production in these systems may result from a combination of increased MHC content (hypertrophy) as well as increased organization of force-producing units. Finally, there may be changes in myosin isoform expression because the 17-kDa regulatory light chain could increase cross-bridge force-producing potential (8). The role of mechanical stress is of particular relevance to smooth muscle cells because mechanical forces are continually influencing smooth muscle-containing tissues such as airways. We have found potential contributions of several strain-induced phenomena that may account for increased smooth muscle cell contractility in certain diseases.

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