Inhibition of the p38 MAP kinase pathway destabilizes smooth muscle length during physiological loading

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Lakser, Oren J., Robert P. Lindeman, and Jeffrey J. Fredberg. Inhibition of the p38 MAP kinase pathway destabilizes smooth muscle length during physiological loading. Am J Physiol Lung Cell Mol Physiol 282: L1117–L1121, 2002; 10.1152/ajplung.00230.2000.—We tested the hypothesis that mechanical plasticity of airway smooth muscle may be mediated in part by the p38 mitogen-activated protein (MAP) kinase pathway. Bovine tracheal smooth muscle (TSM) strips were mounted in a muscle bath and set to their optimal length, where the active force was maximal (F_o). Each strip was then contracted isotonically (at 0.32 F_o) with ACh (maintained at 10^{-4} M) and allowed to shorten for 180 min, by which time shortening was completed and the static equilibrium length was established. To simulate the action of breathing, we then superimposed on this steady distending force a sinusoidal force fluctuation with zero mean, at a frequency of 0.2 Hz, and measured incremental changes in muscle length. We found that TSM strips incubated in 10 μM SB-203580-HCl, an inhibitor of the p38 MAP kinase pathway, demonstrated a greater degree of fluctuation-driven lengthening than did control strips, and upon removal of the force fluctuations they remained at a greater length. We also found that the force fluctuations themselves activated the p38 MAP kinase pathway. These findings are consistent with the hypothesis that inhibition of the p38 MAP kinase pathway destabilizes muscle length during physiological loading.

Methods

Muscle preparations. Bovine tracheas obtained from a local slaughterhouse were sectioned into 4–5-ring segments and stored for no longer than 24 h in cold phosphate-buffered saline. Muscle strips, measuring 2 × 1 × 10 mm, were dissected out by removing the inner and outer layers of connective tissue and adjoining cartilage. Each end of the strip was glued to small brass clips. The strips were then suspended in a glass tissue bath using a fine steel rod. The top of the rod was attached to a servo-controlled lever arm via a miniature force transducer. The lower clip was latched onto a glass hook fused to the bottom of the bath. We established previously that the servo-lever and mounting system add no appreciable compliance compared with the muscle itself (4). Finally, the bath was perfused with Krebs-Henseleit solution, maintained at a volume of 50 ml, aerated with 95% O_2-5% CO_2, and maintained at 37°C. Each bovine tracheal smooth muscle (TSM) strip was mounted in the muscle bath (as described above) and allowed to equilibrate for 1 h. The strip was then set to its optimal length (L_o) using electric field stimulation adjusted for optimal response. Once at L_o, the strip was maximally stimulated with 10^{-4} M ACh establishing the optimal force F_o. After L_o and F_o had been established, the ACh was flushed out of the bath.

Protocol 1: force fluctuations. SB-203580-HCl (10 μM, treatment) or an equivalent volume (0.05 ml) of distilled H_2O (control) was added to the 50-ml muscle bath, and the smooth
Each strip was then isotonically contracted (at 0.32 $F_o$) with fluctuations of amplitude ($\delta F$) (4, 8, 16, 24, 32, and back to 8% $F_o$) with zero mean, at a frequency of 0.2 Hz, were superimposed upon the steady distending force (Fig. 1). We then measured the incremental changes in muscle length that accumulated progressively over the course of many tidal cycles. We also measured resulting changes of muscle length that occurred within each tidal event and that were phasic with tidal changes in the muscle load. From the tidal force and length fluctuations we computed muscle stiffness, a measure of the slope of the force-length loop.

**Protocol 2: p38 MAP kinase activity.** At this time there is no commercially available product that recognizes bovine heat shock protein (HSP) 27. Therefore, we measured activation of a kinase upstream of HSP27, namely, p38 MAP kinase. Muscle strips were set to $L_{SE}$, where $F_o$ was maximal. Each strip was then isotonically contracted (at 0.32 $F_o$) with $10^{-4}$ M ACh and allowed to shorten for 180 min, by which time its static equilibrium length ($L_{SE}$) was established. Sinusoidal $\delta F$ (0, 16, or 32% $F_o$) with zero mean, at a frequency of 0.2 Hz, were superimposed upon the steady distending force. After 15 min, each strip was quickly removed from the apparatus, flash-frozen in liquid nitrogen, and stored at $-80^\circ$C. The tissue was homogenized using a probe homogenizer at 4°C in a buffer containing 20 mM Tris (pH 7.5), 5 mM EGTA, 1 mM Na$_3$VO$_4$, 20 mM $\beta$-glycerophosphate, 10 mM NaF, 1 mM dithiothreitol, 1 $\mu$g/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was spun on a table-top microfuge for 30 s, and 200 $\mu$l of supernatant was removed and boiled in Laemlli buffer containing 5% 2-mercaptoethanol. Protein concentration was determined according to the Bradford method. Samples were resolved on 12% SDS polyacrylamide minigels, transferred to polyvinylidene difluoride membrane, and probed with antibodies against tyrosine-phosphorylated p38 or total p38 (commercially available kit from New England Biolabs, Beverly, MA) according to the manufacturer’s instructions. Protein loading was identical for all samples. Specific bands were detected by electrochemiluminescence.

**RESULTS**

In a representative control strip (Fig. 1, top), stimulation with $10^{-4}$ M ACh caused the muscle to shorten until it reached a steady-state length that was statically equilibrated ($L_{SE}$). Whereas muscle that is contracted isometrically reaches a steady-state tension within minutes, muscle that is contracted isotonically continues to shorten for a much longer time before becoming statically equilibrated. When small force fluctuations (4 and 8% $F_o$) were subsequently superimposed on the steady distending force (0.32 $F_o$), there was little change in muscle length. However, when larger-amplitude force fluctuations were superimposed, the strip lengthened and became dynamically equilibrated at a length that substantially exceeded the $L_{SE}$. When $\delta F$ was dropped back to 8% $F_o$, the strip did not reshorten to the same length as with the initial force fluctuations. The dynamically equilibrated lengths for all control strips showed similar findings (Fig. 2). The strip pretreated with SB-203580-HCl (Fig. 1, bottom) also shortened when activated by ACh. The treated strips tended to a steady-state length that was shorter than the control strips (0.49 vs. 0.59 $L_{SE}$, Fig. 1), but this difference did not achieve statistical significance (Fig. 2, $P = 0.219$, comparing $a = 0.562 \pm 0.037$ for control strips vs. $a = 0.499 \pm 0.030$ for treated strips). There was little change in length with imposition of small force fluctuations (4 and 8% $F_o$) in the treated strips (Fig. 2), but when larger force fluctuations ($\geq 16\%$) were superimposed on the steady distending load, the muscle strips lengthened. The representative strip pretreated with the inhibitor...
lengthened to a greater extent than did the control strip (1.15 vs. 0.75 L₀/a t/H₁₁₀₀₁₅/F/H₁₁₀₀₁₁/H₁₁₀₀₁₉₃₂% in Fig. 1).

Overall, treated strips demonstrated significantly greater lengthening than control strips (Fig. 2, P = 0.013, comparing b = 0.00020 ± 0.00002 for control vs. b = 0.00037 ± 0.00005 for treated strips) despite being activated by the same dose of ACh and being loaded in an identical manner. When the δF was dropped back to 8% F₀, the treated strips did not reshorten to the length observed at the original 8% F₀, indicating an important role for loading history and resulting plasticity. All strips (treated and control) became less stiff with larger force fluctuations (Fig. 3).

Fluctuation-driven lengthening is better appreciated when the same data are normalized by the LSE, rather than L₀ (Fig. 4). There was a greater degree of smooth muscle lengthening, for a given δF, in strips exposed to the p38 MAP kinase inhibitor than for control strips.

The force developed during the isometric contraction was the same in muscle strips treated with the inhibitor and control strips. Similarly, the force was maintained in both groups of muscle strips for the entire 180 min. After the tidal loading maneuvers, the isometric force generation capacity of the muscle was not compromised, retaining 85% or more of its initial capacity.

Activation of airway smooth muscle with ACh results in increased phosphorylation of p38 MAP kinase (12). We found that imposition of load fluctuations on airway smooth muscle resulted in further activation (phosphorylation) of p38 MAP kinase (Fig. 5).

**DISCUSSION**

The principal findings of this report are as follows. We found little change in smooth muscle length with imposition of small load fluctuations but substantial lengthening when δF was increased to 16% or more of F₀. For any given δF, blocking p38 MAP kinase activity with the inhibitor SB-203580-HCl caused the muscle to lengthen to a greater extent. Last, the force fluctuations themselves induced activation of the p38 MAP kinase pathway.

We have shown previously that fluctuation-driven muscle lengthening can be explained in large part by the effect of the load fluctuations on myosin binding (4). That is, when the load fluctuations are large enough...
Fig. 5. Densitometry (in pixels) of the ratio of phosphorylated p38 mitogen-activated protein (MAP) kinase to total p38 MAP kinase in baseline (inactivated) smooth muscle strips and in strips activated with 10^-4 M ACh and exposed to force fluctuations of 0, 16, and 32% of $F_0$. Data are normalized to strips activated with ACh alone, without imposition of force fluctuations (0%).

(≥16% of $F_0$), actomyosin binding becomes perturbed, muscle stiffness decreases, and the muscle lengthens. Muscle length becomes dynamically equilibrated. However, bridge-based mechanisms alone cannot account for the incomplete reshortening of the muscle as $\delta F$ is reduced. It appears that, during the force fluctuation protocol, the smooth muscle strips have undergone a plastic change. This conclusion is consistent with the observations of others, who have suggested that plasticity may play a major role in changes in the muscle contractile state length as a result of imposed stretch (8, 21, 24). Moreover, these findings are consistent with the hypothesis that activation of p38 MAP kinase stabilizes airway smooth muscle and limits the bronchodilating effects of deep inspirations.

Our data demonstrate that the load fluctuations themselves acted as stressors that activate the p38 MAP kinase pathway (Fig. 5). These data suggest, therefore, that in addition to activation of the p38 MAP kinase pathway by thermal, osmotic, and biochemical stressors, the p38 pathway is also activated by mechanical stress. Together, these results suggest that although myosin is the primary effector molecule of the contractile response of smooth muscle, myosin exerts its mechanical effects within cytoskeletal scaffolding that is both deformable and in a continuous state of remodeling.

The increased responsiveness of TSM to load fluctuations in the presence of the p38 MAP kinase inhibitor provides some insight into the type of plastic change that could be occurring. In the control situation, activation of the smooth muscle strips with ACh resulted in the activation of the p38 MAP kinase pathway, as demonstrated here and by others (12, 13), and we have shown here that a p38 MAP kinase inhibitor destabilized muscle mechanics. To explain the mechanical effects that were observed, HSP27 is a logical candidate molecule. Activation of p38 MAP kinase is known to result in the downstream activation of HSP27, which in turn stabilizes the actin cytoskeleton (13, 26, 28). Moreover, Gerthoffer and Gunst (6) have speculated recently that HSP27 may play a key role in cytoskeletal plasticity in airway smooth muscle, and the data in this report are consistent with that possibility.

HSPs are a family of molecules induced by a variety of stressors including elevated temperature, osmotic stress, ultraviolet light, exposure to reactive oxygen species, and some inflammatory cytokines. The activation of these HSPs increases a cell’s capacity to survive these stressors. HSP27, one member of this family, is an actin binding protein that is constitutively expressed at high levels in smooth muscle. It is believed to modulate the polymerization of actin and to remodel the cytoskeleton by binding to and capping barbed ends of microfilaments and stabilizing them (13, 26, 28). Phosphorylation of HSP27 has been shown to be a necessary event for the migration of airway smooth muscle cells (12), and it has been proposed that HSP27 phosphorylation may be necessary for smooth muscle contraction (13). Other investigators have demonstrated that HSP27 is activated upon routine contraction of airway smooth muscle cells (12). HSP27 lies downstream of p38 MAP kinase, which phosphorylates MAP kinase-activated protein kinases-2/3 and ultimately leads to the phosphorylation (activation) of HSP27 (15, 28).

We speculate, but could not demonstrate, that when muscle strips were incubated with the p38 MAP kinase inhibitor before activation with ACh, activation of HSP27 was blocked (12, 15), thereby limiting its stabilizing effect on the cytoskeleton. As such, force fluctuations generated more muscle lengthening. An alternative possibility is some nonspecific effect of the inhibitor on activation of the motor proteins, but muscle strips pretreated with SB-203580-HCl and then activated with ACh demonstrated no difference in phosphorylation of the 20-kDa myosin regulatory light chain compared with control strips activated with ACh.

How does this all relate to airway narrowing in asthma? It has long been thought that spontaneous asthmatic obstruction behaves as if it were caused by an intrinsic impairment of the bronchodilating effect of a deep inspiration (3, 5, 18–20). This bronchodilating effect can be explained in large part by perturbed equilibria of actomyosin binding caused by imposed load fluctuations in the physiological range (4). Muscle stiffness decreases, and the muscle lengthens and becomes dynamically equilibrated. We have shown here that a p38 MAP kinase inhibitor modulates that process.

In that connection, there is evidence in the literature that there are elevated levels of HSP70 in the airways of asthmatic individuals (1, 25). There is also a suggestion that there may be higher levels of HSP27, in particular in allergic asthmatic individuals (11). Our results demonstrate that activation of the p38 MAP kinase pathway limits the ability of force fluctuations...
In summary, we have shown that activation of the p38 MAP kinase pathway may modulate muscle mechanical responses to imposed load fluctuations during contractile stimulation. The presence of SB-203580-HCl increased the degree of smooth muscle lengthening induced by load fluctuations. As such, these data suggest the hypothesis that activation of p38 MAP kinase stabilizes airway smooth muscle and may limit the bronchodilating effect of deep inspirations.

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REFERENCES


