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Original

Augmented bronchial smooth muscle contractility induced by aqueous cigarette smoke extract in rats

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Abstract

Cigarette smoking is the main risk factor for the development of chronic obstructive pulmonary disease (COPD). However, little is known about the mechanisms of cigarette smoke-induced bronchial smooth muscle (BSM) hyperresponsiveness. In the present study, we investigated the effects of aqueous cigarette smoke extract (ACSE) on the BSM contraction in rats. The bronchial strips of rats were incubated with ACSE or control-extract for 24 h. The acetylcholine (ACh), high K^+ depolarization and sodium fluoride (NaF)-induced BSM contraction of the ACSE-treated group was significantly augmented as compared to that of the control one. The expression levels of both myosin light-chain kinase (MLCK) and RhoA were significantly increased in the ACSE-treated BSM. These findings suggest that the water-soluble components of cigarette smoke may cause BSM hyperresponsiveness via an increase in MLCK and RhoA.

Key words: Chronic obstructive pulmonary disease, Bronchial smooth muscle, Airway hyperresponsiveness, Cigarette smoke

Introduction

Current estimates are that over 200 million people worldwide have moderate to severe stages of chronic obstructive pulmonary disease (COPD) (1), a term used to describe a range of chronic lung disorders characterized physiologically by progressive and largely irreversible airflow limitation (2). Cigarette smoking has profound effects on human health and is the major risk factor associated with the development and progression of COPD, which is one of the most important causes of morbidity and mortality in the world. The main systems affected by cigarette smoke directly, as well as by passive exposure, are the respiratory tract and

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the cardiovascular system.

Cigarette smoke is a complex cloud aerosol, containing gases, particulates, and water. More than 4,000 chemical compounds have been identified in mainstream smoke, including arylamines, benzoic acid, and benzamide-like derivatives (3). These toxic compounds can markedly deteriorate such serious diseases as COPD (4). Multicenter clinical trials (Lung Health Study) showed that current smokers with functional evidence of early COPD have airway hyperresponsiveness (5, 6). Similarly, a dose-dependent effect of cigarette smoking on human airway hyperresponsiveness *in vivo* has been reported (7). The latter study supports the concept that cigarette smoke has an effect on airway responsiveness. It is possible that one of the factors that contribute to the exaggerated airway narrowing in patients with COPD is an abnormality in the nature of the airway smooth muscle.

On the other hand, bronchodilators are the mainstay of current management for patients with COPD. Anti-cholinergic bronchodilators, such as tiotropium bromide, have been used as the standard care in the control of COPD (8, 9). There is evidence that the cholinergic tone of the airways is increased in patients with COPD (10, 11).

Experimental evidence has been reported that chronic exposure to cigarette smoke augments the *in vivo* responsiveness of airways to cholinergic agonists in rats (12, 13), guinea pigs (14) and mice (15). Interestingly, subacute expose to inhalation of cigarette smoke *in vivo* caused an augmented acetylcholine (ACh)-induced contraction of isolated bronchial smooth muscle in rats (13). Although the smoke component(s) leading to the induction of bronchial smooth muscle (BSM) hyperresponsiveness has not yet been identified, an involvement of components other than nicotine has been suggested (16). However, the mechanism of smoke components-induced BSM hyperresponsiveness has not been fully understood. In the present study, we employed the water-soluble fraction of whole smoke through a glass fiber filter, and examined the effects of aqueous cigarette smoke extract (ACSE) *in vitro* on BSM contraction in the rats.

Materials and Method

Preparation of aqueous cigarette smoke extract

Cigarette smoke extract was collected from a glass fiber filter membrane (Whatman; EPM2000) through which the main-stream cigarette smoke generated from four cigarettes was passed using a 50 ml syringe, after which the weight of the filter was measured. The wet filter containing the aqueous components was dried for a few days. The dried filter was then extracted with 5 ml phosphate buffered saline for 24 h at room temperature under dark conditions. Aliquots of this aqueous cigarette smoke extract were stored at –35°C. The extract was diluted to 0.02, 0.07 and 0.2 mg/ml in Dulbecco's modified Eagle medium (DMEM). The extract of an air-passed filter was used as a control extract.

Animals

Male Wistar rats (6 weeks of age, specific pathogen-free, 170–190 g; Charles River Japan, Inc.) were used. All experiments were approved by the Animal Care Committee at Hoshi University.

Functional study for intact BSM preparations

To determine whether the ACSE affects the BSM responsiveness *in vitro*, the isometric contraction of the circular smooth muscle of the main bronchus was measured as described previously (13). In brief, the rats were killed by exsanguination from the abdominal aorta under chloral hydrate anesthesia (400 mg/kg,

intraperitoneally). The airway tissues below the larynx to the lungs were immediately removed. A 4-mm length (3 mm diameter) of the left main bronchus was isolated (8–9 cartilages). One bronchial preparation was isolated from each rat. The epithelium was removed as much as possible by gently rubbing with keen-edged tweezers (13). Normal bronchi were randomly selected and divided between control and ACSE groups. The bronchial strips were incubated with 0.02, 0.07 mg/ml ACSE or control-extract (control) in DMEM at room temperature for 24 h. The resultant tissue ring preparations were then suspended in a 5 ml organ bath at a resting tension of 1.0 g. The isometric contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T, Nihon Kohden, Tokyo, Japan). The organ bath contained modified Krebs-Henseleit solution [(mM); NaCl 118.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2 and glucose 10.0 (pH 7.4)]. The buffer solution was maintained at 37°C and oxygenated with 95% O_2-5% CO_2. During an equilibration period in the organ bath, the tissues were washed four times at 15-min intervals and equilibrated slowly to a baseline tension of 1.0 g. Fifteen min after the last washing, higher concentrations of ACh were successively added after attainment of a plateau response to the previous concentration. After measurement of the responsiveness to ACh, the BSM was also depolarized with isotonic high K^+ solution prepared by the iso-osmotic replacement of NaCl by KCl in the presence of 10^{-6} M atropine and 10^{-6} M indomethacin.

Western blot analyses

Protein samples of bronchial tissues were prepared as previously described (13). The resulting tissues were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. The bronchial tissue segments were quickly frozen with liquid nitrogen, and the tissue was crushed to a powder using a Cryopress (CP-100W; Microtec, Co. Ltd., Chiba, Japan) (15 s × 3). The tissue powder was homogenized in ice-cold T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific Inc., IL, USA). The tissue homogenate was centrifuged (3,000 × *g* at 4°C for 15 min), and the resultant supernatant was stored at –85°C until use. To determine the level of myosin light chain kinase (MLCK), myosin light chain (MLC), α-smooth muscle actin (α-SM-actin) and RhoA proteins in BSM preparations, the samples (10 μg of total protein per lane) were subjected to 15% SDS-PAGE, and the proteins were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 3% gelatin, the PVDF membrane was incubated with rabbit anti-MLCK antibody (1:3,000 dilution; Santa Cruz Biotechnology, CA, USA), rabbit anti-MLC antibody (1:1,000 dilution; Santa Cruz Biotechnology, CA, USA), rabbit anti-α-SM actin antibody (1:3,000 dilution; Sigma-Aldrich, MO, USA) or rabbit anti-RhoA antibody (1:3,000 dilution, Santa Cruz Biotechnology, CA, USA). The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 dilution, GE Healthcare, Buckinghamshire, UK), detected by an enhanced chemiluminescent system (GE Healthcare), and analyzed by a densitometry system with cooled CCD camera system (Light-Capture II, ATTO Corporation, Japan). Detection of a house-keeping gene was performed on the same membrane by using monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase antibody (anti-GAPDH, 1:5,000 dilution, Sigma-Aldrich, MO, USA) to confirm that the same amount of proteins had been loaded.

Statistical analyses

Data were expressed as the mean with SEM. Statistical significance was determined by one-way or two-way ANOVA with *post hoc* Bonferroni/Dunn (StatView for Macintosh ver. 5.0; SAS Institute, NC, USA). A value of $P< 0.05$ was considered significant.

Results

Effects of in vitro incubation with ACSE on the contraction induced by ACh, high K^+-depolarization, NaF and calyculin-A

Fig. 1A shows a typical trace of ACh-induced contraction of the rat BSM preparations incubated with control-extract or ACSE (0.07 mg/ml). ACh elicited a concentration-dependent contractile response. The concentration-response curve to Ach (Fig. 1C) was significantly shifted upward in an ACSE concentration-dependent manner (0.02 mg/ml: $P<0.05$ and 0.07 mg/ml: $P<0.001$). The maximal contraction induced by ACh was enhanced 1.4 fold by treatment with 0.07 mg/ml ACSE. Fig. 1B shows a typical trace with high K^+ solution (10, 30 and 60 mM)-induced contraction of the rat BSM preparations incubated with control-extract or ACSE (0.07 mg/ml). Application of isotonic high K^+ solution (10, 30 and 60 mM) also elicited a concentration-dependent contractile response in all preparations. The contraction induced by K^+-depolarization was also augmented by preincubation with ACSE (0.07 mg/ml). The 60 mM K^+-induced contraction was enhanced 1.75 fold by treatment with 0.07 mg/ml ACSE in Fig. 1D.

Sodium fluoride (NaF) is known as an activator of G-proteins (17–20). As shown in Fig. 2A–C, the NaF-induced contraction was also increased 2 fold by ACSE (0.07 mg/ml, $P<0.01$). However, the contraction induced by calyculin-A, a potent myosin light chain phosphatase inhibitor, was not affected by ACSE (0.07 mg/ml, Fig. 2D–F).

Effects of ACSE on the expression of MLCK, MLC, α-SM actin and RhoA in the BSM preparations

Representative immunoblots for each protein and GAPDH of control-extract and ACSE (0.07 mg/ml)-pre-treated bronchial preparations are shown in Fig. 3A. The data was summarized in Fig. 3B. The expressions of MLCK and RhoA proteins in ACSE (0.07 mg/ml)-treated bronchial preparations were significantly increased as compared with that of the control-extract treated preparations (MLCK: $P<0.05$ and RhoA: $P<0.01$). On the other hand, ACSE had no effect on the levels of MLC and α-SM actin.

Discussion

In the present study, the contractions induced by ACh, high K^+-depolarization and NaF were augmented in ACSE-treated BSM preparations. The expression levels of MLCK and RhoA were significantly increased in ACSE-treated BSM preparations, although no significant difference in α-SM actin and MLC expression was observed between control and ACSE-treated groups.

The smooth muscle contraction is regulated not only by cytosolic Ca^{2+}-dependent but also by Ca^{2+}-independent pathways. The former activates calmodulin/myosin light chain kinase (CaM/MLCK) to phosphorylate MLC, while the latter modulates MLC phosphorylation by regulating the RhoA or PKC/myosin light chain phosphatase (MLCP) pathway. The agonists such as carbachol increase the contractile force further even at a given cytosolic Ca^{2+} level (Ca^{2+} sensitization due to the inhibition of MLCP) (21–23). It has become clear that the Ca^{2+} sensitization occurs through an inhibition of myosin phosphatase via an activation of the monomeric GTP-binding protein RhoA (21, 23–25). We previously demonstrated that the RhoA mediated ACh-induced Ca^{2+} sensitization of rat BSM preparations, and that a RhoA inhibitor C3 toxin inhibited the contraction induced by ACh in mouse and rat permeabilized bronchial smooth muscle preparations. Activation of RhoA was also induced by ACh in the BSM preparations (26, 27). In the present study, the level of RhoA,

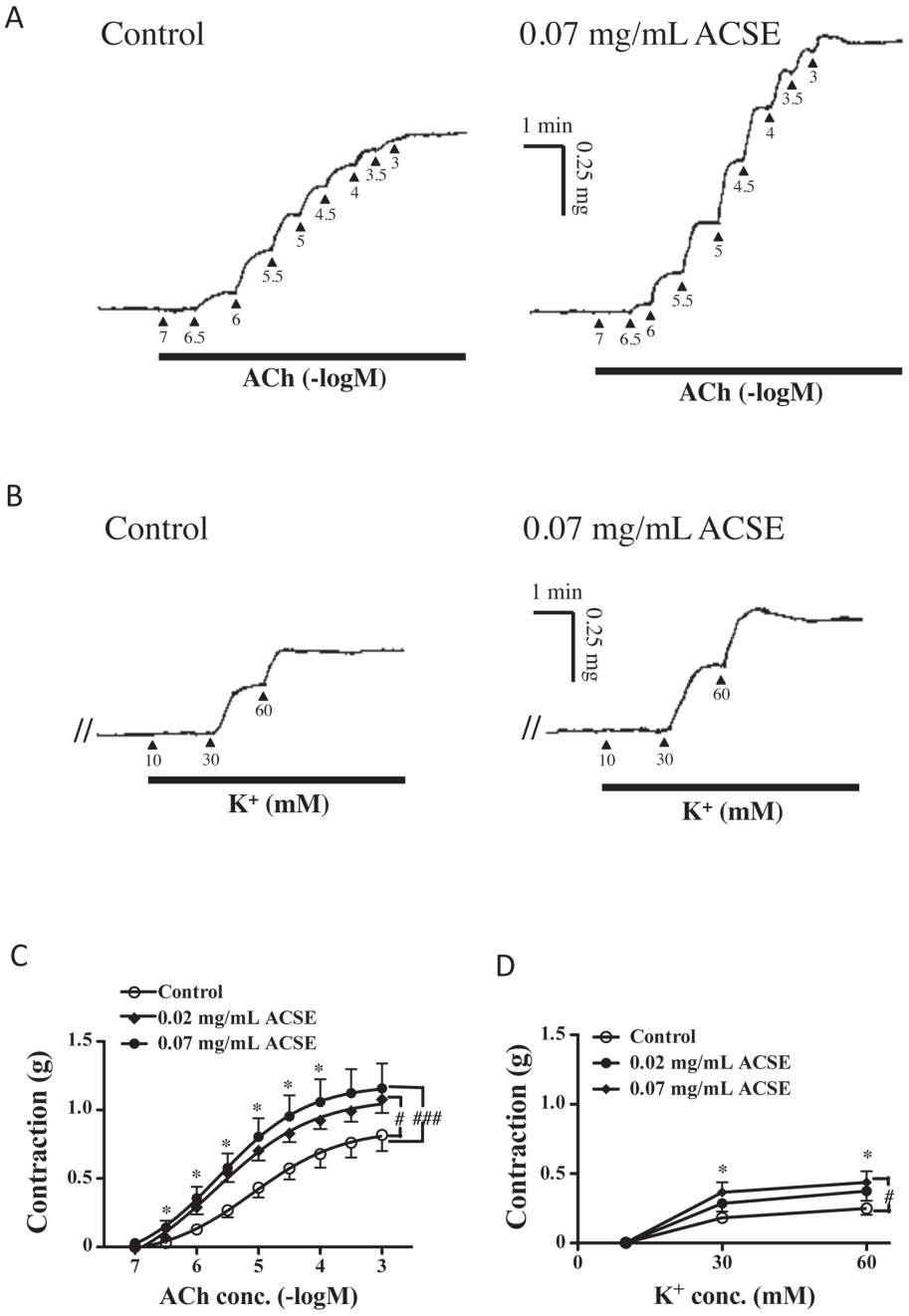

Fig. 1. Effects of aqueous cigarette smoke extract (ACSE) on ACh- and high K^+-depolarization-induced contractile responses of the isolated rat bronchial smooth musclepreparations. Typical traces of both ACh- (A) and high K^+-induced contractions (B) of the rat bronchial smooth muscle incubated with control-extract or ACSE (0.07 mg/ml) are shown. C and D: cumulative concentration-dependent contractile response curves. Each point represents the mean ± SEM from 6 (control), 5 (0.02 mg/ml ACSE) and 6 (0.07 mg/ml ACSE) bronchial preparations. **P*<0.05 (one-way ANOVA with Bonferroni/Dun test), #*P*<0.05 and ###*P*<0.001 (two-way ANOVA with Bonferroni/Dun test) vs. Control.

an important protein that mediates Ca^{2+} sensitization (23), was significantly increased in the bronchial tissue from the ACSE-treated BSM preparations. It is thus possible that the increased RhoA seems to enhance the ACh-induced Ca^{2+} sensitizing signal, which results in an augmentation of the contractile response in cigarette

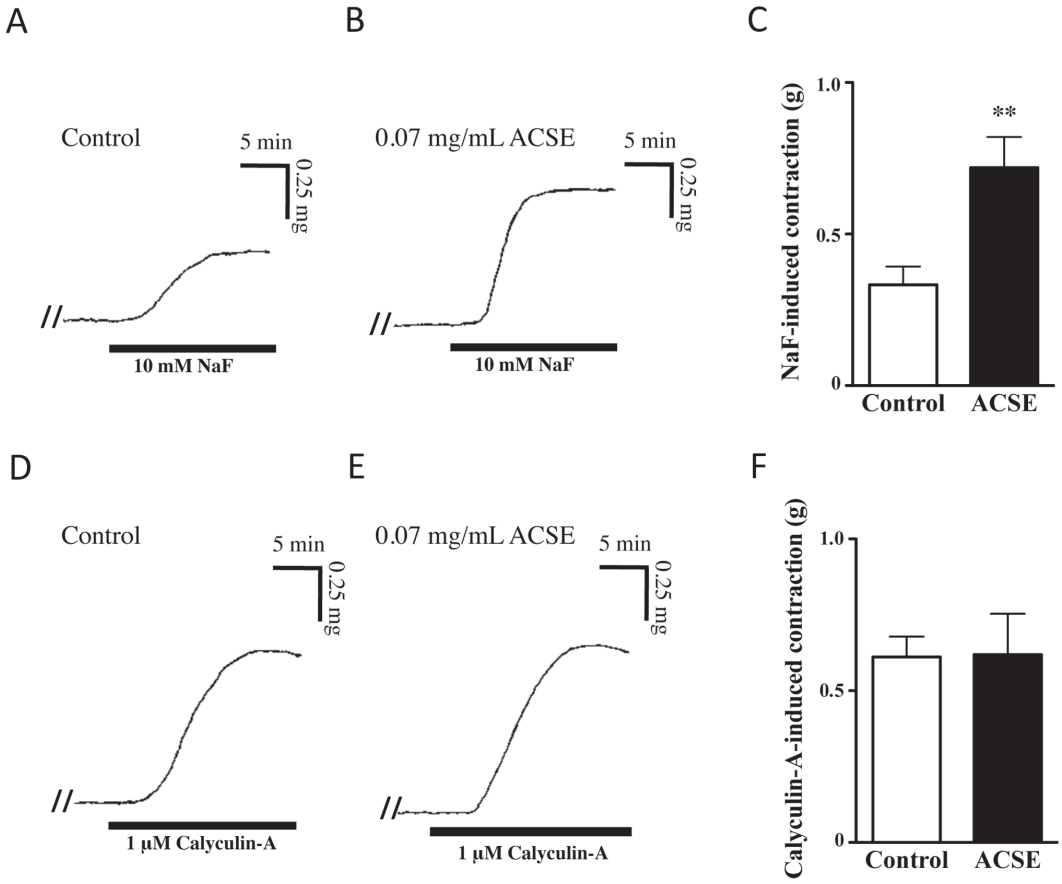

Fig. 2. Effects of ACSE on sodium fluoride (NaF)- and calyculin-A-induced contractile responses of the isolated rat bronchial smooth muscle preparations. Typical traces of both NaF- and calyculin-A-induced contractions are shown in A, B, D and E. Contractions induced by NaF (C) and calyculin (F) are summarized. Each bar represents the mean ± SEM from 6 (control) and 6 (0.07 mg/ml ACSE) bronchial preparation. ***P*<0.01 (one-way ANOVA with Bonferroni/Dun test) vs. Control.

smoke-exposed rats. The contraction induced by calyculin-A was not affected by treatment with ACSE. Thus, it seems unlikely that the treatment of ACSE affects the smooth muscle contractile apparatus, such as the activity of actomyosin ATPase. Zhang et al. (28) suggests that RhoA-mediated regulation of ACh-induced contractile tension in airway smooth muscle results from its role in mediating actin polymerization rather than from effects on MLC phosphatase or MLC phosphorylation. Therefore, further studies are needed to resolve this point.

In our previous *in vivo* study, when rats were exposed to diluted mainstream cigarette smoke for 2 h/every day for 2 weeks, K^+ responsiveness was slightly, but not significantly, augmented by the cigarette smoke exposure (16). In the present study, the contraction induced by K^+-depolarization was significantly augmented by preincubation with ACSE *in vitro*. This inconsistency may be a technical difference. Furthermore it is also possible that the component(s) of the tobacco that enhances the reactivity of K^+ depolarization is metabolized, and the effect may disappear in a living body. Although further studies need to resolve this point, the enhanced Ca^{2+}/CaM-MLCK pathway may be also involved in ACSE-induced BSM hyperresponsivesess *in vitro*.

In conclusion, we found that the ACh-induced contractile responses were augmented in the BSM preparations incubated with ACSE, which coincided with the augmentations of both the expression levels

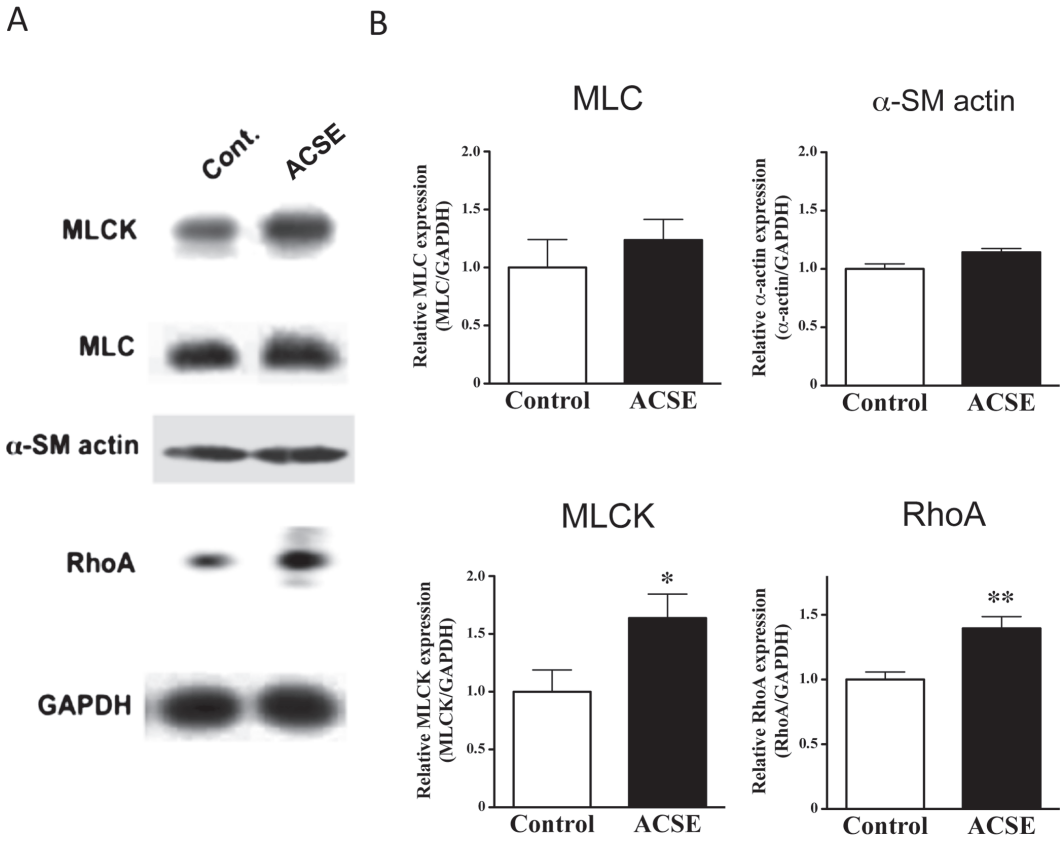

Fig. 3. Effects of ACSE on MLCK, MLC, α-smooth muscle (SM) actin and RhoA protein expressions in rat bronchial smooth muscle preparations. A: typical immunoblots of MLCK, MLC, α-SM actin and RhoA and GAPDH in bronchial smooth muscle preparations from control, 0.07 mg/ml and ACSE exposed rats. B: relative densities of target proteins to GAPDH. Values are mean ± SEM from 5 bronchial preparations. * $P<0.05$ and ** $P<0.01$ vs. Control.

of MLCK and RhoA in the BSM preparations. These findings suggest that the water-soluble components of cigarette smoke may cause BSM hyperresponsiveness via increases in both MLCK and RhoA.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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