Different effects of smoke from heavy and light cigarettes on the induction of bronchial smooth muscle hyperresponsiveness in rats

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Abstract

Cigarette smoking is one of the main risk factors in the development of chronic obstructive pulmonary disease (COPD). It has been suggested that an augmented agonist-induced, RhoA mediated Ca2+ sensitization is responsible for the enhanced bronchial smooth muscle contraction induced by cigarette smoking. In the present study, to determine whether or not these phenomena are dependent on the degree of exposure to the components of cigarette smoke, we examined the effects of exposure to mainstream smoke derived from either light or heavy cigarettes on both the contractile responsiveness and the expression of RhoA in bronchial smooth muscle. Male Wistar rats were exposed to mainstream cigarette smoke for 2 hr/day for 2 weeks. Twenty-four hr after the last cigarette smoke exposure, we measured isometric contractions of the bronchial smooth muscle. The concentration-response curve to ACh was significantly shifted upward after heavy cigarette smoke (HCS) exposure, whereas no significant difference was observed in the case of light cigarette smoke (LCS) exposure compared with control rats. No significant difference in K+ responsiveness was observed between the groups. The expression of RhoA protein in bronchial preparations from rats repeatedly exposed to HCS, but not to LCS, was significantly increased as compared with that of the control animals. On the other hand, inhalation of nicotine had no effect on either the ACh- and high K+ depolarization-induced contractions or the expression of RhoA protein. The increased expression of RhoA seems to have an important role in the augmented contractile responses of the airways in rats, a characteristic feature of early COPD.

Key words: COPD, bronchial smooth muscle, airway hyperresponsiveness, cigarette smoke, RhoA

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Introduction

Cigarette smoking is a major cause of chronic obstructive pulmonary disease (COPD), which is one of the most important causes of morbidity and mortality in the world. Cigarette smoking induces an inflammatory response in the airways that might play a key role in the pathogenesis of COPD. Furthermore, multicenter clinical trials (Lung Health Study) showed that current smokers with functional evidence of early COPD have airway hyperresponsiveness (Tashkin et al., 1992; Tashkin et al., 1996). Similarly, a dose-dependent effect of cigarette smoking on human airway hyperresponsiveness \textit{in vivo} has been reported (Tashkin \textit{et al.}, 1993). The latter study supports the concept that cigarette smoke has an \textit{a priori} effect on airway responsiveness.

Bronchodilators are the mainstay of the current management of patients with COPD. Anti-cholinergic bronchodilators, such as tiotropium bromide, have become the standard for care in the control of COPD (Barnes, 2004b; Gross, 2004). There is evidence that the cholinergic tone of the airways may be increased in patients with COPD (Gross \textit{et al.}, 1989; Nisar \textit{et al.}, 1992). Some of the chemical and oxidizing pollutants generated by cigarette smoking affect airway smooth muscle contractility directly (Ben-Jebria \textit{et al.}, 1993; Marthan \textit{et al.}, 1996). Therefore, it is possible that one of the factors that contribute to the exaggerated airway narrowing in patients with COPD may be an abnormality in the nature of airway smooth muscle. Experimental evidence has been reported that chronic exposure to cigarette smoke augments the \textit{in vivo} responsiveness of airways to cholinergic agonists in rats (Xu \textit{et al.}, 1993), guinea pigs (Wu and Lee, 1999), and mice (Barrett \textit{et al.}, 2002). Although little is known concerning the effect of cigarette smoking \textit{in vivo} on the contractility of airway smooth muscle \textit{ex vivo}, we previously demonstrated that subacute exposure to cigarette smoke \textit{in vivo} caused an augmented ACh-induced contraction with an upregulation of RhoA in bronchial smooth muscle \textit{ex vitro} (Chiba \textit{et al.}, 2005). In the present study, to determine whether or not these phenomena are dependent on the degree of exposure to the components of cigarette smoke, we examined the effects of exposure to mainstream smoke derived from light and heavy cigarettes on both the contractile responselessness and the RhoA expression in bronchial smooth muscle.

Materials and Methods

\textbf{Animal and treatment}

Male Wistar rats (6 wk of age, specific pathogen-free, 170–190 g; Charles River Japan, Kanagawa, Japan) were used. All experiments were approved by the Animal Care Committee at the Hoshi University (Tokyo, Japan). Rats were randomly divided into three groups to be exposed to mainstream smoke of either heavy cigarettes (a total of 1.4 mg nicotine and 17 mg tar/cigarette, HI-LITE) or light cigarettes (a total of 0.1 mg nicotine and 1 mg tar, MILD SEVEN ONE) (Japan Tobacco, Tokyo, Japan) with room air as the control. In the cigarette smoke group, animals were exposed to diluted mainstream cigarette smoke for 2 hr/day everyday for 2 weeks by using an automated smoking machine (Model INH06-CIGR01; Medical Interface Project Station, Osaka, Japan). Each rat was alert while held in an exposure chamber that was connected to the smoking machine. A puff of mainstream cigarette smoke (35 mL) was generated and mixed with 280 mL of
room air before delivery to the chamber. Each cigarette was puffed 40 times with a suction volume of 600 mL/min. Thirty cigarettes were used in a 2 hr period. In another series of experiments, conscious rats were exposed to $1.7 \times 10^{-2}$ M nicotine (41 mg/15 mL) which was inhaled using an ultrasonic nebulizer (TUR-3000, Nihon Koden, Tokyo, Japan) for 2 hr in a plexiglass box (300 × 200 mm, higher: 150 mm). This corresponded to the nicotine content of the smoke of 30 heavy cigarettes. The animals were subjected to inhalation of this amount of nicotine 14 times every 24 hr. A saline solution replaced the nicotine solution in the control rats.

**Functional study using intact bronchial smooth muscle preparations**

To determine whether the cigarette smoke exposure *in vivo* affects the bronchial smooth muscle responsiveness *ex vitro*, isometric contractions of the circular smooth muscle of the main bronchus were measured as described previously (Chiba *et al.*, 2005). In brief, 24 hr after the last cigarette smoke or room air exposure, the rats were killed by exsanguination from the abdominal aorta under chloral hydrate anesthesia (400 mg/kg, intraperitoneally). The airway tissues from 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), and the resultant tissue ring preparation was suspended in an organ bath at a resting tension of 1 g. The organ bath contained modified Krebs-Henseleit solution with the following composition (mM): NaCl 118.0, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 1.2, NaHCO$_3$ 25.0, KH$_2$PO$_4$ 1.2, and glucose 10.0 (pH 7.4). The isometric contraction of the circular smooth muscle was measured with a force-displacement transducer (TB-612T; Nihon Kohden, Tokyo, Japan). During an equilibration period, the preparations were washed three or four times at 15 to 20 min intervals and equilibrated slowly to a baseline tension of 1 g. After the equilibration period, a concentration-response curve to acetylcholine (ACh) ($10^{-7}$–$10^{-3}$ M in final concentrations) was constructed cumulatively, with a higher concentration of ACh being successively added after attainment of a plateau response to the previous concentration. In another series of experiments, isotonic K$^+$ solution (10–90 mM in final concentrations) was cumulatively administered in the presence of atropine and indomethacin (both $10^{-6}$ M) to determine the bronchial smooth muscle responsiveness to high K$^+$ depolarization.

**Western blot analyses**

Protein samples of bronchial tissues were prepared as previously described (Chiba *et al.*, 2005). The airway tissues from below the main bronchi to the lungs were removed and immediately soaked in ice-cold, oxygenated Krebs-Henseleit solution. The airways were carefully cleaned of adhering connective tissues, blood vessels, and lung parenchyma using a stereomicroscope. The epithelium was removed as much as possible by gently rubbing with keen-edged tweezers (Chiba *et al.*, 2005). The bronchial tissue (containing the main and intrapulmonary bronchi) segments were quickly frozen in liquid nitrogen, and the tissue crushed to pieces using a Cryopress (CP-100W; Microtec, Co. Ltd., Chiba, Japan) (15 s × 3). The tissue powder was homogenized in ice-cold T-PER (Thermo Fisher Scientific Inc., IL, USA). The tissue homogenate was centrifuged (3,000 × g at 4°C for 15 min), and the resultant supernatant stored at −85°C until use.

To determine the level of RhoA protein in the bronchial smooth muscle, the samples (10 mg of
total protein per lane) were subjected to 15% SDS-PAGE, and the proteins electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 3% gelatin, the PVDF membrane was incubated with polyclonal rabbit anti-RhoA antibody (1:3,000 dilution) (Santa Cruz Biotechnology, CA, USA). The bronchial smooth muscle responsiveness to ACh (A) or high K⁺ (B) was measured isometrically. Each point represents the mean ± SEM. from 5-6 independent experiments. *, P<0.05 and **, P<0.01 vs. control.

Statistical analyses
Data were expressed as the mean with SE. Statistical significance was determined by using an unpaired Student’s t test or two-way ANOVA with post hoc Bonferroni/Dunn correction (StatView for Macintosh ver. 5.0; SAS Institute, NC, USA). A value of P<0.05 was considered to be significant.

Results
Figure 1 shows the acetylcholine (ACh) responsiveness of bronchial smooth muscle preparations isolated from control rats, and from rats exposed repeatedly to either light cigarette smoke (LCS) or heavy cigarette smoke (HCS).
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ACh elicted a concentration-dependent contractile response. The concentration-response curve to ACh was significantly shifted upward after HCS exposure, whereas no significant difference was observed in the case of LCS exposure compared with control rats. Application of isotonic high K+ solution (10, 30, 60 and 90 mM) also elicited a concentration-dependent contractile response in all tissues used (Fig. 1). No significant difference in K+ responsiveness was observed between the groups.

Anti-RhoA antibody was used to detect RhoA protein in rat bronchial preparations in the present study. Representative immunoblots for both RhoA and GAPDH in bronchial preparations from control rats, and rats exposed to either LCS or HCS are shown in Fig. 2A. Immunoblotting with the antibody against RhoA showed a single 21 kD band. The corresponding RhoA/GAPDH ratios were calculated and are summarized in Fig. 2B. The expression of RhoA protein in bronchial preparations of the repeatedly HCS-exposed rats was significantly increased as compared with that of the control animals ($P<0.05$). On the other hand, no significant change in the RhoA expression of bronchial smooth muscle in repeatedly LCS exposed rats was observed.

To investigate the effect of nicotine, one of the main components of cigarette smoke, on
bronchial smooth muscle contractile responsiveness, we examined the influence of nicotine inhalation on bronchial smooth muscle contraction. Nicotine had no effect on either ACh or high K+ depolarization-induced contraction (Fig. 3). As well as ACh-induced contraction, RhoA expression was at the same level in both control and nicotine exposed rats (Fig. 4).

**Discussion and Conclusion**

In the present study, the concentration-response curve for ACh-induced contraction in bronchial smooth muscle preparations from HCS-exposed rats was significantly augmented as compared to those from control animals, indicating that AHR occurred at the level of bronchial smooth muscle after repeated exposure to HCS. However, the responsiveness to ACh was not changed in the bronchial smooth muscle of the LCS-exposed rats. RhoA expression in bronchial smooth muscle preparations was significantly increased in HCS-exposed animals. No significant differences in the high K⁺-induced contractile responses were observed between the control and the LCS and HCS exposed groups. The augmented ACh responsiveness of bronchial smooth muscle might therefore be related to the increased expression of RhoA in HCS-exposed rats.

It has been known that various contractile agonists can induce the Ca²⁺ sensitization of smooth muscle contraction (Fujita et al., 1995; Gong et al., 1997; Otto et al., 1996). It has also become clear that this Ca²⁺ sensitization occurs through an inhibition of myosin phosphatase via an activation of
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The monomeric GTP-binding protein RhoA (Fujihara et al., 1997; Gong et al., 1996; Hirata et al., 1992; Otto et al., 1996). We have also previously demonstrated RhoA mediated ACh-induced Ca\textsuperscript{2+} sensitization of rat bronchial smooth muscle (Chiba et al., 1999). Translocation of RhoA from the cytosol to the membrane was also shown to be induced by ACh in bronchial smooth muscle (Chiba et al., 1999).

In the current study, the level of RhoA, an important protein that mediates Ca\textsuperscript{2+} sensitization (Otto et al., 1996), was significantly increased in the bronchial tissue from the HCS-exposed animals (Fig. 2). It is thus possible that the increased RhoA seems to enhance the ACh-induced Ca\textsuperscript{2+} sensitizing signal, which results in an augmentation of the contractile response in the HCS-exposed rats.

In contrast, the ACh responsiveness in the bronchial smooth muscle of the nicotine-inhaled rat was within control levels, and the expression of RhoA was no different to that of the control bronchial smooth muscle. These findings also strongly suggest that the augmented ACh responsiveness observed in the main bronchial smooth muscle of the HCS-exposed rats might be due to an increased expression of RhoA.

In this study, the bronchial smooth muscle contractile responsiveness was not changed by the inhalation of nicotine. As well as ACh-induced contraction, RhoA expression in bronchial smooth

Fig. 4. Effects of $1.7 \times 10^{-2}$ M nicotine exposures on RhoA protein expression in rat bronchial smooth muscle preparations. Typical immunoblots of RhoA (upper) and GAPDH (lower) in bronchial smooth muscle preparations from saline or nicotine exposed rats (A). Relative densities of RhoA to GAPDH (RhoA/GAPDH) in bronchial smooth muscle preparations (B). Values are expressed as the mean ± SEM, from 5 experiments.
muscle was also at control levels in the nicotine treated group. Lei et al. (1995) reported that cigarette smoke increased plasma exudation in airways in a dose-related manner, and that nicotine, at a dose calculated to approximate that in the plasma of cigarette-exposed animals, did not increase airway plasma exudation. Therefore the cigarette smoke components other than nicotine, such as tar, might play an important role in HCS-induced airway hyperresponsiveness and inflammation.

Although the underlying mechanism(s) responsible for cigarette smoke-induced hyperresponsiveness and/or RhoA upregulation in bronchial smooth muscle is not clear, there is experimental evidence that reactive oxygen species (ROS) might have the ability to induce upregulation of RhoA by enhancing its transcription in non-smooth muscle cells (Turcotte et al., 2003). ROS are produced by inflammatory cells, such as neutrophils (Barnes, 2004a), which were significantly increased in bronchoalveolar lavage fluids after HCS, but not after LCS exposure (data not shown). Cigarette smoke is also a potent source of ROS (Barnes, 2004a). On the other hand, cigarette smoke contains potent respiratory irritants, such as acrolein, an unsaturated aliphatic aldehyde. It has been reported that inhalation of acrolein at a concentration of 1 ppm induced airway hyperresponsiveness to ACh in guinea pigs (Leikauf et al., 1989). Ben-Jebria et al. (1995) reported that acrolein inhalation in vivo caused hyperresponsiveness of isolated tracheal smooth muscle to cholinergic agonists but not to high K+ depolarization in ferrets. As another factor, Petrescu et al., (2010) demonstrated that the concentration of TNF (tumor necrosis factor)-alpha is elevated in the serum of heavy smokers in a cigarette dose-dependent manner. Moreover, treatment of rat bronchial smooth muscle with TNF-alpha resulted in a significant upward shift in the concentration-response curve to ACh, but not to high K+, compared with control tissues, while RhoA protein was increased in bronchial smooth muscle (Sakai et al., 2004). Taken together, these findings might indicate that TNF-alpha is also one of the essential factors involved in the facilitation of the elevated sensitivity of bronchial smooth muscle to ACh stimulation.

In conclusion, the ACh-induced contractile responses were augmented in the bronchial smooth muscle of rats exposed to HCS, but not to LCS, which was coincident with an increase in RhoA expression in the bronchial smooth muscle. Nicotine had no effect on either RhoA expression or ACh-induced contraction in bronchial smooth muscle. The increased expression of RhoA seems to have an important role in the augmented contractile responses of rat airways, a characteristic feature of COPD.

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References

Barnes, P.J. (2004). Mediators of chronic obstructive pulmonary disease. Pharmacol. Rev. 56: 515–548.
Barnes, P.J. (2004). The role of anticholinergics in chronic obstructive pulmonary disease. Am. J. Med. 117: 24S–32S.
Barrett, E.G., Wilder, J.A., March, T.H., Espindola, T. and Bice, D.E. (2002). Cigarette smoke-induced...
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Airway hyperresponsiveness is not dependent on elevated immunoglobulin and eosinophilic inflammation in a mouse model of allergic airway disease. *Am. J. Respir. Crit. Care Med.* **165**: 1410–1418.

Ben-Jebria, A., Crozet, Y., Eskew, M.L., Rudeen, B.L. and Ultman, J.S. (1995). Acrolein-induced smooth muscle hyperresponsiveness and eicosanoid release in excised ferret tracheae. *Toxicol. Appl. Pharmacol.* **135**: 35–44.

Ben-Jebria, A., Marthan, R., Rossetti, M., Savineau, J.P. and Ultman, J.S. (1993). Effect of in vitro exposure to acrolein on carbachol responses in rat trachealis muscle. *Respir. Physiol.* **93**: 111–123.

Chiba, Y., Takada, Y., Miyamoto, S., Mitsui-Saito, M., Karaki, H. and Misawa, M. (1999). Augmented acetylcholine-induced, Rho-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in antigen-induced airway hyperresponsive rats. *Br. J. Pharmacol.* **127**: 597–600.

Chiba, Y., Murata, M., Ushikubo, H., Yoshikawa, Y., Sai toh, A., Sakai, H., Kamei, J. and Misawa, M. (2005). Effect of cigarette smoke exposure in vivo on bronchial smooth muscle contractility in vitro in rats. *Am. J. Respir. Cell Mol. Biol.* **33**: 574–581.

Fujihara, H., Walker, L.A., Gong, M.C., Lemichez, E., Boquet, P., Somlyo, A.V. and Somlyo, A.P. (1997). Inhibition of RhoA translocation and calcium sensitization by in vivo ADP-ribosylation with the chimeric toxin DC3B. *Mol. Biol. Cell* **8**: 2437–2447.

Fujita, A., Takeuchi, T., Nakajima, H., Nishio, H. and Hata, F. (1995). Involvement of heterotrimeric GTP-binding protein and rho protein, but not protein kinase C, in agonist-induced Ca^{2+} sensitization of skinned muscle of guinea pig vas deferens. *J. Pharmacol. Exp. Ther.* **274**: 555–561.

Gong, M.C., Fujihara, H., Walker, L.A., Somlyo, A.V. and Somlyo, A.P. (1997). Down-regulation of G-protein-mediated Ca^{2+} sensitization in smooth muscle. *Mol. Biol. Cell* **8**: 279–286.

Gong, M.C., Iizuka, K., Nixon, G., Browne, J.P., Hall, A., Eccleston, J.F., Sugai, M., Kobayashi, S., Somlyo, A.V. and Somlyo, A.P. (1996). Role of guanine nucleotide-binding proteins—ras-family or trimeric proteins or both—in Ca^{2+} sensitization of smooth muscle. *Proc. Natl. Acad. Sci. U.S.A* **93**: 1340–1345.

Gross, N.J. (2004). Tiotropium bromide. *Chest* **126**: 1946–1953.

Gross, N.J., Co, E. and Skorodin, M.S. (1989). Cholinergic bronchomotor tone in COPD. Estimates of its amount in comparison with that in normal subjects. *Chest* **96**: 984–987.

Hirata, K., Kikuchi, A., Sasaki, T., Kuroda, S., Kaibuchi, K., Matsuura, Y., Seki, H., Saida, K. and Takai, Y. (1992). Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J. Biol. Chem.* **267**: 8719–8722.

Leikauf, G.D., Leming, L.M., O'Donnell, J.R. and Douplik, C.A. (1989). Bronchial responsiveness and inflammation in guinea pigs exposed to acrolein. *J. Appl. Physiol.* **66**: 171–178.

Lei, Y.H., Barnes, P.J. and Rogers, D.F. (1995). Mechanisms and modulation of airway plasma exudation after direct inhalation of cigarette smoke. *Am. J. Respir. Crit. Care Med.* **151**: 1752–1762.

Marthan, R., Roux, E. and Savineau, J.P. (1996). Human bronchial smooth muscle responsiveness after in vitro exposure to oxidizing pollutants. *Cell Biol. Toxicol.* **12**: 245–249.

Nisar, M., Earis, J.E., Pearson, M.G. and Calverley, P.M. (1992). Acute bronchodilator trials in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* **146**: 555–559.

Otto, B., Steusloff, A., Just, I., Aktories, K. and Pfitzer, G. (1996). Role of Rho proteins in carbachol-induced contractions in intact and permeabilized guinea-pig intestinal smooth muscle. *J. Physiol. (Lond.)* **496**: 317–329.

Petrescu, F., Voican, S.C. and Silosi, I. (2010). Tumor necrosis factor-alpha serum levels in healthy smokers and nonsmokers. *Int. J. Chron. Obstruct. Pulmon. Dis.* **5**: 217–222.

Sakai, H., Otogo to, S., Chiba, Y., Abe, K. and Misawa, M. (2004). Involvement of p42/44 MAPK and RhoA protein in augmentation of ACh-induced bronchial smooth muscle contraction by TNF-alpha in rats. *J. Appl. Physiol.* **97**: 2154–2159.

Tashkin, D.P., Altose, M.D., Bleecker, E.R., Connett, J.E., Kanner, R.E., Lee, W.W. and Wise, R. (1992).
The lung health study: airway responsiveness to inhaled methacholine in smokers with mild to moderate airflow limitation. The Lung Health Study Research Group. *Am. Rev. Respir. Dis.* 145: 301–310.

Tashkin, D.P., Altose, M.D., Connett, J.E., Kanner, R.E., Lee, W.W. and Wise, R.A. (1996). Methacholine reactivity predicts changes in lung function over time in smokers with early chronic obstructive pulmonary disease. The Lung Health Study Research Group. *Am. J. Respir. Crit. Care Med.* 153: 1802–1811.

Tashkin, D.P., Simmons, M.S., Chang, P., Liu, H. and Coulson, A.H. (1993). Effects of smoked substance abuse on nonspecific airway hyperresponsiveness. *Am. Rev. Respir. Dis.* 147: 97–103.

Turcotte, S., Desrosiers, R.R. and Beliveau, R. (2003). HIF-1alpha mRNA and protein upregulation involves Rho GTPase expression during hypoxia in renal cell carcinoma. *J. Cell Sci.* 116: 2247–2260.

Wu, Z.X. and Lee, L.Y. (1999). Airway hyperresponsiveness induced by chronic exposure to cigarette smoke in guinea pigs: role of tachykinins. *J. Appl. Physiol.* 87: 1621–1628.

Xu, L.J., Dandurand, R.J., Lei, M. and Eidelman, D.H. (1993). Airway hyperresponsiveness in cigarette smoke-exposed rats. *Lung* 171: 95–107.