Augmentation of Endothelin-1-Induced Phosphorylation of CPI-17 and Myosin Light Chain in Bronchial Smooth Muscle from Airway Hyperresponsive Rats

Hiroyasu Sakai,* Yoshihiko Chiba, and Miwa Misawa

Department of Pharmacology, School of Pharmacy, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan. Received April 24, 2006; accepted June 15; published online June 16, 2006

Airway hyperresponsiveness (AHR) associated with heightened airway resistance and inflammation is a characteristic feature of bronchial asthma. It has been demonstrated that contractile responsiveness to endothelin-1 (ET-1) in repeated antigen challenge-induced airway hyperresponsive bronchial preparation was significantly increased. ET-1 is a potent contracting substance for various smooth muscles including airways. In addition to the classical Ca2+-mediated contraction, ET-1 also induced Ca2+ sensitization of contraction. However, it is not clear whether ET-1 stimulation also activates the CPI-17 (PKC-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa) pathway in airway smooth muscles. Therefore, the changes in ET-1-induced activation/phosphorylation of CPI-17 and myosin light chain (MLC) in bronchial smooth muscle of repeatedly antigen-challenged rats were examined. The levels of ET-1-induced phosphorylation of CPI-17 and MLC were increased much more markedly in the AHR group than in the sensitized control animals. It might be suggested that the augmented activation of CPI-17 observed in the hyperresponsive bronchial smooth muscle is responsible for the enhanced agonists-induced contraction of bronchial smooth muscle in AHR rats.

Key words airway hyperresponsiveness; asthma; CPI-17; bronchial smooth muscle; endothelin-1

Excitatory agonists initially increase intracellular Ca2+ levels, leading to activation of myosin light chain kinase (MLCK) in smooth muscle and, as a result, MLC phosphorylation. This action is followed immediately by a decrease in MLC phosphatase (MLCP) activity and a resultant increase in MLC phosphorylation, which induces further contraction, that is Ca2+ sensitization.1–4) Multiple second messengers/signaling pathways including the RhoA/Rho-associated coiled-coil forming protein kinase (ROCK)5–7) and protein kinase C (PKC)8,9) pathways, have reportedly been linked to bronchial smooth muscle of repeatedly antigen challenged rats.20) Induction of CPI-17 expression and ACh-induced phosphorylation of CPI-17 was induced in bronchial smooth muscle of repeatedly antigen challenged rats.20) ET-1 is a potent contracting substance for various smooth muscles including airways. In addition to the classical Ca2+-mediated contraction,21) ET-1 also induced Ca2+ sensitization of contraction.22) ET-1 stimulation induces phosphorylation of CPI-17 in rabbit femoral artery.23) and vas deferens.24) However, it is not clear whether CPI-17 is involved in ET-1-induced Ca2+ sensitization in airway smooth muscle of AHR rats. Endothelin-1 is an elevated level of endothelin-1 has been reported in the bronchoalveolar lavage fluids from asthmatic patients.25) Thus, it might be important for asthma therapy to understand the detailed mechanism of endothelin-1-induced airway smooth muscle contraction. In the present study, phosphorylation of CPI-17 and MLC induced by ET-1 was determined in bronchial smooth muscle of rats. Furthermore, the levels of CPI-17 phosphorylation induced by ET-1 in bronchial smooth muscles of the AHR rats were compared with those of control animals.

MATERIALS AND METHODS

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 of Hoshi University (Tokyo, Japan).

Sensitization and Antigenic Challenge Rats were sensitized and repeatedly challenged with 2,4-dinitrophenylated Ascaris suum antigen (DNP-Asc) by the method described previously.18,19) The sensitized control group received the same immunization procedure except for inhaled saline aerosol instead of antigen challenge.

Western Blot Analyses Samples were prepared by the method described previously.20,22) In brief, the bronchial tissue (containing the main and intrapulmonary bronchi) segments isolated from rats were equilibrated in oxygenated Krebs–Henseleit solution (37 °C) for 60 min with 15-min

* To whom correspondence should be addressed. e-mail: sakai@hoshi.ac.jp

© 2006 Pharmaceutical Society of Japan
washout intervals. After the equilibration period, the tissue segments were stimulated by endothelin-1 for 15 min in the presence of atropine and indomethacin (both 10⁻⁶ m). The reaction was stopped by quickly freezing with liquid nitrogen, and the tissue was then homogenized with T-PER™ Tissue Protein Extraction Reagent (Pierce) and centrifuged. Protein concentrations were determined using an Advanced Protein Assay Reagent (Cytoskeleton Inc.) with BSA as a standard. To quantify the phosphorylation of CPI-17 proteins, immunoblotting was performed as described previously. 25) Briefly, the samples (10 µg protein per lane) were subjected to 15% SDS-PAGE. Proteins were then electrophoretically transferred for 4 h onto PVDF membranes (Hybond-ECL, Amersham, Little Chalfont, U.K.) in cold transfer buffer (20% methanol containing Tris 25 mM and glycine 192 mM). After repeated washing with Tris buffer (Tris 20 mM, NaCl 500 mM, pH 7.5) containing 0.1% (v/v) Tween 20 (TTBS), the PVDF membranes were incubated with blocking buffer (3% gelatine in TTBS) for 1.5 h at room temperature. The PVDF membranes were then incubated with primary antibody, polyclonal goat anti-[Thr38]- phospho-CPI-17 (1:5000 dilution, Santa Cruz Biotechnology) in antibody buffer (1% gelatine in TTBS) for 12 h at room temperature. The PVDF membranes were then washed five times (each for 15 min) with TTBS. They were incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG (Amersham) for 1.5 h at room temperature, and then washed five times with TTBS. The blots were detected with an enhanced chemiluminescent peroxidase-conjugated anti-goat immunoglobulin (Ig) G (1:5000 dilution; Santa Cruz Biotechnology, Inc.). Then the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-goat IgG (Amersham) for 1.5 h at room temperature, and then washed five times with TTBS. The blots were detected with an enhanced chemiluminescent method (ECL System; Amersham) and quantified by densitometry (Atto Densitograph Software ver. 4.0; Atto Co., Japan). To normalize the phosphorylated CPI-17 contents to primary antibody, polyclonal mouse anti-β-actin, immunoblotting was also performed on the same gel by using monoclonal mouse anti-β-actin N-terminal (Sigma, St. Louis, Missouri, U.S.A.) and goat anti-mouse IgG (Amersham). The ratios of corresponding phosphorylated CPI-17/β-actin in each lane were calculated as indices of phosphorylated CPI-17 protein level. In the ET-1-induced of MLC phosphorylation study, the bronchial preparation were stimulated by ET-1 10⁻⁶ m for 15 min. Then the samples were homogenized with T-PER™ Tissue Protein Extraction Reagent (Pierce). After the samples (20 µg) were subjected to 15% SDS-polyacrylamide gel electrophoresis, western blot was performed. The membranes were incubated with the primary antibodies. As the primary antibodies were used goat anti-p-MLC (Thr18/Ser19, 1:1000 dilution; Santa Cruz Biotechnology, Inc.) or rabbit anti-myosin light chain (1:1000; Santa Cruz Biotechnology, Inc.). Then the membranes were incubated with horseradish peroxidase-conjugated donkey anti-goat immunoglobulin (Ig) G (1:5000 dilution; Santa Cruz Biotechnology, Inc.), detected by an ECL system. The ratio of corresponding p-MLC/MLC was calculated as an index of p-MLC.

**RATS** We previously suggested that ET-1-induced contraction of bronchial smooth muscle was significantly enhanced in the repeatedly antigen-challenged group. 26) Currently, by using phospo-[Thr38]-CPI-17 specific antibody, the phosphorylation of CPI-17 induced by ET-1 was determined in rat bronchial preparations. Because the expression of CPI-17 is enhanced by the repeatedly antigen-challenged, cellular phosphorylation level of CPI-17 was determined by comparison with the expression level of β-actin. The expression of β-actin is unaffected by the repeatedly antigen-challenged. 20) As shown in Fig. 1, incubation of bronchial preparation with ET-1 (10⁻⁶ m, for 15 min) caused a distinct phosphorylation level of CPI-17 in each group. In the bronchial smooth muscle of repeatedly antigen-challenged rats, the ET-1-induced phosphorylation of CPI-17 was significantly greater (Challenged vs. Sensitized+ET-1: 632.7±107.1% when compared with the sensitized control animals (Sensitized+ET-1: 282.3±29.1%, p<0.001).

**DISCUSSION**

Our previous study demonstrated that repeated challenge with aerosolized antigen to actively sensitized rats (the same methods as those used in the present study) causes distinct...
airway inflammation and remarkable AHR to inhaled acetylcholine (ACH) in vivo.\textsuperscript{17} The isolated bronchial smooth muscles from these animals also exhibited a hyperresponsiveness to ACh and ET-1.\textsuperscript{18,26} A marked augmentation of ACh-induced Ca\textsuperscript{2+} sensitization has been demonstrated in bronchial smooth muscle of this animal model of AHR.\textsuperscript{18}

Moreover, the expression of CPI-17 and ACh-induced phosphorylation of CPI-17 were significantly increased\textsuperscript{20} Here, both the CPI-17 and MLC phosphorylations induced by ET-1, a potent bronchoconstrictor that stimulates receptors other than muscarinic ones, were also augmented. There is report that phosphorylated CPI-17 enhances myosin phosphorylation and contraction of permeabilized smooth muscle.\textsuperscript{12} Taken together, the phosphorylation of CPI-17 induced by ET-1 stimulation is involved in the bronchial smooth muscle hyperresponsiveness. In addition, it is interesting that unlike CPI-17 phosphorylation, the phosphorylation levels of MLC under stimulated condition are the same, even after antigen challenge. It seems that CPI-17 is important for MLC phosphorylation level under agonist stimulation.

We previously reported that ACh-induced Ca\textsuperscript{2+} sensitization in β-escin-permeabilized bronchial smooth muscle was inhibited by a RhoA inhibitor, C3 exoenzyme,\textsuperscript{19} or a ROCK inhibitor, Y-27632.\textsuperscript{4} The RhoA/ROCK pathway seems to be considered as a major signaling pathway of the agonist-induced Ca\textsuperscript{2+} sensitization in rat bronchial smooth muscle. Recently, the bronchial contraction and phosphorylations of CPI-17 and MLC induced by ACh and ET-1 were attenuated by pretreatment with Y-27632 treatment, suggesting that CPI-17 is phosphorylated via activation of ROCK in agonists-induced rat bronchial smooth muscle contraction (Sakai et al., submitted data).\textsuperscript{27} Taken together, RhoA/ROCK/CPI-17 pathway may play an important role in agonist-induced Ca\textsuperscript{2+} sensitization of among airway smooth muscle contraction.

In conclusion, the ET-1-induced CPI-17 phosphorylation was markedly increased in the bronchial smooth muscles of AHR rats as compared with that of the sensitized control group. Our data support that the augmented activation of CPI-17 observed in the hyperresponsive bronchial smooth muscle might be responsible for the enhanced agonists-induced contraction in AHR rats.

**Acknowledgements** We thank Ms Kumiko Numano and Ms Midori Yoshino for their help in technical assistance.

**REFERENCES**
