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Short communication

Endothelin-1-induced translocation of RhoA is mediated by endothelin ET_A receptors in rat bronchial smooth muscle

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Abstract

To clarify the receptor subtype(s) contributing to the RhoA activation by endothelin-1 in bronchial smooth muscle, the effects of BQ-123 [cycro(D-Asp-Pro-D-Val-Leu-D-Trp)], an endothelin ET_A receptor antagonist, and/or BQ-788 [2,6-dimethylpiperidinecarbonyl-g-methyl-Leu-Nin-(Methoxycarbonyl)-D-Trp-D-Nle], an endothelin ET_B receptor antagonist, on the endothelin-1-induced translocation of RhoA to plasma membrane were examined. Incubation of rat bronchial smooth muscle with endothelin-1 induced a distinct translocation of RhoA to plasma membrane, indicating an activation of RhoA by endothelin-1. The endothelin-1-induced translocation of RhoA was completely blocked by treatment with BQ-123, whereas BQ-788 had no effect. Thus, endothelin ET_A but not ET_B receptors might be involved in the endothelin-1-induced translocation of RhoA in rat bronchial smooth muscle.

Keywords: Bronchial smooth muscle; RhoA translocation; Endothelin-1

1. Introduction

Endothelin-1 is a potent constrictor for various smooth muscles including airways. However, the mechanisms of endothelin-1-mediated smooth muscle contraction have not been fully understood. In airway smooth muscles, both endothelin ET_A and ET_B receptor subtypes have been demonstrated in rats (Henry, 1993), mice (Carr et al., 1996) and humans (Fukuroda et al., 1996); both of them reportedly contribute to endothelin-1-induced contraction. The airway smooth muscle contraction induced by endothelin-1 was inhibited in the combined presence of an endothelin ET_A receptor antagonist, BQ-123 [cycro(D-Asp-Pro-D-Val-Leu-D-Trp)], and an endothelin ET_B receptor antagonist, BQ-788 [2,6-dimethylpiperidinecarbonyl-g-methyl-Leu-Nin-(Methoxycarbonyl)-D-Trp-D-Nle], but not by either antagonist alone in rats (Henry, 1993) and mice (Carr et al., 1996). It has also been suggested that the contraction induced by endothelin-1 is mediated by activation of different signal transduction pathways in airway smooth muscle (Henry, 1993): stimulation of endothelin ET_A receptors is linked to contraction via the release of Ca^{2+} from intracellular Ca^{2+} stores, whereas the Ca^{2+} source in endothelin ET_B receptor-mediated contraction is Ca^{2+} influx via an activation of Ca^{2+} channels. In addition to the Ca^{2+}-dependent contraction, endothelin-1 also has an ability to induce Ca^{2+} sensitization of smooth muscle contraction including airways (Yoshii et al., 1999; Yoshimura et al., 2001). To date, the receptor subtype(s) involved in the endothelin-1-induced Ca^{2+} sensitization of airway smooth muscle contraction is not known.

A small GTPase RhoA is a key protein participating in the agonist-induced Ca^{2+} sensitization of smooth muscle contraction including airways (Chiba et al., 1999). In bovine aortic smooth muscle cells, treatment with a selective Rho-kinase inhibitor, Y-27632 [(+)-(R)-trans-4-(1-aminoethyl)-(4-pyridyl)cyclohexanecarboxamide dihydrochloride], inhibited the endothelin-1-induced phosphorylation of myosin light chain (Gohla et al., 2000). An involvement of RhoA in endothelin-1-induced Ca^{2+} sensi-

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tization has also been demonstrated in human bronchial smooth muscle by using the selective inhibitor for Rho-kinase, a downstream target of RhoA (Yoshii et al., 1999). Although the activation pathway of RhoA via membrane receptors is not yet clear in airway smooth muscle, it is known that translocation of RhoA from cytosol to plasma membrane occurs when RhoA is activated (Chiba et al., 2001, 2004). In the present study, the effects of BQ-123 and/or BQ-788 on the endothelin-1-induced translocation of RhoA to membrane were examined to clarify the receptor subtype(s) contributing to the RhoA activation by endothelin-1.

2. Materials and methods

2.1. 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 the Hoshi University (Tokyo, Japan).

2.2. Western blot analysis

Membrane and cytosolic fractions of bronchial tissues were prepared by the method described previously (Chiba et al., 1999, 2001). In brief, the bronchial tissue (containing main and intrapulmonary bronchi) segments isolated from rats were equilibrated in oxygenated Krebs–Henseleit solution (37 °C) for 60 min with 10-min washout intervals. After the equilibration period, the tissue segments were stimulated by endothelin-1 for an indicated time in the presence of atropine and indomethacin (both 10^{-6} M). In some experiments, the tissue segments were incubated with an endothelin ET_A receptor antagonist BQ-123 and/or an endothelin ET_B receptor antagonist BQ-788 (10^{-6} M, respectively) 15 min before stimulation with endothelin-1. The reaction was stopped by quickly freezing with liquid nitrogen, and the tissue was then homogenized and ultracentrifuged to obtain the membrane and cytosolic fractions (Chiba et al., 2001). RhoA contents in each fraction (10 μg protein) were determined by immunoblotting (Chiba et al., 2001).

2.3. Functional study

About 4 mm length of the left main bronchus was isolated. Isometrical contraction of the circular smooth muscle was measured as described previously (Chiba and Misawa, 1995; Chiba et al., 2001). The endothelin ET_A receptor-mediated contraction was induced by treatment of muscle strips with endothelin-1 (10^{-6} M) in the presence of an ET_B receptor antagonist BQ-788, atropine and indomethacin (each 10^{-6} M). After the plateau contraction induced by endothelin-1 was observed, a selective Rho-kinase inhibitor Y-27632 (10^{-8}–10^{-5} M) was administered cumulatively.

2.4. Data analyses

All the data were expressed as the mean with s.e.m. Statistical significance of difference was determined by Dunnett's multiple analysis or two-way analysis of variance (ANOVA). A value of $P<0.05$ was considered significant.

3. Results

Both the membrane and cytosolic fractions of bronchial smooth muscle contained RhoA proteins (RhoA/b-actin, $n=5$: 0.12±0.04 and 0.42±0.18, respectively) at resting state (no endothelin-1 stimulation). The ratio of membrane to total RhoA at resting state was 27.2±6.8%. Endothelin-1 (10^{-6} M) stimulation elicited sustained increases in membrane RhoA (Fig. 1A and B), while cytosolic RhoA was decreased (not shown), i.e., translocation of RhoA to plasma membrane. The peak response of endothelin-1-induced translocation of RhoA was observed at 20 min: the ratio of membrane to total RhoA 20 min after stimulation with endothelin-1 was 79.2±13.3% ($P<0.01$ vs. resting state). Then the level of membrane RhoA was slightly reduced 30 min after endothelin-1 stimulation. Endothelin-1 (10^{-8}–10^{-6} M) stimulation also exhib-

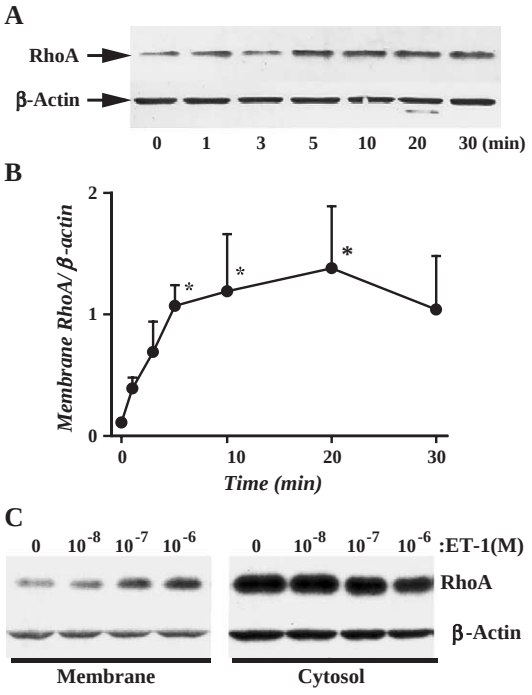

Fig. 1. A) and B) Time course of endothelin-1-induced change in membrane RhoA protein level, i.e., RhoA translocation to membrane, in bronchial smooth muscle of rats. The isolated main and intrapulmonary bronchi were stimulated by endothelin-1 (10^{-6} M), and homogenized to prepare membrane fractions after stopping the reaction by liquid nitrogen at the time indicated. Western blotting was performed by using these fractions on both RhoA and β-actin in the identical transferred membrane. A) Representative Western blots of membrane RhoA (21 kD) and β-actin. B) Values are means with s.e.m. from 5 experiments in duplicate. *$P<0.05$ vs. Time 0 (no stimulation). C) Concentration-dependency of endothelin-1 (ET-1)-induced translocation of RhoA in rat bronchial smooth muscle. The bronchial tissues were treated with indicated concentration of endothelin-1 (10^{-8}–10^{-6} M) for 20 min and homogenated, and the membrane (left) and cytosolic (right) fractions were subjected to Western blotting. The blots shown are representative of three independent experiments. Note that the membrane RhoA was increased concentration-dependently, whereas the cytosolic RhoA was decreased.

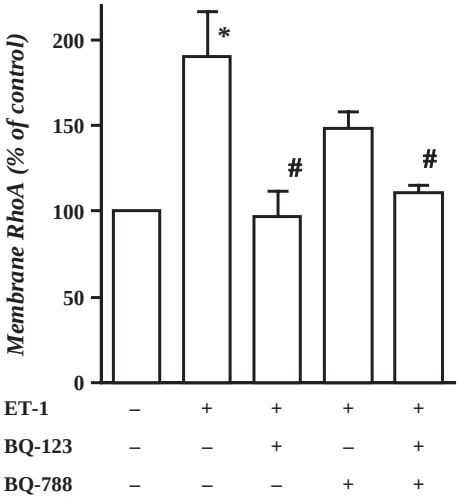

Fig. 2. Effects of an endothelin ET_A receptor antagonist, BQ-123 (10^{-6} M), and an endothelin ET_B receptor antagonist, BQ-788 (10^{-6} M), on endothelin-1 (ET-1: 10^{-6} M)-induced translocation of RhoA to plasma membrane in bronchial smooth muscles of rats. Values are means with s.e.m. from 4–5 experiments in duplicate. $^{*}P<0.05$ vs. control (no endothelin-1 stimulation) and $^{\#}P<0.05$ vs. endothelin-1-stimulated group in the absence of antagonist.

its translocation of RhoA from cytosol to plasma membrane in an endothelin-1 concentration-dependent manner (Fig. 1C).

Fig. 2 shows the effects of BQ-123, BQ-788 and their combination on the endothelin-1-induced translocation of RhoA to plasma membrane. The endothelin-1-induced translocation of RhoA was completely blocked by treatment with BQ-123 and the combination, whereas BQ-788 had no significant effect, indicating that endothelin ET_A receptors might be involved in the endothelin-1-induced translocation of RhoA in rat bronchial smooth muscle.

Next, the effects of a selective Rho-kinase inhibitor, Y-27632, on endothelin ET_A receptor-mediated contraction of rat bronchial smooth muscles were determined. The endothelin ET_A receptor-mediated contraction was induced by treatment of muscle strips with endothelin-1 (10^{-6} M) in the presence of an ET_B receptor antagonist BQ-788, atropine and indomethacin (each 10^{-6} M). As shown in Fig. 3, Y-27632 (10^{-8}–10^{-5} M) inhibited the contraction mediated by endothelin ET_A receptors, concentration-dependently, indicating that the RhoA/Rho-kinase signaling might be involved in the endothelin ET_A receptor-mediated contraction in rat bronchial smooth muscle.

4. Discussion

Endothelin-1 is a potent bronchoconstrictor (Uchida et al., 1988), and an elevated level of endothelin-1 has been reported in the bronchoalveolar lavage fluids from asthmatic patients (Mattoli et al., 1991). Thus, it might be important for asthma therapy to understand the detailed mechanism of endothelin-1-induced airway smooth muscle contraction. In addition to the classical Ca^{2+}-mediated contraction through activation of endothelin ET_A and ET_B receptors (Henry, 1993), endothelin-1 also induces Ca^{2+} sensitization of contraction through activation of RhoA/Rho-kinase pathway in rabbits and humans (Yoshii et al., 1999). Presently, we showed for the first time a direct evidence that the activation of RhoA by endothelin-1 might be mediated by endothelin ET_A receptors in rat bronchial smooth muscle. Inhibition of the endothelin ET_A receptor-mediated contraction by Y-27632, a selective Rho-kinase inhibitor, also supports the hypothesis.

Although the detail is not fully understood in airway smooth muscle, the mechanism of translocation of RhoA has been proposed as follows. In the resting state, GDP-bound inactive form of RhoA exists in the cell cytosol with GDP dissociation inhibitor, called RhoGDI, which buries the geranylgeranylated, hydrophobic tail of RhoA. Activation of RhoA is initiated by guanine nucleotide exchange factors (RhoGEFs) through activation of plasma membrane receptors coupled to certain heterotrimeric G proteins. The active RhoGEFs exchange GDP for GTP on RhoA. Then RhoGDI dissociates from GTP-bound active form of RhoA, resulting in the association of GTP-bound RhoA with membrane (Somlyo and Somlyo, 2003). So in the present study, the membrane-bound RhoA was measured by immunoblotting to determine the activation of RhoA by endothelin-1. The endothelin-1-induced translocation of RhoA was blocked by treatment with BQ-123, an endothelin ET_A receptor antagonist, but not by BQ-788, an endothelin ET_B receptor antagonist, suggesting that endothelin ET_A receptors might be involved in the activation of RhoA in rat bronchial smooth muscle. By using similar methods, Miao et al. (2002) also demonstrated that endothelin-1 increases RhoA activity through activation of endothelin ET_A but not ET_B receptors in rabbit basilar artery. Furthermore, selective activation of RhoA by endothelin ET_A receptors in endothelin-1-stimulated rabbit intestinal smooth muscles has been demonstrated by using Rhotekin binding assay (Hersch et al., 2004). Thus, the endothelin-1-induced activation of RhoA in smooth muscle might be mediated

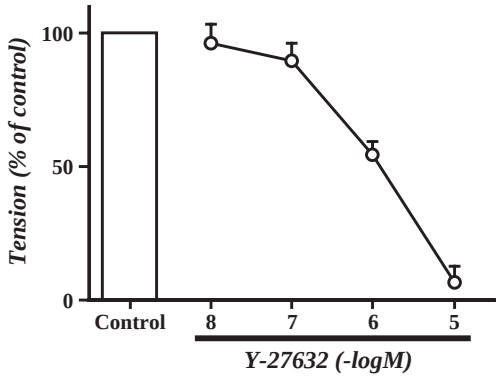

Fig. 3. Effects of a selective Rho-kinase inhibitor, Y-27632, on endothelin ET_A receptor-mediated contraction of rat bronchial smooth muscles. The endothelin ET_A receptor-mediated contraction was induced by treatment of muscle strips with endothelin-1 (10^{-6} M) in the presence of an ET_B receptor antagonist BQ-788, atropine and indomethacin (each 10^{-6} M). After the plateau contraction induced by endothelin-1 was observed (Control), Y-27632 (10^{-8}–10^{-5} M) was administered cumulatively. Values are means with s.e.m. from 4 experiments.

by endothelin ET_A receptors universally over species and organs.

In conclusion, this study clearly showed that endothelin-1 induces translocation of RhoA via an activation of endothelin ET_A receptors in rat bronchial smooth muscle.

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