Involvement of Rho kinase in the pathogenesis of acute pulmonary embolism-induced polystyrene microspheres in rats

M. Toba, T. Nagaoka, Y. Morio, K. Sato, K. Uchida, N. Homma, and K. Takahashi

Department of Respiratory Medicine, Juntendo Univ. School of Medicine, Tokyo, Japan

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Toba M, Nagaoka T, Morio Y, Sato K, Uchida K, Homma N, Takahashi K. Involvement of Rho kinase in the pathogenesis of acute pulmonary embolism-induced polystyrene microspheres in rats. Am J Physiol Lung Cell Mol Physiol 298: L297–L303, 2010. First published November 13, 2009; doi:10.1152/ajplung.90237.2008.—Acute pulmonary embolism (PE) is a life-threatening disease, and several vasoconstrictors, including endothelin-1 (ET-1), play a key role in vasoconstriction and hypoxemia during the development of PE. Rho kinase is activated by various vasoconstrictors resulting in vascular contraction and remodeling. Recent evidence has revealed an important role of Rho kinase in the pathogenesis of systemic and pulmonary vascular diseases. However, contribution of Rho kinase in PE remains unclear. We thus investigated the role of Rho kinase in the PE rat model induced by intrajugular administration of polystyrene microspheres (mean diameter, 26 μm). At 6 h following the administration of microspheres (1.5 ml/kg), right ventricular systolic pressure (RVSP) was higher in the PE than in the control rats (15.8 ± 1.6 vs. 32.9 ± 7.5 mmHg). Arterial oxygen tension was lower (92.3 ± 12.5 vs. 66.0 ± 17.7 Torr), and alveolar-arterial difference in oxygen partial pressure was higher (3.9 ± 3.8 vs. 36.5 ± 26.9 Torr) in the PE rats. Western blotting analysis revealed upregulation and downregulation in expression of vascular cell adhesion molecule-1 and endothelial nitric oxide synthase in lungs from the PE rats, respectively, and radioimmunoassay demonstrated an increase in plasma ET-1 levels. Lung Rho kinase α expression was greater in the PE rats. At 5 h following administration of microspheres (0.75 ml/kg), intravenous Rho kinase inhibitors HA1077 and Y27632 (3 mg/kg each) attenuated elevation of RVSP (22.0 ± 3.7, 17.1 ± 3.2, 14.3 ± 2.6 mmHg, PE, PE+HA1077, PE+Y27632) and the severity of hypoxemia (66.3 ± 16.2, 94.9 ± 23.0, 89.1 ± 8.5 Torr, PE, PE+HA1077, PE+Y27632) in the PE rats. These results suggest that pulmonary endothelial dysfunction and activation of Rho kinase may contribute to the potentiation of vasoconstriction and hypoxemia in the PE rats.

Address for reprint requests and other correspondence: T. Nagaoka, Dept. of Respiratory Medicine, Juntendo Univ. School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan (e-mail: jnagaoka@med.juntendo.ac.jp).

ACUTE PULMONARY EMBOLISM (PE) is a serious and life-threatening disease. PE is responsible for 5–10% of all in-hospital death (8, 11), and untreated PE is associated with a mortality rate of ~30% (5). The major reasons of death in PE are pulmonary hypertension, right ventricular failure, and arterial hypoxemia. Pulmonary hypertension is caused by obstruction of pulmonary arteries with embolization. PE also causes inadequate redistribution of alveolar blood flow and mismatching of ventilation and perfusion. This mismatching is the most common cause of arterial hypoxemia (9, 21). Moreover, recent evidence has shown that upregulation of serotonin (25, 45), thromboxane A2 (17, 18), and endothelin-1 (ET-1) (23, 35, 38) have been confirmed in human and animal PE. These constrictors are thought to play an important role in the vascular and bronchial constriction and provoke the mismatching of ventilation and perfusion, thus resulting in hypoxemia in PE (39). Current treatment for “human” PE is focused on removing the mechanical obstruction with thrombolysis and anticoagulation (1, 3), and recent studies have revealed the presence of residual pulmonary hypertension despite adequate and sufficient anticoagulation (7, 10). Therefore, this may raise the necessity and consideration for an additional strategy for the treatment of PE.

Previous studies have shown that the small GTPase RhoA and its downstream effector Rho kinase are activated by G protein-coupled receptor agonists such as serotonin, thromboxane A2, and ET-1 (15, 40). RhoA/Rho kinase signaling is involved in the pathogenesis of various vascular diseases, including systemic hypertension (27), angina pectoris (37), cerebral vasospasm following subarachnoid hemorrhage (36), and pulmonary hypertension (16). Nagaoka et al. (28, 29) and other researchers (2, 14, 32) recently revealed that RhoA/Rho kinase signaling plays a key role in the development of pulmonary hypertension in several experimental pulmonary hypertensive models. However, contribution of Rho kinase in PE remains unclear. Therefore, the purpose of this study was to investigate whether Rho kinase signaling is involved in a key role in the development of pulmonary hypertension and hypoxemia in the PE rat model. We also explored the mechanism that regulates Rho kinase signaling in the PE rat model. PE was induced by intrajugular administration of polystyrene microsphere beads in the rat, although the pathogenesis of this embolism model may not be completely similar to that of human pulmonary thromboembolism. We determined the plasma ET-1 levels and Rho kinase α protein expression in the PE rat lungs. Additionally, although vascular endothelial dysfunction may exist during the development of vascular disease, there are no studies that have determined the contribution of pulmonary vascular endothelial dysfunction in the pathogenesis of PE. We thus investigated the expression of vascular cell adhesion molecule-1 (VCAM-1) and endothelial nitric oxide synthase (eNOS) to evaluate the pulmonary vascular endothelial dysfunction in the PE rats (22, 24, 26). We also tested whether Rho kinase inhibitor is capable of attenuating the degree of pulmonary hypertension and hypoxemia in the PE rats in vivo.

MATERIALS AND METHODS

Animals. All experimental procedures were approved by the Animal Care and Use Committee of Juntendo University (Tokyo, Japan). Experiments were performed with adult male Sprague-Dawley rats (250–420 g).

Induction of PE and hemodynamic measurements. Male Sprague-Dawley rats were anesthetized with intramuscular pentobarbital sodium (40 mg/kg) for placement of catheters in the right ventricle and carotid artery. Rats were administered either vehicle (saline) or
polystyrene microsphere beads (1.5 ml/kg, mean diameter 26 μm, 7525A; Duke Scientific) into the right ventricle as previously described (41, 48). At 6 h following the administration of polystyrene microsphere beads or vehicle, measurements of right ventricular systolic pressure (RVSP) were performed while the animals were conscious as previously described (20). At the end of the experiment, all rats were euthanized with an overdose of pentobarbital sodium, and blood sample and lung tissue were collected as described below.

Arterial blood gas analysis and plasma ET-1 measurement. Arterial blood samples of the control and PE rats were collected from the catheter in the carotid artery. Collected blood sample was placed on ice, and blood gas analysis was immediately performed. Blood samples for ET-1 measurement were centrifuged, and plasma ET-1 peptide was measured using a radioimmunoassay kit as previously described (20).

Western blot analysis. Lung tissues of the control and PE rats were isolated for Western blot analysis. Isolated lung tissues were immediately frozen in liquid nitrogen. To examine the expression of VCAM-1, eNOS, and Rho kinase α, frozen lung tissues were homogenized in lysis buffer (1% Triton X-100, 50 mM Tris · HCl, 150 mM NaCl) containing protease inhibitor (1% aprotinin and PMSF). Homogenate was centrifuged at 15,000 g for 15 min. Western blotting was performed with anti-eNOS antibody (1:2,500 dilution, BD Transduction) (26), anti-VCAM-1 antibody (1:100 dilution, Santa Cruz Biotechnology) (26), anti-Rho kinase α antibody (1:1,000 dilution, BD Transduction) (29), and anti-β actin (1:5,000 dilution, Sigma) (29), as previously described. Western blotting for VCAM-1 was performed to evaluate the degree of endothelial impairment, since VCAM-1 is an early marker of vascular endothelial dysfunction (22, 24). Relative density of blots was determined using an image software.

Experimental protocol in vivo. In this protocol, we reduced the volume of polystyrene microsphere beads (0.75 ml/kg) since vital conditions of rats administered 1.5 ml/kg of polystyrene microsphere beads were unstable and intolerable for systemic hypotension induced by intravenous Rho kinase inhibitors HA1077 and Y27632 (30). If the heart rate fell below 300 beats/min, it was suspected that the animal’s cardiovascular function had been compromised, and measurements were excluded from analysis. Experiments were performed with four groups of rats. The first group was administered the vehicle at 1 h following vehicle injection. The second, third, and fourth groups were administered the vehicle, HA1077 (3 mg/kg), and Y27632 (3 mg/kg) at 1 h following injection of polystyrene microsphere beads, respectively.

We next investigated the effect of Rho kinase inhibitors HA1077 and Y27632 in the PE rats. After 5 h following vehicle or Rho kinase inhibitor administration, both hemodynamic measurement and blood gas analysis were performed in the control and PE rats treated or not with Rho kinase inhibitors. We also measured the plasma brain natriuretic peptide (BNP) in the control and PE rats treated or not with Y27632 by chemiluminescent enzyme immunoassay to evaluate the right heart strain. Additionally, we measured the expression of lung eNOS, VCAM-1, and the plasma ET-1 of the PE rats treated or not with HA1077 by Western blot analysis and radioimmunoassay, respectively, to evaluate the effect of HA1077 to these parameters.

Statistical analysis. Values are means ± SD. Comparisons between groups were determined with the Student’s t-test or analysis of variance with the Fisher post hoc test for multiple comparisons. Differences were considered significant at \( P < 0.05 \).

RESULTS

Acute PE induced polystyrene microspheres. Polystyrene microsphere beads were evenly distributed in the entire lungs and have occluded the pulmonary artery of ~50 μm of diameter in rats (Fig. 1).

Hemodynamic measurement and arterial blood gas analysis. At 6 h following administration of polystyrene microsphere beads, RVSP was significantly higher in the PE rats than in the control rats (15.8 ± 1.6 vs. 32.9 ± 7.5 mmHg, respectively, control vs. PE, \( P < 0.05 \)). Arterial oxygen tension (PaO\(_2\)) was significantly lower (92.3 ± 12.5 vs. 66.0 ± 17.7 Torr, respectively, control vs. PE, \( P < 0.05 \)) and alveolar-arterial difference in oxygen partial pressure (A-aDO\(_2\)) was significantly higher (3.9 ± 3.8 vs. 36.5 ± 26.9 Torr, respectively, control vs. PE, \( P < 0.05 \)) in the PE rats than in the control rats (Fig. 2).

No significant changes in the systemic arterial systolic pressure, arterial carbon dioxide tension (PaCO\(_2\)), and arterial blood pH were observed in the PE rats compared with the control rats (123.2 ± 7.6 vs. 107.8 ± 18.0 mmHg, 46.4 ± 10.2 vs. 38.0 ± 10.3 Torr, 7.378 ± 0.1 vs. 7.379 ± 0.2, respectively, control vs. PE).

Lung VCAM-1 and eNOS expression in PE rats. Lung VCAM-1 expression determined with Western blotting was significantly increased in the PE rats compared with that of the control rats (Fig. 3). Lung eNOS expression was significantly lower in the PE rats than in the control rats (Fig. 4).

Plasma ET-1 levels and lung Rho kinase α expression in PE rats. Plasma ET-1 levels determined with radioimmunoassay were significantly higher in the PE rats than in the control rats (4.9 ± 0.9 vs. 6.4 ± 1.1 pg/ml, respectively, control vs. PE, \( P < 0.05 \); Fig. 5). Lung Rho kinase α expression determined with Western blotting was significantly increased in the PE rats compared with that of the control rats (Fig. 6).

Effect of intravenous Rho kinase inhibitors in PE rats. Administration of intravenous Rho kinase inhibitors HA1077 and Y27632 significantly attenuated the elevated RVSP in the PE rats (22 ± 3.7, 17.1 ± 3.2, and 14.3 ± 2.6 mmHg, respectively, PE, PE-treated HA1077, and PE-treated Y27632, \( P < 0.05 \) vs. PE rats). HA1077 and Y27632 also prevented the change in worsening of PaO\(_2\) (66.3 ± 16.2, 94.9 ± 23.0, and 89.1 ± 8.5 Torr, respectively, control vs. PE, HA1077 vs. PE, and Y27632 vs. PE, \( P < 0.05 \); Fig. 7). Values of BNP were significantly higher in the PE rats than in the control rats (6.3 ± 0.6 vs. 8.3 ± 0.7 pg/ml, control vs. PE, \( P < 0.001 \), \( n = 4 \) each), but Y27632
did not suppress the elevation of BNP (8.3 ± 0.7 vs. 8.0 ± 0.2 pg/ml, PE vs. PE-treated Y27632, \( P = 0.33, n = 4 \) each). Levels of plasma ET-1 (6.4 ± 1.1 vs. 7.7 ± 0.4 pg/ml, PE vs. PE with HA1077, \( P = 0.09, n = 6 \) and 3, respectively) and lung VCAM-1 (data not shown) of the PE rats treated with HA1077 were not significantly different from those of the PE rats without HA1077. Decreased lung eNOS proteins also remained after treatment of HA1077 similar to those of the PE rats (data not shown).

**Fig. 2.** Right ventricle systolic pressure (RVSP), Arterial oxygen tension (\( \text{PaO}_2 \)), and alveolar-arterial difference in oxygen partial pressure (A-\( \text{aDO}_2 \)) in the control and pulmonary embolism (PE) rats. Values are means ± SE; \( n = 5 \) for the control and \( n = 7 \) for the PE in RVSP; \( n = 5 \) for the control and \( n = 6 \) for the PE in \( \text{PaO}_2 \) and A-\( \text{aDO}_2 \). *\( P < 0.05 \) vs. control.

**Fig. 3.** Protein levels of VCAM-1 in lungs from control and PE rats. *Top:* representative immunoblots. *Bottom:* densitometric assessments. Values are means ± SE; \( n = 5 \) for the control and \( n = 4 \) for the PE. *\( P < 0.05 \) vs. control.

**Fig. 4.** Protein levels of eNOS in lungs from the control and PE rats. *Top:* representative immunoblots. *Bottom:* densitometric assessments. Values are means ± SE; \( n = 4 \) for the control and \( n = 5 \) for the PE. *\( P < 0.05 \) vs. control.
DISCUSSION

The major findings of this study are as follows: 1) VCAM-1 protein expression in the lung tissue was increased in the PE rats; 2) eNOS protein expression in the lung tissue was decreased in the PE rats; 3) plasma ET-1 levels and lung Rho kinase/\(H_9251\) protein expression were increased in the PE rats; and 4) Rho kinase inhibitors HA1077 and Y27632 attenuated the elevation of RVSP and the severity of hypoxemia in the PE rats.

Pulmonary hypertension and hypoxemia are major pathophysiological factors involved in PE. Pulmonary hypertension and subsequent right ventricular failure are caused by mechanical pulmonary vascular occlusion and subsequent vasoconstriction. After vascular occlusion, blood flow from obstructed pulmonary arteries is redirected to other gas exchange units, and ventilation and perfusion become mismatched. Mismatching of ventilation and perfusion is the most common cause of impairment of pulmonary oxygen transfer and hypoxemia (21). Atelectasis, caused by loss of surfactant and alveolar hemorrhage, also contribute to reduced ratios of ventilation to perfusion and hypoxemia (19). Moreover, platelet aggregation surrounding the embolism produces serotonin and thromboxane \(A_2\) (39), and shear stress and endothelial impairment results in upregulation of ET-1 (13). Recent evidence has revealed that these constrictors provoke further ventilation and perfusion mismatching via vascular and bronchial constriction and play a pivotal role in the development of hypoxemia (19, 39). Although the vasoconstriction could regulate the increased vascular tone resulting from endothelial dysfunction as mentioned above, the contribution of vasoconstrictors in the pathogenesis of PE remains unclear. We showed the vasodilator effect of Rho kinase inhibitors in this study, suggesting the important role of pulmonary vasoconstriction in the pathogenesis of PE.

Previous studies have demonstrated the involvement of ET-1 in the pathogenesis of human pulmonary thromboembolism (6), and elevated plasma ET-1 levels and the beneficial effect of nonselective ET-1 receptor antagonist have been revealed in a pulmonary thromboembolism model of canine (23). Furthermore, elevation of plasma ET-1 and effectiveness of selective ET-1 receptor antagonist have also been shown in the experimental pulmonary air embolism model (4, 35, 38). Collectively, it is suggested that ET-1 may contribute to development of several forms of PE. In the present study, we also showed the increase in the level of plasma ET-1 in microsphere PE rats. RhoA/Rho kinase signaling is activated by various vasoconstrictors, including ET-1, serotonin, and thromboxane \(A_2\), the receptors of which are coupled to G proteins (40), and upregulation of these constrictors have already been reported in experimental and human PE (6, 23, 39). RhoA and its downstream Rho kinase play an important role in the pathogenesis of various systemic and pulmonary vascular diseases via vascular constriction and remodeling (15). Thus we hypothesized that Rho kinase-mediated vasoconstriction contributes to the development of pulmonary hypertension and hypoxemia in the PE rats. We first demonstrated the increase in expression of lung Rho kinase \(\alpha\) in the PE rats, and next revealed that HA1077 attenuated the increase of RVSP and suppressed the development of hypoxemia in the PE rats. These results suggest that Rho kinase-mediated pulmonary vasoconstriction induced by ET-1 is involved in the pathogenesis of PE. However, Tsang et al. (43) have recently reported that nonselective ET-1 receptor antagonist attenuated pulmonary hypertension, but did not improve hypoxemia and mismatching of ventilation and perfusion in the porcine pulmonary thromboembolism model. They suggested that the main mechanism for hypoxemia of PE.
was mechanical redistribution of pulmonary regional blood flow away from the embolized vessels and that the blockade of ET-1 receptor could not affect the redistribution of pulmonary regional blood flow. On the other hand, there may be a possibility that Rho kinase inhibitor prevented hypoxemia in our preparation probably via blockade of bronchial constriction (42) similar to the vasoconstriction in PE. Thus, further investigation is required to confirm the effect of pulmonary vasodilator on the ventilation and perfusion mismatching in the present PE model.

Nitric oxide is generated from three nitric oxide synthase isoforms, neuronal, inducible, and endothelial nitric oxide synthase (31). eNOS-mediated NO signaling is speculated to modulate pulmonary vascular responses to a variety of vasoconstrictor stimuli and play a key role in maintaining normal low basal pulmonary vascular tone, including the suppression of ET-1 system (46). Contribution of eNOS in the regulation of vascular tone has been suggested by studies using a variety of inhibitor and mice with targeted gene deletion of eNOS (12, 33, 44) in experimental pulmonary hypertension. However, the role of NO in PE has not been fully understood. Endothelial cell adhesion molecules play a pivotal role in the recruitment and binding of inflammatory cells to the vascular endothelium during the development of vascular disease. Since several reports suggested that elevated VCAM-1 levels reflect the impairment of endothelial function (26), VCAM-1 has been regarded as a marker of endothelial dysfunction. In this study, we demonstrated the augmentation of lung VCAM-1 expression and plasma ET-1 levels, and reduction of lung eNOS expression, suggesting endothelial dysfunction characterized by downregulation of eNOS expression and upregulation of ET-1 levels in the PE rat. We speculated that polystyrene microsphere beads cause endothelial damage mechanically, resulting in downregulation of eNOS expression in the PE rat. Our results suggest that attenuation of eNOS-mediated NO signaling may partly play a role during the development of pulmonary hypertension and hypoxemia in the PE rats. Since recent evidence has shown that cGMP, downstream of eNOS, regulates the activity of RhoA/Rho kinase signaling (34), attenuation of eNOS may contribute to the activation of Rho kinase in the PE rats. On the other hand, treatment with Rho kinase inhibitor HA1077 did not affect the expression of eNOS, VCAM-1, and ET-1 of the PE rats. These results indicate that Rho kinase inhibitor did cause pulmonary vasodilation without any effects on the expression of these parameters, whereas the elevated ET-1 resulting from endothelial dysfunction characterized by decreased eNOS and increased VCAM-1 could activate the upstream of Rho kinase signaling. BNP has been reported as a predictor of adverse outcome in human pulmonary embolism (47). Values of BNP were significantly higher in the PE than in the control rats, but Rho kinase inhibitor did not suppress the elevation of BNP. This result may suggest that Rho kinase inhibitor causes pulmonary vasodilation and improves hypoxemia, although the acute right heart strain remains.

Several limitations should be mentioned for the present study. We used microsphere embolism model to evaluate the role of endothelial markers and Rho kinase in PE because it is very difficult to make the microthromboembolism model in a small animal like a rat. However, the pathogenesis of microsphere embolism model is not completely similar to that of human pulmonary thromboembolism. For example, the role of serotonin generated from aggregated platelet around the thrombosis may be more important in the pathogenesis of human pulmonary thromboembolism than in that of the present microspheres embolism model. In addition, there are several forms of pulmonary embolism including air, fat, tumor, and septic embolization. Although multiple factors such as cytokines, chemokines, and growth factors beyond vascular mediators may be involved in the pathogenesis of PE consequent on above diseases, endothelial dysfunction may still be one of the candidates of common pathogenesis. Acute right heart strain may be greater in the present model than in human pulmonary thromboembolism. Further investigation is needed to determine the detailed mechanism of Rho kinase inhibition in the PE model.

In summary, this study revealed that Rho kinase was involved in the development of pulmonary hypertension and hypoxemia in the PE rats, demonstrating endothelial dysfunction characterized by downregulation of eNOS expression and
upregulation of ET-1 levels. Rho kinase inhibitor decreased RVSP and improved hypoxemia in the PE rats. These effects most likely resulted from the inhibition of pulmonary vasoconstriction and improvement in the mismatching of ventilation and perfusion. We thus propose that activation of Rho kinase might be the common factor in the PE. Although current conventional treatments for human PE are only thrombolysis and anticoagulation, our results suggest that Rho kinase might be a novel target for the treatment of human PE.

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DISCLOSURES
No conflicts of interest are declared by the author(s).

REFERENCES


