Vasoconstrictor effect of endothelin-1 on hypertensive pulmonary arterial smooth muscle involves Rho-kinase and protein kinase C

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Barman SA. Vasoconstrictor effect of endothelin-1 on hypertensive pulmonary arterial smooth muscle involves Rho-kinase and protein kinase C. Am J Physiol Lung Cell Mol Physiol 293: L472–L479, 2007. First published April 27, 2007; doi:10.1152/ajplung.00101.2006.—Although one of the common characteristics of pulmonary hypertension is abnormal sustained vasoconstriction, the signaling pathways that mediate this heightened pulmonary vascular response are still not well defined. Protein kinase C (PKC) and Rho-kinase are regulators of smooth muscle contraction induced by G protein-coupled receptor agonists including endothelin-1 (ET-1), which has been implicated as a signaling pathway in pulmonary hypertension. Toward this end, it was hypothesized that both Rho-kinase and PKC mediate the pulmonary vascular response to ET-1 in hypertensive pulmonary arterial smooth muscle, and therefore, the purpose of this study was to determine the role of PKC and Rho-kinase signaling in ET-1-induced vasoconstriction in both normotensive (Sprague-Dawley) and hypertensive (Fawn-Hooded) rat pulmonary arterial smooth muscle. Results indicate that ET-1 caused greater vasoconstriction in hypertensive pulmonary arteries compared with the normal vessels, and treatment with the PKC antagonists chelerythrine, rottlerin, and Gö 6983 inhibited the vasoconstrictor response to ET-1 in the hypertensive vessels. In addition, the specific Rho-kinase inhibitor Y-27632 significantly attenuated the effect of ET-1 in both normotensive and hypertensive phenotypes, with greater inhibition occurring in the hypertensive arteries. Furthermore, Western blot analysis revealed that ET-1 increased RhoA expression in both normotensive and hypertensive pulmonary arteries, with expression being greater in the hypertensive state. These results suggest that both PKC and Rho/Rho-kinase mediate the heightened pulmonary vascular response to ET-1 in hypertensive pulmonary arterial smooth muscle.

Although one of the common characteristics of pulmonary hypertension is abnormal sustained vasoconstriction of pulmonary arteries (46, 47), the signaling pathways that mediate the heightened pulmonary vascular response are still not clearly defined. Rho-associated serine/threonine kinase (Rho-kinase) is a downstream effector of small GTPase RhoA, which has been identified as a regulator of smooth muscle contraction (61). Recent studies show that Rho-kinase signaling mediates pulmonary vasoconstriction induced by G protein-coupled receptor agonists (18, 28), and RhoA is activated by a variety of pulmonary vasoconstrictors, including endothelin-1 (ET-1), phenylephrine, and serotonin, all having G protein-coupled receptors (42). Rho-kinase mediates monocrotaline-induced pulmonary hypertension (1), hypoxic pulmonary vasoconstriction (20), and ET-1-induced pulmonary arterial contraction in chronically hypoxic rats (63). Hypoxia also activates Rho/Rho-kinase signaling in rat pulmonary arteries and mediates the prolonged phase of acute hypoxic vasoconstriction (48, 61). In addition, Rho-kinase inhibitors cause pulmonary vasodilation in chronically hypoxic perfused rat lungs, suggesting a role for activation of Rho-kinase in the increased basal vascular tone present in hypoxic pulmonary hypertension (42).

In the pulmonary vasculature, protein kinase C (PKC) is a key regulatory enzyme involved in the signal transduction of several cellular functions, including vascular smooth muscle growth and contractility (4, 10). PKC consists of a family of serine/threonine kinases with at least 12 members, and numerous PKC isoforms are expressed in vascular smooth muscle that may be dependent on species, type of vessel, and age of the vessel (27, 31). In addition, studies show that several of these specific isoforms coexist and participate in vasoconstrictor signaling mechanisms in pulmonary vascular smooth muscle cells (7, 8).

ET-1 is a 21-amino acid peptide originally isolated from the supernatants of cultured porcine aortic endothelial cells (65). ET-1 has been found in lung tissue and pulmonary endothelial cells (45), and pulmonary blood vessels possess ET-1 receptors (35). ET-1 causes vasoconstriction through a variety of mechanisms, including PKC activation (34, 58). Specifically, ET-1 enhances the production of 1,2-diacylglycerol, which endogenously activates PKC in vascular smooth muscle (25, 34). ET-1 causes vasoconstriction in the pulmonary circulation of many species, including the cat (36), rat (9), rabbit (9, 37), dog (5, 6, 9), and human (22). In addition, ET-1 has been implicated in cardiopulmonary disease states, because plasma levels and expression of ET-1 are elevated in patients with pulmonary hypertension and cardiogenic shock (15, 22).

In light of these previous investigations, the present study was done to determine the role of both Rho-kinase and PKC on ET-1-induced vasoconstriction in pulmonary arterial smooth muscle. Specifically, the effect of Rho-kinase and PKC signaling was investigated in hypertensive pulmonary arterial smooth muscle of the Fawn-Hooded rat, an animal model of “idiopathic” pulmonary hypertension (50, 57) as exhibited by pulmonary vasoconstriction (3, 30) and right ventricular hypertrophy (50, 57). Subsequently, the effects found in the hypertensive state were compared with that observed in normotensive pulmonary arterial smooth muscle of the Sprague-Dawley rat.

MATERIALS AND METHODS

Animals

All procedures and protocols were approved by the Animal Care and Use Committee at the Medical College of Georgia, and the investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

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Experimental Protocols

For FHR and SDR isolated vessel experiments, concentration-response treatments using ET-1 (1 to 1,000 nM) were done with 1) ET-1 alone (n = 4), 2) pretreatment with 1 μM chelerythrine (PKC antagonist) for 30 min before ET-1 (n = 4), and 3) pretreatment with 1 μM Y-27632 (Rho-kinase antagonist) for 30 min before ET-1 (n = 4). Additional isolated FHR pulmonary artery experiments were done using pretreatments with either 1 μM rrotelin (PKCζ antagonist) or 1 μM Gö 6983 (PKCδ, β, δ, γ, ζ antagonist) for 30 min before ET-1 (n = 4 for each group). For the Western blot experiments, both FHR and SDR denuded pulmonary arterial vessel tissue were treated with either 1) 80 mM KCl alone (n = 4 for each group) or 2) ET-1 alone (1 to 1,000 nM) for 30 min (n = 4 for each treatment).

Drugs

ET-1 was purchased from Peptides International, and all other drugs were purchased from Calbiochem.

Statistical Analysis

All values are expressed as means ± SE. Significance was determined using an analysis of variance for within-group and between-group comparisons. If a significant F ratio was found, then specific statistical comparisons were made using Bonferroni/Dunn post hoc tests. Statistical significance was accepted when P < 0.05.

RESULTS

Identification of Right Ventricular Hypertrophy

The right ventricular-to-left ventricular plus septal weight ratios (RV/LV+S) in FHR (0.48 ± 0.06; n = 52) vs. control SDR (0.27 ± 0.06, P < 0.05; n = 36) was indicative of right ventricular hypertrophy in FHR as reported in other studies (43, 50).

Effect of KCl on Vasconstriction and RhoA Expression

The initial maximal pressor response to 80 mM KCl in both FHR and SDR isolated pulmonary arterial vessels is shown in Fig. 1. KCl elicited vasoconstrictor responses that were an ~85% increase over baseline tension in SDR vessels and an ~70% increase in FHR vessels (Table 1). The pressor response to KCl was prevented by PKC inhibitors, but not by the Rho-kinase inhibitor Y-27632. The inability of acetylcholine to produce endothelium-dependent relaxation. The inability of vessels to relax to acetylcholine after being treated (see Experimental Protocols) for 30 min before ET-1 (1 μM) had no effect on the maximal pressor response to 80 mM KCl.
~115% increase above baseline tension in FHR vessels, which were not blocked by pretreatment with 1 μM Y-27632. These data indicate that the vasoconstrictor response to membrane depolarization is higher in hypertensive pulmonary arteries and that both SDR and FHR pulmonary vasoconstrictor responses to KCl occur independently of the pharmacological concentration of Y-27632 used to inhibit Rho kinase. Western blot analysis also revealed that 80 mM KCl did not increase RhoA expression in either the SDR (Fig. 2A) or FHR (Fig. 2B) pulmonary arteries.

**Effect of PKC Inhibition**

The effect of PKC inhibition on ET-1-induced vasoconstriction (measured as a percentage of maximum KCl contractile response) in isolated normotensive (SDR) and hypertensive (FDR) pulmonary arterial vessels is shown in Fig. 3. ET-1 caused vasoconstriction in both SDR and FHR pulmonary arteries, exhibiting a significantly greater percentage of the maximum KCl contractile response in the hypertensive pulmonary arteries at all concentrations, with the highest concentration of ET-1 increasing vasoconstriction 195.3 ± 12.1% for FHR vs. 135.1 ± 11.2% for SDR. Treatment with the nonspecific PKC antagonist chelerythrine significantly attenuated the vasoconstrictor response at all concentrations of ET-1 in the hypertensive vessels to ~50% of the maximal contractile response to KCl but had little effect on the contractile response to ET-1 in normotensive pulmonary arteries, ranging from 105.8 ± 5.8 to 120.2 ± 7.6% of the maximum KCl contractile response. Further experiments were done with the specific PKC isozyme inhibitors Gö 6983 (α, β, δ, γ, ζ) and rottlerin (δ) to determine which PKC isozymes may be involved in the hypertensive (FHR) pulmonary arterial contractile response to ET-1. As shown in Fig. 4, rottlerin significantly attenuated the vasoconstrictor response to ET-1 to ~75% of the maximum KCl contractile response at each concentration of ET-1, and Gö 6983 decreased the ET-1-induced pressor response further to <50% of the maximum KCl contractile response at each concentration of ET-1.

**Effect of Rho-Kinase Inhibition**

Since RhoA is activated by ET-1 in vascular smooth muscle (33), experiments were done to determine whether Rho-kinase inhibition would affect ET-1-induced pulmonary vasoconstriction. As shown in Fig. 5, ET-1 again caused a greater contraction in the FHR pulmonary arteries compared with the SDR vessels (maximal %KCl response was 210.8 ± 10.3% for FHR vs. 160.3 ± 11.2% for SDR), and treatment with the specific Rho-kinase inhibitor Y-27632 significantly attenuated the effect of ET-1 in both normotensive and hypertensive isolated pulmonary arterial vessels to ~100% maximal KCl contractile response in both phenotypes, with a greater percentage of contractile inhibition to ET-1 occurring in the hypertensive phenotype (~50% inhibition) vs. the normotensive state (~33% inhibition).

**Western Blot Analysis**

Because it was observed that the contractile response to ET-1 was greater and that Rho-kinase inhibition resulted in a greater decrease in ET-1 induced vasoconstriction in hypertensive pulmonary arteries compared with normotensive vessels, experiments were done to measure the effect of ET-1 on RhoA expression in both FHR and SDR pulmonary arteries. As shown in Fig. 6A, ET-1 significantly increased RhoA expression in hypertensive pulmonary arteries at a concentration as low as 1 nM, with peak expression occurring at 10 nM. In contrast, ET-1 elicited a smaller increase in RhoA expression in the normotensive pulmonary arteries at higher concentrations (100 to 1,000 nM; Fig. 6B) compared with the FHR vessels.

**Discussion**

The results of this study demonstrate that 1) ET-1 causes greater vasoconstriction in the FHR (hypertensive) pulmonary arteries compared with the SDR (normotensive) vessels, 2) PKC inhibition significantly blocked the vasoconstrictor response to ET-1 in the hypertensive vessels but had little effect in the normotensive pulmonary arteries, 3) treatment with the specific Rho-kinase inhibitor Y-27632 significantly attenuated the effect of ET-1 in both normotensive and hypertensive isolated pulmonary arterial vessels, with a greater inhibitory response occurring in the hypertensive arteries, and 4) ET-1 caused a significant increase in RhoA expression in both normotensive and hypertensive pulmonary arteries, with the effect of ET-1 being greater in the hypertensive state.

The study of mechanisms of pulmonary hypertension has been problematic because of the lack of relevant experimental...
animal models. In addition, it is difficult to separate changes that may be pathogenic from alterations that occur secondarily to the hypertension (3). As a result, the most commonly used methods to induce pulmonary hypertension include monocrotaline (1, 52), chronic hypoxia (20, 42, 61), thromboxane stimulation (11, 52), overexpression of angiopoietin-1 (16), and aortic/coronary artery banding (17, 19). In the present study, the FHR strain was the experimental model of pulmonary hypertension, which has been used widely to study genetic risk factors for the development of pulmonary arterial hypertension (50, 57). The FHR strain develops significant pulmonary hypertension, which is age dependent (30) and occurs when exposed to mild hypoxic conditions during the first 3–4 wk of life or earlier (43, 50). Evidence also suggests that the genetic locus for the pulmonary hypertensive condition is PH1 on chromosome 1 (57).

Initially, in both FHR and SDR isolated pulmonary arteries, 1 μM Y-27632 did not inhibit KCl-induced pulmonary vasoconstriction. Weigand et al. (63) reported that 10 μM Y-27632 had little effect on KCl-induced pulmonary vasoconstriction in Wistar rat pulmonary arteries. In contrast, 10 μM Y-27632 inhibited depolarization-induced pulmonary constriction in hypoxic but not normoxic isolated rat lungs (42) and in isolated pulmonary vessels of both mice and rats (20, 42), suggesting that the effect of Y-27632 on KCl-induced pulmonary vasoconstriction may be related to both species and/or concentration specificity of the Rho-kinase antagonist.

In the present study, chelerythrine blocking the response to ET-1 in hypertensive vessels but having a small effect in normotensive pulmonary arteries indicates a role for PKC signaling in the heightened vasoconstriction that occurs in pulmonary hypertension. Further experiments showed that the specific PKC isozyme inhibitors Gö 6983 (α, β, δ, γ, ζ) and rottlerin (β) also significantly attenuated the effect of ET-1 in the FHR pulmonary arteries. Rottlerin is a compound isolated from Mallotus philippinensis that selectively inhibits PKCβ at the concentration used in this study (26). Although rottlerin also exhibits selectivity for calmodulin (CaM) kinase III at low concentrations (IC50 = 5.3 μM), the concentration used in this study (1 μM) would suggest that CaM kinase III is minimally affected by rottlerin. In contrast, Weigand et al. (63) reported that ET-1-induced vasoconstriction in pulmonary arteries exposed to chronic hypoxia was not mediated by PKC. However, differences between these two studies include different animal models of pulmonary hypertension (Wistar vs. FHR) and the length of time the pulmonary vasculature was exposed to chronic hypoxia, as well as the size of the pulmonary vessels isolated, which may partly explain the different responses to PKC inhibition observed in the two studies. Several studies report that ET-1 causes pulmonary vasoconstriction via PKC activation (5, 58), and ET-1 has been implicated in pulmonary hypertension, since expression of ET-1 is increased in the lungs of patients with pulmonary hypertension (22) and plasma levels of ET-1 are elevated in patients with idiopathic pulmonary hypertension (22). In blood vessels, ET-1 enhances the production of 1,2-diacylglycerol, which endogenously activates PKC (25, 34), which induces the mobilization of intracellular Ca2+ stores and mediates sensitivity to Ca2+, causing vascular smooth muscle contraction (54, 56). PKC also mediates Ca2+ sensitization through inhibition of myosin light chain phosphatase (MLCP) activity (21). Specifically, PKC phosphorylates and activates CPI-17, a phosphorylation-dependent inhibitory protein of MLCP, which may be involved in PKC-mediated Ca2+ sensitization to cause vascular contraction (32).

Results of this study also suggest that Rho-kinase signaling is important in ET-1-mediated pulmonary vasoconstriction,

**Fig. 3. PKC inhibition significantly attenuates ET-1-induced vasoconstriction in hypertensive (FHR) pulmonary arteries but had little effect in normotensive (SDR) vessels. Pretreatment with the specific PKC antagonist chelerythrine (CHEL; 1 μM) for 30 min (n = 4) decreased the concentration response to ET-1 (1–1,000 nM). Vessels were first maximally preconstricted with 80 mM KCl and then rinsed several times with buffer to return vessels to baseline values before agonist/antagonist treatments. Values are means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001, significantly different from FHR.**

**Fig. 4. PKC inhibition significantly attenuates ET-1-induced vasoconstriction in hypertensive (FHR) pulmonary arteries. Pretreatment with the specific PKC isozyme antagonists rottlerin (1 μM) and Gö 6983 (1 μM) for 30 min (n = 4 for each group) decreased the concentration response to ET-1 (1–1,000 nM). Vessels were first maximally preconstricted with 80 mM KCl and then rinsed several times with buffer to return vessels to baseline values before agonist/antagonist treatments. Values are means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001, significantly different from control.**
because the specific Rho-kinase inhibitor Y-27632 significantly attenuated the effect of ET-1 in both normotensive and hypertensive isolated pulmonary arterial vessels, with a greater inhibitory response occurring in the hypertensive arteries. Recent studies have shown that many agonists, including ET-1, mediate vascular constriction through the calcium-independent Rho/Rho-kinase pathway (21, 59). These substances are believed to couple their receptors to the Gq family of heteromeric G proteins, which is linked to the elevated concentration of calcium via the phosphoinositol cascade and activation of myosin light chain kinase, leading to a subsequent increase in smooth muscle calcium sensitivity (21, 56). Specifically, it is surmised that vasoconstrictors utilize a mechanism that involves Rho-kinase-mediated Ca2+ sensitization of contraction through inhibiting MLCP in vascular smooth muscle (24), which prolongs the increase in the phosphorylation of MLC and subsequent smooth muscle contraction at a constant level of intracellular free Ca2+ (55). Consistent with this supposition are recent studies showing that ET-1 increases the Ca2+ sensitivity of vascular contraction via inhibition of MLCP, with a subsequent increase in MLC phosphorylation at a constant level of intracellular Ca2+ (40, 51). In the present study, the vasoconstrictor response to ET-1 occurred within 30 min, suggesting upregulation of Rho-kinase expression and pulmonary vasoconstriction via MLC phosphorylation within this time frame. Furthermore, ET-1 is also coupled to G12/13, which causes Rho-kinase-dependent smooth muscle contraction (23).

Rho is a member of the Ras superfamily of small GTP-binding proteins (60). The small GTPase RhoA and its downstream effector Rho-kinase mediate vascular smooth muscle contractility (28, 42, 49), and receptor agonists activate Rho-kinase via stimulation of RhoA activity, which results in inhibition of myosin phosphatase (49). Recently, the Rho-kinase pathway has been implicated in a variety of vascular hypertensive states. Asano and Nomura (2) observed that Rho-kinase caused systemic arterial constriction in both small and large mesenteric arteries from normotensive and hypertensive (SHR) rats, with a greater contractile response occurring in the hypertensive strain, and Seko et al. (53) reported that in rats made hypertensive with an inhibitor of NO synthase, the Rho-kinase inhibitor Y-27632 lowered blood pressure. In the pulmonary circulation, Abe et al. (1) reported that Rho-kinase inhibition improved monocrotaline-induced pulmonary hypertension in rats. Specifically, it was observed that the Rho-kinase inhibitor fasudil improved pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular lesions with concomitant suppression of vascular smooth muscle proliferation and macrophage infiltration, as well as improvement of endothelial cell dysfunction and vascular smooth muscle contraction, suggesting that Rho-kinase mediates many pathophysiological characteristics of the disease.

Rho-kinase is involved in hypoxic pulmonary vasoconstriction (20) and ET-1-induced pulmonary arterial contraction in chronically hypoxic rats (63), as well as in the increased contractile sensitivity of pulmonary arteries to Ca2+ in FHR (44). Hypoxia activates Rho/Rho-kinase signaling in rat pulmonary arteries and mediates the prolonged phase of acute hypoxic vasoconstriction (48, 61). In addition, Rho-kinase inhibitors cause pulmonary vasodilation in chronically hypoxic perfused rat lungs, suggesting a role for activation of Rho-kinase in the increased basal vascular tone present in hypoxic pulmonary hypertension (42). Furthermore, Wang et al. (61) observed elevation of MLC phosphorylation.
phosphorylation within 10 min of hypoxia, followed by an increase in Rho-kinase activation within 40 min of hypoxia (61), suggesting that Rho-kinase is not involved in initiating MLC phosphorylation but rather maintains MLC phosphorylation in the sustained phase of vasoconstriction when intracellular Ca\(^{2+}\) decreases. Finally, Rho-kinase signaling may mediate both vasoconstriction and vascular remodeling in a murine model of hypoxic pulmonary hypertension (20).

As previously stated, the pulmonary vasoconstrictor response to ET-1 was greater in the FHR compared with the SDR. Because ET-1 expression and production are greater in FHR (66), a tonic release of endogenous ET-1 in combination with exogenous ET-1 may have elicited a synergistic pulmonary vasoactive response to the peptide. In both normotensive and pulmonary hypertensive arteries, the response to ET-1 was significantly attenuated by the specific Rho-kinase inhibitor Y-27632. Several studies document that Y-27632 is effective in reducing agonist-induced vasoconstriction. For example, evidence suggests that Y-27632 inhibits the hypertension associated with NO synthase inhibition (53), as well as that seen in both spontaneously hypertensive rats (59) and deoxycorticosterone acetate (DOCA)-salt and renal hypertensive rats (62). In addition, Y-27632 blocks the vasoconstrictor response to phenylephrine in both systemic and pulmonary blood vessels (12, 41, 64) and the onset of spontaneous tone in systemic vessels in angiotensin II-hypertensive rats (29). In the pulmonary circulation, Y-27632 inhibits the acute vasoconstrictor effects of angiotensin II (20) and hypoxia (48, 61) and decreases the vasoconstriction and vascular remodeling in mice exposed to chronic hypoxia for 2 wk (20). Furthermore, inhaled Y-27632 may be a more effective vasodilator than inhaled NO in hypoxic pulmonary hypertension (42). Y-27632 is a relatively specific Rho-kinase blocker that selectively inhibits Rho-kinase in the 1 to 10 \(\mu\)M range, having very little effect on MLC kinase (MLCK), because the \(K_i\) to Rho-kinase is 0.14 \(\mu\)M, whereas the \(K_i\) to MLCK is >250 \(\mu\)M (59). Studies suggest that Y-27632 inhibits ET-1-induced MLC phosphorylation and subsequent vasoconstriction (40), and evidence for the specificity of the compound stems from the identification of a single binding site (Rho-associated kinase) within smooth muscle cells and the selectivity for inhibiting agonist-induced vasoconstriction over de-polarization-induced vasoconstriction (59).

In summary, the results of this study show that ET-1 caused a greater vasoconstriction in the FHR pulmonary arteries compared with the SDR vessels, and PKC antagonists significantly blocked the vasoconstrictor response to ET-1 in the hypertensive vessels, whereas the specific Rho-kinase inhibitor Y-27632 significantly attenuated the contractile effect of ET-1 in both normotensive and hypertensive vessels, with a greater inhibitory response occurring in the hypertensive arteries. ET-1 also caused a significant increase in RhoA expression in both normotensive and hypertensive pulmonary arteries, with the effect of ET-1 on expression being greater in the hypertensive state. Collectively, the data obtained with these inhibitors demonstrate that both PKC and Rho/Rho-kinase signaling may be upregulated in hypertensive pulmonary arteries to mediate the heightened pulmonary vascular response caused by agonist (ET-1) stimulation. In contrast, these particular signaling mechanisms may be less important under normotensive conditions. Thus these observed phenomena may be germane toward understanding signaling mechanisms of pulmonary hypertension, as well as employing both PKC and Rho-kinase inhibitors as potential therapeutic treatments for this disease state.

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