Rho kinase and Ca\(^{2+}\) entry mediate increased pulmonary and systemic vascular resistance in L-NAME-treated rats

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Dhaliwal JS, Casey DB, Greco AJ, Badejo AM Jr, Gallen TB, Murthy SN, Nossaman BD, Hyman AL, Kadowitz PJ. Rho kinase and Ca\(^{2+}\) entry mediate increased pulmonary and systemic vascular resistance in L-NAME-treated rats. Am J Physiol Lung Cell Mol Physiol 293:L1306–L1313, 2007. First published August 31, 2007; doi:10.1152/ajplung.00189.2007.—The small GTP-binding protein and its downstream effector Rho kinase play an important role in the regulation of vasconstrictor tone. Rho kinase activation maintains increased pulmonary vascular tone and mediates the vasoconstrictor response to nitric oxide (NO) synthesis inhibition in chronically hypoxic rats and in the ovine fetal lung. However, the role of Rho kinase in mediating pulmonary vasoconstriction after NO synthesis inhibition has not been examined in the intact rat. To address this question, cardiovascular responses to the Rho kinase inhibitor fasudil were studied at baseline and after administration of an NO synthesis inhibitor. In the intact rat, intravenous injections of fasudil cause dose-dependent decreases in systemic arterial pressure, small decreases in pulmonary arterial pressure, and increases in cardiac output. L-NAME caused a significant increase in pulmonary and systemic arterial pressures and a decrease in cardiac output. The intravenous injections of fasudil after L-NAME caused dose-dependent decreases in pulmonary and systemic arterial pressure and increases in cardiac output, and the percent decreases in pulmonary arterial pressure in response to the lower doses of fasudil were greater than decreases in systemic arterial pressure. The Ca\(^{2+}\) entry blocker isradipine also decreased pulmonary and systemic arterial pressure in L-NAME-treated rats. Infusion of sodium nitroprusside restored pulmonary arterial pressure to baseline values after administration of L-NAME. These data provide evidence in support of the hypothesis that increases in pulmonary and systemic vascular resistance following L-NAME treatment are mediated by Rho kinase and Ca\(^{2+}\) entry through L-type channels, and that responses to L-NAME can be reversed by an NO donor.

HA-1077; Rho kinase pathway; Ca\(^{2+}\) sensitization; pulmonary vascular bed; sodium nitroprusside; isradipine; N\(^{\text{ω}}\)-nitro-\(l\)-arginine methyl ester

RECENT ADVANCES in vascular research have identified the small GTP-binding protein RhoA and its downstream effector Rho kinase as important regulators of vasconstrictor tone (3, 4, 24, 28–30, 34, 39, 40, 46, 52). Rho kinase is a potential therapeutic target for the treatment of a number of cardiovascular diseases (1, 2, 12, 14, 16, 27, 31, 32, 37, 38, 51, 56). Rho kinase inhibitors are vasodilator agents that are effective in the treatment of pulmonary hypertension in rodent models and in humans (1, 2, 14, 16, 35, 37, 41, 42, 43, 47). Fasudil, originally described as a cellular calcium antagonist, has been shown to attenuate monocrotaline- and hypoxia-induced pulmonary hypertension in the rat (1, 6, 11, 12, 35, 42, 47). It has been reported that fasudil has selective pulmonary vasodilator activity in the monocrotaline-treated rat, and that Rho kinase inhibitors prevent the increase in pulmonary vascular resistance in response to nitric oxide (NO) synthesis inhibition in the fetal lamb (27, 48). It has also been reported that N\(^{\text{ω}}\)-nitro-\(l\)-arginine-induced increases in perfusion pressure in isolated hypoxia-exposed perfused rat lungs were reversed by Y-27632 and fasudil (43). However, the effects of fasudil on increases in pulmonary and systemic vascular resistance following NO synthesis inhibition have not been investigated in the intact-chest rat. In addition, it has been reported that fasudil inhibited hypoxia and potassium chloride-induced increases in Ca\(^{2+}\) in rat pulmonary arterial vascular smooth muscle cells and inhibited responses to potassium chloride in isolated perfused rat lungs, suggesting that Ca\(^{2+}\) signaling was impaired (54). To determine whether blockade of Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels plays a role in the pulmonary and systemic hypertensive responses to an NO synthesis inhibitor, responses to the Rho kinase inhibitor fasudil and the Ca\(^{2+}\) channel blocker isradipine were compared. The results of the present study show that the NO synthesis inhibitor N\(^{\text{ω}}\)-nitro-\(l\)-arginine methyl ester (l-NAME) causes large increases in total pulmonary and systemic vascular resistance in the intact rat that are reversed by fasudil, isradipine, and sodium nitroprusside. These data suggest that l-NAME-mediated vasoconstriction involves Rho kinase and Ca\(^{2+}\) entry.

METHODS

The experimental protocol used in these experiments was approved by the animal care committee of the Tulane University Medical School, and all procedures were conducted in accordance with institutional guidelines. For these experiments, adult male Sprague-Dawley rats (Harlan Sprague-Dawley) weighing 300–450 g were anesthetized with Inactin (100 mg/kg ip) and placed in the supine position on an operating table. Supplemental doses of Inactin were given as needed to maintain a uniform level of anesthesia. Body temperature was maintained with a heating lamp. The trachea was cannulated with a short segment of polyethylene (PE) tubing to maintain a patent airway, and the animals breathed room air enriched with 100% O\(_2\). A femoral artery was catheterized with PE-50 tubing for the measurement of systemic arterial pressure. The left jugular and femoral veins were catheterized with PE-50 tubing for intravenous (iv) injections or infusions of drugs and fluids. For measurement of pulmonary arterial

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pressure, a specially designed 3F catheter with a radio opaque marker and curved tip was passed from the right jugular vein into the right atrium and ventricle and main pulmonary artery under fluoroscopic guidance (Picker-Surveyor fluoroscope), as described previously (7). Pulmonary and systemic arterial pressures were measured by Namic transducers (Boston Scientific), and the pressure data were digitized by a Biopac MP100 data acquisition system. The vascular pressures were displayed and stored on a Dell personal computer. The cardiac output was determined by the thermodilution technique; 0.2 ml of 0.9% sodium chloride solution at room temperature was injected into the jugular vein catheter with its tip near the right atrium, and changes in blood temperature were measured by a 1.5- or 3.5F thermistor microprobe catheter (Columbus Instruments) positioned in the aortic arch from the left carotid artery. Cardiac output was measured by a Cardiomax-III (Columbus Instruments) cardiac output computer, and temperature dilution curves were stored on the Dell personal computer.

In the first set of experiments, responses to iv injections of fasudil and sodium nitroprusside were investigated under baseline control conditions. In the second set of experiments, responses to iv fasudil injections and iv injections and infusions of sodium nitroprusside were investigated starting 30–45 min after injection of L-NAME (100 mg/kg iv). In the third set of experiments, responses to iv injections of isradipine were determined in animals treated with L-NAME (100 mg/kg iv). The order of injection of the various doses of fasudil, sodium nitroprusside, and isradipine was randomized, and sufficient time was permitted for pressures to return toward baseline value. Fasudil HA-1077 [1-(5-isquinolinesulfonyl)homopiperzine, in HCl salt] (LC Laboratories), sodium nitroprusside, and N^\-nitro-L-arginine methyl ester hydrochloride (Sigma Aldrich) were dissolved in 0.9% sodium chloride solution, and isradipine (Sigma Aldrich) was dissolved in 0.9% sodium chloride with 0.1% Tween 20 (Sigma Aldrich). Working solutions were made on a daily basis or every other day, and vehicles for the drugs had no consistent effect on hemodynamic values.

The hemodynamic data are expressed as means ± SE. For comparison of decreases in pulmonary and systemic arterial pressure under baseline conditions and in L-NAME-treated animals, values are expressed as percent decrease to normalize pulmonary and systemic arterial pressures. Total pulmonary vascular resistance was calculated by dividing the mean pulmonary arterial pressure by the cardiac output. Systemic vascular resistance was calculated by dividing mean systemic arterial pressure by the cardiac output. The data were analyzed using paired or group t-tests or an analysis of variance with a post hoc test. The criterion for statistical significance was P < 0.05.

![Graphs showing decreases in pulmonary and systemic arterial pressures, increases in cardiac output, and decreases in total pulmonary and systemic vascular resistance in response to intravenous (iv) injections of fasudil in the rat under baseline control condition; n indicates no. of experiments. *Value is significantly different from control.](http://ajplung.physiology.org/)

**Fig. 1.** Bar graphs showing decreases in pulmonary and systemic arterial pressures, increases in cardiac output, and decreases in total pulmonary and systemic vascular resistance in response to intravenous (iv) injections of fasudil in the rat under baseline control condition; n indicates no. of experiments. *Value is significantly different from control.
Responses to fasudil under control conditions. Under control baseline conditions, iv injections of fasudil in doses of 0.1–3.0 mg/kg cause significant dose-related decreases in systemic arterial pressure, small decreases in pulmonary arterial pressure, and increases in cardiac output (Fig. 1). Total pulmonary and systemic vascular resistances were decreased significantly by all doses of fasudil studied (Fig. 1).

Responses after L-NAME administration. The administration of L-NAME at a dose of 100 mg/kg iv caused a significant increase in systemic and pulmonary arterial pressure and a significant decrease in cardiac output (Table 1). After administration of the NO synthesis inhibitor, iv injections of fasudil caused significant dose-dependent decreases in systemic and pulmonary arterial pressure and increases in cardiac output (Fig. 2). Total pulmonary and systemic vascular resistances were decreased significantly by injections of fasudil in L-NAME-treated rats (Fig. 2). The iv injections of isradipine in doses of 0.01–0.1 mg/kg caused dose-related decreases in pulmonary and systemic arterial pressure and increases in cardiac output in L-NAME-treated rats (Fig. 3).

Comparison of responses to fasudil. The decreases in pulmonary and systemic arterial pressures in response to iv injections of fasudil were compared in control and L-NAME-treated rats, and responses were expressed on a percent-decrease basis to normalize values in the pulmonary and systemic vascular beds; the results are summarized in Fig. 4. Under control conditions, when baseline tone in the pulmonary vascular bed

Table 1. Effect of L-NAME on systemic and pulmonary arterial pressure and on cardiac output

<table>
<thead>
<tr>
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<th>Systemic Arterial Pressure, mmHg</th>
<th>Pulmonary Arterial Pressure, mmHg</th>
<th>Cardiac Output, ml/min</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>99 ± 5</td>
<td>17 ± 1</td>
<td>102 ± 5</td>
</tr>
<tr>
<td>L-NAME (100 mg/kg iv)</td>
<td>137 ± 7*</td>
<td>31 ± 2*</td>
<td>56 ± 3*</td>
</tr>
<tr>
<td>n</td>
<td>21</td>
<td>15</td>
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Values are mean ± SE; n = no. of experiments. *P < 0.05 compared with corresponding control.

Fig. 2. Bar graphs showing decreases in pulmonary and systemic arterial pressures, increases in cardiac output, and decreases in total pulmonary and systemic vascular resistance in response to iv injections of fasudil after administration of N\textsuperscript{-}nitro-l-arginine methyl ester (L-NAME; 100 mg/kg iv); n indicates no. of experiments. *Value is significantly different from control.
is low, iv injections of fasudil (0.1–3 mg/kg) caused significantly greater decreases in systemic arterial pressure compared with decreases in pulmonary arterial pressure (Fig. 4A). In contrast to responses in control animals, the decreases in pulmonary arterial pressure at the 0.1 and 0.3 mg/kg iv doses of fasudil were significantly greater than the decreases in systemic arterial pressure in l-NAME-treated animals (Fig. 4B). The relative decreases in pulmonary and systemic arterial pressure in response to iv injections of isradipine were compared in l-NAME-treated rats, and these data are summarized in Fig. 4C. The percent decreases in pulmonary arterial pressure in response to iv injections of isradipine were not significantly different from the percent decreases in systemic arterial pressure (Fig. 4C).

Responses to sodium nitroprusside. Injections of sodium nitroprusside in doses of 1 and 3 μg/kg iv under control conditions caused small decreases in pulmonary pressure, large dose-dependent decreases in systemic arterial pressure, and small increases in cardiac output (Fig. 5A). After treatment with l-NAME (100 mg/kg iv), injection of sodium nitroprusside caused significant dose-related decreases in pulmonary and systemic arterial pressures and increases in cardiac output (Fig. 5B).

The iv infusion of sodium nitroprusside (1.5–15 μg/min) in l-NAME-treated animals caused a significant decrease in pulmonary and systemic arterial pressures to values not significantly different from baseline (Fig. 6A). When the sodium nitroprusside infusion was terminated, pulmonary and systemic arterial pressures rose to values similar to those measured before infusion of the NO donor (Fig. 6A). The possible interaction between fasudil and sodium nitroprusside was investigated by comparing decreases in pulmonary arterial pressure in response to fasudil before and after iv injections of sodium nitroprusside, and these data are summarized in Fig. 6B. The decreases in pulmonary arterial pressure in response to iv injections of fasudil were not significantly different when compared before and after injections of sodium nitroprusside in doses of 1–3 μg/kg iv in l-NAME-treated rats or in rats in which pulmonary arterial pressure was increased by an iv infusion of U46619 (Fig. 6C).
DISCUSSION

The major findings of this study are that iv injection of the NO synthesis inhibitor L-NAME produced large increases in total pulmonary and systemic vascular resistance, and the Rho kinase inhibitor fasudil, the L-type Ca$^{++}$ channel blocking agent isradipine, and the NO donor sodium nitroprusside reversed pulmonary and systemic hypertensive responses to L-NAME.

The results of the present study show that when rats are treated with L-NAME, iv injections of fasudil cause dose-dependent decreases in pulmonary and systemic arterial pressures. Inasmuch as cardiac output was increased, these data show that fasudil decreases total pulmonary and systemic vascular resistance in L-NAME-treated animals. The increases in cardiac output were not dose related, and the reason for the lack of a dose-response relationship is unclear. These data are consistent with the results in the fetal lamb pulmonary circulation and in the isolated hypoxia-exposed perfused rat lung, in which vasoconstrictor responses to the NO synthesis inhibitor N$^\omega$-nitro-$l$-arginine were prevented or reversed by Y-27632 or fasudil (43, 48).

Although fasudil caused significant dose-dependent decreases in pulmonary arterial pressure when total pulmonary vascular resistance was increased by L-NAME, the Rhokinase inhibitor produced only modest decreases in pulmonary arterial pressure under control conditions, when baseline tone in the pulmonary vascular bed is low (19–22). It has been reported that fasudil decreases pulmonary arterial pressure without altering systemic arterial pressure in monocrotaline-treated rats (1, 27). However, in the present study, iv administration of fasudil in a wide range of doses decreased both systemic and pulmonary arterial pressures under baseline conditions and in L-NAME-treated animals. This finding is in agreement with results showing a reduction in systemic arterial pressure in response to the Rho kinase inhibitor Y-27632 in endothelial NO synthase knockout mice and spontaneously hypertensive rats (36). The reason for the difference in results is uncertain but may involve the upregulation of Rho kinase in monocrotaline-treated rats or the dose and route of administration of the Rho kinase inhibitor (27, 41). In this regard, inhaled Rho kinase inhibitors have been shown to produce a selective decrease in pulmonary arterial pressure in the rat (42). Although the Rho kinase inhibitor did not have a selective pulmonary vasodilator effect when injected iv, the percent decrease in pulmonary arterial pressure was greater than the percent decrease in systemic arterial pressure in response to the lower doses of fasudil in L-NAME-treated animals. In contrast to results in L-NAME-treated rats, the decreases in systemic arterial pressure were greater than the decreases in pulmonary arterial pressure under baseline conditions at all doses of

![Fig. 5. Bar graphs comparing the effect of iv injections of sodium nitroprusside on pulmonary and systemic arterial pressures and cardiac output in control (A) and in L-NAME (100 mg/kg iv)-treated rats (B); n indicates no. of experiments. *Response is significantly different from control.](http://ajplung.physiology.org/)

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The observation that fasudil and other Rho kinase inhibitors decrease both total pulmonary and systemic vascular resistance in the intact rat is similar to recent observations in the fetal lamb (48).

It has recently been reported that Rho kinase inhibitors impair Ca++ entry in rat pulmonary artery smooth muscle cells exposed to hypoxia or depolarizing potassium solutions (54). These data, along with the observation that the potassium chloride-induced vasoconstrictor is inhibited in the isolated perfused rat lung, suggest that fasudil and Y-27632 inhibit Ca++ signaling (54). To provide information on the role of Ca++ entry, responses to isradipine, an L-type calcium channel blocking agent, and fasudil were compared in L-NAME-treated rats. The results of these studies show that both fasudil and isradipine were capable of reversing pulmonary and systemic vasoconstriction induced by NO synthesis inhibition. The results of these experiments suggest that increases in total pulmonary and systemic vascular resistance following L-NAME treatment are mediated by Rho kinase and Ca++ entry through L-type channels. The comparison of responses to fasudil and isradipine does not directly identify the mechanism of fasudil-induced vasodilation and suggests that both Ca++ entry and Rho kinase inhibition can reverse L-NAME-induced vasoconstriction.

There is disagreement about the effects of NO synthesis inhibitors and the role of endothelial-derived NO in the maintenance of baseline tone in the pulmonary vascular bed of the rat (18). The results of most studies in isolated perfused rat lungs show little or no effect of L-NAME and other NO synthesis inhibitors on pulmonary perfusion pressure (5, 9, 13, 15, 19). Although most studies in intact rats show no significant pulmonary vasoconstrictor response to NO synthesis inhibitors, increases have been reported in some studies, and these have been attributed to “nonspecific effects” (18, 49).

It has been reported that treatment with an NO donor inhibited store and receptor-operated Ca++ entry in isolated rat pulmonary arteries (26). To determine whether an interaction between fasudil and sodium nitroprusside occurred in the present experiment, responses to fasudil were compared before and after injections of sodium nitroprusside. The results of these experiments show that pulmonary vasodilator responses to fasudil were not affected by injections of the NO donor in doses of 1–3 μg/kg iv.

The results in L-NAME-treated rats show that iv administration of fasudil in a wide range of doses caused dose-dependent decreases in pulmonary arterial pressure and total pulmonary vascular resistance and are consistent with results in isolated hypoxia-exposed perfused rat lungs and the fetal lamb pulmonary circulation (43, 48). These data are consistent with the hypothesis that Rho kinase and Ca++ entry mediate the increase in pulmonary vascular resistance after NO synthesis inhibition (48). The present data showing that fasudil decreases systemic vascular resistance in control and in L-NAME-treated rats suggest that baseline vasoconstrictor tone and L-NAME-induced vasoconstriction are mediated in part by Rho kinase in the systemic vascular bed. The observation that isradipine reversed the pulmonary and systemic hypertensive response to L-NAME also suggests a role for Ca++ entry through L-type channels. The finding that both total pulmonary and systemic vascular resistance in control and in L-NAME-treated rats suggest that baseline vasoconstrictor tone and L-NAME-induced vasoconstriction are mediated in part by Rho kinase in the systemic vascular bed.
vascular resistances are decreased by fasudil under baseline conditions is different from the results in the literature, in which Y-27632 had no significant effect on total pulmonary vascular resistance under baseline conditions in the rat (43). The reason for the difference in results is uncertain.

Recent reports in the literature indicate the importance of Rho kinase in the regulation of vascular smooth muscle tone and as a therapeutic target for treatment of a number of cardiovascular diseases (1, 2, 12, 14, 16, 27, 30, 31, 37, 38, 50, 51, 56). The small GTPase Rho and its downstream effector Rho kinase regulate smooth muscle calcium sensitization (3, 4, 24, 25, 28, 29, 30, 34, 39, 52). The myosin-binding unit of smooth muscle myosin phosphatase is a substrate, and Rho kinase activation inhibits smooth muscle phosphorylase activity and increases myosin light chain phosphorylation and smooth muscle contraction (39, 52). Rho kinase inhibitors increase myosin light chain dephosphorylation and cause vasodilatation (6, 11, 12, 44, 53). Fasudil has been shown to be effective in the treatment of monocrotaline- and hypoxia-induced pulmonary hypertension in rodents and primary pulmonary hypertension in humans (2, 16, 17, 23, 24, 27, 41, 42). The observation that fasudil has robust pulmonary vasodilator activity in L-NAME-treated rats suggests that Rho kinase inhibitors may be useful in the treatment of pulmonary hypertensive disorders in which endothelial function is impaired, and it has been shown that fasudil improved endothelial function in humans with coronary artery disease (8, 45). Rho kinase inhibitors reduce systemic arterial pressure and may be effective in the treatment of essential hypertension (36, 37).

In summary, results of the present study show that fasudil has significant vasodilator activity in the pulmonary and systemic vascular beds of the rat. The NO synthesis inhibitor L-NAME produced large increases in total pulmonary and systemic vascular resistance that could be reversed by fasudil, isradipine, and an NO donor. After treatment with L-NAME, pulmonary and systemic vasodilator responses to fasudil were increased, and pulmonary vasodilator responses became dose dependent. Moreover, when decreases in pressure in response to fasudil were compared on a percent-decrease basis to normalize values, decreases in pulmonary arterial pressure in response to lower doses of fasudil were greater than decreases in systemic arterial pressure after L-NAME treatment, whereas a different pattern of response was observed with isradipine. The present data suggest that Rho kinase is involved in the normal physiological regulation of tone in the pulmonary and systemic vascular beds, and that Rho kinase and increased Ca++ entry mediate the increase in vasconstrictor tone when NO synthesis is inhibited in the rat.

GRANTS

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REFERENCES

RESPONSES TO FASUDIL AND ISRADIPINE IN L-NAME-TREATED RATS

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