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L-Arginine Reduces Right Heart Hypertrophy in Hypoxia-Induced Pulmonary Hypertension

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Endothelin (Et) and nitric oxide (NO) may serve as chemical mediators of hypoxia-induced pulmonary hypertension. Plasma levels of Et1 were elevated 2-fold while levels of nitrate, a NO metabolite, decreased in rats exposed to 10 days of hypoxia (10% O_2). Administration of L-arginine, the precursor for NO, decreased Et, increased nitrate, and decreased right ventricular hypertrophy in hypoxic animals. By increasing plasma NO levels, the right ventricular hypertrophy and right heart failure seen in hypoxia-induced pulmonary hypertension in human patients may be prevented.

Pulmonary hypertension, characterized by increased pulmonary vascular resistance, eventually leads to right heart failure and death. The cause of primary pulmonary hypertension is generally unknown, while hypoxia is the most common cause of secondary pulmonary hypertension. It is thought that exposure to hypoxia initially results in vasoconstriction which causes a rapid increase in pulmonary blood pressure followed by the development of polycythemia and remodeling of the pulmonary artery walls (1). A rise in endothelin (Et), the most potent vasoconstrictor known to date (2), may induce the pulmonary vasoconstriction. When released by endothelial cells, Et acts locally to constrict vascular smooth muscle (VSM) cells. Plasma Et levels were increased in patients with primary and secondary pulmonary hypertension (3) and in rats exposed to hypoxia (4). Endothelin 1 (Et1), an isoform of endothelin, has been shown to increase mean arterial blood pressure (2,5). Patients with congestive heart failure and increased pulmonary pressure and vascular resistance, had three-fold greater plasma levels of Et than normal subjects (6). Et1 has mitogenic effects on VSM cells, is a growth factor for VSM cells (7), and has been shown to induce hypertrophy in cultured cardiac myocytes (8). Rats with left ventricular hypertrophy were found to have increased expression of the endothelin precursor prepro Et1 mRNA and increased tissue content of mature Et1 in the left ventricle (9). Et1 may therefore be involved in the development of cardiac hypertrophy due to pressure overload and the vasoconstriction and smooth muscle cell proliferation in hypertension.

Nitric oxide (NO), an endogenous nitrovasodilator produced by endothelial and smooth muscle cells (10), may also play a role in pulmonary hypertension. Chronic hypoxic pulmonary hypertension impairs NO release (11), and reduces NO levels in animals (12) and humans (13). Blockage of NO synthesis in hypoxic rats by NO analogs increased hypoxic pulmonary vasoconstriction, but had no effect on pulmonary arterial pressure in normal rats (14). Return of hypoxic animals to normoxia restored normal pulmonary endothelium-dependent relaxation (12). These results suggest that a decrease in O_2 tension may account for reduced NO levels during hypoxic pulmonary hypertension. We therefore investigated the roles of Et and NO in hypoxia-induced pulmonary hypertension.

METHODS

Animals and hypoxic exposure. Male Sprague-Dawley rats (~200g; Charles River Laboratories, Wilmington, MA) were housed in polycarbonate chambers (size: 51 × 41 × 44 cm; 3-4 rats/chamber) in which humidified gas flowed into the chamber at a rate of 400 ml/minute (15). Control rats breathed air (21% oxygen) and experimental rats inhaled 10% oxygen-90% nitrogen for 10 days followed by three or seven days of recovery in air. Chambers were opened 1-2 times daily for feeding, cleaning and injections. Rats were provided with water and Purina Rat Chow *ad libitum* (Ralston Purina Co., St. Louis, MO).

Animals were randomly placed into one of four groups: controls injected with saline, controls with L-Arg injections, 10 days of hypoxia with saline injections or 10 days of hypoxia injected with L-Arg. Animals were injected with sterile L-Arg (300 mg/kg body weight) intraperitoneally (I.P., 0.2 ml) twice daily, 12 hours apart for 10 days or with sterile 0.9% NaCl (I.P., 0.2 ml) twice daily for 10 days. The first injections were given immediately before the animals were placed in the hypoxic chambers on day one. The last injections were given 12 hours before tissue samples were taken.

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TABLE 1

Effect of Hypoxia on Right Ventricular Hypertrophy, Pulmonary Artery Wall Thickness and Plasma Levels of Et1

	% Packed cell volume	RV/(LV + S)	Pulmonary artery wall thickness (mm)	Et1 pg/ml
Control	40.8 ± 0.3	0.260 ± 0.008	0.083 ± 0.003	102.5 ± 6.5
3 day hypoxia	59.4 ± 0.09[a]	0.291 ± 0.007[a]	0.086 ± 0.003	152.5 ± 21.7[a]
10 day hypoxia	64.5 ± 1.4[a]	0.342 ± 0.023[a,b]	0.118 ± 0.005[a,b]	155.0 ± 35.0[a]

Note. Hematocrit, right heart hypertrophy and pulmonary artery wall thickness were measured as described in the Methods Section following 3 or 10 days of hypoxia. Plasma was analyzed for Et1 as described in the Methods Section. Data represents mean ± SEM. For all groups, n = 5.

[a] $p \leq 0.05$ compared to control; [b] $p \leq 0.05$ compared to 3 days of hypoxia.

Blood sampling. Animals were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. A blood sample for hematocrit was collected from the abdominal aorta in a heparinized hematocrit tube. To measure plasma Et levels, blood was collected in a polypropylene vacuum tube containing EDTA (1 mg/ml blood) and aprotinin (500 KIU/ml blood) by cardiac puncture of the right ventricle. Blood samples were centrifuged (1050 xg, 20 minutes, 0-4° C) and the plasma collected and stored at −70° C until assayed. For amino acid analysis, plasma was acidified with TCA (final concentration of 7.5%) and centrifuged (11,000 xg, 15 min, 25° C). The supernatant was collected, lyophilized, and reconstituted in 300 μl of 0.2 M sodium citrate, pH 3.5. Plasma amino acid concentrations were measured on a Beckman automated amino acid analyzer.

Examination of ventricular weights and pulmonary artery morphology. Hearts were taken out and the atria removed. The right ventricle (RV), septum (S) and left ventricle (LV) were separated and dried. Dry weights were determined and the ratios of RV/(LV+S) were calculated as described previously (15). Pulmonary arteries (PA) were excised and placed in 10% formalin (buffered to pH 6.8 with sodium phosphate), and embedded in paraffin. Sections (10 m) were stained with hematoxylin and eosin and the medial wall thickness of each pulmonary artery was measured using a calibrated ocular micrometer.

Measurement of endothelin and nitrite. Following acidification with trifluoroacetic acid (TFA, HPLC grade, Pierce, Rockford, IL) Et was extracted using 200 mg C-18 Sep-pak columns and levels of Et1 were measured using a radioimmunoassay kit according to the manufacturer's directions (Peninsula Laboratories, Inc. Belmont, CA). Measured Et1 levels varied between experiments (values for controls ranged from 18-225 pg Et1/ml plasma), but were consistently elevated following hypoxia. Nitrite and nitrate levels were measured in plasma or the plasma flow-through from the Sep-pak columns. Plasma samples (300 μl) were acidified with 1 M perchloric acid (final concentration 0.2 M), neutralized to pH 7 with 2.5 M KOH-0.1 M Pipes, and centrifuged (1050 xg, 10 min). Nitrate was converted to nitrite (16) and nitrite was measured spectrophotometrically (17).

Statistical analysis. All data were analyzed using either the unpaired Student's t test or the one way ANOVA and the Fisher's protected least significant difference test.

RESULTS

Hypoxia increases total red blood cell mass and this was reflected by an increase in hematocrit (% packed cell volume) following 3 and 10 days of hypoxia (Table 1). Right ventricular hypertrophy, determined by the ratio of dry ventricular weights [RV/(LV + S)], was observed as early as 3 days of hypoxia and was even more pronounced by 10 days of hypoxia (Table 1). Medial wall thickness of the main pulmonary artery was not different from controls following 3 days of hypoxia but increased by 42% following 10 days of hypoxia (Table 1).

Since Et is a potent vasoconstrictor and promotes smooth muscle cell proliferation, this raised the possibility that Et may play a role in hypoxia-induced pulmonary vasoconstriction and vascular remodeling. Following 3 and 10 days of hypoxia, plasma Et1 levels were significantly elevated compared to controls (Table 1). Plasma levels of nitrite, an end product of the vasodilator NO, were significantly ($p \leq 0.05$, n = 4-5) decreased after 10 days of hypoxia to 4.5 ± 0.4 μM compared to control levels of 6.0 ± 0.5 μM. Thus, the increase in plasma levels of Et and the decrease in NO levels may contribute to the development and maintenance of hypoxic pulmonary hypertension.

The physiological changes which occur during hypoxia are reversible with recovery in normoxia (18). Following 3 and 7 days of recovery in room air, hematocrit levels were elevated above control levels, but were significantly lower than those found in animals exposed to 10 days of hypoxia (Table 2). Hematocrit levels remain elevated even after 14 days of recovery from hypoxia (data not shown). After 7 days of recovery, plasma Et levels had decreased significantly from the

TABLE 2

Et1 Levels Following Recovery from Hypoxia

	% Packed cell volume	pg Et1/ml
Control	39.3 ± 0.7	24.0 ± 4.1
10 day hypoxia	62.9 ± 0.8[a]	59.0 ± 1.7[a]
3 day recovery	58.3 ± 1.4[a,b]	56.7 ± 4.9[a]
7 day recovery	52.3 ± 1.5[a,b,c]	38.7 ± 4.3[a,b,c]

Note. Hematocrit and plasma Et1 levels were determined in animals exposed to hypoxia (10% O_2) for 10 days and following recovery in air for up to 7 days. Data represents the mean ± SEM. For all groups, n = 4–6.

[a] $p \leq 0.05$ compared to control; [b] $p \leq 0.05$ compared to 10 days of hypoxia; [c] $p \leq 0.05$ compared to 3 days of recovery.

TABLE 3

Effects of Hypoxia and L-Arginine Treatment on Right Ventricular Hypertrophy, Plasma Nitrite and Et1

	% Packed cell volume	RV/(LV + S)	μM Nitrite	pgEt1/ml
Control/saline	34.9 ± 0.8	0.303 ± 0.012	7.5 ± 1.4	34.7 ± 7.2
Control/L-Arg	34.2 ± 0.6	0.302 ± 0.010	11.6 ± 1.5[a]	44.3 ± 8.7
10 day hypoxia/saline	51.7 ± 2.0[a]	0.578 ± 0.061[a]	5.9 ± 1.7	66.6 ± 4.1[a]
10 day hypoxia/L-Arg	53.9 ± 1.6[a]	0.440 ± 0.009[a,b]	11.1 ± 4.0[a]	29.1 ± 2.8

Note. Hematocrit, ratio of ventricular weights, plasma nitrite and Et1 was measured in animals exposed to 10% O_2 or room air for 10 days and injected twice daily with either 300 mg/kg body weight L-Arg or saline. Data represents mean ± SEM. For all groups, n = 5–8.
[a] $p \leq 0.05$ compared to control/saline; [b] $p \leq 0.05$ compared to hypoxia/saline.

increased levels measured after 10 days of hypoxia, but were still elevated from control levels. Like the hematocrit, plasma Et levels appear to slowly return to normal during recovery.

Effect of L-Arg administration on hypoxia. Chronic hypoxia results in a decreased release of NO as evidenced by the decrease in plasma nitrite (12). This decrease may contribute to the onset of pulmonary hypertension. Plasma levels of Arg, the substrate for nitric oxide synthase (NOS), were not significantly different during hypoxia, indicating that plasma Arg levels are maintained at normal concentrations during hypoxic exposure (data not shown). It was hypothesized that an increase in the supply of L-Arg would promote NO production. Therefore, L-Arg was administered (300 mg L-Arg/kg body weight or 0.2 ml vehicle, IP twice daily) during hypoxia to determine whether increased plasma Arg levels would increase NO levels and prevent or reduce some of the changes observed with hypoxic pulmonary hypertension. L-Arg treatment did not significantly affect hematocrit levels in control or hypoxic animals (Table 3). Plasma levels of nitrite, measured as an indicator of NO, increased in both control and hypoxic animals administered L-Arg compared to animals injected with vehicle (Table 3) indicating that L-Arg treatment stimulates NO synthesis. Treatment of hypoxic animals with L-Arg prevented the increase in plasma Et levels. L-Arg did not significantly affect Et levels of control animals indicating that L-Arg administration did not affect Et synthesis. These data suggest that there may be a feedback mechanism between NO production and Et release (and/or synthesis) which work in concert to maintain Et levels within a normal range.

Elevated Et levels and decreased NO levels during hypoxia may contribute to the development of right ventricular hypertrophy. Following 10 days of hypoxia, the ratio of ventricular weights were increased 2-fold in the saline-treated group compared to control animals. Hypoxic animals injected with L-Arg showed a 50% decrease in the RV/(LV + S) compared to the hypoxic animals injected with saline (Table 3). Thus, administration of L-Arg during hypoxia increases NO synthesis and diminishes right ventricular hypertrophy.

DISCUSSION

The sequence of events leading to the development of pulmonary hypertension is unknown. One possible scenario is that the presence of low O_2 tension stimulates the release of Et and decreases the production of NO resulting in pulmonary vasoconstriction. In response, pulmonary pressure increases (1) by generating additional mechanical forces on the blood vessel wall and (2) stimulating the expression of Et and other growth factors resulting in the thickening of the blood vessel wall due to hypertrophy and hyperplasia of smooth muscle cells (19) and increased deposition of newly synthesized connective tissue (15, 20). This remodeling serves to decrease the cross-sectional area of the blood vessel lumen, further obstructing the flow of blood (20). As the pressure increases, pulmonary vascular resistance increases which increases the work load for the heart. With time, the heart is unable to meet the increasing work load, right ventricular hypertrophy develops, and eventually, right heart failure occurs.

The finding that levels of plasma Et increase and NO synthesis decrease during hypoxia suggests that perhaps these compounds, which have a direct influence on vascular tone, play a role in the onset or maintenance of pulmonary hypertension. Et and NO may also function to regulate each other's levels. We found that hypoxic animals treated with L-Arg had normal plasma Et levels compared to increased levels in chronic hypoxic animals injected with saline. This indicates that there may be a feedback loop between Et and NO. If Et levels become elevated, increased NO synthesis may be triggered and NO may then act to down regulate the synthesis of Et. The disruption of this loop during hypoxia may contribute to pulmonary hypertension (Figure 1). This interruption results in elevated Et levels and below normal NO levels, leading to vasoconstriction and increased blood pressure. Intravenous infusion of L-Arg to patients with peripheral arterial occlusive disease induced peripheral vasodilation which may have been mediated by NO production (21). Chronic administration of L-Arg may up regulate NOS and result in an increase in NO levels under conditions in which NO synthesis is diminished. Per-

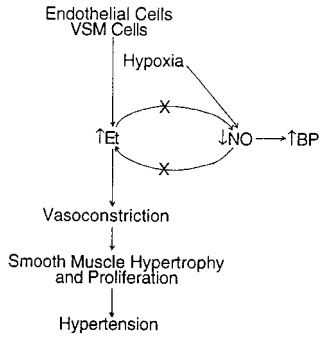

FIG. 1. Possible mechanism for the roles of endothelin and nitric oxide in pulmonary hypertension. Exposure to hypoxia causes increased Et levels which leads to vasoconstriction and then SMC hypertrophy and proliferation. Hypoxia also causes a decrease in NO levels which contributes to increasing blood pressure (BP). The feedback loop between NO and Et may be disrupted during pulmonary hypertension.

haps treatment of patients with pulmonary hypertension should include initial short-term inhalation of NO and L-Arg dosing. Administration of L-Arg may then up regulate NOS and result in an increase in endogenous NO production.

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