# Endothelium-derived relaxing factor activity in rat lung during hypoxic pulmonary vascular remodeling

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ZHAO, LAN, D. E. CRAWLEY, J. M. B. HUGHES, T. W. EVANS, AND R. J. D. WINTER. *Endothelium-derived relaxing factor activity in rat lung during hypoxic pulmonary vascular remodeling.* J. Appl. Physiol. 74(3): 1061–1065, 1993.—We have investigated the role of endothelium-derived relaxing factor in modulating hypoxic pulmonary vasoconstriction by inhibiting its synthesis with the false substrate $N^G$-monomethyl-L-arginine (L-NMMA) in the isolated blood-perfused lungs of Wistar rats after chronic hypoxia (CH, fractional inspiratory $O_2$ concentration 10%) for 15 h, 2 days, and 7 days. Lungs were perfused with blood of normal hematocrit at constant flow (18 ml/min) ventilated with *1*) 95% air-5% $CO_2$ (normoxia) and *2*) 2% $O_2$-5% $CO_2$-93% $N_2$ (hypoxia) and were studied in the absence and presence of L-NMMA (30 and 300 $\mu$M) or L-arginine (L-Arg, 1 and 6 mM) in separate groups. Pulmonary arterial pressure (Ppa) rose incrementally with hypoxic exposure (all $P < 0.05$ vs. normoxic control group). Hypoxic pulmonary vasoconstriction (HPV) was markedly reduced after 15 h and 2 days of CH: the mean increases in Ppa ($\Delta$Ppa) in hypoxia were 15.3, 3.5, 3.8, and 13.6 mmHg in control rats and rats exposed to 15 h ($P < 0.05$ vs. control and 7 days of CH), 2 days ($P < 0.001$ vs. control and 7 days of CH), and 7 days of CH, respectively. Ppa in control rats and rats exposed to 15 h, 2 days, and 7 days of CH were 137, 179, 184, and 166% of control, respectively, after 30 $\mu$M L-NMMA (all $P < 0.05$ when expressed as percent change vs. no L-NMMA). Similar augmentation in HPV was seen after 30 $\mu$M L-NMMA, with all hypoxic groups having a greater response than control groups. Hypoxic Ppa in control rats and rats exposed to 15 h, 2 days, and 7 days of CH were 96, 85 ($P < 0.05$ vs. control), 82 ($P < 0.01$ vs. control), and 91% of control after 1 mM L-Arg and 88, 77 ($P < 0.05$ vs. control), 56 ($P < 0.001$ compared with control and 7 days of CH), and 82% of control after 6 mM L-Arg. The attenuation of HPV at 15 h and 2 days of CH with partial restoration toward normal by L-NMMA suggests that the early phase of CH exposure is associated with release of endothelium-derived relaxing factor.

pulmonary circulation; pulmonary artery; hypoxia; nitric oxide; $N^G$-monomethyl-L-arginine; L-arginine

SMALL MUSCULAR PULMONARY ARTERIES undergo rapid structural changes in response to prolonged hypoxia with concomitant increases in pulmonary arterial pressure (Ppa) (9, 17, 18, 21), and such arteries also constrict in response to airway hypoxia [hypoxic pulmonary vasoconstriction (HPV)] (27). Although the mechanism for both of these responses is unknown, smooth muscle hyperplasia and hypertrophy, the predominant changes in remodeling (9, 17), are accompanied by alteration of the normal appearance of the endothelial cell with the development of edema and subcellular blebs on transmission electron microscopy (18). A role for the endothelial cell in vascular remodeling is further suggested by the finding by Tozzi and colleagues (26) that, in isolated rat pulmonary artery segments, pressure-induced connective tissue synthesis is dependent on intact endothelium. Furthermore, endothelial cells in culture have been shown to release growth factors under conditions of acute hypoxia (14). Studies on intact vessels in vitro indicated that the endothelial cell can influence many vascular responses and has a major role in the regulation of vasomotor tone through relaxing and constricting factors (4, 6).

Endothelium-derived relaxing factor (EDRF) is now considered to be nitric oxide or a related compound (10, 20), and the identification of the biosynthetic pathway of EDRF and the development of stable specific analogues of L-arginine that act as a false substrate (22), thus inhibiting production of EDRF, have allowed studies of the role of EDRF in the normal control of vascular tone (19, 20). In the isolated blood-perfused rat lung, we and others showed that the false substrate for the L-arginine biosynthetic pathway, $N^G$-monomethyl-L-arginine (L-NMMA), is capable of augmenting HPV while having no effect on resting pulmonary artery tone (3, 8, 13, 23). The action of the vasoconstrictor angiotensin II is reportedly also associated with release of EDRF (15, 30), evidenced by increased pressor response after hemoglobin or methylene blue in isolated rings of both rat aorta and bovine carotid artery (7).

Previous work has thus suggested a role for endothelium-derived factors in modulating acute HPV and has indicated that the endothelial cell is involved in the production of growth factors and in the transduction of pressure changes in small pulmonary arteries undergoing rapid structural change. The present experiments were designed to determine whether the release of EDRF occurred during the sustained constrictor stimulus of chronic hypoxia and specifically whether the effect of L-NMMA or L-arginine on HPV was altered during the early stages of pulmonary vascular remodeling.

## METHODS

*Animals and environmental chamber.* Specific pathogen-free albino male Wistar rats (weight 200–250 g) were used throughout. They were placed in a normobaric hyp-

oxic environmental chamber where the fractional inspired $O_2$ concentration was maintained at 10%, with excess humidity and $CO_2$ removed by means of scrub filters (29). Gas was sampled periodically and analyzed by mass spectrometer, and fractional inspired $CO_2$ concentration was shown to be <0.04% at all times. Relative humidity was always less than ambient, and temperature was constantly maintained within 1°C of air temperature. Littermate normoxic controls were placed in the same room and exposed to the same light-dark cycle. Groups were kept in the hypoxic environment for 15 h, 2 days, and 7 days. Food and standard laboratory chow were given to all animals ad libitum.

*Isolated and blood-perfused rat lung.* The isolated rat lung preparation of Emery and colleagues (5) was used with minor modification. Rats were anesthetized (diazepam 0.6 mg/kg ip, fentanyl 0.15 mg im). Blood was taken from a normoxic donor rat, so the hematocrit was identical in all groups. The lungs were left in situ after the trachea had been cannulated. A metal cannula was introduced through the right ventricle and tied with a ligature. The left atrium was cannulated directly with a preformed cannula, the pressure difference across all cannulas used being <1 mmHg up to flow rates of 20 ml/min. Blood from control rats was heparinized and perfused into the pulmonary artery by means of a Watson-Marlow roller pump (model 503U, Falmouth, Devon, UK) and a plastic reservoir suspended in the water bath; blood was returned to the reservoir from the left atrial cannula. Reservoir temperature was maintained at 38°C. The perfusion circuit consisted of the reservoir, plastic tubing, a bubble trap, and a connection to the manometer placed at the level of the pulmonary artery; a short length of silicon rubber was used for injections on the inlet side. Ppa was measured using a Gould pressure transducer (model P23XL) and a three-channel thermal array recorder (model RE 550, Gould). The perfusion blood flow rate was kept constant at 18 ml/min, which gave Ppa within the normal range (15.2 mmHg, 95% confidence interval, $n$ = 18 for control rats).

*Ventilation and reactivity to hypoxia.* The lungs were ventilated with 5% $CO_2$ in air, and blood pH was adjusted by the addition of small quantities of sodium bicarbonate (1 mmol/l) to maintain reservoir pH at 7.40. Hypoxic vasoconstriction was assessed by changing the ventilation gas to 2% $O_2$-5% $CO_2$-93% $N_2$ and allowing Ppa to reach a plateau. The interval between successive tests was 8 min. The lungs were ventilated using a ventilation pump for small animals at a constant rate (32 breaths/min) to a maximum end-expiratory pressure of 4 mmHg, giving a tidal volume of 5–7 ml. Intratracheal pressure was recorded continuously.

*Effects of L-NMMA and L-arginine on vasoreactivity.* Successive hypoxic challenges (usually 3) were given until the increment in Ppa was constant, because the magnitude of the hypoxic pressor response is known to increase during the first two challenges. Pulmonary vascular reactivity to two doses of L-NMMA (reservoir concn 30 and 300 μM, Wellcome, Beckenham, UK) and to L-arginine (Sigma Chemical, reservoir concn 1 and 6 mM) was assessed in separate groups of animals at all time points. Drugs were given in a volume of <0.2 ml added to the 20-ml blood reservoir. The drugs or vehicle was added before a hypoxic challenge to assess their effects on baseline Ppa. This was followed by a hypoxic challenge when the effects of Ppa had peaked (or equivalent time point) to assess the effects of the increment in Ppa produced in hypoxia. Drugs or vehicle was given 5 min before challenge with hypoxia, and their effects on baseline Ppa and on the increment in Ppa (ΔPpa) produced by hypoxia were examined.

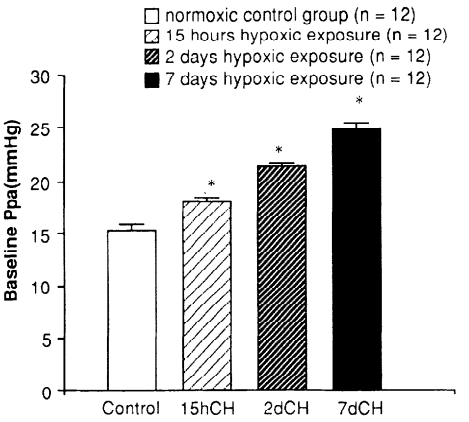


FIG. 1. Significant stepwise increases in baseline pulmonary arterial pressure (Ppa) in normoxic and 3 hypoxic exposure groups. CH, chronic hypoxia. * $P < 0.001$ compared with normoxic group.

*Vasoreactivity to angiotensin II.* Because an altered hypoxic pressor response was seen after 15 h and 2 days of chronic hypoxia, the effect of angiotensin II on resting pulmonary artery tone was studied. Angiotensin II (Sigma Chemical, final reservoir concn 50 nM) was added to the reservoir in the absence and presence of 30 μM L-NMMA to assess the effect on resting Ppa.

*Data manipulation and statistical analysis.* Resting Ppa and ΔPpa were expressed as means ± SD. The effect of L-NMMA and L-arginine on the ΔPpa was expressed as previously (13): ΔPpa with drug/ΔPpa vehicle × 100%. Data after L-NMMA and L-arginine are expressed in both absolute terms (mmHg) and as percent change in ΔPpa. Comparisons between groups were made using analysis of variance and Student's $t$ test as appropriate.

## RESULTS

*Ppa and HPV.* Resting Ppa in the normoxic control group is shown in Fig. 1. Graded increases in Ppa were seen in the chronic hypoxic groups in proportion to the duration of exposure to hypoxia (Fig. 1). The response to an acute hypoxic challenge was markedly attenuated in the early stages of hypoxic exposure (Fig. 2); the mean rise in Ppa (ΔPpa) in the four groups was 15.3 ± 1.6 mmHg in normoxic control compared with 3.5 ± 0.4 mmHg at 15 h and 3.8 ± 0.3 mmHg at 2 days (both $P <$ 0.001 compared with control). After 7 days of hypoxia, ΔPpa was 13.6 ± 1.7 mmHg ($P > 0.05$ compared with control, Fig. 2).

*Effects of L-NMMA on vasoreactivity.* L-NMMA augmented HPV in normoxic controls (Fig. 3*A*). The effect of 30 and 300 μM L-NMMA on HPV ΔPpa for the normoxic control and chronic hypoxic groups is shown in Fig. 4*A*. Augmentation of HPV is seen in all the groups exposed to chronic hypoxia. Although ΔPpa was reduced

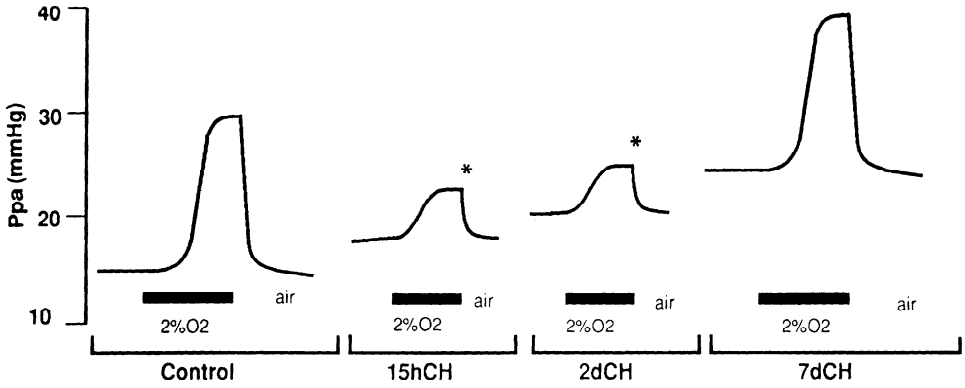


FIG. 2. Representative tracings of resting Ppa and acute hypoxic pulmonary vasoconstrictor (HPV) responses in normoxic control (control) rats and rats exposed to 15 h (15hCH), 2 days (2dCH), and 7 days of hypoxia (7dCH). Acute HPV was attenuated in 15hCH and 2dCH rats. * $P < 0.001$ compared with normoxic group.

after 15 h and 2 days of chronic hypoxic exposure (Fig. 2), HPV ΔPpa L-NMMA/HPV ΔPpa was significantly greater in all chronically hypoxic rats than in the normoxic control group when shown as percent increment (Fig. 5). There were no significant differences between 30 and 300 μM L-NMMA in their effects on ΔPpa. Both 30 and 300 μM L-NMMA caused slight but not significant increases in baseline Ppa in normoxic and hypoxic rats.

*Effects of L-arginine on vasoreactivity.* L-Arginine attenuated HPV in normoxic controls (Fig. 3*B*). Both in normoxic controls and after 7 days of hypoxia (when HPV ΔPpa was similar), there was significant attenuation by 1 and 6 mM L-arginine (Fig. 4*B*). There were no significant differences in L-arginine response between normoxic animals and those submitted to 7 days of hypoxia in terms of percent decrement (Fig. 6). L-Arginine produced a greater percent decrease in Ppa in rats exposed to 15 h and 2 days of chronic hypoxia than in normoxic rats (Fig. 6); 1 and 6 mM L-arginine caused a small but not significant reduction in baseline Ppa in normoxic and hypoxic rats.

*Vasoreactivity to angiotensin II.* There was no difference in the ΔPpa with angiotensin II among the normoxic control rats (8.2 ± 1.7 mmHg, $n = 5$) and the rats exposed to 15 h (7.9 ± 0.5 mmHg) and 2 days of hypoxia (8.2 ± 0.9 mmHg). Addition of 30 μM L-NMMA had no effect on the angiotensin II response (8.3 ± 2.0 mmHg in normoxic control and 8.2 ± 1.0 mmHg in rats exposed to 2 days of hypoxia).

## DISCUSSION

Whereas airway hypoxia raises pulmonary vascular resistance through vasoconstriction of precapillary vessels (5, 27), chronic hypoxic pulmonary hypertension is considered to develop as a consequence of increased vasomotor tone and structural remodeling of the pulmonary vascular bed (9, 17, 18, 21). Much evidence now exists for a physiological role for the endothelial cell in acutely modulating pulmonary vascular tone through release of EDRF. Brashers et al. (3) and Mazmanian et al. (15) reported that EDRF inhibition potentiates hypoxic vasoconstriction in the perfused rat lung. Furthermore, L-NMMA has been shown to enhance hypoxic vasoconstriction in isolated perfused rat lungs (13), an effect considered to be due to competitive inhibition of the synthesis of nitric oxide from L-arginine. Our results showing that L-NMMA augmented HPV in control rat lungs confirm these findings and suggest that EDRF is released during acute HPV. It is not known whether factors that modify acute pulmonary artery constriction in response to hypoxia are also those that are involved in the earliest stages of vascular remodeling, but it is possible that the release of EDRF, previously shown during acute HPV (3, 8, 13, 15), might be sustained in response to the persistently increased tone and thus might act to modify pulmonary vascular remodeling.

We studied periods of chronic hypoxia up to 7 days as the stimulus for pulmonary vascular remodeling, because previous work has shown that the changes in right ventricular hypertrophy and the changes in the small pulmonary arteries are maximal during this time (31). Thickening of the media of small pulmonary arteries begins after a few hours and is well established by about the 10th day in the rat exposed to this level of hypoxia (9, 17, 18, 25). On the basis of the data of Hunter and colleagues (9), the changes in pulmonary vessels are ~70% completed

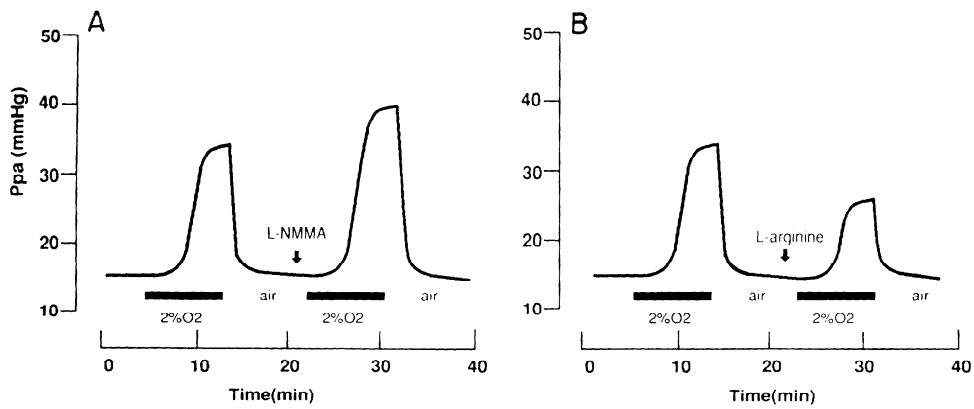


FIG. 3. Representative tracings showing the effects of 30 μM *N*-monomethyl-L-arginine (L-NMMA, *A*) and 1 mM L-arginine (*B*) on pulmonary vasoreactivity in normoxic control lungs.

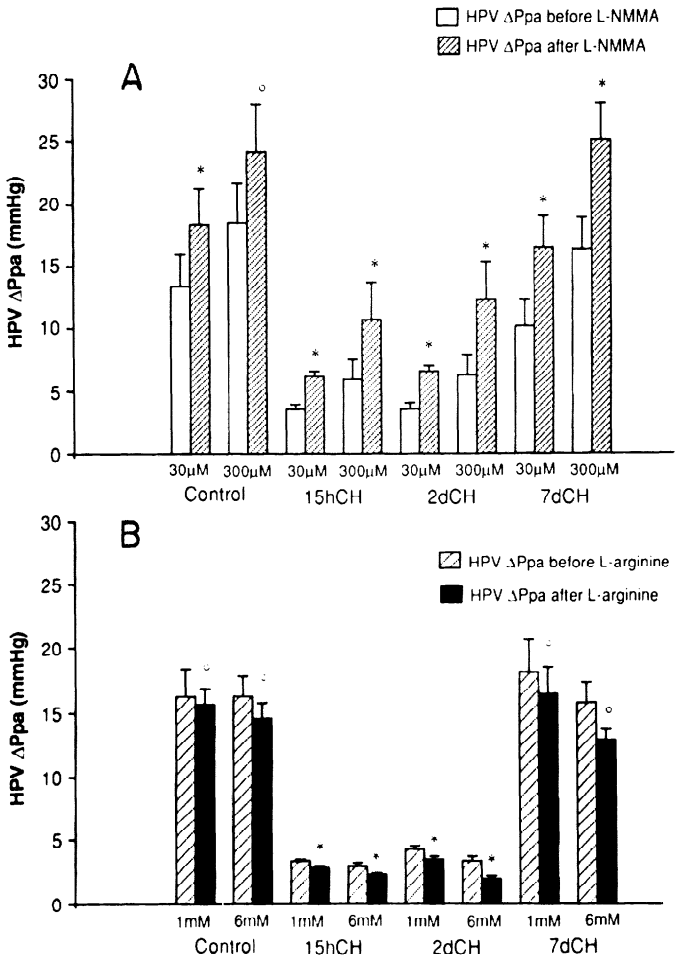


FIG. 4. *A*: HPV responses before and after 30 and 300 μM L-NMMA. *B*: HPV responses before and after 1 and 6 mM L-arginine ($n$ = 6/group). ° $P < 0.05$ compared with vehicle control HPV ΔPpa before L-NMMA or L-arginine. * $P < 0.001$ compared with vehicle control HPV ΔPpa before L-NMMA or L-arginine.

within the exposure period used. Thus, any contribution of altered EDRF release to pulmonary vascular remodeling is likely to occur within the time points used in this study. The increase in resting (normoxic) Ppa observed after 15 h, 2 days, and 7 days of exposure to hypoxia confirms that pulmonary hypertension and vascular remodeling developed in a time-dependent manner.

Reduced HPV response has been described after 40–48 h of exposure to chronic hypoxia (2, 16), in keeping with

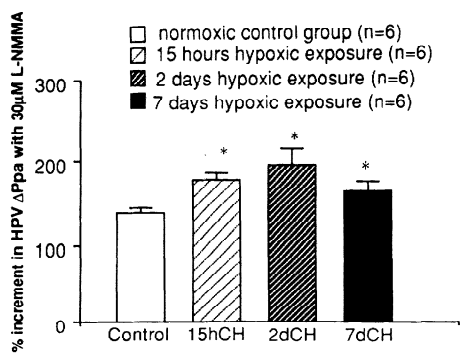


FIG. 5. Percent increase in hypoxic pulmonary vasoconstriction (ΔPpa) in normoxic control, 15hCH, 2dCH, and 7dCH rats after 30 μM L-NMMA. * $P < 0.05$ compared with normoxic group.

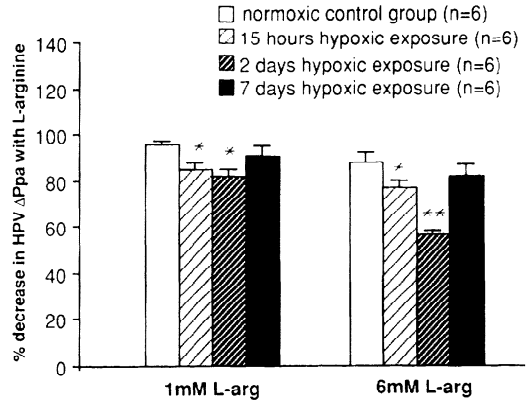


FIG. 6. Percent decrease in hypoxic pulmonary vasoconstriction in normoxic control rats and rats exposed to 15 h, 2 days, and 7 days of hypoxia after 1 and 6 mM L-arginine. * $P < 0.05$ compared with normoxic group. ** $P < 0.001$ compared with normoxic rats and rats exposed to 7 days of hypoxia.

the diminished HPV seen in the present study at 15 h and 2 days and suggestive of rapid early biochemical adaptation to hypoxia. In contrast to the altered response to HPV, responsiveness to the constrictor angiotensin II was not different in the pulmonary circulation of chronically hypoxic animals. Thus a period of hypoxic exposure of only 2 days is associated with specific change in the constrictor response to hypoxia. Although the HPV response was greatly diminished at 15 h and 2 days, it had returned toward normal after 7 days of hypoxia. Augmentation of HPV by L-NMMA was greater in rat lungs exposed to 7 days of hypoxia than in normoxic controls, but the attenuating effect of L-arginine was similar. These findings are consistent with the hypothesis that there is a sustained release of EDRF after 7 days of hypoxia. Adnot et al. (1) challenged isolated perfused lungs from rats exposed to chronic hypoxia (7 and 21 days) with acetylcholine or ionophore A23187. After 21 days of exposure the vasodilator responses were lost, but after 7 days of exposure the vasodilator response was preserved, although slightly less than in the normoxic controls. These findings also suggest sustained release of EDRF in the initial period of exposure to hypoxia.

Previous in vitro studies in isolated pulmonary artery preparations have suggested that severe acute hypoxia by itself inhibits EDRF activity (11, 24, 28). The present study in chronic hypoxia shows that the rise in Ppa is associated with continuing release of EDRF, in agreement with the concept of stretch receptors on endothelial cells (12).

In this study, we have provided evidence that both L-NMMA and L-arginine had a greater effect on the pulmonary circulation of chronically hypoxic animals. After 15 h and 2 days of hypoxic exposure, HPV was markedly reduced, and there was a proportionally greater potentiation of L-NMMA and a significant attenuation of HPV with L-arginine. These data suggest continuing release of EDRF during the earliest stages of pulmonary vascular remodeling, which may act to modulate vascular remodeling in the pulmonary circulation. Changes in acute HPV at 15 h and 2 days of chronic hypoxic exposure without alteration of reactivity to angiotensin II suggests that the smooth muscle itself maintains its

functional capacity during the earliest stages of remodeling. Further studies using continuous infusion of physiological antagonists such as L-NMMA during the process of remodeling may provide additional information about the role of EDRF during pulmonary vascular remodeling.

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