Effects of chronic hypoxia and altered hemodynamics on endothelial nitric oxide synthase expression in the adult rat lung.

T D Le Cras, … , R A Johns, S H Abman

J Clin Invest. 1998;101(4):795-801. https://doi.org/10.1172/JCI786.

Research Article

Mechanisms that regulate endothelial nitric oxide synthase (eNOS) expression in normal and hypoxic pulmonary circulation are poorly understood. Lung eNOS expression is increased after chronic hypoxic pulmonary hypertension in rats, but whether this increase is due to altered hemodynamics or to hypoxia is unknown. Therefore, to determine the effect of blood flow changes on eNOS expression in the normal pulmonary circulation, and to determine whether the increase in eNOS expression after chronic hypoxia is caused by hemodynamic changes or low oxygen tension, we compared eNOS expression in the left and right lungs of normoxic and chronically hypoxic rats with surgical stenosis of the left pulmonary artery (LPA). LPA stenosis in normoxic rats reduced blood flow to the left lung from 9.8+/-0.9 to 0.8+/-0.4 ml/100 mg/min (sham surgery controls vs. LPA stenosis, $P < 0.05$), but there was not a significant increase in right lung blood flow. When compared with the right lung, eNOS protein and mRNA content in the left lung was decreased by 32+/-7 and 54+/-13%, respectively ($P < 0.05$), and right lung eNOS protein content was unchanged. After 3 wk of hypoxia, LPA stenosis reduced blood flow to the left lung from 5.8+/-0.6 to 1.5+/-0.4 ml/100 mg/min, and increased blood flow to the right lung from 5.8+/-0.5 to 10.0+/-1.4 ml/ 100 mg/min (sham surgery controls vs. LPA stenosis, […]

Effects of Chronic Hypoxia and Altered Hemodynamics on Endothelial Nitric Oxide Synthase Expression in the Adult Rat Lung

Timothy D. Le Cras,* Robert C. Tyler,‡ Marilee P. Horan,* Ken G. Morris,‡ Rubin M. Tuder,§ Ivan F. McMurtry,‡ Roger A. Johns,‖ and Steven H. Abman*

**Pediatric Heart Lung Center, Department of Pediatrics; ‡Cardiovascular Pulmonary Research Laboratory, Department of Medicine; and §Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado 80262; and ‖Department of Anesthesiology, University of Virginia Health Sciences Center, Charlottesville, Virginia 22908*

Abstract

Mechanisms that regulate endothelial nitric oxide synthase (eNOS) expression in normal and hypoxic pulmonary circulation are poorly understood. Lung eNOS expression is increased after chronic hypoxic pulmonary hypertension in rats, but whether this increase is due to altered hemodynamics or to hypoxia is unknown. Therefore, to determine the effect of blood flow changes on eNOS expression in the normal pulmonary circulation, and to determine whether the increase in eNOS expression after chronic hypoxia is caused by hemodynamic changes or low oxygen tension, we compared eNOS expression in the left and right lungs of normoxic and chronically hypoxic rats with surgical stenosis of the left pulmonary artery (LPA). LPA stenosis in normoxic rats reduced blood flow to the left lung from 9.8±0.9 to 0.8±0.4 ml/100 mg/min (sham surgery controls vs. LPA stenosis, $P < 0.05$), but there was not a significant increase in right lung blood flow. When compared with the right lung, eNOS protein and mRNA content in the left lung was decreased by 32±7 and 54±13%, respectively ($P < 0.05$), and right lung eNOS protein content was unchanged. After 3 wk of hypoxia, LPA stenosis reduced blood flow to the left lung from 5.8±0.6 to 1.5±0.4 ml/100 mg/min, and increased blood flow to the right lung from 5.8±0.5 to 10.0±1.4 ml/100 mg/min (sham surgery controls vs. LPA stenosis, $P < 0.05$). Despite reduced flow and pressure to the left lung and increased flow and pressure to the right lung, left and right lung eNOS protein and mRNA contents were not different. There were also no differences in lung eNOS protein levels when compared with chronically hypoxic sham surgery controls ($P > 0.05$). We conclude that reduction of pulmonary blood flow decreases eNOS mRNA and protein expression in normoxic adult rat lungs, and that hypoxia increases eNOS expression independently of changes in hemodynamics. These findings demonstrate that hemodynamic forces maintain eNOS content in the normoxic pulmonary circulation of the adult rat, and suggest that chronic hypoxia increases eNOS expression independently of changes in hemodynamics. (*J. Clin. Invest.* 1998. 101:795–801.) Key words: pulmonary hypertension • vasoreactivity • pulmonary blood flow • pulmonary vascular resistance • shear stress

Introduction

The endothelium contributes to local regulation of vascular smooth muscle tone by releasing various vasoactive products, including nitric oxide (NO).[1] The enzyme responsible for NO production in the endothelium of the vascular wall is type III endothelial NO synthase (eNOS; 1). NO is produced by a reaction using the amino acid L-arginine and molecular oxygen as substrates, and other cofactors including NADPH, tretrahydrobiopterin, FAD, and FMN. NO, or a closely related molecule, stimulates vascular smooth muscle relaxation through activation of soluble guanylate cyclase to produce cGMP (2). Although constitutively expressed by endothelial cells, eNOS gene expression is regulated by various stimuli, including shear stress and hypoxia (3).

In normal adult rats, NO does not appear to play a major role in the maintenance of low basal pulmonary vascular tone (4). After exposure to chronic hypoxia, however, eNOS expression is induced in small resistance vessels (5), and inhibition of NO synthesis causes marked vasoconstriction (6). NO plays a more active role in regulating basal pulmonary vascular tone in humans, as reflected by the increase in pulmonary vascular resistance with NO synthase inhibition (7). In addition to its direct effect on vascular tone, NO may play other roles in the vasculature, including regulating vascular smooth muscle cell proliferation (2).

Inhaled NO acutely reduces high vascular tone in experimental and clinical pulmonary hypertension (8). Chronic inhalation of NO reduces hypertensive structural remodeling of small pulmonary arteries in hypoxic rats (9). Although it has been hypothesized that eNOS expression and NO production might be decreased in pulmonary hypertension, several studies have found that NO production and eNOS expression is increased in rats after chronic hypoxic pulmonary hypertension (10, 11, 5). The mechanism for eNOS upregulation with chronic hypoxia is unknown. It is possible that altered hemo-

Address correspondence to Timothy D. Le Cras, Ph.D., Department of Pediatrics, Box C218, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Denver, CO 80262. Phone: 303-315-6225; FAX: 303-315-3851; E-mail: timothy.lecras@uchsc.edu

Portions of this work have been submitted in Abstract form (1997. *FASEB J.* 11:A134, 781).

Received for publication 2 June 1997 and accepted in revised form 18 December 1997.

J. Clin. Invest.

0021-9738/98/02/0795/07 $2.00
Volume 101, Number 4, February 1998, 795–801
http://www.jci.org

1. *Abbreviations used in this paper:* CO, cardiac output; eNOS, endothelial nitric oxide synthase; LPA, left pulmonary artery; NO, nitric oxide; MPA, main pulmonary artery; PAP, pulmonary artery pressure; TPR, total pulmonary vascular resistance.

dynamic stimuli, such as increased pressure or shear stress, or decreased oxygen tension upregulates eNOS expression.

The eNOS gene contains a shear stress response element, and eNOS expression is increased by shear stress in vitro (12). However, in vivo data concerning regulation of eNOS expression is less clear. Chronic exercise increases eNOS expression in the endothelium of adult dogs, but whether this expression is due to increased flow, shear stress, or to other effects of exercise was not determined (13). Increases in blood flow in the systemic circulation caused by an arteriovenous shunt also increased aortic eNOS expression (14). Recently we have shown that chronic increases in pulmonary artery pressure decrease eNOS expression in the ovine fetal lung (15). Thus, lung eNOS expression may be modified by alterations in hemodynamic forces independently of the effects of hypoxia.

To examine the relative roles of hemodynamic forces and chronic hypoxia in eNOS expression in the pulmonary circulation, we performed a series of experiments using rats with altered left pulmonary artery blood flow. Surgical banding of the left pulmonary artery (LPA) was performed on adult male rats, reducing blood flow to the left lung. The use of rats with LPA stenosis allowed us to study the effect of decreased pulmonary blood flow on lung eNOS expression. Since LPA banding protects the left lung from hypertensive changes induced by chronic hypoxia (16), we used this model to help differentiate the direct effects of chronic hypoxia from hemodynamic forces on lung eNOS expression.

Methods

Animals

All procedures and experiments were approved by the University of Colorado and the University of Virginia Health Sciences Center Animal Care Committees. LPA banding surgery was performed on Sprague Dawley adult male rats (250–300 g) at Zivic-Miller Laboratories (Portersville, PA). Stenosis was produced by tying a 3-0 silk suture around a blunt needle and the LPA, and then removing the needle. The gauge of the needle was used to determine the approximate diameter of the silk band, and to ensure uniformity in the degree of stenosis. 25-gauge needles were used for LPA banding (16). Sham surgery was performed on age-matched male rats to control for any effects of surgery itself. Surgery in the sham surgery control animals was performed in the same manner as for the LPA stenosis rats, but the ligature was removed before closing around the LPA. Rats were shipped 2 d after surgery.

Normoxic protocol

Normoxia groups (LPA stenosis rats and sham surgery controls) were kept in room air for 4 wk with a 12-h light/12-h dark cycle, and were allowed free access to rat chow and water.

Hypoxic protocol

Chronic hypoxia groups (LPA stenosis rats and sham surgery controls) were placed in a hypobaric chamber and exposed to progressive increases in simulated altitude over 1 wk until they reached 17,000 ft. ($FiO_2 = 0.10$), and were then kept at that altitude for 3 wk. The chamber was ventilated continuously with room air to prevent accumulation of carbon dioxide, ammonia, and water vapor, as previously described (6). The chronically hypoxic rats were exposed to 12-h light/12-h dark cycles, and were allowed free access to rat chow and water in the hypobaric chamber. Hypoxic rats were exposed to $FiO_2 = 0.10$ for 24 h a day, except for brief periods (< 10 min) when chambers were returned to atmospheric pressure for cage cleaning and replenishing of food and water.

At the end of the study period, rats were anesthetized with ketamine (100 mg/kg; Fort Dodge Labs, Fort Dodge, IA) and xylazine (12 mg/kg; Miles, Shawnee, KS) and a thoracotomy was performed. A catheter was inserted into the main pulmonary artery (MPA), and the lungs were perfused with heparinized saline (1 U/ml), until the effluent was clear of blood. After perfusion the lungs were either (*a*) removed and snap-frozen in liquid nitrogen, stored at −70°C, and subsequently used for extraction of RNA and protein for Northern and Western blot analysis; or (*b*) perfused with paraformaldehyde through the MPA, and then molten agarose–injected through the trachea to inflate the airways, and then placed in formalin for paraffin embedding. Paraffin-embedded tissue was used for histology and eNOS immunolocalization.

Chronically hypoxic rats used for hemodynamic studies were removed from the high altitude chamber after 20 d at 17,000 ft. for surgery and instrumentation, and were then returned to the chamber for the remaining time period.

Hemodynamic studies

Surgery and instrumentation. Hemodynamic measurements were performed as previously described (17). Catheters were placed in the right carotid artery, MPA, and jugular vein. Rats were either housed in room air or returned to the high altitude hypoxia chamber ($FiO_2 = 0.10$) for the remainder of the time period. Upon removal from normoxia or hypoxia, conscious rats were placed in a ventilated clear plastic chamber during hemodynamic measurements and pulmonary and systemic blood pressure determinations. Heart rates were measured, and mean pressures were calculated. Cardiac outputs (CO) were measured by the standard dye-dilution technique. Hemodynamic measurements were performed while all the rats were breathing room air, and also subsequently in the hypoxic groups while breathing hypoxic gas ($FiO_2 = 0.10$).

Measurement of lung blood flow using radiolabeled microspheres. Radiolabeled microspheres were used to measure lung and kidney blood flows as previously described (18). ^{46}Sc-labeled 15-μm microspheres were injected into the right jugular vein for measurement of blood flows while the rats were exposed to room air. The hypoxic rat groups were then exposed to hypoxic gas for 5 min, at which time hemodynamic measurements were again taken and ^{113}Sn-labeled 15-μm microspheres were injected. The heart was removed for measurement of right ventricular hypertrophy (RVH). Left and right lungs and left and right kidneys were collected for counting of radioisotope content. Kidney flow was measured to determine systemic escape of any microspheres. Organ flows in ml/min were calculated as [(cardiac output) × (organ cpm)]/(total injected cpm).

Northern blot analysis

Northern blot analysis was performed according to previously published methods (5, 19), with 20 μg of total RNA per rat lung and a rat cDNA probe for eNOS (a kind gift from Dr. Hideki Takahashi, Dept. of Respiratory Medicine, Juntendo University School of Medicine, Tokyo, Japan). 18S rRNA levels were measured by hybridization with an oligonucleotide probe (ACGGTATCTGATCCGTCTTCGAACC) labeled with ^{32}P-dCTP using terminal deoxytransferase (Boehringer Mannheim Biochemicals, Indianapolis, IN). Densitometry was performed using a scanner and National Institutes of Health Image software (Bethesda, MD).

Western blot analysis

Western blot analysis was performed using 25 μg of lung protein according to a previously published method (5) with a monoclonal antibody to eNOS (Transduction Laboratories, Lexington, KY). Densitometry was performed with a scanner and National Institutes of Health Image software. Western blot analysis with increasing amounts of lung protein from a hypoxic sham control rat showed that there was a linear increase in eNOS protein signal, and that the amount of protein (25 μg) used for comparison of control and treatment groups fell within the linear range of the Western blot analysis.

Table I. Lung Weight, Right Ventricular Hypertrophy, and Body Weight Changes

	No. of rats	Right lung wt	Left lung wt	Left/right lung wt	Right ventricle wt/ left ventricle + septum wt	Body wt increase
		g	*g*			*g*
Normoxic						
Sham surgery	14	1.21±0.10	0.66±0.05	0.55±0.01	0.28±0.01	122±9
LPA stenosis	13	1.32±0.04	0.48±0.03*	0.37±0.02*	0.28±0.01	104±4
Hypoxic						
Sham surgery	13	1.39±0.07	0.76±0.05	0.55±0.01	0.43±0.03	98±8
LPA stenosis	12	1.62±0.06*	0.56±0.05*	0.34±0.02*	0.49±0.03	109±6

*Significant difference from sham surgery group value, $P < 0.05$.

Histology and immunohistochemical staining for eNOS protein

Small pieces of lung (2–6 mm) were placed in 10% buffered formalin and paraffin-embedded. Paraffin sections 5 μm thick were serially mounted onto Superfrost Plus™ slides (Fisher Scientific Co., Fairlawn, NJ). Hemotoxylin and eosin staining was performed on left and right lungs from three animals in each study group. At least three sections per lung, and left and right lungs for each animal were assessed for histologic changes. For eNOS immunostaining, slides were dewaxed in 100% xylene. Sections were rehydrated by immersion in 100% ethanol; 95% ethanol/5% water; 70% ethanol/30% water; and then 100% water. Antigen retrieval was performed by boiling the slides in 0.01 M citric acid, pH 6.0. Slides were washed in PBS (2.7 mM KCl, 1.2 mM KH_2PO_4, 138 mM NaCl, 8.1 mM Na_2HPO_4 = 1× PBS). Endogenous biotin in the tissue sections was blocked by glucose, glucose oxidase treatment (0.2 M glucose, 1.5 U/ml glucose oxidase [Boehringer Mannheim Biochemicals] in 1× PBS). The slides were washed in 1× PBS. Sections were blocked with Super Block™ (Sky Tek, Logan, Utah) diluted 1:10 (vol/vol) in 1× PBS, and were then incubated with anti-eNOS monoclonal antibody (Transduction Laboratories) diluted 1:10,000, or an IgG_1 negative control (Jackson Laboratories, West Grove, PA) diluted 1:10,000 in 1× PBS, 2% (wt/vol) BSA, 0.1% (wt/vol) NaN_3. After incubation with the primary antibodies, sections were washed in 1× PBS. Biotin-labeled anti–mouse secondary antibody (Vector Laboratories, Inc., Burlingame, CA) was incubated with the sections at a dilution of 1:200 in 1× PBS, 2% (wt/vol) BSA, 0.1% (wt/vol) NaN_3. Again, the slides were washed in 1× PBS. Slides were incubated in streptavidin-biotin-horseradish peroxide solution and developed with diaminobenzidine (DAB) and hydrogen peroxide, with nickel chloride for enhancement (Vector). The NiCl enhancement–DAB color development reaction was stopped by washing with water, and then the slides were dehydrated in 70% ethanol/30% water, 95% ethanol/5% water; 100% ethanol, and finally 100% xylene before coverslipping.

Data analysis

All data is expressed as mean±SEM. Unpaired *t* tests were performed on the quantitative data to test for statistical differences between the groups. Data groups were considered as significantly different if $P < 0.05$. Each group contained at least four rats.

Results

All study groups showed a gain in total body weight of 98–122 g (Table I), and there were no differences among the groups. Hemodynamic measurements were made while the four groups were breathing room air (Table II), and also with the hypoxic rats exposed to $FiO_2 = 0.10$. COs were not different among the groups.

Normoxic groups

Organ weights and hemodynamics. In comparison with sham surgery control animals, LPA stenosis reduced left lung weight by 26%, but did not significantly alter right lung weight (Table I). The ratio of the left/right lung weight was reduced in the LPA stenosis rats. Right ventricle/left ventricle + septum weights were similar in sham and LPA stenosis groups. In normoxic rats, LPA stenosis reduced blood flow to the left lung, and there was no significant change in right lung blood flow compared with sham surgery controls (Table II). When calculated as percentage flow, LPA stenosis reduced blood flow to the left lung from 52±5% to 7±3% (sham surgery controls vs. LPA stenosis). Main pulmonary artery pressure (PAP) was increased by LPA stenosis compared with the sham surgery con-

Table II. Pulmonary Blood Flows and Other Hemodynamics

	No. of rats	Left lung blood flow	Right lung blood flow	PAP	CO	TPR
		ml/100 mg/min	*ml/100 mg/min*	*mmHg*	*ml/min*	*mmHg/ml/min*
Normoxic						
Sham surgery	4	9.8±0.9	9.0±1.3	20±1	186±24	0.11±0.01
LPA stenosis	6	0.8±0.4*	11.1±2.0	25±1*	162±22	0.17±0.02
Hypoxic						
Sham surgery	7	5.8±0.6‡	5.8±0.5‡	34±1‡	134±9	0.26±0.02‡
LPA stenosis	8	1.5±0.4*	10.0±1.4*	43±2*‡	158±16	0.30±0.04‡

*Significantly different from sham surgery group value, $P < 0.05$. ‡Significantly different from normoxic group value, $P < 0.05$.

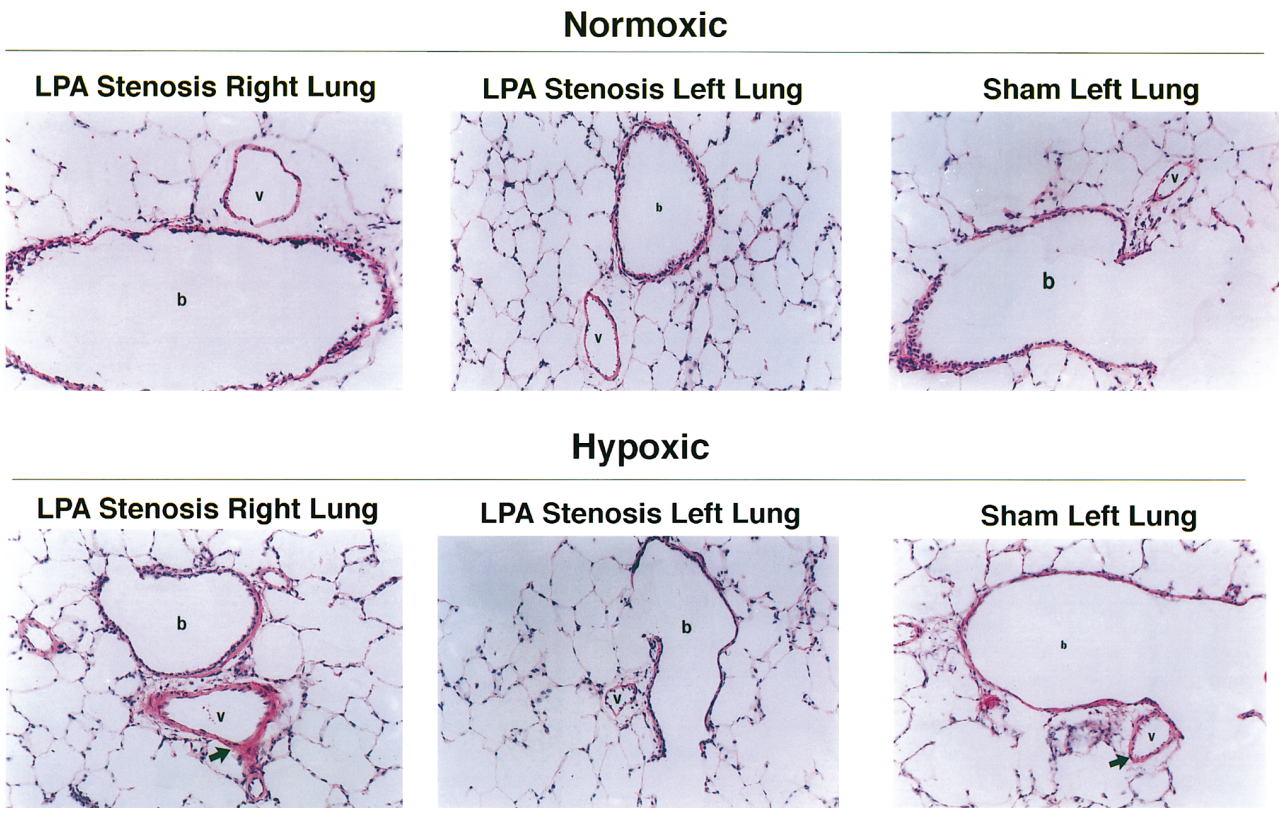

Figure 1. Morphology of the right and left lungs of a normoxic LPA stenosis rat and left lung of a normoxic sham surgery control rat (*Normoxic*). Morphology of the right and left lungs of a chronically hypoxic LPA stenosis rat and left lung of a chronically hypoxic sham surgery control rat (*Hypoxic*). Note the thickening of the muscular layer of the arterial vessels (*v*) at the terminal bronchiole (*b*) level in the right lung of hypoxic LPA stenosis rat and left lung of the hypoxic sham surgery control (*arrows*). In the left lung of the hypoxic LPA stenosis lung, pulmonary arteries at the terminal and respiratory levels showed no remodeling (hemotoxylin-eosin staining; 400×).

trols, but total pulmonary vascular resistance (TPR) was not different.

Histology. Muscularized pulmonary arteries in the right and left lungs of the LPA stenosis group under normoxia showed normal vascular structure, as also shown by the sham surgery controls (Fig. 1).

eNOS mRNA, protein levels, and localization. In normoxic rats, LPA stenosis decreased eNOS mRNA levels in the left lung by 54±13% ($P < 0.05$) compared with the right lung (Fig. 2). In normoxic rats, LPA stenosis decreased eNOS content in the left lung by 32±7% ($P < 0.05$) compared with sham surgery controls (Fig. 3). Immunohistochemical staining localized eNOS protein to large and medium vessels in normoxic rat lungs. In the LPA stenosis rats, a similar pattern of staining was seen in both left and right lungs (Fig. 4).

Hypoxic groups

Organ weights and hemodynamics. In rats exposed to chronic hypoxia, LPA stenosis increased right lung weight and decreased left lung weight, resulting in a lower left/right lung weight ratio (Table I). Although RVH increased with hypoxia, there was not a further increase after LPA stenosis (Table II). PAP and TPR increased in both hypoxic groups compared with normoxic groups. In hypoxic rats, LPA stenosis reduced blood flow to the left lung and increased blood flow to the right lung. When calculated as percentage blood flows, LPA stenosis reduced blood flow to the left lung from 50±5 to 13±3% (sham surgery controls vs. LPA stenosis, respectively; $P < 0.05$), and increased right lung blood flow from 50±4 to 87±12% (sham surgery controls vs. LPA stenosis, respectively; $P < 0.05$). Left and right lung blood flows in the chronically hypoxic LPA stenosis rats were not different from blood flows in the normoxic LPA stenosis rats. The hypoxic LPA stenosis rats had higher PAP compared with the hypoxic sham surgery controls, but TPR was not different (Table II). When the hypoxic groups were exposed to $FiO_2 = 0.10$ and hemodynamic measurements were repeated, there was a significant increase in PAP in the LPA stenosis group (43±2 to 54±3 mmHg). Blood flows, CO, and TPR were not different from measurements made while the rats were breathing room air.

Histology. With exposure to chronic hypoxia, the right lungs of the LPA stenosis rats showed thickening of the muscular medial layer, and were similar in structure to the lungs of the chronically hypoxic sham surgery controls (Fig. 1). After LPA stenosis, the left lungs did not develop the hypertensive changes seen in the right lungs and the hypoxic sham surgery controls (Fig. 1). Histology of the left lung of the LPA stenosis group was similar to that of the normoxic animals.

eNOS mRNA, protein levels, and localization. In hypoxic rats, there was no difference in eNOS mRNA levels between

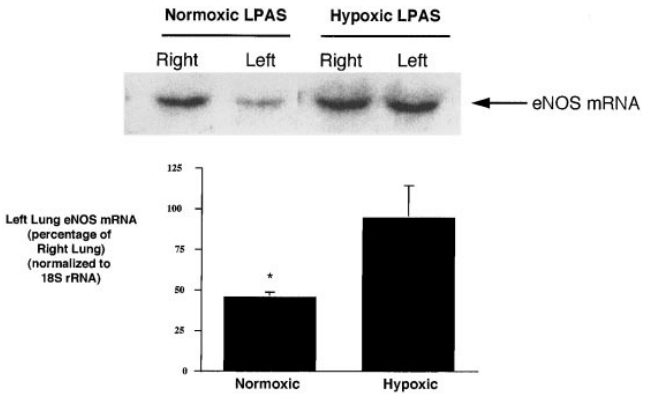

Figure 2. Northern blot analysis of eNOS mRNA levels of right and left lungs of normoxic and hypoxic LPA stenosis rat lungs. The eNOS mRNA was determined to be 4.4 kb. Quantitation of the eNOS mRNA levels normalized to the signal for 18S rRNA is shown below. The results depict eNOS mRNA expression in four animals. $*P <$ 0.05 vs. right lung.

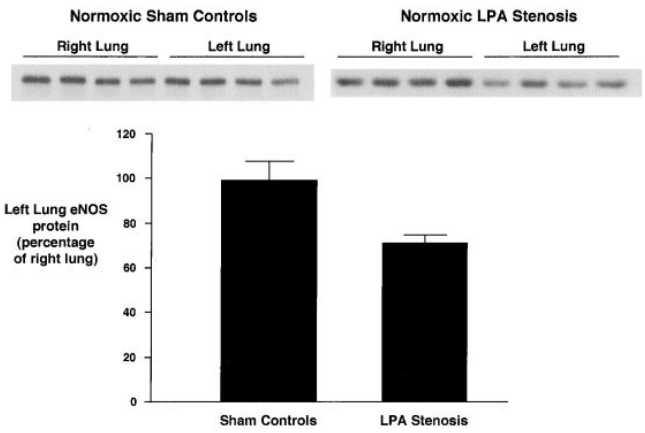

Figure 3. Western blot of eNOS protein content of normoxic LPA stenosis and sham surgery control rats, right and left lungs. The eNOS monoclonal antibody detected a single band in the rat lung homogenates of 140 kD. Quantitation of the eNOS protein signal is shown below. The results depict eNOS protein expression in four animals. LPA stenosis vs. sham, $P < 0.05$.

left and right lungs with LPA stenosis (Fig. 2). After 3 wk of hypoxia, lung eNOS protein content increased 1.5-fold in sham surgery controls compared with normoxic controls (Fig. 5). Lung eNOS protein expression also increased in both left and right lungs of the hypoxic LPA stenosis rats (Fig. 6), and was not different from eNOS protein levels in the hypoxic sham surgery controls (Fig. 7). There was no difference between left and right lung eNOS protein content in either the hypoxic

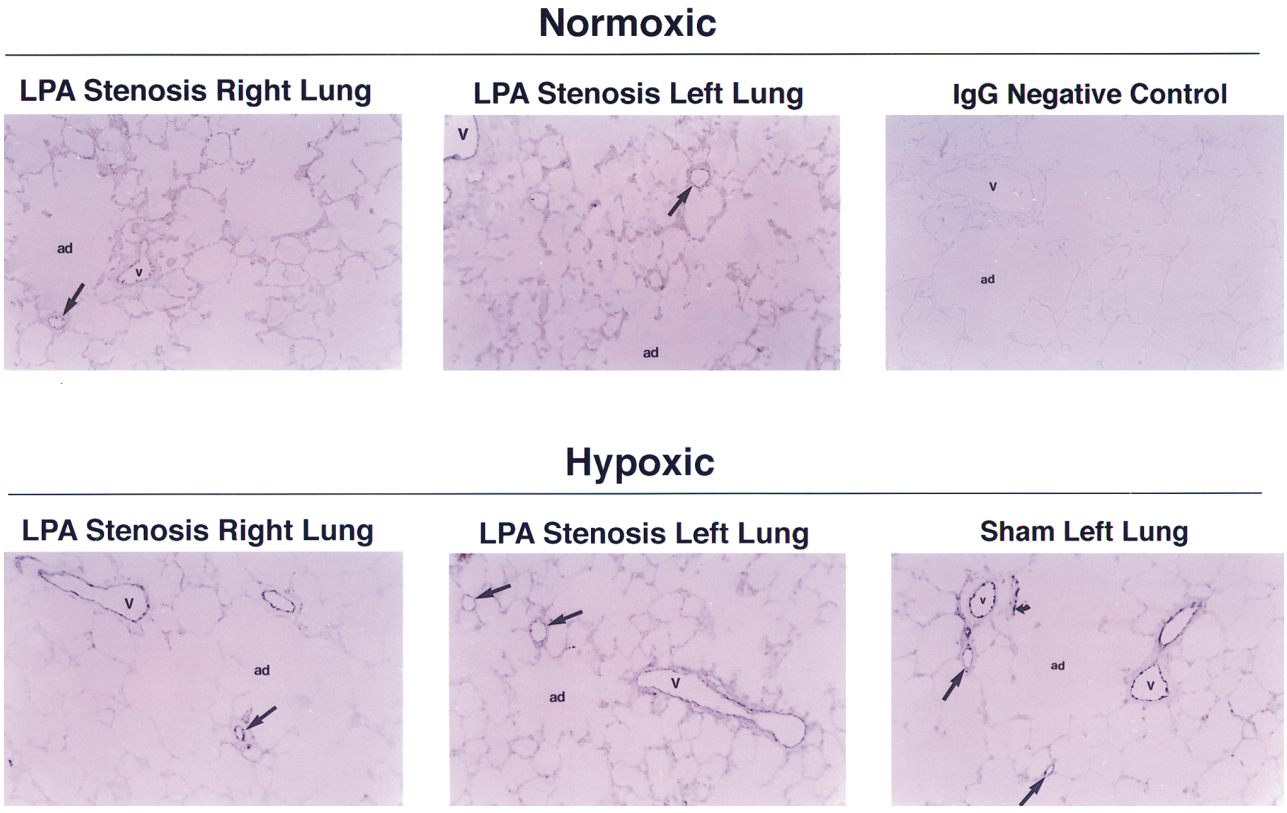

Figure 4. Immunohistochemical localization of eNOS expression in the right and left lungs of normoxic and hypoxic LPA stenosis rats, and left lung of a hypoxic sham surgery control rat. (*Normoxic*) The expression of eNOS in the normoxic right and left lungs of the LPA stenosis rats was present predominately in the endothelium of large and medium-sized pulmonary vessels (*v*), with precapillary vessels showing either faint (*arrows*) or lack of immunoreactivity. (*Hypoxic*) The right lungs of the hypoxic LPA stenosis rats demonstrated expression of eNOS in the endothelium of large and medium vessels (*v*), and also small arterial vessels (precapillary; *arrows*) located along the septal wall of alveolar ducts (*ad*). A similar pattern of eNOS staining was seen in the right and left lungs of LPA stenosis and left lungs of the sham surgery controls. The right lung of a hypoxic sham surgery control rat incubated with an isotype-matched control antibody (IgG) in place of the eNOS monoclonal antibody (*upper right*). All slides 200×.

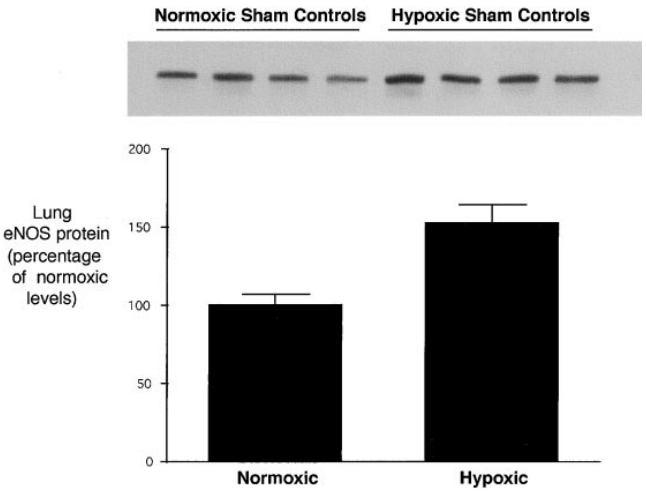

Figure 5. Western blot of lung eNOS protein content of normoxic and chronically hypoxic sham surgery controls. The eNOS monoclonal antibody detected a single band in the rat lung homogenates of 140 kD. Quantitation of the eNOS protein signal is shown below. The results depict eNOS protein expression in four animals. Normal vs. hypoxic, $*P < 0.05$.

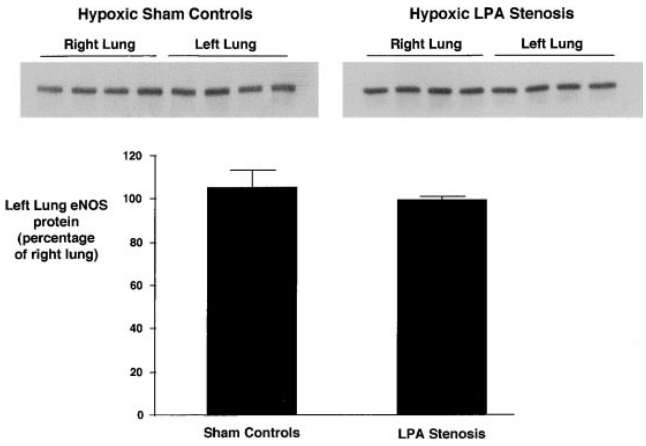

Figure 7. Western blot of eNOS protein content of chronically hypoxic LPA stenosis and sham surgery control rats, right and left lungs. Quantitation of the eNOS protein signal is shown below. The results depict eNOS protein expression in four animals.

LPA stenosis rats or hypoxic sham surgery controls (Fig. 7). Chronically hypoxic rats showed staining for eNOS in small vessels as well as medium and large vessels (Fig. 4). The pattern of eNOS staining was similar in left and right lungs of both LPA stenosis rats and sham surgery control lungs. There were no differences in the distribution of eNOS staining between LPA stenosis rats and sham surgery controls. Staining for eNOS was also detected in the bronchial epithelium, particularly on the apical surface of the epithelial cells. No differences in bronchial staining for eNOS could be seen between the different treatment groups.

Discussion

We found that reduction of pulmonary blood flow due to LPA stenosis decreased eNOS mRNA and protein expression in the normoxic adult rat lung. Although blood flow was decreased by 8.6-fold in the left lung of normoxic LPA stenosis rats, there was a 32% decrease in eNOS protein and a 54% decrease in eNOS mRNA expression. Blood flow to the right lung did not change with LPA stenosis, and there was no change in eNOS expression. Exposure of LPA stenosis rats to chronic hypoxia increased eNOS gene expression in both left and right lungs despite marked reduction of blood flow to the left lung (4.0-fold) and increased flow to the right lung (1.7-fold). After chronic hypoxia, there was no difference in eNOS mRNA and protein content between left and right lungs. Hence, chronic hypoxia increased lung eNOS gene expression despite altered hemodynamics as assessed by direct measurements of reduced blood flow and by histologic evidence of reduced hypertensive remodeling in the left lung. These findings suggest that (*a*) normal pulmonary blood flow maintains eNOS mRNA and protein content in the normoxic rat lung, and (*b*) that chronic hypoxia increases lung eNOS gene expression primarily because of hypoxia or hypoxia-induced factors rather than hypertension or other hemodynamic factors.

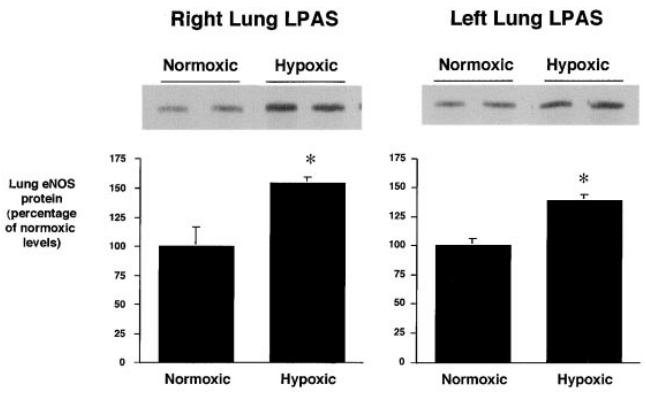

Figure 6. Western blot of eNOS protein content of normoxic and chronically hypoxic LPA stenosis rats, right and left lungs. Quantitation of the eNOS protein signal is shown below. The results depict eNOS protein expression in four animals. $*P < 0.05$.

In vitro studies have shown that increased shear stress upregulates eNOS expression (12), implying that this mechanism may also be operative in vivo. In in vivo studies of the systemic circulation where shear stress may have been increased, such as in chronically exercised dogs, eNOS expression was increased in the aortic endothelium (13). In a rat model of high blood flow created by an arteriovenous shunt, eNOS mRNA and protein were also found to be increased in the aorta (14). Our study showed that in the chronically hypoxic pulmonary circulation, increases in blood flow or pressure had no effect on eNOS expression. Although decreased pulmonary blood flow reduced eNOS expression in normoxic rats, it is possible that this decrease in eNOS expression may also be influenced by other mechanisms such as altered growth factor activity, which may be associated with reduction in the size of the left lungs after LPA stenosis. In a recent paper, Resta et al. (20) reported that eNOS expression was increased in the arteries, but not in the veins of chronically hypoxic rat lungs. They suggested that hypertension or increased shear stress may be responsible for selective upregulation of pulmonary arterial eNOS, but could not preclude a role for hypoxia.

The model used in this study was originally described by

Rabinovitch et al. (16). They reported that banding the left pulmonary artery decreased blood flow and pressure in the left lung of chronically hypoxic rats. This decrease prevented both muscular extension into previously nonmuscular peripheral pulmonary arteries and medial hypertrophy of normally muscular arteries. Histologic and hemodynamic results in our study were comparable to those reported by Rabinovitch et al. (16), and confirmed that LPA banding prevents the hypertensive changes usually associated with chronic hypoxia. Therefore, despite altered blood pressure and flow in the left lung of the hypoxic LPA stenosis rats, chronic hypoxia increased eNOS expression to a similar degree in left and right lungs.

Potential limitations of this study include lack of bronchial blood flow measurements. Results reported are for a single time point. Because of the small size of the banded left pulmonary artery, we were not able to measure pressure distal to the band. Potential effects of changes in the bronchial circulation on lung eNOS expression are unknown. Hemodynamic effects on lung eNOS expression at earlier and later time points were not examined in this study, but will be the subject of future studies. Staining for eNOS was also detected in the bronchial epithelium, particularly on the apical surface of the epithelial cells. No differences in bronchial staining for eNOS could be seen among the different treatment groups. The contribution that bronchial eNOS protein makes towards whole-lung eNOS protein levels is not known. In conclusion, this study suggests that hemodynamics contribute to the regulation of eNOS expression in the normal pulmonary circulation as evidenced by the fall in lung eNOS expression after reduction of pulmonary blood flow in normoxia. With chronic hypoxia, eNOS expression was increased to similar levels in left and right lungs with LPA stenosis despite large differences in blood flows and presumably pressures. We suggest that the increase in eNOS expression after chronic hypoxic pulmonary hypertension may be due to the direct effects of hypoxia or hypoxia-induced factors independently of changes in hemodynamics.

Acknowledgments

The authors would like to thank Nan Zhou, Lisa Hepler, and Cheryl Oliver-Pickett for technical assistance.

References

1. Pollack, J.S., U. Forstermann, J.A. Mitchell, T.D. Warner, H.H. Schmidt, M. Nakane, and F. Murad. 1991. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl. Acad. Sci. USA*. 88:10480–10484.

2. Griendling, K.K., and R.W. Alexander. 1996. Endothelial control of the cardiovascular system: recent advances. *FASEB J.* 10:283–292.

3. Forstermann, U., and H. Kleinert. 1995. Nitric oxide synthase: expression and expressional control of the three isoforms. *Naunyn-Schmiedebergs Arch. Pharmacol.* 352:351–364.

4. Crawley, D.E., S.F. Lui, T.W. Evans, and P.J. Barnes. 1990. Inhibitory role of endothelium-derived nitric oxide in rat and human pulmonary arteries. *Br. J. Pharmacol.* 101:166–170.

5. Le Cras, T.D., C. Xue, A. Rengasamy, and R.A. Johns. 1996. Chronic hypoxia upregulates endothelial and inducible nitric oxide synthase gene and protein expression in rat lung. *Am. J. Physiol.* 270(1 Pt. 1):L164–L170.

6. Oka, M., K. Hasunuma, S.A. Webb, T.J. Stelzner, D.M. Rodman, and I.F. McMurtry. 1993. EDRF suppresses an unidentified vasoconstrictor mechanism in hypertensive rat lungs. *Am. J. Physiol.* 264:L587–597.

7. Stamler, J.S., E. Loh, M.-A. Roddy, K.E. Currie, and M.A. Creager. 1994. Nitric oxide regulates basal systemic and pulmonary vascular resistance in healthy humans. *Circulation.* 89:2035–2040.

8. Moncada, S., and E.A. Higgs. 1995. Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB J.* 9:1319–1330.

9. Roos, C.M., D.U. Frank, C. Xue, R.A. Johns, and G.F. Rich. 1996. Chronic inhaled nitric oxide: effects on pulmonary endothelial function and pathology in hypoxic and normoxic rats. *J. Appl. Physiol.* 80:252–260.

10. Isaacson, T.C., V. Hampl, E.K. Weir, D.P. Nelson, and S.L. Archer. 1994. Increased endothelial-derived NO in hypertensive pulmonary circulation of chronically hypoxic rats. *J. Appl. Physiol.* 76:933–940.

11. Shaul, P.W., A.J. North, T.S. Brannon, K. Ujiie, L.B. Wells, P.A. Nisen, C.J. Lowenstein, S.H. Snyder, and R.A. Star. 1995. Prolonged in vivo hypoxia enhances nitric oxide synthase type I and type III gene expression in adult rat lung. *Am. J. Respir. Cell Mol. Biol.* 13:167–174.

12. Ranan, V., Z. Xiao, and S.J. Diamond. 1995. Constitutive NOS expression in cultured endothelial cells is elevated by fluid shear stress. *Am. J. Physiol. (Heart Circ. Physiol.* 38) 269:H550–H555.

13. Sessa, W.C., K. Pritchard, N. Seyedi, J. Wang, and T.H. Hintze. 1994. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* 74:349–353.

14. Nadaud, S., M. Phillippe, J.F. Arnal, J.B. Michel, and F. Soubrier. 1996. Sustained increase in aortic endothelial nitric oxide synthase expression in a model of chronic high blood flow. *Circ. Res.* 79:857–863.

15. Villamor, E., T.D. Le Cras, M.P. Horan, A.C. Halbower, R.M. Tuder, and S.H. Abman. 1997. Chronic intrauterine pulmonary hypertension impairs endothelial nitric oxide synthase in the ovine fetus. *Am. J. Physiol.* (*Lung Cell Mol. Physiol.* 16) 272:L1013–L1020.

16. Rabinovitch, M., M.A. Konstam, W.J. Gamble, N. Papanicolaou, M.J. Arnovitz, S. Treves, and L. Reid. 1983. Changes in pulmonary blood flow affect vascular response to chronic hypoxia in rats. *Circ. Res.* 52:432–441.

17. Stevens, T., K. Morris, I.F. McMurtry, M. Zamora, and A. Tucker. 1993. Pulmonary and systemic vascular responsiveness to TNF-μ in conscious rats. *J. Appl. Physiol.* 74:1905–1910.

18. Heymann, M.A., B.D. Payne, J.I.E. Hoffman, and A.M. Rudolph. 1977. Blood flow measurements with radionuclide-labeled particles. *Prog. Cardiovasc. Dis.* 20:55–79.

19. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Plainview, NY. 7.40–7.42, 7.49.

20. Resta, T.C., R.J. Gonzales, W.G. Dail, T.C. Sanders, and B.R. Walker. 1997. Selective upregulation of arterial endothelial nitric oxide synthase in pulmonary hypertension. *Am. J. Physiol. (Heart Circ. Physiol.* 41) 272:H806–H813.