Developmental differences in pulmonary eNOS expression in response to chronic hypoxia in the rat

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Chicoine, Louis G., Jose W. Avitia, Cody Deen, Leif D. Nelin, Scott Earley, and Benjimen R. Walker. Developmental differences in pulmonary eNOS expression in response to chronic hypoxia in the rat. J Appl Physiol 93: 311–318, 2002. First published April 5, 2002; 10.1152/japplphysiol.01083.2001.—Chronic hypoxia (CH) increases pulmonary endothelial nitric oxide synthase (eNOS) protein levels in adult rats but decreases eNOS protein levels in neonatal pigs. We hypothesized that this differing response to CH is due to developmental rather than species differences. Adult and neonatal rats were placed in either hypobaric hypoxia or normoxia for 2 wk. At that time, body weight, hematocrit, plasma nitrite/nitrate (NOx⁻), and right ventricular and total ventricular heart weights were measured. Percent pulmonary arterial wall area of 20–50 and 51–100 μm arteries were also determined. Total lung protein extracts were assayed for eNOS levels by using immunoblot analysis. Compared with their respective normoxic controls, both adult and neonatal hypoxic groups demonstrated significantly decreased body weight, elevated hematocrit, and elevated right ventricular-to-total ventricular weight ratios. Both adult and neonatal hypoxic groups also demonstrated significantly larger percent pulmonary arterial wall area compared with their respective normoxic controls. Hypoxic adult pulmonary eNOS protein and plasma NOx⁻ were significantly greater than levels found in normoxic adults. In contrast, hypoxic neonatal pulmonary eNOS protein and plasma NOx⁻ were significantly less compared with normoxic neonates. We conclude that there is a developmental difference in eNOS expression and nitric oxide production in response to CH.

neonatal; nitric oxide; pulmonary hypertension

EXPOSURE TO CHRONIC HYPOXIA (CH) leads to pulmonary hypertension (PH) via hypoxic pulmonary vasoconstriction, increased pulmonary vascular muscularization, and polycythemia (9, 29). These changes are associated with elevated right ventricle (RV) afterload and, if severe, can lead to right heart failure (31, 39). Nitric oxide (NO) possesses vascular smooth muscle antimitogenic (10, 17, 25, 27, 37) and vasodilatory (1, 4, 28) properties that are important in modulating the vascular changes that occur with the development of PH. NO is produced from the oxidation of L-arginine to L-citrulline by NO synthase (NOS). Endothelial NOS (eNOS) expression is known to play a major role in the control of pulmonary vascular tone in the fetus, neonate, and adult (12). Although in vitro eNOS expression is upregulated by several factors including vascular shear stress (30) and oxygen exposure (8, 19, 21), the effect of in vivo CH exposure on pulmonary eNOS expression remains controversial.

Conflicting data exist concerning the effects of CH on pulmonary eNOS regulation and NO production in vivo. For example, several groups have reported that NO-dependent vasodilation is impaired in models of CH-induced PH (1, 3, 5, 20), whereas others have reported that NO-dependent vasodilation is unchanged or augmented under similar conditions (6, 36, 40, 41). Pulmonary eNOS expression increases with CH exposure in the adult rat (15, 32, 33). Some of this controversy may be attributed to study design or species differences. The ontogeny of pulmonary vascular eNOS has been described in several species (13, 26), and, in general, pulmonary eNOS expression peaks at mid to late gestation and decreases after birth. However, the response of pulmonary eNOS expression is variable in neonatal pigs exposed to CH (7, 14, 42).

It is unclear whether these disparate findings represent differences in experimental design, species differences, or developmental differences in eNOS regulation during CH. Therefore, to clarify this issue, we performed the following studies to test the hypothesis that eNOS expression in response to CH within a species changes with development. Adult and neonatal rats were exposed to 2 weeks of CH or normoxia; we then assessed whole lung eNOS protein, plasma nitrite/nitrate (NOx⁻), and stained fixed lung sections for eNOS protein localization. In addition, to assess the physiological response to CH, we measured hematocrit (Hct), RV weight-to-total ventricular weight ratios (RVT), and percent pulmonary arterial wall area (%wall area).

METHODS

Experimental Groups

All protocols employed in this study were reviewed and approved by the Institutional Animal Care and Use Com-
mittee of the University of New Mexico Health Sciences Center.

Adult male Sprague-Dawley rats (250–275 g; Harlan Industries) and neonatal rats (on day 2 of life; Harlan Industries) were placed in a hypobaric hypoxic chamber with barometric pressure maintained at 380 ± 5 mmHg for a period of 2 weeks (n = 6 for adults, n = 2 litters of 10–12 neonates each). Neonates were housed with their birthing dam. The chamber was opened three times per week to provide animals with fresh food, water, and clean bedding. Animals were removed from the hypoxic chamber and immediately placed in a hypoxic (12% O2-88% N2) gas mixture to maintain hypoxic exposure until the time of death. Age-matched normoxic controls were housed in similar conditions except under ambient barometric pressure (630 mmHg) (n = 6 for adults and n = 2 litters of 10–12 neonates each). All animals were housed on a 12:12-h light-dark cycle.

Tissue and Plasma Preparation

On the day of study, adult or neonatal rats were anesthetized with intraperitoneal injections of pentobarbital sodium (32.5 mg per adult or 3.2 mg per neonate). After a median sternotomy, heparin (100 U per adult and 10 U per neonate) was injected into the RV. Whole blood was drawn from the left ventricle (LV) for plasma NOx and Hct determinations. The lungs were removed, rinsed in ice-cold phosphate-buffered saline (PBS), pH 7.4, blotted dry, and snap frozen in liquid N2. Lungs from neonates were pooled in groups of three for immunoblot analysis (see eNOS Immunoblot Analysis). The hearts were removed, rinsed in cold PBS, and placed in 10% buffered formalin for RV/T determination (see below).

Assessment of RV Hypertrophy

Determination of RV hypertrophy (RVH) was performed, as previously described (32), by personnel masked to the treatment. Briefly, the atria and major vessels were removed from the fixed hearts, and the RV wall was dissected from the LV and septum. The RV and the LV + septum were weighed, and the RV/T calculations were used to assess the degree of RVH.

Assessment of Vascular Remodeling

Adult rat pulmonary vascular remodeling was assessed in an additional set of animals, as previously described (34, 35), and neonatal rat pulmonary vascular remodeling was assessed in an additional set of neonates, as described in Preparation of neonatal lung sections.

Preparation of adult lung sections. Adult rats were anesthetized with pentobarbital sodium (32.5 mg ip). After tracheal cannulation with a 17-gauge needle stub, the lungs were ventilated with a Harvard positive-pressure rodent ventilator (model 683) at a frequency of 55 breaths/min and a tidal volume of 2.5 ml with a warmed and humidified gas mixture (6% CO2 in room air). Inspiratory pressure was set at 9 cmH2O, and positive end-expiratory pressure was set at 3 cmH2O. After a median sternotomy, heparin (100 U) was injected directly into the RV, and the pulmonary artery was cannulated with a 13-gauge needle stub. The preparation was immediately perfused at 0.8 ml/min via a microprocessor-controlled pump (model 7524-10, Masterflex) with 250 ml of a physiological saline solution (PSS) containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO4, 19 NaHCO3, 1.8 CaCl2, 5.5 glucose with 10% (wt/vol) albumin, and 10−4 M papaverine (all from Sigma Chemical, St. Louis, MO). The LV was cannulated with a plastic tube (4 mm OD), and the heart and lungs were removed en bloc and suspended in a humidified chamber maintained at 38° C. Perfusion rate was gradually increased to 60 ml-min−1 kg body wt−1. PSS perfusion was followed with 250 ml of fixative (0.1 M PBS with 4% paraformaldehyde, 0.1% glutaraldehyde, and 10−4 M papaverine). During both PSS and fixative perfusion, venous pressure was maintained at 12 mmHg to maximally recruit and distend the vascular surface area. To optimally distend the lungs, the trachea was filled with fixative to a pressure of 23 cmH2O during fixation. The trachea was then ligated, the arterial and venous ends were simultaneously clamped, and the lungs were immersed in fixative for 30 min. A transverse section (2- to 3-mm thickness) of tissue from the hilar level of the left lung was removed, rocked in fixative for an additional 3.5 h, rinsed, and stored in 70% ethanol for paraffin embedding and sectioning. Transverse sections of lung were cut (4 μm thick), mounted on Superfrost Plus slides (Fisher Scientific), and elastin stained (Sigma Chemical).

Preparation of neonatal lung sections. Neonatal rats were anesthetized with pentobarbital sodium (3.2 mg ip). The trachea was cannulated via tracheotomy, and the lungs were ventilated at a tidal volume of 0.25 ml, without positive end-expiratory pressure and with a room air-gas mixture. The heart and lungs were exposed, heparin (10 U) was injected directly into the RV, and the pulmonary artery and LV were cannulated. The preparation was perfused through the pulmonary artery first with 5 ml of PSS and then with 5 ml of fixative at 20 cmH2O. The lungs were inflated with fixative via the trachea, also at 20 cmH2O, and the preparation was allowed to stand for an additional 15 min. The heart and lungs were then removed en bloc and placed in fixative overnight. A transverse section (2- to 3-mm thickness) of tissue from the hilar level of the left lung was removed, washed with 70% ethanol, sectioned, and elastin stained (Sigma Chemical).

Elastin-stained vessel images from lung sections were used by personnel masked to the treatment to determine pulmonary vascular remodeling with MetaMorph Imaging System hardware and software (Universal Imaging) by using a Nikon Diaphot 300 microscope, as previously described (34, 35). The integrity and fullness (undulation free) of the internal elastic lamina identified artery from vein and indicated optimal lung fixation volume and pressure. The medial circumference, assessed from the outer margin of the external elastic lamina, luminal circumference, and vessel diameter, calculated from the outer edge of the medial circumference, were obtained for analysis. Percent arterial wall area was determined by subtracting the calculated area circumscribed by the luminal circumference from that area within the medial circumference and then dividing by the total area of the vessel. Vessels sectioned at oblique angles were excluded from analysis. Fifty vessels (20–50 μm thick) and 84 vessels (51–100 μm thick) were obtained from 12 adult rats. Two hundred fifty-two vessels (20–50 μm thick) and 103 vessels (51–100 μm thick) were obtained from 50 neonatal rats.

eNOS Immunoblot Analysis

eNOS immunoblot analysis was carried out as previously described (32, 33). Adult (n = 6) and neonatal (n = 6, where n is a pooled group of lungs from three neonates) frozen lungs from normoxic and chronically hypoxic animals were crushed with a precooled mortar and pestle, then homogenized on ice in Tris buffer [10 mM Tris-HCl (pH 7.4) containing 255 mM sucrose, 2 mM EDTA, 12 μM leupeptin, 1 μM pepstatin A, 0.3 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride (all

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from Sigma Chemical]. Tissue homogenates were centrifuged at 1,500 g at 4°C for 10 min. Protein concentrations were determined by using a commercially available assay (Bio-Rad). Samples (20 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 7.5% acrylamide, along with molecular-weight standards (Bio-Rad) and an eNOS standard (Transduction Laboratories). The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) and blocked overnight at 4°C with 5% nonfat milk (Carnation), 3% bovine serum albumin (Sigma Chemical), and 0.05% Tween 20 (Bio-Rad) in a Tris-buffered saline solution (TBS) containing 10 mM Tris-HCl and 50 mM NaCl (pH 7.5). Blots were incubated for 4 h at room temperature with a mouse monoclonal antibody raised against human eNOS (1:10,000; Transduction Laboratories) in TBS. Immunohistochemical labeling was achieved by incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; Bio-Rad) in TBS. Chemiluminescence labeling was performed per kit instructions (Amersham). eNOS protein bands were detected by exposure to chemiluminescence-sensitive film and quantitated by densitometric analysis (Sigma Gel, Jandel Scientific). Membranes were stained with Coomassie brilliant blue to confirm equal protein loading per lane (34).

**NOx**− Assay

Personnel masked to the treatment assayed duplicate plasma samples spectrophotometrically for NOx−, as previously described (24). All reagents were obtained from Sigma Chemical. Plasma samples were diluted 1:10 with Krebs-Ringer buffer. Reduced NADP (50 μl, 0.8 mg/ml phosphate buffer) and nitrate reductase (10 μl, 5 U/ml phosphate buffer) were added to 500 μl of diluted plasma, and the mixture was incubated for 3 h at room temperature. Then 300 μl of Griess reagent [1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% phosphoric acid] were added and incubated for 10 min at room temperature. Absorbance was measured at 546 nm against a blank consisting of Krebs-Ringer buffer with added reduced NADP, nitrate reductase, and the Griess reagent. Absorbance values were compared with a standard curve generated by using NaNO3 with added nitrate reductase.

**eNOS Immunohistochemistry**

Immunohistochemistry was performed as previously described (33). Lung sections, 4 μm thick, were cut from fixed and paraffin-embedded tissue prepared as described above and mounted on Super Frost Plus slides (Fisher Scientific). Sections were deparaffinized and hydrated by using xylene, an ethanol gradient, and washed into PBS with Triton X-100 (PBS-TX; 50 mM Na2HPO4, 140 mM NaCl, 0.3% Triton X-100, at pH 7.4). Endogenous peroxidases were blocked with 0.3% H2O2 in PBS-TX. Sections were then rinsed in PBS-TX and incubated with 4% horse serum in PBS-TX, followed by incubation for 24 h at 4°C with mouse monoclonal antibody for eNOS (1:2,500; Transduction Laboratories) in PBS-TX. Then the sections were incubated for 2 h at 37°C with rat-absorbed biotinylated horse anti-mouse IgG (1:400; Vector Laboratories). After rinsing in PBS, the sections were treated with Autoprobe Peroxidase (Biomed) for 20 min at 37°C and rinsed. Working Chromagen (Biomed) was used to visualize antibody-labeled proteins. Sections were washed in distilled H2O and mounted with a combination of Crystal/ Mount (Biomed) and Permount (Sigma Chemical). Control sections were prepared by incubation with mouse IgG (1:2,500; Sigma Chemical) instead of primary antibody.

**Statistics**

All data are expressed as means ± SE, and n refers to the number of animals in each group, except for the neonatal eNOS immunoblot of lung homogenates, where n refers to the number of pooled samples of lungs from three neonates. Data were compared by Student’s t-test or where appropriate by two-way ANOVA. If differences were detected by ANOVA, individual groups were compared by using the Student Newman-Keuls test. All data expressed as percentages were normalized by using arcsine transformation before statistical analysis with appropriate parametric tests. A level of P < 0.05 was accepted as statistically significant for all comparisons.

**RESULTS**

After 2 wk of hypoxia, both adult and neonatal CH rats weighed less than the corresponding normoxic rats (Table 1). Mean Hct of adult hypoxic rats was ~27% greater than Hct of adult normoxic rats (Table 1). Mean Hct of neonatal hypoxic rats was ~33% greater than Hct of neonatal controls (Table 1).

**Assessment of RVH**

The adult hypoxic mean RV/T was 56% greater than that in adult normoxic rats. The neonatal hypoxic mean RV/T was 90% greater than that in neonatal normoxic rats (Table 1). There was no difference in total heart weight between the adult hypoxic and adult normoxic rats; however, there was an increase in total heart weight in the neonatal hypoxic rats compared with the neonatal normoxic rats (Table 1).

**Assessment of Vascular Remodeling**

The %wall area of 20- to 50-μm and 51- to 100-μm diameter pulmonary arteries was greater in the adult CH group compared with the adult normoxic group (Fig. 1, A and B). Similarly, the %wall area of 20- to 50-μm and 51- to 100-μm diameter pulmonary arteries

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<td>Body weight, g</td>
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Values are means ± SE, n, number of rats; Hct, hematocrit; RV/T, right ventricular weight-to-total ventricular weight ratio. *P < 0.01 from corresponding control.
was greater in the neonatal CH group compared with the neonatal normoxic group (Fig. 1, C and D).

**eNOS Immunoblot Analysis**

Immunoblot analysis of eNOS protein levels in whole lung homogenates from normoxic and CH adult and neonatal rats demonstrated a band that comigrated with the eNOS standard at ~140 kDa (Fig. 2, A and B, respectively). Lung homogenates from CH adult rats exhibited more intense eNOS protein staining compared with lungs from normoxic adult rats. In contrast, lung homogenates from CH neonatal rats exhibited less intense eNOS protein staining compared with normoxic neonatal controls. Membranes stained with Coomassie brilliant blue confirmed equal protein loading per lane (data not shown) (34). eNOS protein levels were greater in the CH adult group compared with adult normoxic controls, as measured by densitometry (Fig. 3A). In contrast, eNOS protein levels in the CH neonatal group were lower than in normoxic neonatal controls (Fig. 3B).

**NOx^− Assay**

Consistent with the immunoblot data, plasma NOx^− concentrations obtained from CH adult rats were greater than those of normoxic adult rats, (Table 2). In contrast, plasma NOx^− concentrations from CH neo-
normoxic controls, suggesting that eNOS activity paralleled the changes in eNOS protein levels in CH neonates. Furthermore, we found both adult and neonatal rats responded to CH with poor weight gain, polycythemia, RVH, and increased %wall area, which are consistent with the development of PH.

It is possible that the neonatal increase in RV/T and %wall area with CH exposure are a result of the elevated RV and pulmonary arterial mass associated with the fetal state. Thus the CH response in the neonate may represent a failure of normal postnatal adaptation, i.e., thinning of the RV muscle mass and similar thinning of the pulmonary arteries. Whether this difference in RV/T and vascular remodeling is caused by hypoxia per se or other unrecognized processes remains for further investigation.

Endothelial cells represent a single layer of cells in the pulmonary vasculature. As lung vessel remodeling occurs, with medial thickening, a decrease in the contribution of the endothelial cells to total lung protein might explain our results. However, the finding that pulmonary vascular remodeling is similar in hypoxic adults and hypoxic neonates, yet eNOS goes up in adults and down in neonates, suggests that vascular remodeling alone did not account for the observed changes in pulmonary eNOS levels.

Plasma NOx\textsuperscript{-} is a reflection of total body NO formation, which may include tissues other than the lung as sources and may also be influenced by a variety of NOS-independent factors, such as dietary intake, bowel flora nitrate synthesis, and denitrifying liver enzymes. Because we could not control for differences in maturity of enzyme systems, diet, and enteric bacterial load between pups and adults, we focused our comparisons between age-matched CH and normoxic groups. Furthermore, we found that both adult and neonatal CH groups lost weight compared with their respective normoxic controls, but the levels of plasma NOx\textsuperscript{-} paralleled the changes in eNOS expression.

The greater difference in weights between the CH and normoxic neonates compared with adults suggests that an effect of CH on the birthing dam may be contributory. However, Mortola et al. (23) demonstrated that, in rats, neonatal growth retardation during severe (10%) hypoxic exposure can be almost entirely attributed to the effects of hypoxia on the newborn and is not mediated by the maternal response. Furthermore, Moromisato et al. (22) demonstrated that decreased nutritional intake was not the sole cause of the weight difference between chronically

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**Table 2. Plasma NOx\textsuperscript{-} concentration responses to chronic hypoxia**

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<th>Adult Hypoxic</th>
<th>Neonatal Control</th>
<th>Neonatal Hypoxic</th>
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<tr>
<td>(n)</td>
<td>5</td>
<td>6</td>
<td>17</td>
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<td>Plasma NOx\textsuperscript{-}, (\mu M)</td>
<td>17.5 ± 0.8</td>
<td>24.9 ± 2.0\textsuperscript{a}</td>
<td>37.2 ± 2.6</td>
<td>21.4 ± 1.3\textsuperscript{a}</td>
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\(\text{Values are means ± SE. NOx}^{-}, \text{ plasma nitrate/nitrate.} *P < 0.02\) from corresponding control.
hypothesis that the ability to upregulate eNOS expression with CH exposure is briefly inhibited after birth.

Hypoxia and mechanical forces, such as shear stress, are known regulators of eNOS expression. However, in lungs from CH adult rats, the contribution of hypoxia vs. changes in shear stress in the regulation of eNOS remains controversial. For example, in cultured endothelial cells not exposed to shear stress, expression of eNOS mRNA and eNOS protein levels decreases with exposure to hypoxia. In contrast, in vivo studies with adult rats suggest a stimulatory role of hypoxia per se in CH-induced pulmonary eNOS upregulation. These results suggest contradictory roles of hypoxia in the regulation of eNOS expression in vivo and in vitro that are independent of changes in shear stress.

On the other hand, recent data from our laboratory support the involvement of shear stress in the CH-induced upregulation of pulmonary eNOS in adult rats. For example, eNOS levels and activity are increased selectively within the pulmonary arterial circulation where shear forces are increased due to the combination of polycythemia and vascular remodeling that occurs with CH exposure. Furthermore, our laboratory has recently reported that pulmonary arterial eNOS expression remains elevated in rats 2 wk after exposure to CH, when there is persistence of polycythemia and pulmonary vascular remodeling and no hypoxic stimulation. These findings support a role of mechanical factors such as shear stress rather than hypoxia per se in CH-induced increases in eNOS expression in the adult rat.

In the present study, we observed that CH stimulated polycythemia in both adult and neonatal groups. Both adult Hct and eNOS levels were greater under conditions of CH compared with neonates. Thus one possible explanation for the observed differences in CH-induced changes in eNOS expression between adults and neonates may be the degree of polycythemia. Polycythemia can increase shear stress on the vessel wall by increasing blood viscosity, and increased shear stress has been shown to increase eNOS expression.

In addition, our laboratory has recently reported that polycythemia-induced polycythemia alone was an insufficient stimulus to increase pulmonary eNOS expression or activity in the adult rat pulmonary vasculature. Thus it is unlikely that differences in Hct between adults and neonates under the same CH conditions led to the observed disparity in eNOS expression.

Finally, conditions specific to the neonate might be expected to contribute to the differences in eNOS expression between CH adults and neonates reported in the present study. For example, neonatal pulmonary blood flow could be affected by the ductus arteriosus. In the fetus, the ductus arteriosus directs blood flow away from the lung. The closure of the ductus arteriosus is partially regulated by O2 levels, and neonatal hypoxia has been shown to delay its closure. Therefore, it is possible that in the CH neonatal group there may have been right-to-left blood flow through the ductus arteriosus, which would result in decreased pulmonary blood flow. The potential for decreased pulmonary blood flow in the neonatal CH group may explain the difference in eNOS response to CH between neonates and adults. Further studies are required to determine the potential role of pulmonary blood flow on the differential response of eNOS expression to CH in neonates and adults.

In conclusion, despite similar physiological responses to CH, neonatal rat lungs exhibited less eNOS expression, whereas adult rat lungs exhibited greater eNOS expression than their respective normoxic controls. These results support our hypothesis of a developmental difference in the response of eNOS expression and NO production to CH. This divergent response may contribute to increased hypoxia-induced vasoconstriction in the neonate that leads to the apparent greater RVH in neonates compared with adults.

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