Inhaled Nitric Oxide Effects on Lung Structure and Function In Chronically Ventilated Preterm Lambs

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Online Data Supplement

METHODS

Preparation of Animals for Experiments

Briefly, time-dated pregnant ewes received spinal anesthesia for a midline hysterotomy, through which catheters were placed in a carotid artery, jugular vein, and hindlimb artery and vein of the fetus under 1% lidocaine local anesthesia. Residual lung liquid was withdrawn from the fetal trachea, followed by injection of 10 ml of calf lung surfactant (35 mg/ml Infasurf, gift of Ony Inc, Amherst, NY) into the lung lumen through a 4.5- or 5-mm endotracheal tube just before delivery. The lambs were mechanically ventilated for 3 weeks, initially with a high-frequency jet ventilator (Bunnell Inc, Salt Lake City, UT) set at a frequency of 420 cycles/min, mean airway pressure of 7-8 cmH_2O, sufficient inflation pressure to keep the $PaCO_2$ at ~35-45 mmHg, and sufficient supplemental O_2 to keep the PaO_2 at 50-90 mmHg. We used high-frequency mechanical ventilation immediately after birth in order to minimize tidal volumes and peak inflation pressures, thereby reducing the risk of extrapulmonary air leaks and early death while attaining adequate respiratory gas exchange in the lungs of these extremely premature lambs.

After an initial period of stabilization, usually 24-72 hours after birth, the lambs were switched to conventional mechanical ventilation with a time-cycled, pressure-limited infant respirator (model IV-100B, Sechrist Inc, Anaheim, CA) set at a rate of 20 breaths/min, inspiratory time of 0.75 sec, and end-expiratory pressure of ~5 cmH_2O. Peak inflation pressure was adjusted to keep $PaCO_2$ at ~35-45 mmHg, and the fraction of inspired oxygen (FiO_2) was adjusted to keep PaO_2 at ~50-90 mmHg. The intent of the switch from early high frequency ventilation to sustained mechanical ventilation using a

slow rate, long inspiratory time and relatively low target level of $PaCO_2$ was to induce long-term cyclic stretch of the lungs with O_2-rich gas over a prolonged period and thereby create chronic lung injury.

The lambs were managed on a padded platform bed beneath a radiant warmer. Their arterial blood pressure was monitored continuously with a calibrated pressure transducer connected to an electronic recorder, and during the stabilization period they received a solution of glucose and saline intravenously (iv) to maintain normal blood glucose and sodium concentrations. The lambs also received buprenorphine (Buprenex, Recritt and Coleman Pharmaceuticals, Richmond, VA), 0.01 mg/kg iv, soon after birth and as often as needed thereafter to prevent agitation.

Each lamb had surgery within 2 days of birth to allow subsequent measurements of PVR. They received general anesthesia with iv fentanyl (Abbott Laboratories, North Chicago, IL), an initial dose of 15-20 μg/kg followed by supplemental doses of 10 μg/kg as often as needed to prevent intraoperative tachycardia or hypertension. As previously described (E1), we did a left thoracotomy to ligate the ductus arteriosus and place catheters in the pulmonary artery and left atrium and a thermistor wire (model SP 5003, Gould, Statham Instruments, Oakland, CA) directly into the pulmonary artery for subsequent measurement of cardiac output by thermodilution. We placed an 8-Fr catheter in the left pleural space for postoperative drainage of air and liquid, in addition to a silicone rubber balloon catheter that was used for subsequent measurement of pleural pressure in conjunction with assessment of lung mechanics.

The lambs received buprenorphine, 0.01 mg/kg iv, every 4-6 hours for postoperative analgesia, and they later received both buprenorphine and either 3-5 mg/kg iv

pentobarbital sodium (Vet Lab, Lenexa, KS) or 10 mg/kg iv phenobarbital (Wyeth Laboratories, Philadelphia, PA) as needed for sedation. We sampled arterial blood hourly to measure pH, PaO_2 and $PaCO_2$, and adjusted ventilator settings accordingly. We measured hematocrit by blood centrifugation twice daily and gave transfusions of filtered maternal blood if the hematocrit was <35%. We infused iv maternal plasma or isotonic saline if mean arterial blood pressure was <40 mmHg.

The lambs received iv sodium penicillin, 100 mg/kg every 12 hours, and gentamicin, 2.5 mg/kg every 24 hours for at least a week after birth. If signs of sepsis developed thereafter, alternative broad-spectrum antibiotics were given. We provided nutrition with iv solutions containing glucose, protein (trophamine), electrolytes, trace metals and vitamins, and with ewe's milk that was given through an orogastic tube. The lambs were weighed daily to monitor their fluid balance and nutritional status. Serum electrolytes were measured at least once each day with ion-selective electrodes (Na/K/Cl Stat Analyzer, model 644, Ciba Corning Diagnostics, Medfield, MA) as a guide to help adjust fluid and electrolyte management, and blood glucose concentrations were monitored with a rapid detection device (Exactech Medisense, Waltham, MA). Urine output was determined from diaper weights before and after each voiding.

Physiological Studies.

Physiological studies included weekly measurements of steady-state pulmonary arterial and left atrial pressures and cardiac output (measured in triplicate by thermodilution using model SP 1425 Cardiac Output Computer, Gould Inc, Statham Intruments, Oxnard, CA) to determine PVR. We measured vascular pressures with calibrated pressure transducers (BT3DC, Statham Instruments) connected to an 8-channel

amplifier-recorder (model 7D, Grass Instruments, Quincy, MA). Respiratory variables were assessed weekly from simultaneous measurements of proximal airway and pleural pressures and gas flow that was measured using a calibrated pneumotachograph connected to a PEDS Pulmonary Evaluation and Diagnosis System (Medical Associated Services, Hatfield, PA) **(**E2**)**. Measured variables included tidal volume (V_T), minute ventilation (V_E), dynamic lung compliance (C_L) and expiratory resistance (R_E). We sampled arterial blood for measurement of pH, PaO_2 and $PaCO_2$ before and after measurements of PVR and lung mechanics.

Postmortem Studies

Lung Sampling. After 3 weeks of continuous mechanical ventilation with or without iNO, the lambs received iv 1000 U of heparin and 35 mg/kg body weight of pentobarbital sodium. We rapidly split the sternum, double-clamped the lung lobes, and excised the tissue with the clamps still attached. Lung lobes were clamped at the prevailing peak inflation pressure so that we could relate lung histopathology to the physiological studies just before death. Clamping the lungs retained air volume, as well as vascular and airspace contents. We placed the clamped right middle lobe in Carnoy's fixative for subsequent assessment of quantitative histology, and the left lingula was immersed in 10% buffered formalin for subsequent immunohistochemistry.

Quantitative Histopathology. We used a design-based method to randomly sample the lung for quantitative histopathology **(3)**. Briefly, we cut each piece of lung into 3-mm thick slabs along parasagittal planes. Large tissue blocks, 2-4 cm^2, were embedded in paraffin and serially sectioned at a thickness of 5 μm. Three stains were applied to the tissue sections to reveal the various structural features of the lung: 1) hematoxylin and

eosin for radial alveolar counts, 2) Masson's trichrome stain to distinguish between smooth muscle and connective tissue elements, and 3) Hart's elastic fiber stain to define the external and internal elastic lamina around the smooth muscle in blood vessels and airways, and to assess elastin accumulation in distal lung parenchyma. A systematic sampling method was used to evaluate random, non-overlapping calibrated fields **(**E3**)** to assess each structural variable described below. The Bioquant True Color Windows Image Analysis system (R & M Biometrics, Nashville, TN) was used to make measurements. Tissue sections were analyzed without knowledge of the lamb group from which the tissue was taken.

To assess abundance of vascular smooth muscle, we traced the external perimeter (vessel area) and the internal perimeter of the media (endothelium plus lumen area). We subtracted the area of the vessel lumen and endothelium from the vessel area to obtain the media area. Results are expressed as the ratio of media area to vessel area. Tissue sections that were processed with Hart's elastic fiber stain were used because elastic fibers delineated the outer and inner boundaries of the vascular smooth muscle layer. Several steps were taken to select small pulmonary arteries that were of comparable generation, shape and size. First, we used terminal bronchioles as the independent landmark for selecting small arteries. Second, only circular cross-sectional profiles of the selected arteries were analyzed. Circular profiles were defined as having an X-Y projection length ratio between 0.8 and 1.2 (circle has a ratio of 1.0). The X-Y projection lengths (diameters) and ratio were calculated automatically from the external perimeter tracing of each vessel. Third, the external diameter measurements of the arteries enabled verification that the vessels were of similar size within and between lung specimens from

different animals. We used the same methods as described above to estimate smooth muscle abundance around small airways that were < 300 μm external diameter. An average of 5 circular profiles of small arteries and 15 circular profiles of terminal bronchioles were analyzed per lamb.

To estimate the number of small blood vessels per unit area of lung tissue, arteries and veins that were 20-100 μm external diameter were counted at a video-projected, color image magnification of x136. The number of blood vessels in 15 non-overlapping calibrated fields was counted in one random tissue section per lamb. The reference space for vessel counts was the surrounding lung parenchyma, the area of which was estimated by point-counting using a 192-point coherent square lattice **(**E4**)**. Using this reference space minimized the effect of different degrees of lung inflation because the air space volume density was excluded.

Alveolar number across terminal respiratory units was estimated by the radial alveolar count method described by Emery and Mithal **(**E5**)**. To apply this method, a line was drawn from the center of a respiratory bronchiole to the nearest interlobular septum, to which an intercept line was drawn in a perpendicular plane. We counted the number of distal air spaces that were transected by the line, and repeated this measurement for 10 terminal respiratory units in one random tissue section per lamb.

We used immunohistochemistry and morphometry to estimate the surface density (S_v) of capillaries in the walls of distal air spaces. Endothelial cells of capillaries were highlighted by immunostaining with anti-bovine PECAM-1 antibody (platelet-endothelial cell adhesion molecule-1, generous gift of Dr Steven Albelda, University of Pennsylvania, Philadelphia, PA) and immunoperoxidase stain **(**E6**)**. Color images

(magnification x2860) of 15 to 20 random fields of distal lung tissue per lamb were video-projected onto a cycloid grid (178 points, P_T, 2.0 cycloid length, D_{grid}, for intersection and point counting (3). Intersections (I) were counted for the immunoperoxidase-stained endothelium of capillaries (I_{cap}), and at the air-tissue interface for determination of epithelial surface density (I_{epi}), which served as a reference for capillary surface density. Points overlying lung tissue (P_{lu}) were counted for volume density (V_v) of lung parenchyma (V_v *lu*). Capillary surface density was calculated from the formula, $S_v = 2 \times \Sigma I/V_v\ lu$, where $V_v\ lu = \Sigma Plu \times (D_{grid}/\text{Magnification})$ **(**E3**)**.

Immunoblot Analysis of eNOS Protein. Segments of third and fourth generation lung arteries and airways were placed in a tissue homogenizer containing Tris·HCl buffer (50 mM Tris, pH 7.5, 4°C) with 10 mM 3-[(3-cholamidopropyl) dimethylammoniol]-1-propane sulfonate (CHAPS), 3 mM dithiothreitol, 20 μM tetrahydrobiopterin, and protease inhibitors (2 μg/ml of pepstatin A, 20 μg/ml of leupeptin, 40 μg/ml of N∝-*p*-tosyl-L-lysine chlormethylketone, and 20 μg/ml of aprotinin). The homogenized tissues were centrifuged, and aliquots of the supernatants were saved at -80°C. Protein content was determined by the bicinchoninic acid assay **(**E7**)** with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL). We performed SDS-PAGE on 50 μg total protein/sample, using the method described by Laemmli **(**E8**)**. The proteins were transferred electrophoretically to nitrocellulose filters and blocked for 1.5 hours in buffer containing 150 mM NaCl, 50 mM Tris·HCl and 0.1% Tween 20. The filters were incubated overnight at 4°C in the presence of a primary antibody specific for eNOS (1:500 dilution in blocking buffer; Transduction Laboratories, Lexington, KY). After incubation with the primary antibody, the nitrocellulose filters were washed with a

solution containing 150 mM NaCl, 50 mM Tris·HCl and 0.1% Tween 20, followed by 90 minutes incubation with a secondary antibody that was linked to horseradish peroxidase (Amersham, Little Chalfont, UK). Bands were visualized by chemiluminescence (ECL Western blotting analysis system; Amersham) and quantified by densitometry (NIH Image software).

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