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# Altered reactivity and nitric oxide signaling in the isolated thoracic duct from an ovine model of congenital heart disease with increased pulmonary blood flow

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**Datar SA, Oishi PE, Gong W, Bennett SH, Sun CE, Johengen M, Maki J, Johnson RC, Raff GW, Fineman JR.** Altered reactivity and nitric oxide signaling in the isolated thoracic duct from an ovine model of congenital heart disease with increased pulmonary blood flow. *Am J Physiol Heart Circ Physiol* 306: H954–H962, 2014. First published February 15, 2014; doi:10.1152/ajpheart.00841.2013.—We have previously shown decreased pulmonary lymph flow in our lamb model of chronically increased pulmonary blood flow, created by the in utero placement of an 8-mm aortopulmonary shunt. The purpose of this study was to test the hypothesis that abnormal lymphatic function in shunt lambs is due to impaired lymphatic endothelial nitric oxide (NO)-cGMP signaling resulting in increased lymphatic vascular constriction and/or impaired relaxation. Thoracic duct rings were isolated from 4-wk-old shunt ( $n = 7$ ) and normal ( $n = 7$ ) lambs to determine length-tension properties, vascular reactivity, and endothelial NO synthase protein. At baseline, shunt thoracic duct rings had 2.6-fold higher peak to peak tension and a 2-fold increase in the strength of contractions compared with normal rings ( $P < 0.05$ ). In response to norepinephrine, shunt thoracic duct rings had a 2.4-fold increase in vascular tone compared with normal rings ( $P < 0.05$ ) and impaired relaxation in response to the endothelium-dependent dilator acetylcholine (63% vs. 13%,  $P < 0.05$ ). In vivo, inhaled NO (40 ppm) increased pulmonary lymph flow (normalized for resistance)  $\sim$ 1.5-fold in both normal and shunt lambs ( $P < 0.05$ ). Inhaled NO exposure increased bioavailable NO [nitrite/nitrate ( $\text{NO}_x$ );  $\sim$ 2.5-fold in normal lambs and  $\sim$ 3.4-fold in shunt lambs] and cGMP ( $\sim$ 2.5-fold in both) in the pulmonary lymph effluent ( $P < 0.05$ ). Chronic exposure to increased pulmonary blood flow is associated with pulmonary lymphatic endothelial injury that disrupts NO-cGMP signaling, leading to increased resting vasoconstriction, increased maximal strength of contraction, and impaired endothelium-dependent relaxation. Inhaled NO increases pulmonary lymph  $\text{NO}_x$  and cGMP levels and pulmonary lymph flow in normal and shunt lambs. Therapies that augment NO-cGMP signaling within the lymphatic system may provide benefits, warranting further study.

lymphatic endothelial function; nitric oxide; nitric oxide-cGMP signaling

LYMPHATIC ABNORMALITIES are associated with many congenital heart defects (2, 14, 17, 19, 36), but the pathophysiological mechanisms are not known. A number of these defects, such as ventricular septal defects, truncus arteriosus, and atrioventricular canals, result in chronic increases in pulmonary blood flow (PBF). Recently, using our lamb model of such a congenital heart defect created by the in utero placement of a large

aortopulmonary graft (shunt), we demonstrated impaired pulmonary lymph flow with decreased transit kinetics and decreased bioavailable nitric oxide (NO) in lymphatic effluent at 4 wk of age compared with normal age-matched control lambs. In addition, the intrapulmonary lymphatic architecture was abnormal; lymphatic vessels appeared dilated and to have more smooth muscle (15).

It is well established that chronic exposure to increased PBF and pressure disrupts the normal regulatory function of the pulmonary vascular endothelium, resulting in abnormal pulmonary vascular constriction, impaired relaxation, and smooth muscle cell proliferation (1, 16, 22). Multiple mechanisms have been identified, including alterations in NO-cGMP signaling (4–7, 20, 29, 34, 40–42, 44, 45). Interestingly, emerging data have suggested that the lymphatic endothelium plays a similar prominent role in normal lymphatic function and development (30, 33). Furthermore, both in vitro and in vivo data have suggested that, like the pulmonary blood endothelium, NO-cGMP signaling pathways have an important role in promoting lymphatic flow (9, 25, 27, 31, 49). For example, in acute ex vivo studies, increased lymph flow stimulated endothelium-dependent NO production and relaxation in rat (25, 27) and canine thoracic ducts (49), responses that were blocked by the inhibition of NO synthase (NOS) enzyme activity.

Therefore, the purpose of this study was to test the hypothesis that abnormal lymphatic function in shunt lambs is due, in part, to impaired lymphatic endothelial NO-cGMP signaling resulting in increased lymphatic vascular constriction and/or impaired relaxation. To test this hypothesis, we isolated thoracic duct rings from 4-wk-old normal and shunt lambs. We determined and compared length-tension properties, agonist-induced contraction [in response to norepinephrine (NE)], and agonist-induced relaxation (in response to endothelium-dependent and endothelium-independent vasodilators). In addition, we determined endothelial NOS (eNOS) protein expression by immunohistochemistry. Finally, as an initial proof of concept, we tested whether exogenously delivered NO could augment NO-cGMP signaling within the pulmonary lymphatic system and its potential effects on pulmonary lymph flow. To this end, we measured pulmonary lymph flow in normal and shunt lambs before and during administration of inhaled NO and quantified levels of bioavailable NO [nitrite/nitrate ( $\text{NO}_x$ )] and cGMP in the pulmonary lymph effluent.

## METHODS

*Chronic model of increased PBF.* As previously described in detail (44), an 8.0-mm Gore-tex vascular graft,  $\sim$ 2 mm length (W. L. Gore

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and Associates, Milpitas, CA), was anastomosed between the ascending aorta and main pulmonary artery in an anesthetized late-gestation fetus (137–141 days gestation, term: 145 days) from nine mixed-breed Western ewes. Twin pregnancies (where one fetus underwent the shunt procedure) provided all of the normal age-matched control lambs. Six normal lambs and six shunt lambs underwent the inhaled NO administration protocol (described below). At the end of the in vivo experiments, while the lamb was still under general and regional anesthesia, the thoracic duct was isolated by dissection for ex vivo analysis to determine length-tension properties, vascular reactivity, and eNOS protein levels. Lambs were then euthanized as described below. To ensure that exogenous inhaled NO treatment did not affect these ex vivo experiments, three normal lambs and three shunt lambs that did not receive inhaled NO were included in the ex vivo thoracic duct ring experiments.

**Isolated thoracic duct experiments.** Four weeks after spontaneous delivery, normal ( $n = 6$ ) and shunt ( $n = 6$ ) lambs were anesthetized, mechanically ventilated, and instrumented to continuously measure hemodynamics and PBF (44). After completion of the hemodynamic measurements (see below), a segment of the thoracic duct between T5 and T7 was harvested, and thoracic duct rings were isolated and mounted as previously described (48). Rings obtained from lambs that had received inhaled NO were harvested after a minimum of 1 h after the termination of NO. Ring segments of the thoracic duct (2 mm long, lacking valves) were prepared and mounted on 40- $\mu$ m wires in multichannel myographs (DMT610M, Danish Myo Technology, Aarhus, Denmark) for dynamic measurements of isometric force development. Up to four vessel segments from a single animal were mounted for recording. Vessels were maintained at 37°C in physiologic saline solution (PSS) equilibrated with a mixture of 21% O<sub>2</sub> and 5% CO<sub>2</sub> throughout the experiments (pH 7.4). Isometric force (in mN) was sampled at 20 Hz with a Powerlab8/30 (AD Instruments) using LabChart version 6.1.1 software and reported as tension (in mN/mm) by dividing by [2  $\times$  segment length (in mm)].

The mounted ring segments were allowed to equilibrate for 15 min (under zero tension) in PSS equilibrated with 21% O<sub>2</sub> and 5% CO<sub>2</sub>. To normalize thoracic duct rings from normal and shunt lambs, ring segments were loaded with a passive resting force of 0.2 mN. Pilot experiments, performed as previously described (48), revealed that this passive resting force yielded a maximum tension development to PSS with NE and K<sup>+</sup> (NE-KPSS) in both normal and shunt lambs. After normalization, ring segments were allowed to equilibrate for a further 60 min.

The ring segment constriction response protocol included a maximal response to KPSS and a dose response to NE (with 9 progressive molar concentrations of  $1 \times 10^{-9}$ ,  $3 \times 10^{-9}$ , and up to  $1 \times 10^{-5}$  M NE) relative to the passive force. The relaxation response was evaluated by pretreatment with NE to 80% maximum constriction (EC<sub>80</sub>) followed by a dose response to ACh (5 doses:  $10^{-7}$ – $10^{-4}$  M) or *S*-nitroso-*N*-acetylpenicillamine (SNAP; 4 doses:  $10^{-8}$ – $10^{-5}$  M). Only segments that developed peak active tension > 0.05 mN/mm were included for statistical analysis (thus, 8 rings from 5 normal lambs and 10 rings from 3 shunt lambs were eventually excluded from the final analysis).

Lymph vessel rings demonstrate two dynamic components of developed force upon active contraction/relaxation: 1) a low-frequency trend that reaches a steady state over time similar to arteries (34) plus 2) superimposed spontaneous pulsations, reflective of lymphatic pumping (24). The low-frequency trend was evaluated by subjecting the original sampled data to a low-pass digital filter with a cutoff frequency of 0.02 Hz (Fig. 1, A and B). The frequency pulsation trend was evaluated by subjecting the original data to a high-pass digital filter with a cutoff of 0.02 Hz. From the high-pass filtered data, the peak to peak value of pulsations was evaluated, along with the first derivative of tension, as a contractility index under conditions of isometric tension.

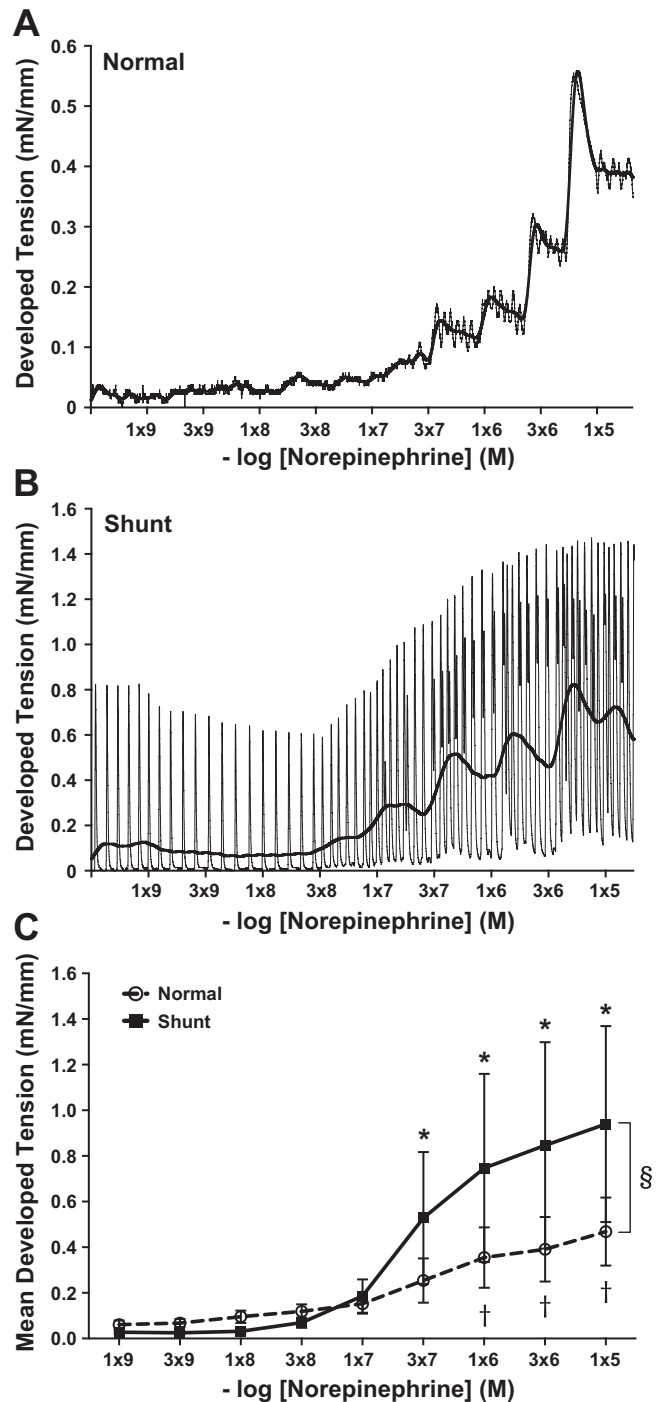


Fig. 1. A: representative graph of a normal (A) and shunt (B) thoracic duct ring in response to increasing log doses of norepinephrine (NE). Active contractions of lymph vessels demonstrate two dynamic components: 1) a low-frequency dynamic trend in developed force (thick solid tracing) that reaches a steady state over time similar to arteries (34) plus 2) a superimposed spontaneous pumping-like force of contraction that is pulse like, reflective of a lymph's pumping ability (24). Dynamic contraction behavior was evaluated from the dose response to NE at selected doses (baseline or passive stretch to 0.2-mN force followed by 9 progressive molar concentrations of  $1 \times 10^{-9}$  to  $1 \times 10^{-5}$  M NE). Note the different y-axis scales. C: shunt thoracic duct rings have 2.4 times greater response to log fold increases in NE than normal rings ( $\$P < 0.05$ ). Values are means  $\pm$  SE;  $n = 7$  normal lambs and 7 shunt lambs. \* $P < 0.05$ , significant difference from baseline for shunt rings; † $P < 0.05$ , significant difference from baseline for normal rings.

Chemicals were all purchased from Sigma Aldrich. Stock solutions of NE, ACh, and SNAP were dissolved in distilled water and stored in aliquots at 20°C.

**Immunohistochemistry on the isolated thoracic duct.** The thoracic duct was harvested at the end of the hemodynamic experiments (as described above) and was fixed in 4% paraformaldehyde for 24 h at 4°C, rinsed in cold PBS, and then transferred to a 30% sucrose-PBS solution. After 24 h at 4°C, these samples were embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Torrance, CA) and cryosectioned at 10  $\mu$ m. Serial sections were collected onto Superfrost Plus slides (VWR Scientific, West Chester, PA), allowed to air dry at room temperature, and stored at -80°C until needed. Hematoxylin and eosin (H&E) staining of representative sections was performed using standard techniques. Multilabeling immunofluorescence was performed as previously described (15): tissue sections were allowed to come to room temperature, washed briefly in Tris-buffered saline (TBS) to remove residual OCT, treated with 20  $\mu$ g/ml proteinase K (Invitrogen, Life Technologies, Carlsbad, CA) in deionized water for 5 min, washed in TBS and 0.03% Tween (TBST) three times for 5 min, and blocked with Dako Antibody Diluent (Dako, Carpinteria, CA). Slides were placed in primary antibody diluted in blocking serum overnight at 4°C. Antibody dilutions were as follows: goat anti-lymphatic vessel endothelial hyaluronan receptor (LYVE)-1, 1:100 (AF2089, R&D Systems); mouse anti-eNOS, 1:100 (no. 610296, BD Transduction Laboratories); and rabbit anti-actin, 1:10,000 (sc-1616-R, Santa Cruz Biotechnology). Slides were then washed three times for 5 min in TBST and placed in the appropriate secondary antibodies, Alexa fluor (Invitrogen, Life Technologies) or Dylight (Thermo Fisher Scientific, Rockford, IL), in blocking serum for 60 min. Appropriate negative controls were performed using each primary and secondary antibody alone. Slides were washed in TBS four times for 5 min. Slides were mounted in Vectashield (Vector, Burlingame, CA) containing 4',6-diamidino-2-phenylindole (DAPI) and then coverslipped.

Images were taken with a Hamamatsu c10600 ORCA-R<sup>2</sup> Digital Camera on a Zeiss Axio Imager Z2 using a  $\times$ 10 DIC objective and the X-cite 120 Mercury/Halide system and then analyzed using ZEN pro 2012 software (Carl Zeiss Microimaging, Thornwood, NY). Identical exposure times were used for each channel for each sample. All vessels were stained for LYVE-1 to confirm lymphatic identity and with primary antibodies alone (data not shown). Secondary-alone negative controls were also performed, and the appropriate images are included in Fig. 4. Images of H&E-stained sections were captured using bright-field illumination. All images were subsequently processed using Adobe Photoshop CS5 software (Adobe, San Jose, CA).

**Preparation of protein extracts and Western blot analysis.** Preparation of thoracic duct protein for Western blot analysis was performed as previously described (5, 37, 51). The thoracic duct from individual animals did not yield enough total protein for adequate analysis; therefore, pooled homogenized extracts from shunt ( $n = 7$ ) and normal control ( $n = 7$ ) thoracic ducts were run, using 105  $\mu$ g/pooled sample. Pooled homogenate was divided equally over three lanes for each group to accommodate the sample volume. Primary antibodies to mouse anti-eNOS (no. 610297, BD Transduction Laboratories) were used at 1:500 dilution. The appropriate Li-COR secondary antibody was used: IRDye 800CW donkey anti-mouse (926-32212). The blot was scanned and analyzed using the Odyssey Infrared Imaging System (LI-COR Biosciences), and protein bands were determined to be in the dynamic range of the system. To normalize for protein loading, blots were reprobated with 1:10,000 rabbit anti- $\beta$ -actin (ab8227, Abcam).

**Inhaled NO experiments.** Four weeks after spontaneous delivery, normal ( $n = 6$ ) and shunt ( $n = 6$ ) lambs were anesthetized, mechanically ventilated, and instrumented to continuously measure hemodynamics and PBF (44). Through a right thoracotomy in the 10th intercostal space, the tail of the caudal mediastinal lymph node was clamped and resected to remove the systemic lymph contribution.

Through a right thoracotomy in the sixth intercostal space, the caudal mediastinal lymph node efferent duct was cannulated as previously described (15, 47).

Animals' vital signs, including core temperature, were monitored throughout the study, and they were given intravenous fluids and prophylactic antibiotics per protocol (44). After a 60-min recovery period, baseline blood samples were collected and hemodynamics were recorded; subsequently, lymph drainage was collected every 15 min for the duration of the study period. Hemodynamic variables were monitored and recorded continuously as well. After a pretreatment period of 90–120 min, inhaled NO was started at 40 ppm for 45 min. After lymph collection was completed, a section of the thoracic duct between T5 and T7 was harvested for immunohistochemistry or isolated ring analysis (as described below). Lymph fluid aliquots were centrifuged at 3,500 rpm to separate cells from the lymph fluid. The cell-free lymph was kept at 4°C for immediate use in assays or stored at -80°C.

At the end of each protocol, all lambs were euthanized with a lethal injection of pentobarbital sodium followed by bilateral thoracotomy, as described in the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. All protocols and procedures were approved by the Committees on Animal Research of the University of California-San Francisco and University of California-Davis.

**Hemodynamic measurements.** Pulmonary and systemic arterial pressures and right and left atrial pressures were measured using Sorenson neonatal transducers (Abbott Critical Care Systems, Chicago, IL). Mean pressures were obtained by electrical integration. Heart rate was measured by a cardiometer triggered from the phasic systemic arterial pressure pulse wave. Left PBF was measured with an ultrasonic flowmeter (Transonic Systems, Ithaca, NY). All hemodynamic variables were measured continuously using the Pomonah Physiology Platform (version 4.9-SP2) with the ACQ-7700 acquisition interface (Data Sciences, St. Paul, MN) and recorded with a Dell Vostro 3500 computer (Dell, Round Rock, TX). Blood gases and pH were measured on a Radiometer ABL5 pH/blood gas analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin concentration and oxyhemoglobin saturation were measured by a co-oximeter (model 682, Instrumentation Laboratory, Lexington, MA). Pulmonary vascular resistance was calculated using standard formulas. Pulmonary flow to systemic flow was determined using the Fick principle. A modified pulse-waveform analysis was performed on the pulmonary artery pressure tracing to estimate pulmonary capillary pressure as previously described (13, 15). Five pulse waveforms sampled at 1,000 Hz during the steady-state expiratory phase and recorded the average value. Body temperature was monitored continuously with a rectal temperature probe.

**Measurement of NO<sub>x</sub> and cGMP.** To quantify bioavailable NO, the concentration of NO and its metabolites were determined in lymph. In solution, NO reacts with molecular oxygen to form nitrite and with oxyhemoglobin and superoxide anion to form nitrate. NO<sub>x</sub> was reduced using vanadium (III) and hydrochloric acid at 90°C. NO was purged from the solution, resulting in a peak of NO for subsequent detection by chemiluminescence (NOA 280, Sievers Instruments, Boulder, CO), as previously described (8, 38, 56). The sensitivity was  $1 \times 10^{-12}$  mol, with a concentration range of  $1 \times 10^{-9}$  to  $1 \times 10^{-3}$  M of nitrate.

cGMP in lymph was quantified using an immunoassay ELISA kit per the manufacturer's instructions (ADI-900-013, Enzo). Samples were not acetylated. To remain in the linear range of the assay, lymph samples were diluted fivefold. Assays were run in duplicate.

**Statistical analysis.** Means  $\pm$  SD were calculated for NO<sub>x</sub> and cGMP levels. Means  $\pm$  SE were calculated for all hemodynamic variables; changes in variables before and after an intervention (i.e., the initiation of inhaled NO) were compared by a nonparametric Kruskal-Wallis test. If significant, Dunn's multiple-comparisons test was used to determine differences between times and groups. Re-

sponses from multiple thoracic duct rings taken from the same animal were averaged. Dose responses were analyzed by a nonparametric Friedman test. If significant, multiple comparisons were performed (by Dunn's multiple-comparison test, multiple *t*-tests with the Bonferroni correction, or the Mann-Whitney *U*-test with appropriate correction for multiple comparisons). *P* values of <0.05 were considered statistically significant. The fold change was calculated between baseline and the maximum dose response. Analysis was done using Prism 6 (Graphpad Software, La Jolla, CA).

## RESULTS

**Reactivity experiments in thoracic duct rings.** Representative reactivity tracings of normal and shunt thoracic duct rings are shown in Fig. 1, *A* and *B*. In response to NE, thoracic duct rings from shunt lambs (*n* = 7 animals, 14 rings) generated a 2.4 times larger increase in mean developed tension compared with rings from normal lambs (*n* = 7 animals, 22 rings, *P* < 0.05; Fig. 1*C*). Consistent with a previous report (48), normal and shunt thoracic duct rings had spontaneous pulsatile contractions (Fig. 1, *A* and *B*). We analyzed the frequency pulsation trend under baseline conditions of isometric contraction (EC<sub>80</sub>) and found that shunt thoracic duct rings had 2.6-fold higher peak to peak tension compared with normal thoracic duct rings (*P* < 0.05; Fig. 2, *A* and *B*) and that the strength of these contractions in shunt rings was 2-fold greater than in rings from normal lambs (*P* < 0.05; Fig. 2, *C* and *D*). With increasing doses of SNAP (Fig. 2, *B* and *D*) but not ACh (Fig. 2, *A* and *C*), the peak to peak tension and strength of contractions in shunt rings decreased to levels similar to normal rings.

Compared with normal rings, shunt rings had impaired relaxation in response to ACh (63% vs. 13%, *P* < 0.05;

Fig. 3*A*). However, both normal and shunt rings relaxed similarly in response to SNAP (72% vs. 80%; Fig. 3*B*), suggesting that shunt thoracic duct rings had a selective impairment of endothelium-dependent relaxation. Importantly, no differences were noted between rings isolated from lambs that had received inhaled NO and those that did not.

**Immunohistochemistry.** We compared relative eNOS protein expression in isolated thoracic ducts from shunt and normal lambs (Fig. 4) by staining for eNOS (red). eNOS expression was increased markedly in shunt thoracic ducts compared with normal control thoracic ducts (Fig. 4, *G* and *H*). By Western blot analysis, eNOS protein was also increased in pooled isolated thoracic duct homogenates from shunt lambs (*n* = 7) compared with normal control lambs (*n* = 7; Fig. 4*K*). Interestingly, thoracic ducts from shunt lambs also had increased cellularity and more smooth muscle based on H&E (Fig. 4, *A* and *B*), DAPI (Fig. 4, *C* and *D*), and actin (Fig. 4, *I* and *J*) staining.

**Inhaled NO experiments.** Hemodynamic data for intact normal and shunt lambs at baseline and during exposure to inhaled NO are shown in Table 1. Consistent with previous studies, shunt lambs had significantly increased PBF, pulmonary artery pressure, and left atrial pressure compared with normal lambs, and calculated left pulmonary vascular resistance was lower (Table 1). Mean systolic arterial pressure was lower in shunt lambs due to lower diastolic blood pressure (data not shown). In response to 40 ppm of inhaled NO, there was a significant decrease in pulmonary artery pressure and left pulmonary vascular resistance in both normal and shunt lambs. In addition, the calculated pulmonary capillary pres-

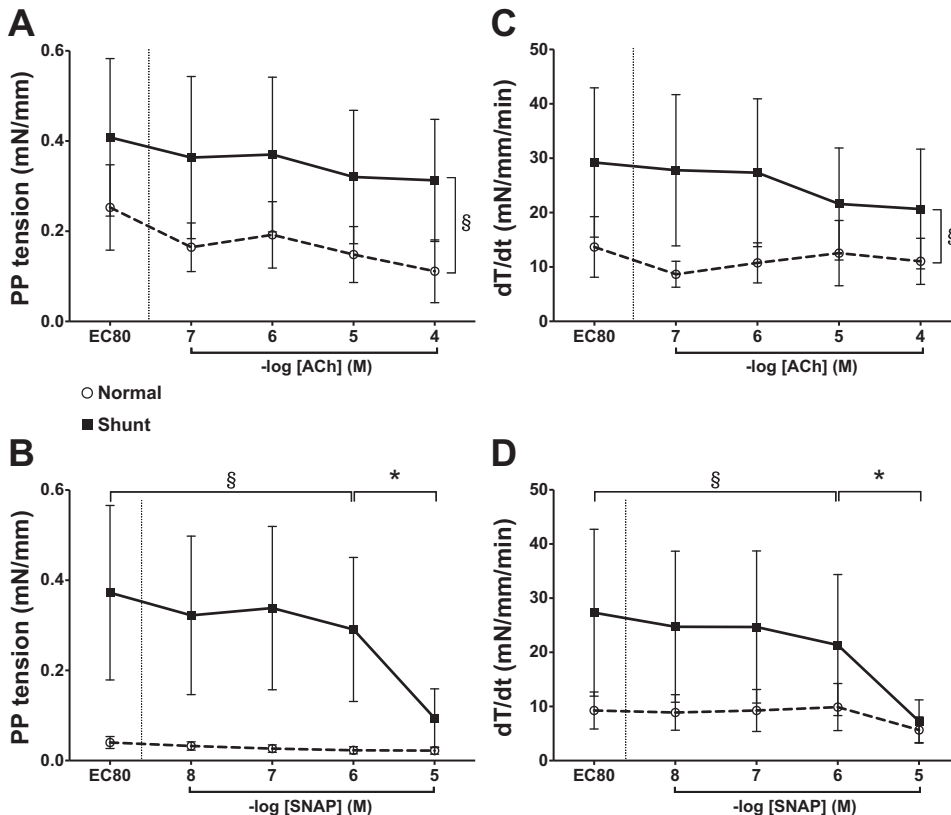


Fig. 2. Peak to peak (PP) tension (*A* and *B*) and strength of spontaneous contractions (*dT/dt*) (*C* and *D*) in normal and shunt thoracic duct rings at baseline [80% maximum constriction (EC<sub>80</sub>)] and in a dose-response relationship to ACh (*A* and *C*) or *S*-nitroso-*N*-acetylpenicillamine (SNAP; *B* and *D*). Analysis was based on the high-frequency component of developed tension. §*P* < 0.05, significant difference in peak to peak tension or *dT/dt* between normal and shunt rings. Shunt thoracic duct rings have 2.6-fold higher peak to peak tension and 2-fold greater *dT/dt* than normal rings (*P* < 0.05). Values are means ± SE; *n* = 6 normal lambs and 7 shunt lambs. \**P* < 0.05, significant difference from baseline for shunt rings. Note that at higher doses of SNAP but not ACh, the behavior of normal and shunt rings is equivalent.

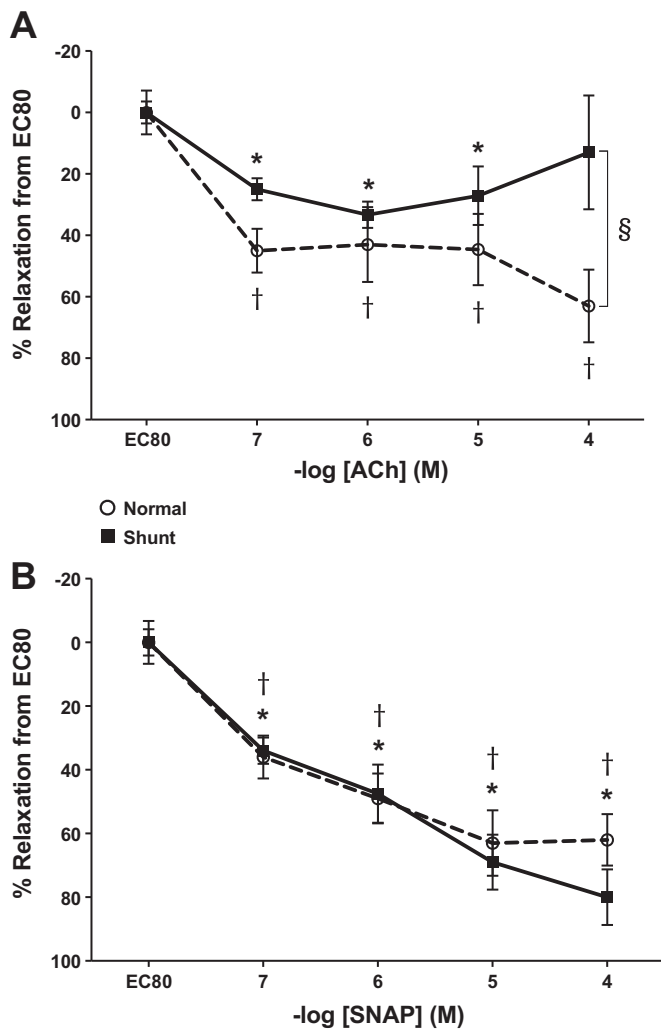


Fig. 3. Dynamic relaxation behavior was evaluated by precontraction with NE to EC<sub>80</sub> and a dose-response relationship to ACh (5 doses from 10<sup>-7</sup> to 10<sup>-4</sup> M; A) and SNAP (4 doses from 10<sup>-8</sup> to 10<sup>-5</sup> M; B). Shunt thoracic duct rings have an attenuated response to ACh compared with normal rings (A; §P < 0.05) but have a similar response to SNAP, an NO donor (B; P > 0.05). Values are mean ± SE; n = 6 normal lambs and 7 shunt lambs. \*P < 0.05, significant difference from baseline for shunt rings; †P < 0.05, significant difference from baseline for normal rings.

sure decreased (from 16 to 12 mmHg, P < 0.05) in shunt lambs but was unchanged (from 7 to 6 mmHg) in normal lambs. PBF, heart rate, and left and right atrial pressures were unchanged (Table 1).

**Lymph flow.** At baseline, shunt lambs had higher absolute lymph flow than normal lambs (6.5 ± 0.9 vs. 3.8 ± 0.6 μl·min<sup>-1</sup>·kg<sup>-1</sup>, P < 0.05). In response to inhaled NO therapy, absolute lymph flow in normal lambs (3.9 ± 0.5 μl·min<sup>-1</sup>·kg<sup>-1</sup>) and shunt lambs (6.4 ± 1.1 μl·min<sup>-1</sup>·kg<sup>-1</sup>) was preserved, despite the drop in resistance. Therefore, when normalized for pulmonary artery pressure (data not shown) or left pulmonary vascular resistance, lymph flow in both normal and shunt lambs increased (Fig. 5). In fact, the change in pulmonary lymph flow (normalized to resistance) was 1.5 times higher in normal lambs and 1.4 times higher in shunt lambs compared with baseline after 15 min of treatment with inhaled NO (P < 0.05). These changes were maintained during the 45-min study period (Fig. 5).

**NO<sub>x</sub> and cGMP levels in the pulmonary lymph effluent.** NO<sub>x</sub> (a measure of bioavailable NO) and cGMP levels were determined in the lymph effluent collected from normal and shunt lambs before and during exposure to inhaled NO. We have previously shown that baseline NO<sub>x</sub> levels are lower in the pulmonary lymph effluent from shunt lambs than normal control lambs by 36% (15). During the 45-min study period, NO<sub>x</sub> levels increased in both shunt and normal lambs (Fig. 6A). In

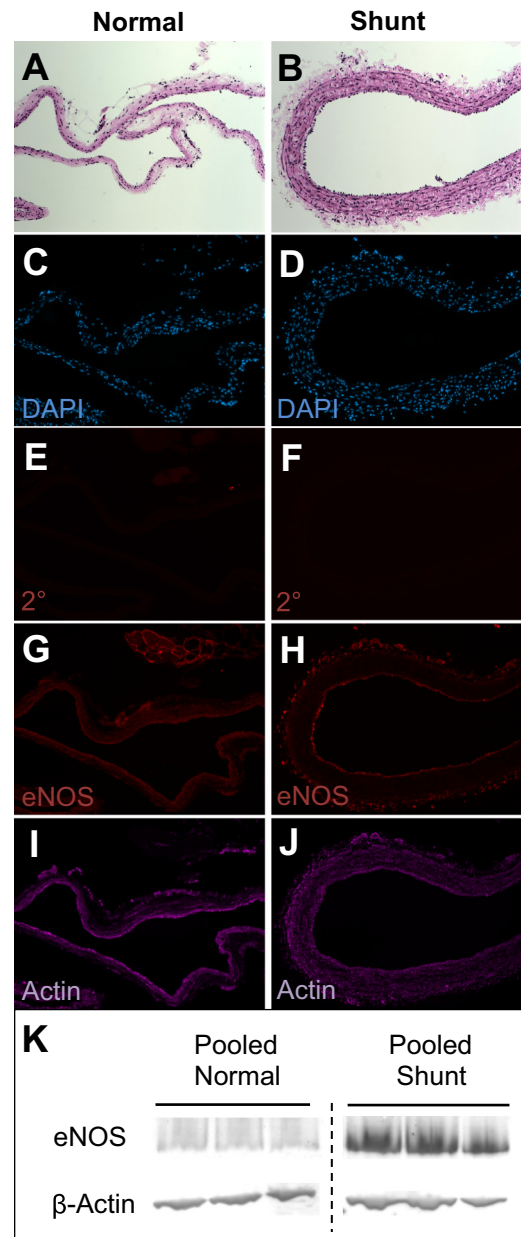


Fig. 4. Staining of isolated and paraformaldehyde-fixed thoracic duct sections from normal (A, C, E, G, and I) and shunt (B, D, F, H, and J) lambs. A and B: hematoxylin and eosin staining. C–J: immunostaining with 4',6-diamidino-2-phenylindole (DAPI; blue; C and D), secondary antibody (2°) alone (red; E and F), endothelial nitric oxide (NO) synthase (eNOS; red; G and H), and actin (pink; I and J). K: eNOS protein expression in pooled homogenates from normal (n = 7) or shunt (n = 7) isolated thoracic duct tissue normalized to β-actin. The dashed line denotes the discontinuity between normal and shunt lanes. eNOS expression was higher in the shunt thoracic duct, and there was increased cellularity and smooth muscle.

Table 1. Hemodynamic changes before and during exposure to 40 ppm of inhaled NO

	Normal Lambs	Normal Lambs + Inhaled NO	Shunt Lambs	Shunt Lambs + Inhaled NO
Mean pulmonary arterial pressure, mmHg	14.5 ± 1.2	12.3 ± 1.2†	26.1 ± 1.2‡	22.4 ± 1.6*
Mean systemic arterial pressure, mmHg	63.7 ± 4.5	63.5 ± 5	54.4 ± 6.5‡	54.3 ± 6.5
Mean left atrial pressure, mmHg	3.6 ± 1.6	3.5 ± 31.3	10.6 ± 41.6‡	10.8 ± 1.6
Mean right atrial pressure, mmHg	2.2 ± 0.9	1.1 ± 0.9	2.8 ± 0.8	2.6 ± 0.8
Heart rate, beats/min	170 ± 16	150 ± 6	151 ± 9	152 ± 9
Left pulmonary arterial blood flow, (ml/min)/kg	58 ± 7	61 ± 8	158 ± 17‡	159 ± 16
Left pulmonary vascular resistance, mmHg·kg/(ml/min)	0.19 ± 0.03	0.16 ± 0.03†	0.10 ± 0.01‡	0.075 ± 0.004*
Ratio of pulmonary to systemic blood flow			3.5 ± 0.9	3.5 ± 0.9

Values are means ± SE; n = 6 normal lambs and 6 shunt lambs except for the ratio of pulmonary to systemic blood flow, where n = 4 shunt lambs, and mean right atrial pressure, where n = 5 normal lambs and 5 shunt lambs. Hemodynamic changes were evaluated in normal and shunt lambs with increased pulmonary blood flow before and during exposure to 40 ppm of inhaled nitric oxide (NO). \**P* < 0.05, shunt lambs vs. shunt lambs + inhaled NO; †*P* < 0.05, normal lambs vs. normal lambs + inhaled NO; ‡*P* < 0.05, shunt lambs vs. normal lambs.

fact, 45 min after the start of inhaled NO (40 ppm), NO<sub>x</sub> levels in the pulmonary lymph effluent had increased 2.6-fold in normal lambs and 3.4-fold in shunt lambs (*P* < 0.05 for each).

At baseline, cGMP levels in the pulmonary lymph effluent from normal and shunt lambs were similar (123 ± 60 vs. 103 ± 16 μmol/l). During the 45-min study period, cGMP levels also increased in the pulmonary lymph of both shunt and normal lambs (Fig. 6B). Forty-five minutes after the start of inhaled NO, cGMP levels increased 2.6- and 2.7-fold in the pulmonary lymph effluent from normal and shunt lambs, respectively (*P* < 0.05 for each).

## DISCUSSION

Alterations in pulmonary lymphatic function in patients with congenital heart defects that cause increased PBF likely contribute in important ways to the pathophysiology but are not yet understood. In a recent study (15), we demonstrated that adaptive increases in lymph flow that occur in response to acute increases in PBF were lost in shunt lambs with chronic exposure to increased PBF. Associated with this decrease in

lymph flow, we identified structural and functional aberrations of the pulmonary lymphatic system including altered transit kinetics, abnormal lymphatic architecture, alterations in the expression of proteins known to be important in lymphatic

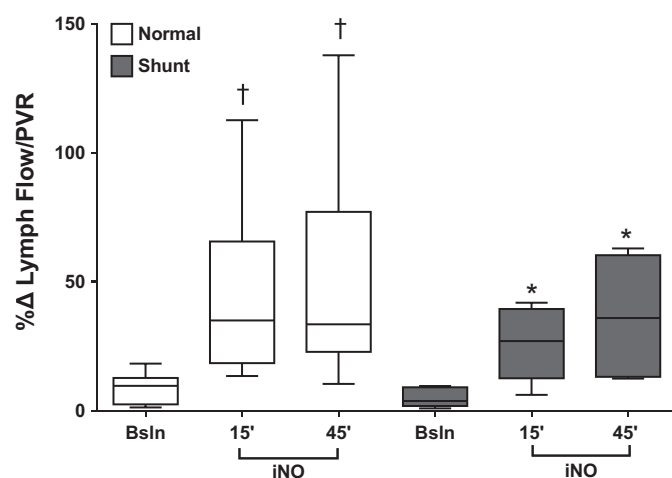


Fig. 5. Lymph flow normalized for resistance (pulmonary vascular resistance) increased significantly from baseline (Bsln) in both normal (†) and shunt (\*) lambs by 15 min (15') after the start of inhaled NO (iNO) at 40 ppm and continued for the duration of the study period [45 min (45')] (*P* < 0.05 for each). Values are mean percent changes ± SE from Bsln; n = 6 normal lambs and 6 shunt lambs. Note that the Bsln percent change (%Δ) represents the variation in lymph flow over the 45-min "steady-state" period and before any study interventions. Lymph flow increased in both normal and shunt lambs in response to iNO.

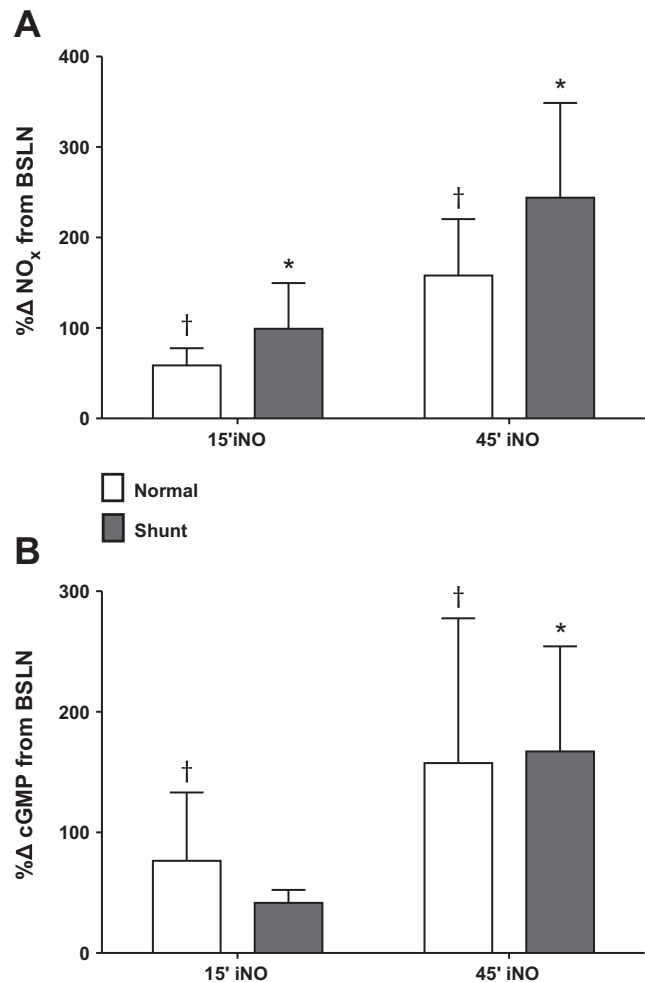


Fig. 6. A and B: bioavailable NO [nitrite/nitrate (NO<sub>x</sub>); A] and cGMP (B) increased in the pulmonary lymph effluent 15 and 45 min after the start of iNO at 40 ppm in both normal and shunt lambs. \**P* < 0.05, significant increase from Bsln for shunt lambs; †*P* < 0.05, significant increase from Bsln for normal lambs. Values are mean percent changes ± SD from Bsln in NO<sub>x</sub> (A) or cGMP (B); n = 6 normal lambs and 6 shunt lambs. NO<sub>x</sub> and cGMP increased in the pulmonary lymph in both normal and shunt lambs in response to iNO.

development, and decreased bioavailable NO in the lymph fluid. The present study was designed to investigate a potential mechanism for this decreased lymph flow, testing the hypothesis that abnormal lymphatic function in shunt lambs is due to impaired lymphatic endothelial NO-cGMP signaling resulting in increased lymphatic constriction and impaired relaxation. We found that thoracic duct rings isolated from shunt lambs had increased tension and strength of contraction at baseline and greater maximum force generation in response to NE compared with rings isolated from normal age-matched control lambs. This correlated with increased cellularity and smooth muscle in shunt thoracic duct rings. Furthermore, we found that rings from shunt lambs, but not normal lambs, had a selective impairment in endothelium-dependent relaxation. These findings are novel and, to our knowledge, provide the first description of aberrant lymphatic endothelial function associated with a pathophysiological state and provide a mechanistic link between increased PBF and pulmonary lymphatic dysfunction.

Increased constriction and impaired relaxation are hallmark findings of pulmonary vascular disease (10). Increased PBF, secondary to congenital heart defects, is a well-established cause of pulmonary vascular disease (22). In fact, in patients with cardiac defects that cause increased PBF, a selective impairment in pulmonary blood endothelium-dependent relaxation occurs early, even before abnormal baseline hemodynamics manifest clinically (11). The present findings in thoracic duct lymphatic rings are striking in their similarity to this established pulmonary vascular pathology. Multiple studies (3, 12, 18, 28, 35, 39, 47) have demonstrated that under acute transient conditions, increased PBF leads to an increase in the filtration of protein-poor fluid across the pulmonary capillary endothelium (due to hydrostatic forces) into the interstitial space with an associated increase in lymph flow. Our previous study (15) demonstrated that with chronic exposure to increased PBF, this adaptive increase in lymph flow was lost. Therefore, a potential factor contributing to the lymphatic endothelial dysfunction demonstrated in the present study is the chronic exposure to increased lymph flow. In studies (25, 27) of isolated lymphatic vessels, it has been demonstrated that acute increases in lymph flow resulted in NO-mediated relaxation with decreased lymphatic vessel contraction frequency and strength. Interestingly, although baseline tension was increased in lymphatic rings in shunt lambs, SNAP (a NO donor) resulted in decreased contraction frequency and strength, suggesting that the lymphatic smooth muscle was capable of responding to NO signaling. The blunted response to agonist-induced endothelium-dependent relaxation is consistent with more selective lymphatic endothelial injury. At the same time, it is notable that thoracic duct rings from shunt lambs appeared to have increased cellularity by DAPI and H&E staining and increased actin staining compared with thoracic duct rings from control lambs, consistent with increased smooth muscle layer thickness. In addition, lymphatic rings from shunt lambs contracted more strongly in response to NE compared with rings from normal lambs, again consistent with increased smooth muscle layer mass. It remains speculative, but plausible, that the regulatory function of the lymphatic endothelium is disrupted by chronic exposure to increased lymph flow, resulting in abnormal smooth muscle cell growth, increased constriction, and impaired relaxation. Indeed, recent studies

(43, 46) in cell culture and in mice suggest that shear stress is important for lymphatic development and remodeling.

Importantly, we found selective impairment in endothelium-dependent lymphatic relaxation and decreased NO<sub>x</sub> levels, both suggestive of decreased bioavailable NO, despite increased eNOS expression in thoracic duct vessels from shunt lambs. Interestingly, we have consistently demonstrated a similar finding in pulmonary arteries harvested from shunt lambs. In those studies (41, 50, 52), we demonstrated decreased bioavailable NO despite increased eNOS protein, due in part to eNOS uncoupling and NO scavenging by superoxide to form peroxynitrite. It is unclear whether similar mechanisms participate in lymphatic endothelial dysfunction under chronic conditions of increased PBF and increased pulmonary lymph flow. Furthermore, the vascular endothelium produces factors that promote smooth muscle cell proliferation and constriction, such as endothelin-1 and thromboxane (which are increased in shunt lambs) (5, 21, 23, 32, 40, 42, 45, 53, 54). Whether the lymphatic endothelium in shunt lambs elaborates these factors is not known. Further studies into these areas are warranted.

Although the potential similarities between the pulmonary arterial and lymphatic systems are intriguing, there are important differences. Indeed, studies using microscopic visualization of pressurized lymphatic vessels have demonstrated an important intrinsic pumping mechanism that influences lymph flow in rodents (26, 55), and the human thoracic duct has been shown to have spontaneous contractile activity as well (48). Our findings in isolated rings also captured these spontaneous pulsatile contractions. Several *ex vivo* and *in vivo* studies (9, 25, 27, 31, 49) have indicated that endothelial NO signaling is an important modulator of lymphatic pump activity and lymph flow. For example, in an acute *ex vivo* setting, increased flow stimulated endothelium-dependent NO production and vessel relaxation in rats (25, 27) and canine thoracic ducts (49) with decreased pump activity, responses blocked by NOS inhibition. Similarly, inhibition of eNOS and NO decreased lymph flow *in vivo* (31). Therefore, a prevailing view is that increases in interstitial fluid requiring increased clearance, result in increased lymph fluid that triggers NO-mediated inhibition of the active lymph pump that maintains increased lymph flow through a decrease in resistance (30). However, this sequence may be altered by disease processes, such as chronically increased PBF. In our study, shunt rings demonstrated increased peak to peak tension with stronger spontaneous contractions compared with control rings, consistent with more active lymph pumping and impaired relaxation. We speculate that this mechanism could increase pulmonary lymphatic resistance and account for the decreased pulmonary lymph flow that we observed in shunt lambs.

Although endothelium-dependent relaxation was impaired in shunt thoracic duct rings, SNAP decreased peak to peak tension and strength of contraction to levels similar to normal control rings. Therefore, we were interested in the potential effects of increasing NO signaling *in vivo*, using our lamb model. However, little is known about the potential therapeutic augmentation of NO signaling within lymphatic vessels. To that end, we performed a proof of concept experiment to begin to examine whether exogenous NO delivered by inhaled NO could augment pulmonary lymphatic NO signaling. We hypothesized that inhaled NO would improve pulmonary lymph flow *in vivo* in shunt lambs. Interestingly, we found that



inhaled NO increased lymph flow in both normal and shunt lambs. Importantly, we also found that NO<sub>x</sub> and cGMP levels increased in the pulmonary lymph fluid with increasing exposure to inhaled NO. This demonstrates that inhaled NO can augment NO-cGMP signaling within the pulmonary lymphatic space. It is unclear whether the primary site of production of NO<sub>x</sub> and cGMP is blood vascular smooth muscle cells (with subsequent diffusion from blood into lymph fluid) or whether NO is able to diffuse into the interstitial space from the alveoli and then into lymphatic vessels, directly activating soluble guanylyl cyclase to produce cGMP. Furthermore, whether the mechanism of action of NO in lymphatic vessels is similar or different between normal and shunt lambs is unclear. Based on our isolated vessel findings, we speculate that in shunt lambs, NO increases lymph flow by decreasing constriction and therefore resistance. In normal lambs, however, it is possible that lymph flow is augmented by this mechanism or by a NO-mediated improvement in lymphatic pumping. Further studies are needed to elucidate the precise mechanisms. However, given the increasing availability of therapies that augment NO-cGMP signaling, our findings are exciting (while preliminary) in that they suggest that these therapies might have the potential to improve pulmonary lymphatic function under conditions of chronically increased PBF.

The primary purpose of the study was to evaluate responses in isolated lymphatic rings as an important step for understanding our previous findings in whole animal studies. While isolated vessel experiments are powerful in their focus, interpretations are inherently limited given the ex vivo nature of the studies. Nonetheless, when combined with our prior whole animal data, the results are intriguing. Further studies will focus on testing various other signaling pathways both in vivo and in vessel baths. In addition, experiments using pressurized lymphatic vessel preparations that allow for variable flow will provide additional information about lymphatic function under normal and abnormal hemodynamic conditions.

We conclude that chronic exposure to increased PBF and increased pulmonary lymph flow is associated with pulmonary lymphatic endothelial dysfunction that disrupts NO-cGMP signaling, leading to increased resting vasoconstriction and increased maximal strength of contraction as well as impaired endothelium-dependent agonist-induced relaxation. Furthermore, we have demonstrated the ability of inhaled NO to increase NO<sub>x</sub> and cGMP levels in the pulmonary lymph fluid. Therapies that augment NO-cGMP signaling within the lymphatic system may prove to benefit patients with cardiac defects associated with increased PBF by maintaining normal lymphatic function.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

#### AUTHOR CONTRIBUTIONS

Author contributions: S.A.D., P.E.O., and J.R.F. conception and design of research; S.A.D., W.G., C.E.S., M.J.J., J.M., R.C.J., and G.W.R. performed experiments; S.A.D., W.G., S.H.B., and C.E.S. analyzed data; S.A.D., S.H.B., and J.R.F. interpreted results of experiments; S.A.D. and C.E.S. prepared figures; S.A.D. drafted manuscript; S.A.D., P.E.O., and J.R.F. edited and revised manuscript; S.A.D., P.E.O., W.G., S.H.B., C.E.S., M.J.J., J.M., R.C.J., G.W.R., and J.R.F. approved final version of manuscript.

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