Chronic hypoxia decreases arterial and venous compliance in isolated perfused rat lungs: an effect that is reversed by exogenous L-arginine

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Jin Y, Chen B, Calvert TJ, Chicoine LG, Liu Y, Nelin LD. Chronic hypoxia decreases arterial and venous compliance in isolated perfused rat lungs: an effect that is reversed by exogenous L-arginine. Am J Physiol Heart Circ Physiol 304: H195–H205, 2013. First published October 26, 2012; doi:10.1152/ajpheart.00188.2012.— Chronic hypoxia (CH)-induced pulmonary hypertension is characterized by vasoconstriction and vascular remodeling, leading to right ventricular dysfunction. Given the role of arterial compliance (C_a) in right ventricular work, a decrease in C_a would add to right ventricular work. Nitric oxide (NO) is a potent vasodilator made by NO synthases from L-arginine (L-Arg). However, little is known of the effect of L-Arg on vascular compliance (C_v) in the lung. We hypothesized that exposure to CH would decrease C_a and that this effect would be reversed by exogenous L-Arg. Sprague-Dawley rats were exposed to either normoxia or CH for 14 days; the lungs were then isolated and perfused. Vascular occlusions were performed and modeled using a three-compartment, two-resistor model. Pressure-flow curves were generated, and a distensible vessel model was used to estimate distensibility and a vascular resistance parameter (R_a). Hypoxia resulted in the expected increase in arterial resistance (R_a) as well as a decrease in both C_a and C_v. L-Arg had little effect on R_a, C_a, or C_v in isolated lungs from normoxic animals. L-Arg decreased R_a in lungs from CH rats and redistributed compliance to approximately that found in normoxic lungs. CH increased R_a, and L-Arg reversed this increase in R_a. L-Arg increased exhaled NO, and inhibition of L-Arg uptake attenuated the L-Arg-induced increase in exhaled NO. These data demonstrate that the CH-induced decrease in C_a was reversed by L-Arg, suggesting that L-Arg may improve CH-induced right ventricular dysfunction.

vascular compliance; cationic amino acid transporter; endothelial nitric oxide synthase; isolated perfused lung; right ventricular function

EXPOSURE TO CHRONIC HYPOXIA (CH) causes vasoconstriction and vascular remodeling, leading to increased right ventricular (RV) afterload, resulting in RV hypertrophy and eventually to RV dysfunction or cor pulmonale. These effects are caused at least in part by decreases in the activity of endothelial nitric oxide (NO) synthase (eNOS), which results in decreased NO production during hypoxia. Indeed, in patients with pulmonary hypertension, NO production has been shown to be decreased (41). In fact, exogenous NO gas given by inhalation has been used as a therapy for lowering pulmonary vascular resistance in patients with pulmonary hypertension (20, 33). NO is endogenously synthesized from L-arginine (L-Arg) by three isoforms of NOS. Plasma levels of L-Arg have been reported to be low in a rat model of pulmonary hypertension (22) and in patients with pulmonary hypertension (21). Studies (5, 10) have demonstrated an increase in arterial resistance (R_a) in lungs from rats exposed to CH using double occlusion methods. In isolated perfused rat lungs, Emery et al. (6) found that static compliance was decreased after CH, and Vanderpool et al. (41) found that pulmonary arterial compliance (C_a) using microcomputed tomography was decreased after CH. However, to the best of our knowledge, there are no reports of the effects of exogenous L-Arg on vascular compliance after CH in the rat lung using occlusion methods. Since RV work is inversely proportional to C_a (18), understanding the effects of CH on C_a may have important implications for RV function. We therefore tested the hypothesis that CH would result in decreased pulmonary C_a and that this decrease in C_a would be reversed by exogenous L-Arg administration. We used arterial, venous, and double occlusions and a five-compartment model to estimate total pulmonary vascular resistance (R_L), R_v, venous resistance (R_v), total pulmonary vascular compliance (C_L), C_v, microvascular compliance (C_v), and venous compliance (C_a) as previously described (2, 17, 29). We also examined pressure-flow (P-Q) curves and used a distensible vessel model to estimate distensibility (α) and a vascular resistance parameter (R_a) (17, 29, 32). It has been shown that CH leads to increased vasoconstrictor responsiveness (5, 33). Therefore, to examine the effect of vasoconstriction on hemodynamics, we performed experiments with the sequential addition of Nω-nitro-L-arginine methyl ester (L-NAME; a NOS inhibitor), KCl, and diethylamine (DETA)-NONOate (an NO donor). We also examined hemodynamics before and after the addition of L-Arg to the perfusate. L-Lysine (L-Lys) was used as a competitive antagonist of L-Arg uptake. We began to examine if L-citrulline (L-Cit) via the endogenous synthetic pathway for L-Arg would have the same effect as L-Arg in the isolated lung. However, to our surprise, L-Cit had little effect on exhaled NO (exNO) production in the isolated, perfused rat lung, and, therefore, we measured mRNA levels of argininosuccinate synthase (AS) and argininosuccinate lyase (AL) in the whole lung. Finally, we used an in vivo model to examine the effect of L-Cit on exNO production.

MATERIALS AND METHODS

All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children’s Hospital. Sprague-Dawley rats (Harlan, Indianapolis, IN) were provided with fresh water, food, and clean bedding 3 times/wk. All animals were housed on a 12:12-h light-dark cycle. Rats were placed in either a normoxic (N) or hypobaric hypoxic (H) cycle. Rats were placed in either a normoxic (N) or hypobaric hypoxic (H) cycle. Rats were placed in either a normoxic (N) or hypobaric hypoxic (H) cycle. Rats were placed in either a normoxic (N) or hypobaric hypoxic (H) cycle. Rats were placed in either a normoxic (N) or hypobaric hypoxic (H) cycle.

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tracheotomy tube was connected to a piston-type ventilator (Harvard Apparatus, Holliston, MA). Lungs were ventilated with a NO-free gas mixture of 5% CO₂-21% O₂-balance N₂ using a tidal volume of 2.5 ml and a rate of 55 breaths/min. A median sternotomy was performed, and heparin was injected directly into the RV. The pulmonary artery and left ventricle were cannulated. The preparation was immediately perfused with physiological saline solution (PSS) with 4% albumin (wt/vol) and 300 μM meclofenamate (Sigma-Aldrich, St. Louis, MO) at a rate of 30 ml kg⁻¹ min⁻¹ using a Masterflex roller pump. The perfusion rate was maintained at this rate for the duration of the experiment. The initial perfusion was nonrecirculating until the perfusate exiting the lung was nearly free of blood, at which point recirculating perfusion was established with PSS, and the total volume of the circulation system was ~40 ml. Lungs were maintained in zone 3 conditions throughout the experimental protocol by maintaining venous pressure (Pv) at 3 mmHg and end-expiratory pressure at 1 mmHg. Pulmonary arterial pressure (Pa), Pv, and airway pressure were recorded continuously using Codas data-acquisition software (CODAS, Dataq Instruments, Akron, OH).

Segmental vascular resistances and vascular compliances. Three vascular occlusion maneuvers (arterial, venous, and double occlusion) were performed, and the resultant pressure data were modeled using a five-compartment model as previously described (28). Briefly, the occlusion of both the arterial inflow and venous outflow (double occlusion) caused Pa and Pv to rapidly equilibrate at the double occlusion pressure (Pd), which approximates the microvascular pressure. Using these three pressures and the perfusate flow rate (Q), vascular resistances can be calculated using the following equations:

\[ R_L = \frac{P_a - P_v}{Q} \]
\[ R_a = \frac{P_a - P_d}{Q} \]
\[ R_v = \frac{P_v - P_d}{Q} \]

Vascular compliances can be assessed using the vascular resistance data in conjunction with the venous occlusion and arterial occlusion data. C_L, C_a, C_c, and C_v were estimated from steady-state P_d and P_v, Q, the area (A_d) encompassed by the Pa curve [P_d(t), where t is time] after arterial occlusion, the equilibrium pressure P_v after double occlusion, and the average slope (m) of the Pa(t) and P_v(t) curves after venous occlusion using the following equations:

\[ C_L = C_a + C_c + C_v = \frac{Q}{m} \]
\[ C_c = C_L - (R_a C_a / R_v) \]
\[ C_a = \frac{A_d}{(P_a - P_d)R_v} \]
\[ C_v = C_L - C_a - C_c \]

Vascular volume. Vascular volume (QL) was measured in isolated perfused lungs using thermodilution. An injector allowed the introduction of 0.35 ml ice-cold saline into the arterial inflow. Serial QL measurements were completed, the lungs were removed, and the cannula was connected directly together to measure the volume of the tubing system alone. The mean transit time (t) from the injector to the venous outflow point was calculated as follows:

\[ t = \int_0^t \frac{C(t) dt}{\int_0^t C(t)} \]

where C(t) is the amplitude of the thermistor signal at time t, t = 0 is the time of the injection, and a is the time when C(t) had returned to 1% of its peak value. Vascular mean transit time (t_m) was calculated by subtracting the t obtained after the lungs were removed from the t that was obtained with the lungs in the system. Qa was then Q × t_m. Values of Qa measured at different P_v were used to calculate the static vascular compliance (C_v) of the lung from the slope of the Qa versus P_v curve (29).

P–Q curve. P–Q curves were constructed as previously described (17, 29). Briefly, Q was decreased to 4.5 ml/min, and the height of the venous reservoir was adjusted to maintain Pa at 3 mmHg, the ventilator was turned off, Pa was recorded, and the ventilator was then turned on. This procedure was repeated with Q at 8, 12, 18, and 25 ml/min, and the Pa value with P_v maintained at 3 mmHg at each Q point was recorded. After the P–Q data were collected, Q was returned to the standard flow rate.

Two parameters, α (which represents the fractional change in mean transit time per mmHg change in pressure) and R0 (which represents the vascular resistance that would exist if the resistance vessels were at their respective diameters obtained when the vascular pressure were zero), were estimated using a distensible vessel model as previously described (17, 29). Briefly, P–Q curves were fit with the following equation using nonlinear regression:

\[ P_a = \left[\frac{(1 + \alpha P_v)^5 + 5 \alpha R_0 Q^5}{\alpha}\right] \]

exNO measurement. In the isolated, perfused lung, exNO was measured as previously described (3, 30). Briefly, 25 min after the equilibration of the system, the baseline exhaled gas was collected for 5 min into a mylar balloon attached to the ventilator exhaust port. The gas collected in the mylar balloon was analyzed using a chemiluminescence NO analyzer (model 280i, Sievers, Boulder, CO). The analyzer was calibrated using a standard curve generated daily with authentic NO (1 ppm in N₂, Matheson, Chicago, IL) mixed with NO-free nitrogen using precision flow meters to obtain concentrations ranging from 0 to 500 ppb (vol/vol). The NO detection limit was 0.5 ppb. After baseline gas collection, L-Arg was added into the perfusate reservoir to achieve the desired final concentration, and the exhaled gas was collected after 25 min for 5 min for NO measurement.

In whole animals, exNO was measured as previously described (5). Briefly, rats were anesthetized with 50 mg/kg ip pentobarbital, intubated, and mechanically ventilated using a NO-free gas mixture of 21% O₂-balance N₂ with a tidal volume of 2.5 ml and a respiratory rate of 55 breaths/min. The carotid artery was cannulated using polyethylene-50 tubing and connected to a blood pressure transducer (Columbus Instruments, Columbus, OH). Blood pressure was continuously monitored (Cardiomax, Columbus Instruments). The jugular vein was cannulated for the administration of medications. The baseline exhaled gas was collected for 5 min into a mylar balloon attached to the ventilator exhaust port after a 25-min equilibration period. The collected exhaled gas was analyzed using a chemiluminescence NO analyzer as described above. After the baseline gas collection, treatment was given intravenously, and the exhaled gas was collected after 25 min for NO measurement as described above.

Protein isolation. Lung tissue was collected at the end of the experiment and frozen at −80°C. Lungs were homogenized in ice-cold Dulbecco’s PBS (pH 7.4) containing protease inhibitors and phosphatase inhibitors. Samples were centrifuged at 12,000 g for 15 min, and the supernatants were collected and analyzed for total protein content using the Bradford assay (Bio-Rad, Hercules, CA). Supernatants were stored at −80°C for further study.

Immunoblot analysis. Tissue homogenates were assayed by Western blot analysis as previously described (5, 30) using the following antibodies: eNOS (1:1,000, BD Transduction, San Jose, CA), neuronal NOS (nNOS; 1:500, BD Transduction), inducible NOS (1:500, BD Transduction), AS (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), AL (1:200, Santa Cruz Biotechnology), and β-actin (1:5,000, Sigma-Aldrich).
RESULTS

Vascular resistances. To evaluate the effect of CH exposure on \( R_L \) and \( C_L \), lungs from N or CH rats were isolated and perfused. The baseline venous, arterial, and double occlusion maneuvers were performed after a 25-min equilibration period. To assess vasoactivity, isolated perfused lungs were treated sequentially with L-NAME (final perfusate concentration: 3 mM), KCl (final perfusate concentration: 30 mM), and DETA-NONOate (final perfusate concentration: 100 \( \mu \)M), and each condition lasted 30 min. \( R_L \) was greater in lungs from CH animals than in those from N animals (Table 1). \( R_L \) was \( \sim \)80% greater and \( R_a \) was \( \sim \)50% greater in lungs from CH animals than in lungs from N animals (Table 1). The addition of L-NAME to the perfusate had little effect on \( R_L \) in either N or CH lungs. The addition of DETA-NONOate to the perfusate significantly decreased \( R_L \) in both groups, although the values for \( R_L \) did not return all the way to basal levels (Table 1). The main contributor to the KCl-induced increase in vascular resistance was \( R_L \) in both N and CH lungs, although the KCl-induced increase in \( R_L \) was substantially greater in CH lungs than in N lungs (Table 1). Although lower in absolute magnitude than in \( R_a \), the addition of DETA-NONOate significantly increased \( R_L \) in both N and CH lungs, and again the increase in \( R_L \) was greater in CH lungs than in N lungs (Table 1). The addition of the NO donor DETA-NONOate significantly decreased both \( R_L \) and \( R_a \), although \( R_L \) and \( R_a \) remained above basal levels in both N and CH lungs (Table 1).

Vascular compliance. The vascular occlusion data were also used to calculate \( C_L \) and its distribution among \( C_a \), \( C_c \), and \( C_\varpi \). During baseline conditions, \( C_L \) did not differ between isolated lungs from N and CH animals (Fig. 1A). The addition of L-NAME
had little effect on $C_L$ in either N or CH lungs. However, $C_L$ was significantly lower in CH lungs after KCl-induced vasoconstriction, and the addition of DETA-NONOate increased $C_L$ in CH lungs to baseline levels (Fig. 1A). $C_v$ was significantly lower in isolated lungs from CH animals compared with N animals (Fig. 1B). The addition of l-NAME had little effect on $C_v$ in either N or CH lungs, whereas the addition of KCl resulted in a significant decrease in $C_v$ in both N and CH lungs (Fig. 1B). The addition of DETA-NONOate resulted in a modest increase in $C_v$ in N lungs, whereas the addition of KCl resulted in a significant decrease in $C_v$ in both N and CH lungs (Fig. 1B). The majority of the total vascular compliance was in $C_v$ in both N and CH lungs under all conditions (Fig. 1C). There were no significant effects of any of the vasoactive agents on $C_v$ in either N or CH lungs (Fig. 1C). $C_v$ was significantly lower in isolated lungs from CH animals than in those from N animals (Fig. 1D). The addition of l-NAME had little effect on $C_v$, whereas the addition of KCl significantly decreased $C_v$ only in lungs from CH animals. The addition of DETA-NONOate had little effect on $C_v$ in either N or CH lungs (Fig. 1D).

The $C_L$ measured during occlusions is a measure of dynamic vascular compliance. To determine the effect of hypoxia on $C_L$ in the isolated rat lung, total $Q_L$ in the lungs was measured at various outflow pressures ($P_v$), including 1, 4, 8, and 12 mmHg. $Q_L$ at a $P_v$ of 1 mmHg was greater in isolated lungs from CH animals than in those from N animals (Fig. 2). In N lungs, $Q_L$ increased significantly with each increase in $P_v$, whereas in CH lungs, the $Q_L$ increase with $P_v$ was smaller than in N lungs and only increased with the first two $P_v$ steps (Fig. 2). $C_L$, the slope of the $Q_L$ versus $P_v$ curve, was significantly lower in isolated lungs from CH animals than in those from N animals (Fig. 2).

**Lung P-Q curves.** To further examine the effect of CH on pulmonary hemodynamics, P-Q curves were constructed using values from isolated perfused lungs from N and CH rats. $R_0$ and $\alpha$ were calculated from P-Q curves (Table 2). No obvious differences in $\alpha$ were detected between N and CH lungs at baseline. $\alpha$ in N lungs did not change upon the addition of l-NAME, KCl, or DETA-NONOate. However, KCl-induced vasoconstriction resulted in a significant decrease in $\alpha$ in CH lungs, and the addition of DETA-NONOate caused an increase in $\alpha$ back to basal levels (Table 2). Similar to what was found with $R_L$, isolated lungs from CH animals had significantly greater $R_0$ at baseline and under all vasoactive conditions studied than isolated lungs from N animals under the same conditions (Table 2). The addition of l-NAME had little effect on $R_0$ in either N or CH lungs. The addition of KCl resulted in ≈14-fold and ≈21-fold increases in $R_0$ in N and CH lungs, respectively (Table 2). The addition of DETA-NONOate decreased $R_0$ in both N and CH lungs but did not return values to baseline levels (Table 2).

**Lung NO production.** To determine the effect of CH on exogenous NO production and lung levels of eNOS and nNOS protein, lungs from N and CH rats were isolated, perfused, and ventilated with a normoxic gas mixture. exNO was measured at baseline and then 30 min after each subsequent addition of l-NAME to the perfusate (final concentrations of 10, 30, 100, 300, and 1,000 μM, respectively). The lungs were then frozen in liquid nitrogen and stored at −80°C until used for protein extraction for eNOS and nNOS with immunoblot analysis. exNO production from isolated perfused lungs from CH rats was significantly greater than from lungs of N rats (Fig. 3A). l-NAME inhibited exNO production in isolated lungs from both N and CH rats in a concentration-dependent manner, reaching a maximal effect at ≈100 μM in both N and CH lungs (Fig. 3A). There was significantly more eNOS protein in lungs from CH rats than in lungs from N rats (Fig. 3B). Although there was a trend to greater nNOS expression in CH lungs, there was no statistically significant difference in lung nNOS protein levels between N and CH rats (Fig. 3C).

**l-Arg and pulmonary hemodynamics.** The effects of exogenous l-Arg on hemodynamics were determined in isolated perfused lungs. Vascular occlusions were performed at baseline and after the addition of l-Arg to the perfusate to achieve a final concentration of 3 mM. The addition of l-Arg decreased $R_L$ only in lungs from CH rats (Table 3). This decrease in $R_L$ was due to a decrease in $R_a$ with the addition of l-Arg with little effect on $R_v$ (Table 3). The addition of exogenous l-Arg had little effect on the distribution of vascular resistance in lungs from N rats. However, the addition of l-Arg changed the distribution of vascular resistance in CH lungs so that it was essentially equally distributed between arteries and veins and not different from that seen in N lungs (Table 3).

<table>
<thead>
<tr>
<th>Table 2. Pressure-flow results</th>
<th>$\alpha$, %/mmHg</th>
<th>$R_0$, mmHg·ml⁻¹·s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>Baseline</td>
<td>3.7 ± 1.0</td>
<td>4.4 ± 1.3</td>
</tr>
<tr>
<td>l-NAME</td>
<td>3.1 ± 0.6</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>KCI</td>
<td>3.8 ± 2.2</td>
<td>1.4 ± 0.3‡</td>
</tr>
<tr>
<td>DETA-NONOate</td>
<td>3.0 ± 0.6</td>
<td>4.4 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. $\alpha$, distensibility; $R_0$, vascular resistance parameter. *Hypoxia different from normoxia ($P < 0.05$); †hypoxia different from normoxia ($P < 0.005$); ‡different from the previous condition with the same exposure ($P < 0.05$).
There were no differences in $C_L$ between lungs isolated from N or CH rats, and the addition of L-Arg had no effect on $C_L$ in either N or CH lungs (Table 3). However, $C_a$ and $C_c$ in lungs from CH rats were significantly lower than in lungs from N rats, and the addition of L-Arg had little effect on $C_a$ and $C_c$ in either N or CH lungs (Table 3). Again, $C_c$ was significantly greater in CH lungs than in N lungs, and the addition of L-Arg to the perfusate eliminated the differences in $C_a$, $C_v$, and $C_c$ between N and CH lungs (Table 3). The majority of $C_L$ in the lungs was in $C_c$ for both N and CH animals, although the percentage of the vascular compliance contributed by $C_c$ was significantly greater in lungs from CH rats than in lungs from N rats (Table 3). In lungs from CH animals, the addition of L-Arg to the perfusate decreased the percentage of the vascular compliance contributed by $C_c$.

Table 3. Hemodynamics after l-arginine

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>l-arginine</th>
<th>Baseline</th>
<th>l-arginine</th>
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<tbody>
<tr>
<td></td>
<td>Normoxia</td>
<td>Hypoxia</td>
<td>Normoxia</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>$R_L$, mmHg·ml⁻¹·s⁻¹</td>
<td>28 ± 4</td>
<td>26 ± 3</td>
<td>53 ± 10*</td>
<td>38 ± 4†</td>
</tr>
<tr>
<td>$R_a$, mmHg·ml⁻¹·s⁻¹</td>
<td>15 ± 2</td>
<td>13 ± 1</td>
<td>32 ± 7*</td>
<td>19 ± 2†</td>
</tr>
<tr>
<td>$R_c$, mmHg·ml⁻¹·s⁻¹</td>
<td>14 ± 2</td>
<td>13 ± 2</td>
<td>21 ± 3</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>$R_a$, % of total $R_L$</td>
<td>53 ± 2</td>
<td>51 ± 2</td>
<td>60 ± 1*</td>
<td>51 ± 2†</td>
</tr>
<tr>
<td>$R_c$, % of total $R_L$</td>
<td>47 ± 2</td>
<td>49 ± 2</td>
<td>40 ± 1*</td>
<td>49 ± 2†</td>
</tr>
<tr>
<td>$C_L$, ml/mmHg</td>
<td>0.15 ± 0.01</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>0.12 ± 0.01</td>
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<tr>
<td>$C_a$, ml/mmHg</td>
<td>0.041 ± 0.004</td>
<td>0.040 ± 0.004</td>
<td>0.020 ± 0.005*</td>
<td>0.030 ± 0.007</td>
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<tr>
<td>$C_c$, ml/mmHg</td>
<td>0.061 ± 0.006</td>
<td>0.055 ± 0.008</td>
<td>0.089 ± 0.010*</td>
<td>0.058 ± 0.013</td>
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<tr>
<td>$C_v$, ml/mmHg</td>
<td>0.047 ± 0.007</td>
<td>0.038 ± 0.008</td>
<td>0.024 ± 0.005*</td>
<td>0.029 ± 0.006</td>
</tr>
<tr>
<td>$C_a$, % of total $C_L$</td>
<td>28 ± 2</td>
<td>31 ± 3</td>
<td>15 ± 4*</td>
<td>26 ± 4†</td>
</tr>
<tr>
<td>$C_c$, % of total $C_L$</td>
<td>41 ± 5</td>
<td>42 ± 4</td>
<td>67 ± 7*</td>
<td>49 ± 8†</td>
</tr>
<tr>
<td>$C_v$, % of total $C_L$</td>
<td>31 ± 3</td>
<td>27 ± 5</td>
<td>18 ± 3*</td>
<td>25 ± 4†</td>
</tr>
</tbody>
</table>

Values are means ± SE. $C_L$, total pulmonary vascular compliance; $C_a$, arterial compliance; $C_c$, venous compliance; $C_v$, microvascular compliance. *Hypoxia different from normoxia ($P < 0.05$); †different from the previous condition with the same exposure ($P < 0.05$).
compliance contributed by $C_C$, such that the distribution of vascular compliance in CH lungs after the addition of 3 mM L-Arg resembled that seen in lungs from N rats (Table 3).

To further examine the effect of exogenous L-Arg on pulmonary hemodynamics, P-Q curves were constructed in isolated perfused lungs from N and CH rats at baseline and after the addition of L-Arg to the perfusate to achieve a final concentration of 3 mM. The addition of L-Arg to the perfusate in N lungs had little effect on the P-Q curve; however, the addition of L-Arg to the perfusate of CH lungs resulted in vascular pressures at each flow rate that were not different from baseline values in N lungs (data not shown). $\alpha$ was not significantly different between lungs from N and CH rats, and the addition of L-Arg to the perfusate had little effect on $\alpha$ (Fig. 4A). $R_0$ was significantly greater in lungs from CH rats than in lungs from N rats, and the addition of L-Arg to the perfusate significantly decreased $R_0$ in CH lungs (Fig. 4B). Indeed, $R_0$ values in CH lungs with L-Arg added to the perfusate were not significantly different from $R_0$ values in N lungs at baseline (Fig. 4B).

**Effect of exogenous L-Arg on exNO production in isolated perfused lungs.** The effect of exogenous L-Arg on exNO production from isolated perfused lungs from N and CH rats was determined at baseline and after the addition of L-Arg to the perfusate to achieve final perfusate concentrations of 1, 3, and 10 mM. The production of exNO increased in an L-Arg concentration-dependent manner in lungs from both N and CH rats, and lungs from CH animals produced more exNO than did lungs from N animals (Fig. 5A). In one set of experiments, D-Arg, which is not actively taken up by the lung, was substituted for L-Arg. The addition of D-Arg to the perfusate had no effect on exNO production at any of the concentrations studied (Fig. 5A). To demonstrate that the L-Arg effect on exNO production was due to active L-Arg uptake, L-Lys, a competitive inhibitor of L-Arg transporters, was added to the perfusate in a set of N lungs. Since 10 mM L-Arg was the largest concentration studied, we chose a concentration of 30 mM L-Lys. exNO production in the presence of L-Lys was significantly lower at all L-Arg concentrations studied (Fig. 5A). In three lungs, the effect of 30 mM D-Lys, which is not transported by cationic amino acid transporters (CATs), on exNO production in the presence of increasing L-Arg concentrations was studied. D-Lys had no effect on the L-Arg-induced increase in exNO production in the isolated perfused rat lung (data not shown).

The exNO production served as a surrogate marker for vasoactive NO production in these isolated lungs, since $R_0$ fell with increasing doses of L-Arg in lungs from both N and CH rats (Fig. 5B). The addition of D-Arg had no effect on $R_0$ in isolated perfused lungs (Fig. 5B). The L-Arg-induced decrease in $R_0$ was attenuated in N lungs when 30 mM L-Lys was added to the perfusate (Fig. 5B). When the change in $R_0$ was plotted against the exNO production from all conditions studied (Fig. 5C), there was a significant negative correlation ($R = -0.89, P < 0.001$).

**Effect of acute hypoxia on exNO production in isolated rat lungs.** To determine the effect of acute hypoxia on exNO production in rat lungs, isolated lungs from N or CH rats were ventilated with a NO-free gas mixture of 5% CO$_2$-21% O$_2$-balance N$_2$ for 30 min, and the ventilating gas mixture was then changed to 5% CO$_2$-5% O$_2$-balance N$_2$. After a 30-min equilibration period, L-Arg was added to the perfusate to achieve final concentrations of 1, 3, and 10 mM. The data shown in Fig. 5 for N and CH lungs ventilated with 21% O$_2$ was used for comparison. Isolated lungs ventilated with 5% O$_2$ had significantly lower exNO production than lungs ventilated with 21% O$_2$, although lungs from CH rats ventilated with 5% O$_2$ had significantly greater exNO production than lungs isolated from N rats ventilated with 5% O$_2$ (Fig. 6).

**Effect of L-Cit on exNO production in isolated lungs.** L-Cit and L-aspartate are metabolized to L-argininosuccinate via AS, and L-argininosuccinate is metabolized to L-Arg with fumarate as a coproduct by AL. It has been suggested that in endothelial cells, AS and AL are involved in a complex with eNOS, such that L-Arg derived from L-Cit is preferentially metabolized by eNOS (9, 37). Therefore, we measured exNO in isolated lungs from N and CH rats at final perfusate concentrations of 0, 1, 3, and 10 mM L-Cit. There were no detectable changes in exNO levels with increasing concentrations of L-Cit (Fig. 7A). We then added both L-Cit and L-aspartate at equal final perfusate concentrations of 0, 1, 3, and 10 mM while measuring exNO production in lungs from N rats. The simultaneous addition of L-Cit and L-aspartate had no discernable effect on exNO production (Fig. 7A). Given these somewhat surprising findings, we measured mRNA levels of AS and AL in lung, liver, and kidney homogenates from N and CH rats using RT-PCR. We found very low levels of AS1 and AL mRNA in the lungs, whereas there were easily detectable mRNA levels of AS1 and AL in the liver and kidney (Fig. 7B). We also measured protein levels of AS1 and AL in lung, liver, and kidney homogenates.

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Fig. 4. Addition of L-arginine (L-Arg) to achieve a final perfusate concentration of 3 mM decreased vascular resistance in CH lungs such that it was not different from that in N lungs. Using a distensible vessel model for the pressure-flow (P-Q) data generated values for vascular distensibility ($\alpha$; A) and a vascular resistance parameter ($R_0$; B). There were no differences in $\alpha$ between N ($n = 7$) and CH ($n = 6$) lungs, and the addition of L-Arg had little effect on $\alpha$. $R_0$ was greater in isolated perfused lungs from CH rats than from N rats, and L-Arg significantly decreased $R_0$ in CH lungs. #CH different from N under the same condition ($P < 0.05$); *CH different from N ($P < 0.05$); #CH different from CH ($P < 0.05$).
Fig. 5. L-Arg caused a concentration-dependent increase in exNO production that was dependent on L-Arg transport. A: exNO measured in isolated perfused lungs from N (n = 19) and CH (n = 12) rats with increasing concentrations of L-Arg. d-Arg was used in N lungs since it is not actively transported, and 30 mM L-lysine (L-Lys), a competitive inhibitor of L-Arg transport, was added in N lungs. *Different from N at the same concentration of L-Arg (P < 0.005). Two-way ANOVA using N and CH data showed a significant effect of both L-Arg concentration and exposure on exNO production (P < 0.001 for both). B: L-Arg caused a concentration-dependent decrease in vascular resistance. Vascular resistance is shown as a percentage of vascular resistance with no added arginine. *Different from no added L-Arg (P < 0.01). C: vascular resistance was negatively correlated with exNO production (R = -0.89, P < 0.001).

DISCUSSION

The major new findings in this study were that 1) CH decreased C_a and C_v, while increasing C_w, 2) CH increased Q_T, 3) CH increased C_m, 4) L-Arg reversed CH-induced alterations in vascular compliance, 5) L-Arg also decreased CH-induced vascular resistance by decreasing R_a, and 6) L-Arg-induced reversal of hemodynamic parameters in CH lungs was due to...
augmented NO production and depended on active transport of \( L\)-Arg from the vascular space. These results support our hypothesis that exogenous \( L\)-Arg results in increased NO production and reverses CH-induced alterations in hemodynamics in the lung. There were no differences between CH and N lungs in \( C_L \) by occlusions or in \( \alpha \) from the P-Q data. However, the vascular occlusion data revealed a significant redistribution of pulmonary vascular compliance after CH; \( C_a \) and \( C_v \) decreased, whereas \( C_c \) increased. The CH-induced redistribution of vascular compliance from arteries and veins to the microvasculature resulted in an increase in lung blood volume at resting \( P_v \). However, the lung vessels were stiffer, such that the increase in \( Q_v \) for a given increase in \( P_v \) was lower after CH. A larger blood volume at low \( P_v \) that is mainly accommodated in the microvasculature may represent a physiological compensatory mechanism to maintain gas exchange under hypoxic conditions. The increase in \( C_c \) would be consistent with angiogenesis during exposure to CH, as has been previously described (10, 12). The redistribution of vascular compliance could also be due to the larger arteries and veins becoming stiffer after CH, which would be a consequence of the vascular remodeling that is a hallmark of CH (5, 15, 35, 42). We (2) have previously reported that in neonatal pigs, chronic pulmonary overcirculation induced by placement of an aorta-to-lobar pulmonary artery shunt resulted in significant pulmonary arterial remodeling that was associated with a significant reduction in \( C_a \).
However, it is of interest to note that in hypoxic lungs, the acute addition of L-Arg redistributed vascular compliance to resemble the vascular compliance distribution found in N lungs, suggesting that rather than vascular remodeling the decrease in $C_a$ was due to vasoconstriction, since it is unlikely that changes in vessel structure would occur in the time course of these isolated lung experiments. This concept is consistent with a recent study by Vanderpool et al. (41), wherein acute Rho kinase inhibition after CH exposure in mice essentially normalized pulmonary vascular resistance while the proximal arterial thickening and stiffening persisted.

Traditionally, pulmonary $P_a$ and pulmonary vascular resistance are assessed as the markers of pulmonary hypertension. However, since blood flow is pulsatile, $C_a$ will also affect pulmonary hemodynamics and RV afterload. The total energy that the RV must use to propel the stroke volume is inversely proportional to the vascular compliance (18). This may have clinical ramifications given that Mahapatra et al. (19) found that $C_a$ (estimated by the investigators as the stroke volume divided by the pulse pressure during right heart catheterization) was inversely correlated with survival in patients with pulmonary arterial hypertension.

We found that eNOS protein expression and exNO production was higher in lungs from CH rats than in those from N rats. This finding is consistent with previous reports using the CH rat model (5, 15, 33), and LeCras et al. (15) found that it was CH and not alterations in lung blood flow that led to the increase in eNOS expression in the rat. We also found that acute hypoxia resulted in decreased exNO production in isolated lungs from both CH and N rats. This finding is consistent with a study by Sato et al. (35) in isolated lungs from hypoxic rats ventilated with either a normoxic or hypoxic gas mixture. This decrease in exNO production could be secondary to the decreased production of NO by eNOS, or it could be secondary to decreased bioavailability of NO within the lung. For example, Jernigan et al. (13) found that the production of ROS was greater in CH lungs than in N lungs and that treatment with ROS scavengers improved endothelium-derived NO-dependent vasodilation. Taken together, we speculate that the elevated eNOS expression in lungs from hypoxic rats represents a compensatory response to the decreased NO production and/or bioavailability caused by hypoxia.

L-Arg resulted in a concentration-dependent increase in exNO production in isolated lungs from both N and CH rats. The L-Arg-induced increase in exNO production depended on L-Arg transport into the lung, since D-Arg did not increase exNO production and L-Lys, but not D-Lys, attenuated the L-Arg-induced increase in exNO production. In the lung, the majority of L-Arg uptake is due to the activity of CATs, specifically CAT-1 and CAT-2, which are encoded by the genes $slc7a1$ and $slc7a2$ (20). The L-Arg-induced increase in exNO was associated in these isolated lungs with a decrease in total pulmonary vascular resistance, demonstrating that exNO is a measure of vasoactive NO production in the isolated perfused lung. It has been found that the administration of exogenous L-Arg to rats during CH exposure can attenuate the resultant pulmonary hypertension (7, 39). Our results demonstrate that L-Arg can also lower pulmonary vascular resistance in established CH-induced pulmonary hypertension. In human studies (22, 27, 31), it has been reported that exogenous L-Arg causes systematic vasodilation in healthy subjects, and in one study (26), the short-term administration of L-Arg improved pulmonary artery pressure and pulmonary vascular resistance in patients with pulmonary hypertension. Thus, increasing L-Arg uptake may represent a potential therapy for pulmonary hypertension that may increase the production of NO and thereby reduce pulmonary vascular resistance. It is of interest to note that a study by Howell et al. (11) found that the administration of L-Arg during CH exposure in rats ameliorated pulmonary hypertension and promoted angiogenesis, suggesting another possible mechanism whereby L-Arg may benefit patients with pulmonary hypertension.

In pulmonary vascular endothelial cells, it has been suggested that the enzymes required for recycling of L-Cit to L-Arg, AS and AL, are essential for eNOS-dependent NO production (9, 37). These findings have led to the notion that exogenous L-Cit may be a more efficient way of increasing NO production by eNOS (9). There have been two small studies examining L-Cit in patients with pulmonary hypertension (1, 36). Given the effect of L-Arg on hemodynamics and exNO production in the isolated rat lung, we repeated our experiments using L-Cit. To our surprise, there was little effect of exogenous L-Cit on exNO production in the isolated perfused rat lung. However, we found low levels of expression of AS1 and very low levels of expression of AL in the rat lung, with relatively robust expression in the kidney and liver, a finding that is consistent with a report in Wistar rats (25). Given these findings regarding the organ-specific distribution of AS and AL in the rat, we sought to determine if supplementing the whole animal would result in an increase in exNO production. When we administered either L-Arg or L-Cit to anesthetized rats, we found a significant increase in exNO production in both N and CH animals. Taken together, these data suggest that L-Cit can be used in rats to increase lung NO production via its conversion to L-Arg in organs other than the lung.

In summary, this study shows that CH resulted in higher pulmonary $R_a$ and lower pulmonary $C_a$. The effects of hypoxia on pulmonary $C_a$ may have important implications for right heart function in pulmonary hypertension. We found that L-Arg administration to isolated lungs from CH-exposed rats resulted in a redistribution of vascular compliance such that it was not different from the vascular compliance distribution found in N lungs, suggesting that the alterations in vascular compliance seen with CH are due to vasoconstriction. L-Arg administration caused a concentration-dependent increase in exNO production in the lung that depended on active L-Arg transport, and this L-Arg transport-dependent increase in NO production was associated with reductions in pulmonary vascular resistance. Taken together, our findings suggest that interventions to increase L-Arg uptake by the lung will increase NO production, resulting in improved $C_a$, and the improved vascular compliance may have beneficial effects on RV function.

GRANTS

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DISCLOSURES

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