Pulmonary vascular iNOS induction participates in the onset of chronic hypoxic pulmonary hypertension

Václav Hampl,¹,⁵ Jana Bíbová,¹,⁵ Alena Baňasová,²,⁵ Jiří Uhlík,³ Dana Miková,¹,⁵ Olga Hniličková,¹,⁵ Věra Lachmanová,⁴,⁵ and Jan Herget¹,⁵

Departments of ¹Physiology, ²Pathophysiology, ³Histology, and ⁴Anesthesiology, Charles University Second Medical School; and ⁵Centre for Cardiovascular Research, Prague, Czech Republic

Submitted 12 January 2005; accepted in final form 3 August 2005

Pulmonary vascular iNOS induction participates in the onset of chronic hypoxic pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 290: L11–L20, 2006. First published August 19, 2005; doi:10.1152/ajplung.0023.2005.—Pathogenesis of hypoxic pulmonary hypertension is initiated by oxidative injury to the pulmonary vascular wall. Because nitric oxide (NO) can contribute to oxidative stress and because the inducible isoform of NO synthase (iNOS) is often upregulated in association with tissue injury, we hypothesized that iNOS-derived NO participates in the pulmonary vascular wall injury at the onset of hypoxic pulmonary hypertension. An effective and selective dose of an iNOS inhibitor, L-N⁶-(1-iminoethyl)lysine (L-NIL), for chronic peroral treatment was first determined (8 mg/kg in drinking water) by measuring exhaled NO concentration and systemic arterial pressure after LPS injection under ketamine + xylazine anesthesia. A separate batch of rats was then exposed to hypoxia (10% O₂) and given L-NIL or a nonselective inhibitor of all NO synthases, N⁵-g-nitro-L-arginine methyl ester (L-NAME), increases perfusion pressure in lungs isolated from chronically hypoxic animals more than in normoxic controls (reviewed in Ref. 23). On the other hand, due to its radical nature, NO may contribute to the oxidative injury to the walls of the pulmonary vessels that appears to initiate their morphological remodeling (27, 28, 35). Such a hypertension-promoting effect may more or less negate the tone- and proliferation-reducing effects.

Evide...
anesthetized animals (40). However, no data were available regarding effective and selective dose for a prolonged, peroral administration. To find it, we gave one group of rats L-NIL in drinking water at a dose of 3 mg/l (n = 7); another group received 8 mg/l (n = 7). The third group received plain water to serve as controls (n = 8). As a comparison with a well-described, nonselective NOS blocker, the fourth group received L-NAME (500 mg/l, n = 7). Because inhibition of eNOS is known to increase systemic arterial pressure (SAP) (47), we measured systolic arterial pressure noninvasively with a tail cuff ultrasonic flow meter (2.5 mm SS-series with J reflector). Of the rats (ketamine 100 mg/kg body wt ip) and xylazine (16 mg/kg im) and intubated via tracheotomy. Exhaled gas was collected via a two-way valve into a small bag (made from a condom). The groups did not differ in the time needed to fill the bag (~2 min). NO concentration in the collected gas was analyzed with a chemiluminescence analyzer (CLD 77 AM; EcoPhysics, Duernten, Switzerland).

After the first NO measurement, the rats were injected with LPS (5 mg/kg body wt) into the jugular vein to induce iNOS expression. Exhaled gas NO analysis was then repeated every 60 min.

**Inhibition of NOS during 1-wk hypoxia.** To test the hypothesis that NO contributes to the pathogenesis of the initial phase of pulmonary hypertension, 24 rats were exposed to 1 wk hypoxia (10% O₂) in a normobaric hypoxic chamber (22). During the exposure, their drinking water was supplemented either with a nonselective inhibitor of all NOS isoforms, L-NAME (500 mg/l, n = 8), or with a selective iNOS inhibitor, L-NIL (8 mg/l, n = 9). The treatment with NOS blockers started 3 days before the hypoxic exposure to make sure that effective NOS inhibition had been achieved by the time hypoxia began (Fig. 1). The doses were based on literature data (L-NAME) (45, 54) and our experiment described above (L-NIL). The groups treated with NOS inhibitors were compared with untreated hypoxic (10% O₂, 1 wk; n = 7) and normoxic (n = 8) controls. In all experiments, the hypoxic chamber was opened for up to 30 min every 2–3 days for cleaning and feeding.

Immediately after termination of the hypoxic exposure, exhaled NO was measured to confirm the effectiveness of NOS inhibition. Each rat was sealed individually in a glass jar (2.1 l) flushed with NO-free air. After 15 min, NO accumulated in the jar was measured with chemiluminescence NO analyzer.

After completing the exhaled NO measurement, we anesthetized the rats (ketamine + xylazine as above). Their carotid artery was cannulated to measure mean SAP. Under oscilloscopic guidance, the pulmonary artery was then catheterized with a thermoplastically shaped polyethylene catheter (1.1 mm outer and 0.75 mm inner diameter) via a right jugular vein introducer, as previously described (22, 26). After we obtained a stable reading of mean pulmonary arterial pressure (PAP), the rats were intubated via tracheotomy and ventilated with room air at ~60 breaths/min (peak inspiratory pressure 10 cmH2O, peak expiratory pressure 0 cmH2O). The chest was opened in midline, and as much as possible extra care was taken to avoid the rats’ bleeding. Ascending aorta blood flow was measured with an ultrasonic flow meter (2.5 mm SS-series with J reflector + T106 flow meter; Transonic Systems, Ithaca, NY) as an estimate of cardiac output (20, 42). This value relative to body weight is reported as cardiac index (CI). The values obtained with this method are known to be lower than those in vivo due to the anesthesia and especially the thoracotomy; however, this error systematically affects all experimental groups, so that meaningful intergroup comparisons are possible.

Finally, the heart was removed, and right ventricle-to-left ventricle plus septum wet weight ratio (RV/LV+S) was used as an index of right ventricular hypertrophy associated with pulmonary hypertension (18).

**NOS inhibition during the first week of a 3-wk hypoxia.** To test whether the effects of NOS inhibition at the beginning of hypoxia are overcome by continued, prolonged hypoxia after discontinuation of the NOS blockade, an experiment was performed in which three groups of rats were exposed to 3-wk hypoxia (10% O₂) and compared with a normoxic control group (n = 6). Of the groups exposed to hypoxia, two received NOS blocker in drinking water (L-NAME 500 mg/l, n = 7; or L-NIL 8 mg/l, n = 8) for the last 3 days before the hypoxic exposure and during the first week of hypoxia (Fig. 1). The third hypoxic group received no treatment (n = 8). We did not observe any differences among the groups in the amount of water consumed. After 3 wk of hypoxia, exhaled NO, SAP, PAP, CI, and RV/LV+S were measured as described above.

**NOS inhibition during the last 10 days of a 3-wk hypoxia.** This experiment was performed to see whether chronic NOS inhibition starting after pulmonary hypertension has already developed would have effects similar to those seen with NOS inhibition during the first week of hypoxia. Rats were exposed to hypoxia for 3 wk as described above. For the last 10 days of hypoxia, the animals received either L-NIL (8 mg/l, n = 8) or L-NAME (500 mg/l, n = 10) in their drinking water (Fig. 1). Eight rats exposed to hypoxia received no treatment to serve as controls. Measurements were the same as in the 1-wk hypoxia study (exhaled NO, SAP, PAP, CI, RV/LV+S).

**iNOS immunohistochemistry.** Rats exposed to hypoxia (10% O₂) for 4 days (n = 10) or 20 days (n = 8) were compared with untreated normoxic controls. After killing the rats by cutting their cervical vertebral column in deep chloral hydrate anesthesia (300 mg/kg body wt ip; Tama, Olomouc, Czech Republic), we fixed the whole left lung with Baker’s fluid and embedded it in paraffin. Slides (4–6 μm
creased lung NO production at the beginning of hypoxic exposure in rats given 3 mg/l L-NIL in drinking water for 3 days before LPS. A higher L-NIL dose (8 mg/l) did not have any significant additional inhibitory effect, as also indicated by maximally effective dose determined in rats (not treated with NOS inhibitor), LPS injection elicited a marked, time-dependent rise in NO concentration in exhaled breath. This increase was substantially reduced in rats treated with L-NAME (500 mg/l). Note that exhaled NO did not differ between rats treated with L-NAME and the higher L-NIL dose (8 mg/l).

Fig. 2. Selective prolonged inducible NOS (iNOS) inhibition with oral L-NIL. Data are means ± SE. Unpaired t-test or one-factor ANOVA followed with Fisher’s protected least-significant difference post hoc test were used for group comparisons as appropriate. *P < 0.05 was considered statistically significant. Because not all measurements were successful in all animals (e.g., bleeding prevented CI measurement in some rats), the actual n for each value is given in the figures and tables. RESULTS Dose of l-NIL for peroral administration. Before the LPS injection, the concentration of NO in the collected exhalate was close to the detection limit of the chemiluminescence NO analyzer (~1 ppb), and no differences among the groups could be discerned (Fig. 2). Exhaled NO did not change 1 h after LPS injection, but it was elevated by 2 h after LPS. In rats that were not treated with any NOS blocker, the exhaled NO continued to rise for the remainder of the experiment (4 h post-LPS) (Fig. 2A). The increase in exhaled NO was blunted by ~50% by 3 mg/l L-NIL. The higher l-NIL dose (8 mg/l) did not have any further inhibitory effect compared with 3 mg/l L-NIL, implying that maximally effective dose was reached (Fig. 2A). l-NAME reduced exhaled NO even more than L-NIL (Fig. 2A). Systolic arterial pressure was significantly elevated by l-NAME treatment (confirming eNOS inhibition), but not by either l-NIL dose (Fig. 2B). This suggests that the maximally effective l-NIL dose against iNOS (8 mg/l) did not cause any measurable nonselective inhibition of eNOS. Because IC_{50} of l-NIL for the third NOS isoform, nNOS (NOS I), is almost 30× higher than that for iNOS (40), we surmised that the dose of l-NIL used in our experiments had no effect on NOS.
**Table 1.** Body weight, cardiac index, right and left ventricle plus septum wet weight, and their ratio (RV/LV+S) in normoxic and 1-wk hypoxic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>CI, ml·min⁻¹·kg⁻¹</th>
<th>RV, mg</th>
<th>LV+S, mg</th>
<th>RV/LV+S</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>297±6*(8)</td>
<td>101±7 (6)</td>
<td>182±10</td>
<td>604±19*</td>
<td>0.30±0.02 (8)</td>
</tr>
<tr>
<td>H 1 wk</td>
<td>227±5 (7)</td>
<td>107±6 (5)</td>
<td>153±7§</td>
<td>440±8</td>
<td>0.35±0.02 (7)</td>
</tr>
<tr>
<td>H 1 wk + l-NAME</td>
<td>223±3 (8)</td>
<td>74±3*§ (7)</td>
<td>149±9§</td>
<td>460±7§</td>
<td>0.32±0.02(8)</td>
</tr>
<tr>
<td>H 1 wk + l-NIL</td>
<td>209±4† (9)</td>
<td>99±15 (3)</td>
<td>160±4</td>
<td>420±14</td>
<td>0.38±0.02(9)</td>
</tr>
</tbody>
</table>

Data are means ± SE, *ns are in the parentheses. BW, body wt; CI, cardiac index; RV, right ventricle; LV + S, left ventricle plus septum; N, normoxic; H, hypoxia; H 1 wk + l-NAME, rats treated with N⁵-nitro-L-arginine methyl ester (500 mg/l) for 3 days before and during a 1-wk exposure to hypoxia; H 1 wk + l-NIL, rats treated with l-NAME, rats treated with N⁵-(1-iminoethyl) lysine (8 mg/l) for 3 days before and during a 1-wk exposure to hypoxia. *P < 0.05 group differs from all other groups, †P < 0.05 group differs from other hypoxic groups, ‡P < 0.05 vs. H 1 wk, §P < 0.05 vs. all other groups.

Inhibition of NOS during 1-wk hypoxia reduces pulmonary hypertension. As expected, all rats exposed to 1-wk hypoxia had lower body weight than normoxic controls. In addition, the rats treated in hypoxia with l-NIL were slightly but significantly lighter than the remaining hypoxic groups (Table 1). After a 15-min accumulation of exhaled NO, its concentration in a 2.1-l jar was significantly higher in 1-wk hypoxic rats compared with normoxic controls. This hypoxia-induced elevation of exhaled NO was prevented by each of the NOS inhibitors (Fig. 3A). Selectivity of l-NIL was indirectly confirmed by the fact that it did not elevate SAP, unlike the nonselective NOS inhibitor L-NAME (Fig. 3B).

One week of hypoxia was sufficient to significantly elevate PAP compared with normoxic controls (Fig. 3C). In rats treated with the NOS inhibitors during hypoxia, PAP was slightly but significantly lower than in hypoxic controls, albeit still higher than in normoxic controls (Fig. 3C). PAP did not differ between the l-NAME- and l-NIL-treated hypoxic groups. Treatment with l-NAME caused a significant CI reduction (Table 1), as reported previously (20). This was not seen with l-NIL; however, the number of successful CI measurements in this group was low. One week of hypoxia was not sufficient to produce statistically significant right ventricular hypertrophy, and there were no differences among the hypoxic groups in the weight of the right ventricle (Table 1). Probably because of the l-NAME-induced systemic hypertension, the left ventricle plus septum weight was higher in the l-NIL-treated hypoxic rats, leading to higher RV/LV+S in the latter group (Table 1).

Reduction of pulmonary hypertension by NOS inhibition during the first week of a 3-wk hypoxia. Rats exposed to hypoxia for 3 wk had lower body weight than corresponding controls living in room air (Table 2). At the end of the exposure, NO concentration in the exhaled air was similarly elevated in all hypoxic groups (Fig. 4A). Chronic hypoxic pulmonary hypertension was evident from markedly elevated PAP at the end of 3-wk hypoxic exposure compared with normoxic controls (Fig. 4C). As expected, PAP was higher after 3 wk than after 1 wk of hypoxia. At the end of the 3-wk hypoxic exposure, PAP was higher in untreated, hypoxic controls than in rats treated during the first week of exposure with l-NIL, despite the 2-wk lag from the end of the l-NIL treatment (Fig. 4C). Although the PAP was numerically similar in the l-NAME group to that in the l-NIL group, the difference between the former and the untreated hypoxic controls did not reach statistical significance, perhaps due to the lower number of successful PAP measurements in the l-NAME group. If both NOS blockade groups were pooled, their PAP was significantly lower than that of the untreated hypoxic group (23.1 ± 1.5 vs. 28.1 ± 1.7 mmHg, P = 0.026). The groups did not differ in SAP and CI (Fig. 4B and Table 2). Compared with normoxic controls, all hypoxic groups had similarly increased RV/LV+S (Table 2).

---

**Fig. 3.** NOS inhibition during a 1-wk hypoxia reduces pulmonary hypertension. Data are means ± SE, *ns are in the parentheses. A: elevation of NO concentration in the exhaled air, induced by 1-wk hypoxia, is blocked by both l-NAME and l-NIL treatment, proving their effectiveness. B: mean SAP is elevated by l-NAME, but not l-NIL, treatment, excluding eNOS inhibition by l-NIL. C: increase in mean pulmonary arterial pressure (PAP), induced by 1-wk hypoxia, is blunted by concomitant NOS inhibition. N, rats kept in normoxia; H 1 wk, rats exposed to hypoxia for 1 wk; H 1 wk+l-NAME, rats treated with l-NAME (500 mg/l) for 3 days before and during a 1-wk exposure to hypoxia; H 1 wk+l-NIL, rats treated with l-NIL (8 mg/l) for 3 days before and during a 1-wk exposure to hypoxia. *P < 0.05 vs. N, †P < 0.05 vs. H 1 wk, ‡P < 0.05 vs. all other groups.
Table 2. BW, CI, RV, and LV+S wet weight and their ratio (RV/LV+S) in normoxic rats and rats treated with NOS inhibitors during the 1st week of a 3-wk hypoxia

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>CI mL min⁻¹ kg⁻¹</th>
<th>RV, mg</th>
<th>LV+S, mg</th>
<th>RV/LV+S</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>385±15 (6)</td>
<td>127±15 (6)</td>
<td>183±7*</td>
<td>703±21*</td>
<td>0.26±0.01 (6)</td>
</tr>
<tr>
<td>H 3 wk</td>
<td>253±8 (8)</td>
<td>104±5 (7)</td>
<td>285±15</td>
<td>620±23</td>
<td>0.46±0.02 (8)</td>
</tr>
<tr>
<td>H 3 wk + l-NAME 1st wk</td>
<td>265±5 (7)</td>
<td>127±12 (4)</td>
<td>275±12</td>
<td>627±17</td>
<td>0.44±0.03 (7)</td>
</tr>
<tr>
<td>H 3 wk + l-NIL 1st wk</td>
<td>249±3 (8)</td>
<td>109±13 (8)</td>
<td>238±15</td>
<td>525±23‡</td>
<td>0.45±0.03 (8)</td>
</tr>
</tbody>
</table>

Data are means ± SE, ns are in the parentheses. NOS, nitric oxide synthase; H 3 wk + l-NAME 1st wk, rats treated with l-NAME (500 mg/l) for 3 days before and during the 1st week of the exposure; H 3 wk + l-NIL 1st wk, rats exposed to hypoxia for 3 wk and treated with l-NIL (8 mg/l) for 3 days before and during the 1st week of the exposure. *P < 0.01 normoxic group differs from all hypoxic groups, †P < 0.05 l-NIL-treated group differs from the untreated hypoxic group, ‡P < 0.05 l-NIL-treated group differs from the l-NAME-treated group.

NOS inhibition during the third week of hypoxia has little effect on pulmonary hypertension. Just before the initiation of the NOS blocker treatment, the exhaled NO levels were similarly elevated in all hypoxic groups. At the end of the NOS blockade during the third week of hypoxia, the exhaled NO was markedly reduced by both l-NAME and l-NIL (Fig. 5A).

As expected, SAP was elevated by 1-wk l-NAME administered just before the measurement, whereas l-NIL had no effect on SAP (Fig. 5B). The groups treated with NOS blockers in the last week of hypoxia did not differ significantly in PAP from the untreated hypoxic controls, although there was a tendency for increase in the group given l-NAME and for reduction in the l-NIL group (Fig. 5C). There were no differences among the groups in CI (Table 3).

Surprisingly, both the right ventricle weight and RV/LV+S were lower in the group given l-NAME during the last week of hypoxia than in the remaining hypoxic groups (Table 3). We do not have any explanation for this perplexing observation, except perhaps some nonspecific effect of l-NAME (2, 3, 8, 46, 53). Left ventricular weights did not differ between these groups. l-NIL had no effect on the ventricular weights (Table 3).

Hypoxia transiently elevates iNOS expression in lung vessels. Expression of iNOS was not detected in lung sections of control rats living in normoxia (Fig. 6A). By contrast, almost all pulmonary arteries of all sizes showed strong positive iNOS immunostaining after 4 days of hypoxia (Figs. 6B and 7). The staining was localized predominantly to the smooth muscle layer, although, to a lesser degree, it was detected also in the intima and adventitia. In addition to the vessels, iNOS immunostaining was positive also in the pleura, airway walls, and focally in the interalveolar septa. The positive staining in the pleura and airways was also evident in the lungs of rats kept in hypoxia for 3 wk. However, the iNOS immunostaining in the walls of pulmonary vessels of all sizes was no longer apparent in these animals and did not differ from normoxic controls (Figs. 6C and 7). We observed an increase in the total number of large pulmonary vessels per mm² in the 3-wk hypoxic group (0.70 ± 0.05) compared with both the 4-day hypoxic (0.45 ± 0.06) and control rats (0.42 ± 0.07). There were no statistically significant differences in the numbers of other vessels among the groups.

Exhaled NO increases early in chronic hypoxia. Concentration of NO in exhaled air was low when the animals lived in normoxia but increased markedly on the first day of hypoxic exposure. It continued to rise until day 5, then leveled off, and, despite daily and individual variations, remained essentially stable for the rest of the hypoxic exposure (Fig. 8A). The NO levels started to drop on the first day of room air recovery and approached the normally low values by day 4 of recovery (Fig. 8A). In the supplementary experiment, exhaled NO measured directly from the tracheal tube doubled after 4-day hypoxia compared with the normoxic group (Fig. 8B). The difference between the tracheal tube collection and jar accumulation,
reflecting the contribution of the upper airways, did not differ between the hypoxic and normoxic groups (Fig. 8B), indicating that the source of the elevated NO exhalation in hypoxia is the lower respiratory tract. Although the contribution of the upper airways remains remarkable, it does not rise in hypoxia.

Nitrotyrosine concentration in the lungs is elevated after 4 days of hypoxia. The concentration of nitrotyrosine was higher \((P = 0.023)\) in extracts from lung tissue of rats exposed to hypoxia for 4 days \((1,065 \pm 96 \text{ mmol/g of extracted protein; } n = 6)\) than in extracts from control lungs \((791 \pm 57 \text{ mmol/g; } n = 8)\). This implies greater exposure to peroxynitrite, the product of reaction of NO with superoxide \((5)\). Increased nitrotyrosine levels in lung tissue after 4 days of hypoxia was also confirmed by immunoblot analysis (data not shown).

**DISCUSSION**

This study shows that increased NO production in (or near) the pulmonary vascular wall during the first days of hypoxia, mostly by the iNOS isofrom, contributes to the initiation of a process that eventually results in pulmonary hypertension. Of our results, especially the following support this conclusion: the PAP rise in the initial days of hypoxic exposure was partly inhibited by concomitant NOS inhibition. In this regard, selective iNOS inhibitor was as effective as the nonselective blocker of all NOS isoforms. If iNOS blockade was then discontinued, the hypoxic exposure continued, the pulmonary hypertension did not completely “catch up”; it was still reduced at the end of a 3-wk hypoxia. On the other hand, 10 days’ treatment with NOS inhibitors, started after the hypoxic pulmonary hypertension had already developed, did not have significant effect on PAP. The role of iNOS in the first days of hypoxia is further supported by the finding of markedly and transiently elevated expression of iNOS in lung vessels on the fourth day of hypoxia. Our exhaled NO data document the elevated lung NO production at the very onset of hypoxia.

NO is best known for its marked vasodilator activity. As such, exogenous NO is useful in clinical management of acute pulmonary hypertensive crises (for review, see e.g., Ref. 19). However, NO is also a reactive radical capable of tissue injury either directly or after reacting with other radicals. For example, an extremely fast reaction of NO with superoxide yields a highly cytotoxic peroxynitrite \((5)\). There is evidence that oxidative injury of the pulmonary vascular wall at the beginning of hypoxia initiates the process of vascular remodeling that eventually results in pulmonary hypertension \((28, 35)\). Our present data show that endogenous NO affects the initial development of chronic hypoxic pulmonary hypertension. The vasodilator and antiproliferative properties of NO do not prevail at this initial phase because otherwise the NOS inhibitors would have to aggravate, rather than reduce, pulmonary hypertension. The conclusion that increased NO production by iNOS at the initial stage of hypoxia promotes the development of pulmonary hypertension by contributing to oxidative vascular wall injury is supported by our observation of increased

---

Table 3. BW, CI, RV and LV+S wet weight and their ratio \((RV/LV+S)\) in rats treated with NOS inhibitors during the last week of a 3-wk hypoxia

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g</th>
<th>CI, ml/min (\cdot\text{kg}^{-1})</th>
<th>RV, mg</th>
<th>LV+S, mg</th>
<th>RV/LV+S</th>
</tr>
</thead>
<tbody>
<tr>
<td>H 3 wk</td>
<td>253±5</td>
<td>81±6</td>
<td>269±18</td>
<td>531±18</td>
<td>0.51±0.02</td>
</tr>
<tr>
<td>H 3 wk + L-NAME 3rd wk</td>
<td>265±4</td>
<td>97±9</td>
<td>214±9*†</td>
<td>551±22</td>
<td>0.39±0.02*†</td>
</tr>
<tr>
<td>H 3 wk + L-NIL 3rd wk</td>
<td>247±6</td>
<td>97±15</td>
<td>275±14</td>
<td>509±8</td>
<td>0.54±0.02</td>
</tr>
</tbody>
</table>

Data are means ± SE, ns are in the parentheses. H 3 wk + L-NAME 3rd wk, rats treated with L-NAME \((500 \text{ mg/l})\) during the last week of a 3-wk hypoxic exposure; H 3 wk + L-NIL 3rd wk, rats treated with L-NIL \((8 \text{ mg/l})\) during the last week of a 3-wk hypoxic exposure. *P < 0.05 group treated with L-NAME differs from L-NIL-treated group, †P < 0.05 group treated with L-NAME differs from untreated hypoxic group.
lung tissue levels of peroxynitrite marker, nitrotyrosine, after 4 days of hypoxia. Further support is provided by our previous finding that serum levels of nitrotyrosine are elevated after 4 days of hypoxia but normalized at the end of a 3-wk exposure (27). Consistently elevated lung nitrotyrosine was reported in human patients with pulmonary hypertension (7).

Because our conclusions rely to a large extent on the L-NIL selectivity for iNOS, it was necessary first to determine an effective and selective L-NIL dose for chronic peroral administration. The effectiveness of various L-NIL doses in inhibiting NO production was assessed by measurements of exhaled NO levels after a challenge with a well-known iNOS inducer, LPS (41), whereas selectivity toward iNOS rather than eNOS was inferred from the absence of an effect on SAP. L-NIL at 8 mg/l in drinking water markedly reduced exhaled NO levels after LPS, albeit not as much as the nonselective NOS blocker, L-NAME, at 500 mg/l. This is consistent with reports that, in addition to iNOS induction, LPS treatment can increase expression of the remaining NOS isoforms (12, 24, 30, 32). Somewhat surprisingly, even the high L-NAME dose used did not prevent the rise in exhaled NO after LPS completely. It is possible that some of the NO is formed in compartments not well accessible from the circulation. For example, macrophages could encounter LPS in the bloodstream, begin the process of iNOS induction, and then leave the vessels toward alveolar spaces, where the penetration of L-NAME may be limited.

The reduction in PAP by NOS inhibition during the first week of hypoxia was not accompanied by a corresponding mitigation of right ventricular hypertrophy. Enlargement of the right ventricle in chronic hypoxia is a recognized consequence of PAP elevation. However, several studies suggest that right ventricular weight and PAP must not always be closely tied. For example, various treatments of experimental pulmonary hypertension reduced right ventricular hypertrophy less (and at higher doses) than PAP (56, 57). Chronic pulmonary hypertension can be elicited experimentally without an accompanying right ventricular hypertrophy (10). Right ventricular weight was reported to rise in proportion to PAP elevation in rats exposed to chronic intermittent hypoxia from fourth day of age, but no significant correlation could be detected in older animals (34). Thus it is quite likely that chronic hypoxia has other effects on the ventricular size in addition to those mediated by increased PAP. This possibility is supported by observations that left ventricle can also be somewhat enlarged (relative to body weight) in chronic hypoxia (34, 43). Because NO has antiproliferative effects, it is also possible that NOS inhibition causes some degree of heart enlargement that may offset the reducing effect of the lessened afterload. This possibility is strongly supported by our earlier observation that chronic L-NAME treatment in normoxia enlarges both ventricles (20).

The effects of NOS inhibition on chronic hypoxic pulmonary hypertension have been studied previously, but not specifically at the initial stage of the exposure. Numerous studies demonstrated that acutely applied, nonselective NOS inhibitors cause considerably greater vasoconstriction in lungs of chron-
L18

**Inos Induction Initiates Hypoxic Pulmonary Hypertension**

Fig. 8. Concentration of NO in exhaled breath increases at the very beginning of CH exposure. A: accumulation of breath NO during a 15-min stay of awake rats in a closed jar (n = 8 in the 1st wk, 5 thereafter) is negligible before exposure to CH but is significantly increased on the 1st day of exposure, remains elevated throughout a 3-wk hypoxia, and drops toward minimal, control level within days of normoxic recovery. All data points in hypoxia differ significantly from day 0. B: total exhaled NO, measured as 10-min accumulation in a closed jar, is higher in 4-day hypoxic rats than in normoxic controls, as is the contribution from lower airways, estimated from breath collection into a small bag through tracheal tube. The difference between these 2 measurements, corresponding to the contribution from the upper airways, is large but unchanged by hypoxia. Data are means ± SE. *P < 0.05 vs. normoxia.

Physically hypoxic compared with normoxic rats, implying elevated lung NO activity in chronic hypoxia (for review, see Ref. 23). In an apparent contrast, rats given l-NAME during the entire 3-wk hypoxic exposure did not have more severe pulmonary hypertension than rats exposed to the same hypoxia, but with no inhibitor treatment (20). Our current results offer one possible interpretation of that paradox. It could be a combination of a sustained reduction of pulmonary hypertension by iNOS blockade in the first week (Fig. 3D) counteracted by augmentation of pulmonary hypertension by inhibition of elevated eNOS activity in the more advanced phase of the hypoxic exposure.

There are also studies of chronic administration of selective iNOS inhibitors (l-NIL and aminoguanidine) during the entire 3- to 4-wk hypoxic exposure, showing no effect on pulmonary hypertension (48, 55). In our study, iNOS blockade during the first week of hypoxia reduced pulmonary hypertension. A possible explanation of this discrepancy could be the fact that we started the administration of NOS blockers 3 days before hypoxic exposure to make sure that their blood and tissue levels were at effective levels at the moment of the onset of hypoxia. It is thus possible that the role of iNOS in vascular wall injury promoting pulmonary hypertension is confined to the very beginning of hypoxia. In addition, ours is the only study demonstrating the selective effectiveness of the iNOS blocker at the dose and route of administration used.

As far as we know, this is the first description of a chronic, peroral use of l-NIL for selective iNOS inhibition in vivo. Continuous infusion or repeated injections of l-NIL were reported previously (48, 49); however, our method of adding l-NIL to drinking water is much more practical. It has a possible disadvantage of variations in water consumption. This is especially important at the beginning of hypoxia, which is typically associated with reduced drinking. However, in our study the l-NIL intake in 1-wk hypoxia was sufficient, as evidenced by the suppression of exhaled NO to the low level similar to that in normoxic controls (Fig. 3A). Furthermore, the exhaled NO was similar after l-NIL treatment in 1-wk hypoxia and at the end of a 3-wk hypoxia, when water intake is known to be normalized (Fig. 5A).

Many authors have reported elevated NOS mRNA and protein in lungs and pulmonary vessels in chronic hypoxia (for review, see Ref. 23). Most of those studies either focused on eNOS or did not discriminate between NOS isoforms. Several studies show elevated iNOS mRNA or protein at the end of a prolonged hypoxia (2–4 wk) in whole lung homogenates (16, 29, 36, 44, 48). We are aware of two publications that focus on lung iNOS expression during the first week of hypoxia (51, 59); using rats, both show increased iNOS protein in whole lung homogenates. Our present data further supplement this information by showing that the iNOS induction in the first days of hypoxia is localized to a major extent in the pulmonary vessels, especially their media (Fig. 6). Furthermore, our data indicate that with prolonged hypoxia, iNOS remains expressed in airway epithelia, but its expression in the pulmonary vascular wall returns to the barely detectable baseline level. Thus it appears that the chronic hypoxia-induced elevation of whole lung iNOS protein in some (29, 36) but not all (16) studies can be attributed to the extravascular iNOS expression. Together, our data are consistent with the reports of others and with the hypothesis that the pulmonary vascular wall iNOS expression rises during the first few days of hypoxia and returns towards baseline thereafter.

Enhanced iNOS expression not accompanied by NO overproduction has been occasionally reported (9, 13, 37). Thus to support our hypothesis of a causative role of elevated NO in the initiation of hypoxic pulmonary hypertension, it was necessary not only to show early iNOS induction in lung vessels (Figs. 6 and 7), but also to prove that NO synthesis actually rises rapidly after the onset of hypoxia. Elevated pulmonary NO production in prolonged hypoxia has been reported previously (for review, see Ref. 23). High-altitude residents have higher exhaled NO compared with lowlanders (4). However, changes in NO production during the first days of hypoxia have not been studied. We show rapidly rising NO production into the exhaled air in the first days of hypoxia (Fig. 8A). Although this measurement is relatively easy to perform, its main limitation is ambiguity in respect to the source of the detected NO. It has been demonstrated that a major source of exhaled NO are the upper airways, especially the nasal and paranasal cavities (14, 39). Therefore, we performed a supplementary experiment to show that while the majority of NO in exhaled air comes from upper airways both in normoxia and hypoxia, the respiratory tract below the level of the trachea is responsible for the increase in exhaled NO seen in the first days of hypoxia (Fig. 8B). Furthermore, our data show that most of the exhaled NO increase in hypoxia is attributable to iNOS, as it was similarly and almost completely inhibited by l-NAME and l-NIL, both at the beginning of hypoxia (Fig. 3A) and after a more pro-
longed exposure (Fig. 5A). In normoxia, almost all exhaled NO is derived from eNOS (58).

The measurement of NO accumulation in an airtight container is a simple method for checking NO production in an intact, awake rat. Although we use it as an indicator of lung NO production, other possible NO sources, such as colonic denitriﬁcation bacteria or acidic nitrate reduction in the stomach, should be considered. However, the hypoxia-induced elevation of exhaled NO was completely prevented by each of the NOS inhibitors, implying that the collected NO was mostly exhaled since the alternative NO sources are NOS independent (38).

Although our data prove iNOS involvement in the initial phase of the development of hypoxic pulmonary hypertension, they do not show whether iNOS induction is a cause or a consequence of injury to the pulmonary vessels that underlies their remodeling. It is possible that the aggravating inﬂuence of NO on the initial phase of pulmonary hypertension can contribute to the ﬁndings that inhaled NO does not improve long-term outcome in acute respiratory distress syndrome (31) and in persistent pulmonary hypertension of the newborn (11, 33). The most clinically relevant correlate to our experimental situation are probably acute exacerbations of chronic hypoxic conditions. Our data suggest that in these situations, adding even more NO (by inhalational therapy) may have drawbacks. This issue should also be considered in attempts to treat high-altitude pulmonary edema with inhaled NO (1).

ACKNOWLEDGMENTS

The authors are grateful to Dr. Ludek Cervenka for invaluable help with systolic arterial pressure measurements in awake rats, to Dr. Richard Vytasek for performing peroxynitrite ELISA, to Michal Snorek and Petr Zdaril for assistance with exhaled NO measurements in awake rats, and to Kveta Vencilková for general technical and logistical support.

GRANTS

This study was supported by the Grant Agency of the Czech Republic Grant 305/05/0672.

REFERENCES


12. Contois AS, El-Dwiari Q, Laubach VE, and Hussain SNA. Lipo-


