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

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Hampl, Václav, Jana Bíbová, Alena Baňasová, Jiří Uhlík, Dana Miková, Olga Hniličková, Věra Lachmanová, and Jan Herget. 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.00023.2005.—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/l in drinking water) by measuring exhaled NO concentration and systemic arterial pressure after LPS injection under ketamine+xylozine 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, N^G-nitro-L-arginine methyl ester (L-NAME, 500 mg/l), in drinking water. Both inhibitors, applied just before and during 1-wk hypoxia, equally reduced pulmonary arterial pressure (PAP) measured under ketamine+xylozine anesthesia. If hypoxia continued for 2 more wk after L-NIL treatment was discontinued, PAP was still lower than in untreated hypoxic controls. Immunostaining of lung vessels showed negligible iNOS presence in control rats, striking iNOS expression after 4 days of hypoxia, and return of iNOS immunostaining toward normally low levels after 20 days of hypoxia. Lung NO production, measured as NO concentration in exhaled air, was markedly elevated as early as on the first day of hypoxia. We conclude that transient iNOS induction in the pulmonary vascular wall at the beginning of chronic hypoxia participates in the pathogenesis of pulmonary hypertension.

pulmonary circulation; nitric oxide; rat; inducible nitric oxide synthase

SINCE THE DISCOVERY THAT NITRIC OXIDE (NO) is formed in mammalian cells as an endogenous mediator, many attempts were made to define its possible role in the pathogenesis of pulmonary hypertension (reviewed in Ref. 23). Although the capacity of lung vessels to produce NO can be reduced in terminal phases of severe pulmonary hypertension (15), possibly due to the progressive endothelial damage, less advanced stages (at least in adults) are associated with increased expression of NO synthase (NOS) and augmented NO production (reviewed in Ref. 23). This is particularly well documented in

the frequently used and clinically relevant model of pulmonary hypertension elicited by chronic hypoxia.

In principle, as the actions of NO in the body are multifaceted, two main functional consequences of the elevated lung NO synthesis in chronic hypoxic pulmonary hypertension are possible. On one hand, the vasodilator and antiproliferative effects of NO may limit the extent of pulmonary vascular resistance elevation. This possibility is supported by numerous reports that acute administration of NOS blockers, such as N^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.

Evidence is accumulating that the principal free radical insult that initiates the process of vascular remodeling occurs during the first few days of chronic hypoxia. For example, indexes of pulmonary hypertension, determined at the end of a prolonged hypoxia, are reduced in rats that were treated with antioxidants at the beginning of the exposure (28, 35). By analogy, the present study was designed to test the hypothesis that elevated lung NO production in the first few days of chronic hypoxia contributes to the development of pulmonary hypertension, presumably by contributing to oxidative vascular wall injury. We reasoned that if this hypothesis were true, then treatment with NOS inhibitor only during the first week of hypoxia would result in reduced pulmonary hypertension.

Chronic hypoxia increases NOS expression in the lung vessels (reviewed in Ref. 23). Traditionally, functional role was implicitly assumed for the endothelial NOS isoform (eNOS). The possible role of the inducible NOS (iNOS, also called NOS II) is unknown, yet there are analogies from other organs that iNOS is functionally significant in situations characterized by tissue injury. Therefore, we also hypothesized that NO participating in the pathogenesis of pulmonary hypertension at the beginning of chronic hypoxia is produced mostly by iNOS. Treatment of hypoxic rats with a relatively selective iNOS inhibitor, L-N⁶-(1-iminoethyl)lysine (L-NIL) (21, 40), was used to test this hypothesis. In addition, iNOS expression was evaluated with immunohistochemistry at the initial and advanced stages of hypoxia (*days 4 and 20*, respectively). The results were reported in a preliminary form (6, 21).

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METHODS

The project was performed on adult male Wistar specific pathogen-free rats (Anlab, Prague, Czech Republic). The experiments conformed to the European Community and National Institutes of Health guidelines for using experimental animals and were approved by the Animal Research Committee of the Charles University Second Medical School. Unless stated otherwise, all drugs were from Sigma (Prague, Czech Republic).

Dose of L-NIL for long-term, peroral use in rats. L-NIL has been reported to relatively selectively inhibit iNOS, but not other NOS isoforms, in *in vitro* experiments and when administered acutely to 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 (25) after 3 days of treatment to make sure that L-NIL at the selected doses did not inhibit eNOS. Subsequently, the rats were anesthetized with 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 begun (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 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

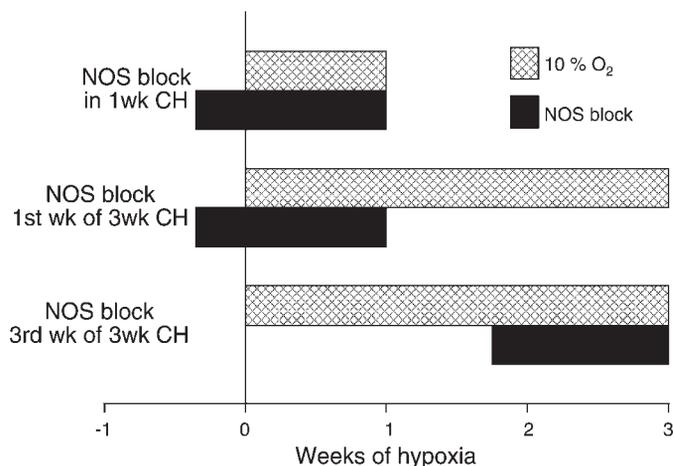


Fig. 1. Schematic of the timing of chronic nitric oxide synthase (NOS) blockade in relation to hypoxic exposure. In one set of experiments, rats received NOS blockers [^{N^G}-nitro-L-arginine methyl ester (L-NAME) or L-N^G-(1-iminoethyl)lysine (L-NIL)] just before and during a 1-wk hypoxic exposure (top pair of bars). In another set, the treatment with NOS blockers was the same, but hypoxia continued after cessation of NOS blockade for 2 more wk (middle pair of bars). In the 3rd set, the hypoxic exposure was the same as in the middle set (3 wk), but NOS was blocked only during the last 10 days (bottom pair of bars). Note that the duration of the treatment with the NOS inhibitors was always the same. See METHODS for detailed description. CH, chronic hypoxia.

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 nine normoxic controls. After killing the rats by cutting their cervical vertebral column in deep chloral hydrate anesthesia (300 mg/kg body wt ip; Tamda, Olomouc, Czech Republic), we fixed the whole left lung with Baker's fluid and embedded it in paraffin. Slides (4–6 μm

thick) were stained with hematoxylin and eosin, cresyl fast blue, aldehyde fuchsin, Gomori silver stain, and toluidine blue. After microwave oven antigen retrieval (52), endogenous alkaline phosphatase was inhibited by levamisole, and nonspecific binding was blocked by 10% BSA in Tris-buffered saline. The sections were then incubated for 24 h with monoclonal anti-iNOS antibody (1:25) at room temperature in a humid chamber. Rabbit anti-mouse polyclonal antibody labeled with alkaline phosphatase (1:50) was used as a secondary antibody for 30 min and then visualized with Fast Red TR/Naphthol AS-MX (Sigma *FAST* tablets). The slides were counterstained with hematoxylin and mounted in gelatin. The primary antibody was omitted in control measurements. Using an image-analyzing software LUCIA General (Laboratory Imaging, Prague, Czech Republic), an observer (J. Uhlík) blinded to the group assignment of the specimens determined separately the percentage of vessels with positive immunostaining for small prealveolar arteries (20–50 μm in diameter), small muscular arteries (50–100 μm), and larger arteries (>100 μm). The slide areas (23–81 mm^2) and the total numbers of vessels per slide (141–498) did not differ significantly among the groups.

Exhaled NO in chronic hypoxia. Evidence is available for increased pulmonary NO production in chronic hypoxia (reviewed in Ref. 23). However, it is unknown whether this increase occurs soon enough to be able to contribute to the radical injury at the beginning of the pulmonary vascular remodeling. To find out, we measured exhaled NO in intact, conscious rats as described in *Inhibition of NOS during 1-wk hypoxia* (15-min collection in 2.1-l jar). The measurements were performed once every 1–2 days during a 26-day exposure to hypoxia (10% O_2) and in subsequent 4 days recovery in room air. To distinguish the contribution of upper airways and lung tissue to the exhaled NO, a supplementary experiment was performed in which normoxic control rats and 4-day hypoxic rats were anesthetized (thiopental 40 mg/kg body wt ip) and intubated via a tracheotomy. NO in their exhaled breath was first measured after placing them for 10 min into the 2.1-l jar, as described above. Because NO production in the nose and paranasal sinuses is known to be very large (14, 39), we expected that NO would diffuse into the jar even in the absence of nasal ventilation. To measure NO production into the exhaled air from the lung tissue and lower airways, the exhalate for NO measurement was then collected directly from the tracheal tube as in the experiments with LPS (see *Dose of L-NIL for long-term, peroral use in rats*).

Nitrotyrosine in lung tissue. To support our hypothesis that increased lung NO production at the beginning of hypoxic exposure contributes to oxidative tissue injury, we used competitive ELISA to

measure 3-nitrotyrosine concentration in soluble extract of lyophilized lung tissue of control and 4-day hypoxic rats. Nitrotyrosine is considered a suitable marker of peroxynitrite production, which originates from a very rapid reaction between NO and superoxide (5). The details of lung tissue preparation and ELISA determination, using our own mouse monoclonal antibody against nitrotyrosine and commercial anti-mouse Ig rabbit antibody conjugated with peroxidase, are described elsewhere (17). Nitrotyrosine concentration was expressed per gram of extracted protein, determined by the bicinchoninic acid method (50).

Statistical analysis. Data were analyzed statistically using StatView 5.0.1 software (SAS Institute, Cary, NC) and presented as means \pm 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 $\sim 50\%$ 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 $30\times$ higher than that for iNOS (40), we surmised that the dose of L-NIL used in our experiments had no effect on nNOS.

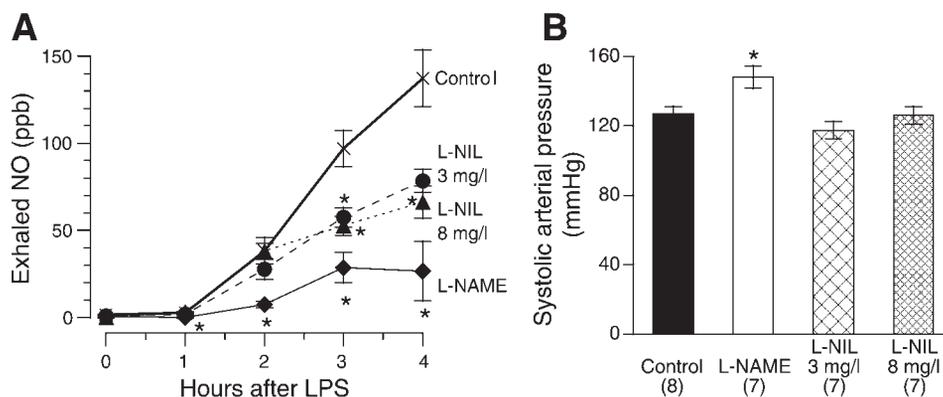


Fig. 2. Selective prolonged inducible NOS (iNOS) inhibition with oral L-NIL. Data are means \pm SE; *ns* for both panels are in the parentheses in *B*. *A*: in control rats (not treated with NOS inhibitor), LPS injection elicited a marked, time-dependent rise in NO concentration in exhaled breath. This increase was substantially smaller 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, suggesting that a maximally effective dose had been reached. Exhaled NO concentration was reduced the most in rats treated with a nonselective NOS inhibitor, 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). *B*: systolic systemic arterial blood pressure (SAP) measured in awake rats is elevated by the nonselective NOS inhibitor, L-NAME, but not by either of the tested doses of L-NIL, suggesting that L-NIL at these doses does not inhibit endothelial NOS (eNOS). * $P < 0.05$ value differs from the corresponding value in the control group.

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

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

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, normoxia; H, hypoxia; H 1 wk + L-NAME, rats treated with N^G-nitro-L-arginone 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-N⁶-(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 group differs from normoxic group, §*P* < 0.05 group differs from L-NIL-treated group.

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-NAME-

than 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).

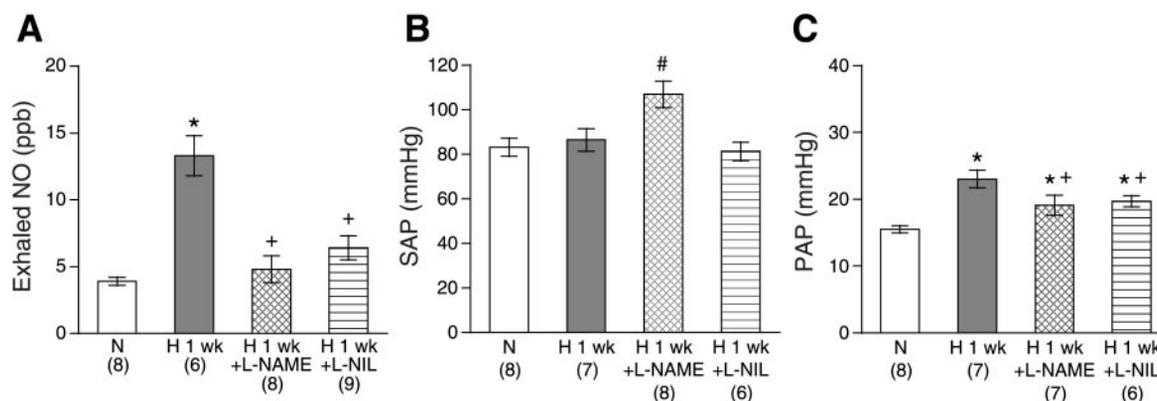


Fig. 3. NOS inhibition during a 1-wk hypoxia reduces pulmonary hypertension. Data are means ± SE, *ns* are in the parentheses. **A:** elevation of NO concentration in the exhaled air, induced by 1-wk hypoxia, is blocked by both L-NAME and L-NIL treatment, proving their effectiveness. **B:** mean SAP 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-week hypoxia

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

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 a 3-wk hypoxic exposure; H 3 wk + L-NIL 1st wk, rats treated with L-NIL (8 mg/l) for 3 days before and during the 1st week of a 3-wk hypoxic 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/L+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 from 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,

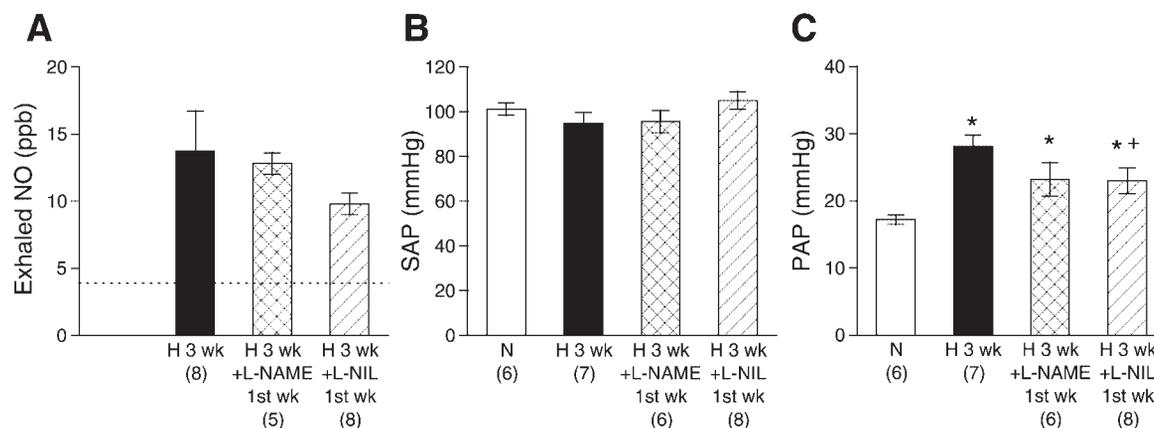


Fig. 4. NOS inhibition during the 1st wk of a 3-wk hypoxia reduces pulmonary hypertension. Data are means ± SE, *ns* are in the parentheses. A: 2 wk after cessation of NOS blockade there are no significant differences in exhaled NO levels among hypoxic animals. The dotted horizontal line indicates the level in normoxic controls from Fig. 3A. B: 2 wk after cessation of NOS blockade there are no significant differences in SAP. C: increase in PAP, induced by 3-wk hypoxia, is smaller in rats treated with L-NIL at the beginning of the exposure. H 3 wk, rats exposed to hypoxia for 3 wk; H 3 wk+L-NAME 1st wk, rats exposed to hypoxia for 3 wk and 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* < 0.05 vs. N, †*P* < 0.05 vs. H 3 wk.

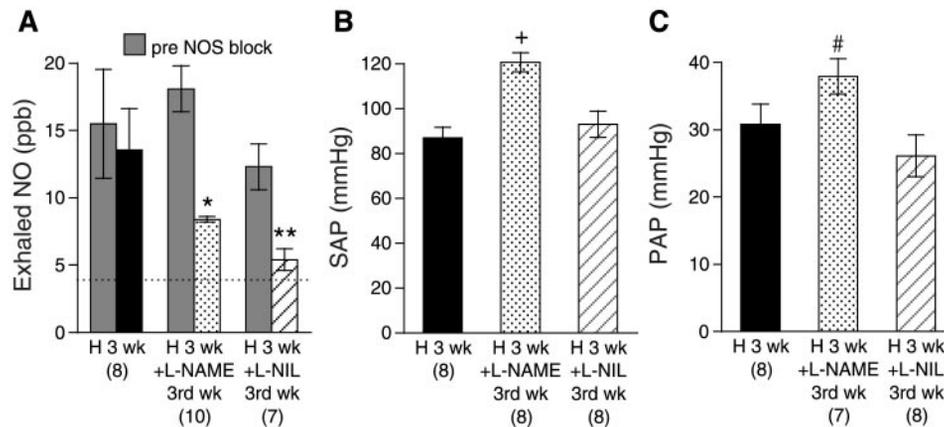


Fig. 5. NOS inhibition during the last 10 days of a 3-wk hypoxia. Data are means \pm SE, *ns* are in the parentheses. *A*: in 3 groups exposed to hypoxia, NO concentration in exhaled air was similar before the initiation of NOS blockade after 2 wk of hypoxia and decreased significantly after 10 days of L-NIL or L-NAME treatment. The dotted horizontal line indicates the level in normoxic controls from Fig. 3*A*. *B*: SAP in rats kept in hypoxia for 3 wk was increased by L-NAME treatment during the last 10 days before the measurement. L-NIL had no effect. *C*: PAP, elevated by 3 wk of hypoxia, was not significantly reduced by NOS inhibition during the last 10 days of the exposure. H 3 wk +L-NAME 3rd wk, rats exposed to 3-wk hypoxia and treated with L-NAME (500 mg/l) during the last 10 days; H 3 wk +L-NIL 3rd wk, rats exposed to 3-wk hypoxia and treated with L-NIL (8 mg/l) during the last 10 days. * $P < 0.0005$ vs. pre NOS block, ** $P < 0.05$ vs. pre NOS block, + $P < 0.05$ vs. all other groups, # $P < 0.05$ vs. H 3 wk +L-NIL 3rd wk.

reflecting the contribution of the upper airways, did not differ between the hypoxic and normoxic groups (Fig. 8*B*), 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 ($1,065 \pm 96$ nmol/g of extracted protein; $n = 6$) than in extracts from control lungs (791 ± 57 nmol/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 isoform, 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 and 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

Group	BW, g	CI, ml·min ⁻¹ ·kg ⁻¹	RV, mg	LV+S, mg	RV/LV+S
H 3 wk	253 ± 5 (8)	81 ± 6 (7)	269 ± 18	531 ± 18	0.51 ± 0.02 (8)
H 3 wk + L-NAME 3rd wk	265 ± 4* (10)	97 ± 9 (5)	214 ± 9*†	551 ± 22	0.39 ± 0.02*† (10)
H 3 wk + L-NIL 3rd wk	247 ± 6 (8)	97 ± 15 (6)	275 ± 14	509 ± 8	0.54 ± 0.02 (8)

Data are means \pm SE, *ns* are in the parentheses. H 3 wk + L-NAME 3rd wk, rats treated with L-NAME (500 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 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.

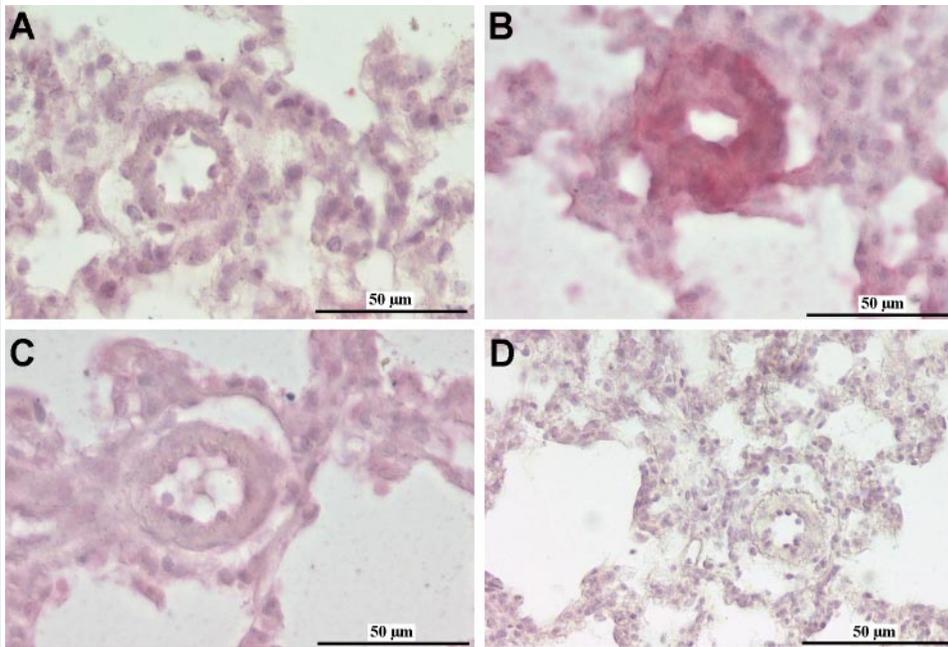


Fig. 6. Transient elevation of iNOS immunostaining in pulmonary vessels at the beginning of CH. *A*: small prealveolar arteriole from a normoxic rat showing relatively thin wall, single elastic lamina and negative iNOS immunostaining. *B*: small prealveolar arteriole from a rat exposed to hypoxia for 4 days demonstrating distinct wall thickening and strongly positive iNOS immunostaining. *C*: small prealveolar arteriole from a rat exposed to hypoxia for 20 days showing wall thickening, double elastic laminae, and negative iNOS immunostaining. *D*: small prealveolar arteriole from a rat exposed to hypoxia for 4 days with primary antibody omitted (negative control). Original magnification was $\times 40$ in all panels, and the scale bars represent 50 μm .

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-

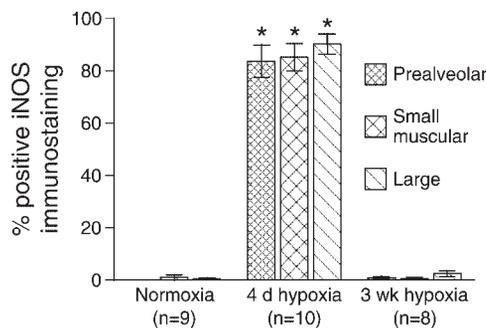
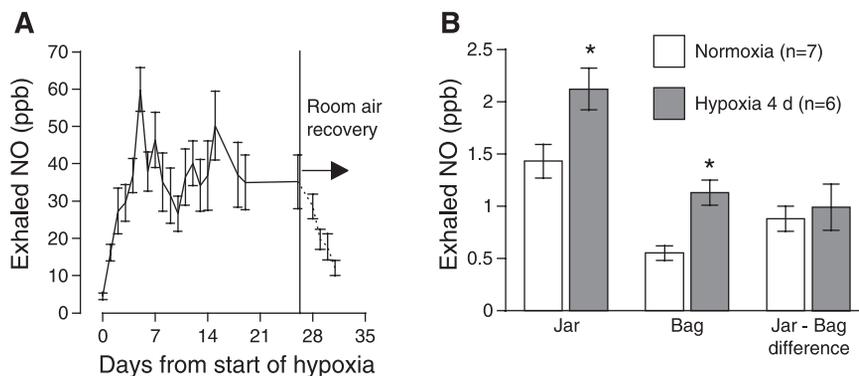


Fig. 7. Transient elevation of iNOS immunostaining in pulmonary vessels at the beginning of CH. Percentage (means \pm SE) of prealveolar, small muscular, and large pulmonary vessels showing iNOS immunostaining in normoxia, after 4 days of hypoxia, and after 3 wk of hypoxia. d, Days. * $P < 0.0001$ vs. the other groups.

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 \pm SE. * $P < 0.05$ vs. normoxia.



ically 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).

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