Induction of arginase isoforms in the lung during hyperoxia

LORETTA G. QUE, STEPHEN P. KANTROW, CHRISTOPHER P. JENKINSON, CLAUDE A. PIANTADOSI, AND YUH-CHIN T. HUANG

Induction of arginase isoforms in the lung during hyperoxia. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L96–L102, 1998.—L-Arginine can be metabolized by nitric oxide (NO) synthase (NOS) to produce NO or by arginase to produce urea and L-ornithine. In the liver, arginase (the AI isoform) is a key enzyme in the urea cycle. In extrahepatic organs including the lung, the function of arginase (the AII isoform) is less clear. Because we found that lung AII was upregulated during 100% O2 exposure in preliminary experiments, we sought to characterize expression of the arginase isoforms and inducible NOS and to assess the functions of arginase in hyperoxic lung injury. Male Sprague-Dawley rats were exposed to 100% O2 for 60 h. Protein expression of AI and AII and their cellular distribution were determined. The activities of arginase and NOS were also measured. Expression of arginase was correlated with that of ornithine decarboxylase, a biochemical marker for tissue repair, in a separate group of rats allowed to recover in room air for 48 h. We found by Western blot analyses that both AI and AII proteins were upregulated after 60 h of hyperoxic exposure (403 and 88% increases by densitometry, respectively) and, like ornithine decarboxylase, remained elevated during the recovery phase. Arginase activity increased by 37%. Immunostaining showed that increases in AI and AII were mainly in the peribronchial and perivascular connective tissues. NOS activity was unchanged and inducible NOS was not induced, but the level of nitrogen oxides in the lung decreased by 67%. Our study showed in vivo induction of arginase isoforms during hyperoxia. The strong expression of arginase in the connective tissues suggests that the function of pulmonary arginase may be linked to connective tissue elements, e.g., fibroblasts, during lung injury and recovery.

nitric oxide; nitric oxide synthase; ornithine decarboxylase; oxygen

Extrahepatic arginase may be involved in the regulation of cell growth and tissue repair (23). L-Ornithine, the amino acid metabolite of arginase, may be converted to glutamate and Δ-pyrroline-5-carboxylate by a mitochondrial enzyme, ornithine aminotransferase. Through a variety of transamination reactions, glutamate can be converted either to amino acids for protein synthesis or to α-ketoglutarate, which then serves as a substrate for the citric acid cycle. Δ-Pyrroline-5-carboxylate can be metabolized further to L-proline, an amino acid essential for the synthesis of many structural proteins including collagen. L-Ornithine is also a precursor for the synthesis of polyamines (putrescine, spermine, and spermidine), which have been shown to play an important role in cell proliferation and growth (21). The activity of ornithine decarboxylase (ODC), the rate-limiting enzyme of the ornithine-polyamine pathway, parallels the intensity of the repair processes in the injured tissues (16).

Because the substrate of arginase, L-arginine, is also the amino acid precursor for nitric oxide (NO) synthesis by nitric oxide synthase (NOS), it has been suggested that increased arginase activity may decrease NO production by limiting the availability of intracellular L-arginine, especially during high-output NO production associated with upregulation of the inducible form of NOS (iNOS) (9). Because the iNOS pathway is considered proinflammatory and injurious, whereas the arginase pathway may be related to cell proliferation and tissue repair, the regulation of these two pathways must be highly coordinated. This hypothesis was supported by in vitro studies in which differential induction of arginase and iNOS was demonstrated with inflammatory or anti-inflammatory mediators such as interferon-γ, transforming growth factor-β (TGF-β), interleukin (IL)-4, IL-10, and PGE 2 (7, 12, 18, 20, 32). In vivo data in inflammatory tissue injury, however, are lacking.

Carraway et al. (10) have previously shown that AII was the predominant isoform in the normal lung. The expression of AII was inhibited, whereas that of iNOS was upregulated in sepsis induced by cecal-ligation puncture (10). In the present study, we sought to investigate the relationship between arginase and iNOS in lung injury induced by hyperoxia. We chose hyperoxia as the model for the following reasons. 1) The lung pathology of prolonged exposure to 100% O2 in animals and humans is characterized by early infiltration of inflammatory cells followed by proliferation of fibroblasts and alveolar type II cells, indicative of activity of the repair processes (6, 13). Therefore, we expect an
early increase in iNOS activity followed by a late increase in arginase activity. 2) Although adult rats exposed to 100% O₂ uniformly die, they can recover completely if removed to room air by 60 h. The repair processes are associated with increased ODC activity and polyamine contents in the lung (31). Thus ODC can be used as an index marker of tissue repair. 3) A high O₂ concentration minimizes the possibility that an inadequate supply of molecular O₂ limits the biosynthesis of NO.

In preliminary experiments, we found that immunoreactive AII was upregulated 60 h after hyperoxic exposure. The objective of this study was to characterize the induction of arginase isozymes during 100% O₂ exposure. The study design. The animals were handled in accordance with the American Association for the Accreditation of Laboratory Animal Care Guidelines with a protocol approved by the Duke University (Durham, NC) Institutional Review Board and Institutional Animal Care and Use Committee.

Methods and Materials

Hyperoxic Exposure

Specific pathogen-free male rats weighing 250–300 g (Charles River Laboratories, Wilmington, MA) were used for these studies. The animals were handled in accordance with the American Association for the Accreditation of Laboratory Animal Care Guidelines with a protocol approved by the Duke University (Durham, NC) Institutional Review Board and Institutional Animal Care and Use Committee.

The rats were exposed to 100% O₂ in polystyrene chambers for 60 h as previously described (13). Control rats were placed in room air (fraction of inspired O₂ = 0.21) for 60 h. Both groups of rats were allowed access to water and rat chow during exposure. A separate group of rats was removed from the O₂ exposure chamber after 60 h and allowed to recover in room air for 48 h. Rats were killed at 12, 36, and 60 h of O₂ exposure and at 48 h of recovery. The objective of this study was to assess changes in NOS activity and NO production in the presence of arginase upregulation, and to determine the functional significance of arginase upregulation in hyperoxic lung injury.

Measurement of NOx Concentration

The supernatants of lung homogenate were precipitated with 10% TCA and assayed for NOx (nitrite and nitrate) with a modified catalytic method for the reduction of NO oxidation products to NO gas (24). Samples were injected into a refluxing glass reaction chamber containing vanadium (III) chloride (0.1 M) and HCl (2 N) at 85°C. NO was sparged from the chamber and carried in nitrogen gas to a chemiluminescence detector (TEA model 543 analyzer, Thermedics, Woburn, MA). The output from the detector was integrated to determine total peak area with a Hewlett-Packard HP-3395 integrator. Measurements of known concentrations of nitrite
and nitrate were quantitatively linear between 25 and 500 pmol with this method. The NOx concentration was normalized to dry lung weight.

Measurement of Total NOS Activity

Snap-frozen lungs were homogenized in 20 mM Tris buffer, pH 7.4, containing 1 mM EDTA, 5 μM FAD, 5 μM flavin mononucleotide, 5 μM BH4, 1.74 mg/ml of phenylmethylsulfonyl fluoride, 1 μg/μl of leupeptin, and 0.1 μg/μl of soy trypsin inhibitor. The supernatant samples were assayed for total NOS activity by measuring the conversion of [3H]arginine to [3H]citrulline according to methods described by Xue et al. (33). Total NOS activity is expressed as picomoles of [3H]citrulline generated per milligram of protein per minute.

Measurement of Arginase Activity

The arginase activity in lung homogenates was determined with standard procedures for the detection of urea by reaction with diacetyl monoxime (15, 22, 28). Tissues were homogenized in four volumes of storage buffer at 4°C and centrifuged at 15,000 g for 15 min. Ninety microliters of the supernatant were incubated for 5 min at 55°C with 10 μl of MnCl2 (100 mM). Samples were incubated for 10 min at 37°C with L-arginine (0.25 M). The reaction was stopped by adding 1 ml of diacetyl monoxime in concentrated phosphoric acid-sulfuric acid. The samples were then vortexed, sealed, and heated in boiling water for 30 min. The urea concentration in each sample was then measured with a spectrophotometer at 490 nm. Baseline urea concentration in each sample was also measured without incubation with L-arginine. The arginase activity was calculated as (sample urea concentration – baseline urea concentration)/10 and is expressed in units per gram of dry lung. One unit of arginase activity is equivalent to the conversion of 1 µmol urea/min at 37°C.

Statistical Analysis

Data are means ± SE. Statistical analysis was performed with commercially available software (Statview 512-T, Brainpower, Calabasas, CA). Differences between two groups were evaluated with two-tailed Student's t-test. For multiple comparisons, analysis of variance was used followed by Tukey's test for comparison between groups. A P value of ≤0.05 was considered statistically significant.

RESULTS

Physiological Effects of Hyperoxic Exposure and Room Air Recovery

During the 60-h exposure to 100% O2, the animals ate and drank poorly and demonstrated reduced grooming, generalized lassitude, and tachypnea. At 60 h, O2-exposed animals showed an increased lung wet-to-dry weight ratio (5.88 ± 0.12 vs. 4.85 ± 0.46 in room air; P < 0.001) and developed pleural effusions (pleural fluid volume of 7.1 ± 1.3 ml vs. 0 ml in room air; Fig. 1). When the rats were removed from the O2 chamber at 60 h and placed in room air for 48 h, they gradually resumed normal activity. The lung wet-to-dry weight ratio normalized (4.87 ± 0.13), and pleural effusion resolved (0 ml).

Protein Expression of AI and AII

By Western blot analyses, AII was detectable (as a band at 40 kDa) in the lungs exposed to 60 h of room air, consistent with the previous findings of Carraway et al. (10). After exposure to 100% O2 for 60 h, AI expression was increased (Figs. 2 and 3). The increase in AII protein was ~88% (0.843 ± 0.091 densitometry units in O2 vs. 0.449 ± 0.024 densitometry units in room air; P < 0.01). The AI level in the control lung was very low (detectable as a band at 39 kDa). After 60 h of 100% O2 exposure, AI expression increased dramatically (Figs. 2 and 3). The increase was >400% (0.533 ± 0.066 densitometry units in O2 vs. 0.106 ± 0.001 densitometry units in room air; P < 0.001). The protein expression of both AI and AII remained elevated in the lungs during recovery in room air (Fig. 3).

Total Arginase Activity

Arginase activity was determined in lung tissues obtained from rats exposed to 60 h of room air and 60 h
of hyperoxia. Total arginase activity increased by 37% after 60 h of hyperoxia (13.88 ± 0.34 U/g dry weight for O2 vs. 10.11 ± 0.68 U/g dry weight for air; Fig. 2). The in vitro arginase activity assay, however, may overestimate the in vivo arginase activity because natural arginase inhibitors, e.g., N ω-hydroxy-L-arginine, are “diluted” during the process of lung homogenization. Thus increases in arginase activity in vivo during hyperoxia might be >37% because the hyperoxic lungs had lower NOx and, presumably, lower N ω-hydroxy-L-arginine than the room air lungs (see NOx Concentration).

Cellular Distributions of AI and AII During Hyperoxic Exposure

The distributions of AI and AII in the lungs at 60 h of room air and hyperoxic exposure are summarized in Table 1. By immunocytochemistry, the main changes in AI and AII immunostaining with hyperoxia were noted in the interstitial connective tissues (Fig. 4). Increases in AI were both peribronchial and perivascular, whereas increases in AII were more localized to the peribronchial regions. Alveolar epithelial cells and macrophages contained AI normally (10), and the expression of AI did not change appreciably with hyperoxic exposure. Bronchial epithelial cells also expressed low levels of AI and AII constitutively (Fig. 4). The AI staining increased, but AII staining did not change appreciably during hyperoxic exposure. The endothelial cells of the pulmonary arterioles and venules also had both AI and AII staining, which was unchanged with hyperoxic exposure.

Total NOS Activity and iNOS Expression

Total activity of NOS in the control lung was low. The control lung converted [3H]arginine to [3H]citrulline at a rate of 0.16 ± 0.01 pmol·min⁻¹·mg protein⁻¹. Total NOS activity remained low after 60 h of hyperoxic exposure (0.18 ± 0.01 pmol·min⁻¹·mg protein⁻¹; Fig. 5). We observed no iNOS expression at 12, 36, or 60 h during hyperoxic exposure, suggesting that the NOS activity was most likely due to the constitutive isoforms of NOS.

NOx Concentration

NOx concentrations were measured in the lung homogenates obtained from rats exposed to 60 h of room air and 60 h of hyperoxia. The average concentration of NOx in the lung homogenates was lower in hyperoxia (0.29 ± 0.02 nM/g dry lung compared with 0.81 ± 0.18 nM/g dry lung during normoxia; P < 0.001). This is shown in Fig. 5.

Protein Expression of ODC

ODC was present at a low concentration in the lungs exposed to room air (detectable as a band at 55 kDa). The protein expression of ODC was upregulated at 60 h after 100% O2 exposure and remained elevated during the recovery phase (Fig. 6).

DISCUSSION

Our study showed that both isoforms of arginase (AI and AII) in the lung were induced along with ODC during 100% O2 exposure and recovery. By immunocytochemistry, the induction of AI and AII was noted mainly in the peribronchial and perivascular connective tissues. The increase in total arginase activity, however, was less than the combined increase in immunoreactive AI and AII, suggesting that the induced AI and AII proteins were not fully functional at 60 h of hyperoxic exposure. The induction of arginase was associated with a lower NO concentration in the lung homogenates despite no change in NOS activity.

Carraway et al. (10) previously showed that AI was the predominant arginase isoform in the normal rat lung. The constitutive expression of AI in the lung was downregulated in sepsis induced by cecal-ligation puncture (10). In the present study, we extended those findings to lungs exposed to hyperoxia. We found that hyperoxia induced expression of both arginase isoforms, especially AI. The induction of AI could not be attributed to red blood cell accumulation in lung injury because rat red blood cells contained no detectable arginase activity (1, 30). The timing of the upregulation of arginase corresponded to the fibroproliferative phase of hyperoxic lung injury (6, 13, 31). Increased arginase activity was observed during the healing of skin wounds,
although specific cell types responsible for the increase were not identified (2–4). Increases in arginase activity were found in the mesangial cells in experimental glomerulonephritis (11, 25). In these models as well as in ours, increased activity of arginase could provide more L-proline via ornithine aminotransferase to support the biosynthesis of collagen, an important element of the tissue matrix. The putative repair function of arginase is further supported by our findings that the increased arginase expression persisted into the recovery phase of hyperoxic lung injury, and the induction patterns of arginase were similar to those of ODC. Whether AI and AII have different functions in hyperoxic lung injury is unclear. Because the lung lacks a complete urea cycle, one function of AI, which is a cytosolic isoform, might be to provide L-ornithine as a substrate for ODC, also a cytosolic enzyme. On the other hand, AII is a mitochondrial enzyme. L-Ornithine produced by AII might serve as a substrate for other ornithine-metabolizing enzymes such as ornithine ami-

Fig. 4. Immunostaining of AI (A and B) and AII (C and D) in lung exposed to 60 h of room air (A and C) and 100% O₂ (B and D). E: nonimmune serum control. Magnification ×220.
The regulation of arginase expression during hyperoxia.

\[ \text{Enzyme activity} = \frac{\text{conversion of L-arginine to L-citrulline}}{\text{reaction time}} \]

B or other transcription factors in nuclear factor-κB (NF-κB) signaling, e.g., oxidant-sensitive transcription factors. The time course of TGF-β mRNA induction makes TGF-β a candidate mediator of lung arginase also may be mediated by oxidant stress of hyperoxia, upregulation of lung arginase may require the use of specific inhibitors against AI and AII, which are currently unavailable.

The regulatory mechanisms for the induction of lung arginase during hyperoxia are unknown. Of the mediators known to stimulate arginase activity, such as TGF-β, cAMP, PGF2, IL-4, and IL-10 (7, 12, 18), TGF-β has been studied more extensively during O₂ toxicity. Rat lungs exposed to 48 h of 100% O₂ and 24 h of recovery show dramatic increases in TGF-β peptide activity in the lavage fluid (8). In mouse lungs exposed to hyperoxia, TGF-β1 mRNA in bronchial epithelial cells is upregulated within 3 h of O₂ exposure and continues to increase for 48 h before decreasing to control levels by 72 h (27). The time course of TGF-β mRNA induction makes TGF-β a candidate mediator for the induction of arginase during hyperoxia. Because of the increased oxidative stress of hyperoxia, upregulation of lung arginase also may be mediated by oxidant signaling, e.g., oxidant-sensitive transcription factors. In RAW cells, lipopolysaccharide-induced arginase enzyme activity is not blocked by an inhibitor of nuclear factor-κB (NF-κB) activation, pyrrolidine dithiocarbamate (32); however, such studies do not definitely exclude a role for nuclear factor-κB or other transcription factors in the regulation of arginase expression during hyperoxia.

We also observed that hyperoxia was associated with lower NOₓ in the lung. Because total NOS activity was unchanged and the essential cosubstrate L-arginine should be adequate during 100% O₂ exposure, the decrease in NOₓ might be attributable to a limitation in intracellular L-arginine. This mechanism would be plausible in the presence of increased activity of arginase, although arginase activity increased by only 37%, whereas NO concentration decreased by 64%. The size of the intracellular L-arginine pool available to NOS in injured lungs cannot be determined until all the arginine-dependent pathways are quantified, including the arginine transporters (systems y⁻ and B₀⁺) on the plasma membrane (17). The effects of hyperoxia on arginase transport are also unknown. Limitations of cofactors for NOS, e.g., tetrahydrobiopterin and NADPH, also are possible; however, it is not clear why O₂ would deplete these cofactors. We also cannot exclude the possibility that the activity of constitutive NOS isoforms (endothelial cell NOS and neuronal NOS) may have been affected differentially by hyperoxia because we measured total NOS activity in lung homogenates. Recently, hyperoxia was shown to decrease endothelial cell NOS activity in mouse lungs but to slightly increase nitrite/nitrate concentration in bronchoalveolar lavage fluid (5). The discrepancy between these results and ours is unclear but may be related to how NO activity and NO concentration was measured. Because the lung contains a variety of cell types, small local changes in NOS activity and NO concentration may not be reflected in the whole lung measurements.

Differential activation of arginase and NOS pathways in cells could have important implications in tissue responses to inflammation and injury. In a model of inflammatory wound healing, an early increase in iNOS activity is followed by enhanced arginase activity after 3 days of healing (3, 4). Enhanced arginase activity was associated with a decreased arginine level in the wound fluid. In a rat model of glomerulonephritis, sequential activation of NOS and arginase was noted, with the highest NOS activity on day 1 and the highest arginase activity on day 4 (11, 25). The glomerular macrophages were a major source of NOS activity, whereas the glomerular mesangial cells contained high arginase activity. In our study, increased arginase expression was seen mainly in the peribronchial and perivascular connective tissues, including fibroblasts, during hyperoxic exposure. Because fibroblasts usually contain little NOS activity, a lower NO concentration in the lung homogenates must have been the result of decreased NO production by other cell types.

In summary, our study demonstrated in vivo induction of both the cytosolic and mitochondrial isoforms of arginase along with ODC during hyperoxic lung injury and recovery. By immunocytochemistry, the induction was mainly in the peribronchial and perivascular connective tissues, including fibroblasts, during hyperoxic exposure. Because fibroblasts usually contain little NOS activity, a lower NO concentration in the lung homogenates must have been the result of decreased NO production by other cell types.

As an example, the graph shows the nitric oxide (NO) concentration in the lung during hyperoxic exposure. Animals exposed to 60 h of room air served as controls; n = 4–6 animals/group. *P < 0.001.
lungs may be linked to connective tissue elements, e.g., fibroblasts, during acute lung injury and repair.

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Address for reprint requests: Y.-C., T. Huang, PO Box 31206, Duke Univ. Medical Center, Durham, NC 27710.

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