Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia

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1Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan; and 2Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Hoshikawa, Yasushi, Sadafumi Ono, Satoshi Suzuki, Tatsuo Tanita, Masayuki Chida, Chun Song, Masafumi Noda, Toshiharu Tabata, Norbert F. Voelkel, and Shigefumi Fujimura. Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia. J Appl Physiol 90: 1299–1306, 2001.—Chronic hypoxia causes pulmonary hypertension and right ventricular hypertrophy associated with pulmonary vascular remodeling. Because hypoxia might promote generation of oxidative stress in vivo, we hypothesized that oxidative stress may play a role in the hypoxia-induced cardiopulmonary changes and examined the effect of treatment with the antioxidant N-acetylcysteine (NAC) in rats. NAC reduced hypoxia-induced cardiopulmonary alterations at 3 wk of hypoxia. Lung phosphatidylcholine hydroperoxide (PCOOH) increased at days 1 and 7 of the hypoxic exposure, and NAC attenuated the increase in lung PCOOH. Lung xanthine oxidase (XO) activity was elevated from day 1 through day 21, especially during the initial 3 days of the hypoxic exposure. The XO inhibitor allopurinol significantly inhibited the hypoxia-induced increase in lung PCOOH and pulmonary hypertension, and allopurinol treatment only for the initial 3 days also reduced the hypoxia-induced right ventricular hypertrophy and pulmonary vascular thickening. These results suggest that oxidative stress produced by activated XO in the induction phase of hypoxic exposure contributes to the development of chronic hypoxic pulmonary hypertension.

N-acetylcysteine; phosphatidylcholine hydroperoxide; xanthine oxidase; allopurinol; pulmonary vascular remodeling

CHRONIC HYPOXIA CAUSES PULMONARY hypertension and right ventricular hypertrophy associated with pulmonary vascular remodeling in humans (13) and in various animal species (25, 26, 28). These pathophysiological aspects are regarded as important factors that may determine the outcome in patients with various respiratory diseases presenting with chronic hypoxemia (6, 11). Hypoxia may generate oxidative stress as suggested by in vitro (2) and in vivo (4) studies. Hypoxic exposure has also been shown to increase production of platelet-activating factor (PAF) in vivo (3, 24). Because reactive oxygen species stimulate the synthesis of PAF by pulmonary arterial endothelium (16), and, because PAF induces the oxidative burst in macrophages (10) and plays a role in the development of pulmonary vascular remodeling induced by hypoxia (22), we hypothesized that oxidative stress might contribute to pulmonary hypertension and vascular remodeling induced by chronic hypoxia.

To test this hypothesis, we first examined the effect of an antioxidant agent, N-acetylcysteine (NAC) (19, 27), on the development of pulmonary hypertension, right ventricular hypertrophy, and pulmonary arterial wall thickening in rats after 3 wk of normobaric hypoxia exposure. We then measured lung phosphatidylcholine hydroperoxide (PCOOH) of the hypoxia-exposed rats as a marker of oxidative stress of the lung tissue.

The xanthine oxidase (XO)-hypoxanthine system has been known to be one of the important pathways to generate oxidative stress in vivo (18), and XO, the final enzyme of purine catabolism, can be activated by hypoxia in cultured pulmonary arterial endothelial cells (23). However, it is not known whether the lung XO activity undergoes a change during hypoxic exposure in vivo and whether XO plays a role in the hypoxia-induced cardiopulmonary changes. We investigated the changes in lung XO activity of hypoxia-exposed rats and the effect of the XO inhibitor allopurinol on lung PCOOH levels and hypoxic pulmonary hypertension.

METHODS

Animals and hypoxic exposure. Male Sprague-Dawley rats (Funabashi Farm, Sendai, Japan) weighing 175–335 g were used in this study. Rats were exposed to hypoxia in a normobaric hypoxic chamber (60 × 70 × 50 cm). The hypoxic environment was maintained by 5.5 l/min flow of hypoxic air (10% oxygen). This low-oxygen-containing air was produced by a hypoxic gas generator (Teijin, Sendai, Japan) utilizing exhaust air from an absorption-type oxygen concentrator.
At 55 breaths/min with 8 cmH2O

by use of a small animal respirator (Model SN-480-7, a tracheal cannula (15-gauge Luer stub adapter, Clay Adams). The rats received water or 1% NAC solution ad libitum. Twenty-four hours after the initiation of hypoxic exposure, the rats were killed and their lungs were collected as described below. Fourteen rats were maintained in room air (normoxia), and their lungs were collected on days 1 (n = 6), 7 (n = 4), and 21 (n = 4) of the study.

At the day of lung sampling, the rats were anesthetized with pentobarbital sodium (100 mg/kg ip), the chest was opened via a median sternotomy, and the lungs were removed en bloc and quickly frozen in liquid nitrogen. All frozen tissues were stored at −80°C until extraction of total lipid.

Total lipid was extracted from the rat lung by a modification of the method of Miyazawa et al. (20, 21), using a mixture of chloroform and methanol (2:1 vol/vol). Two milliliters of 0.15 M NaCl containing 0.002% butylated hydroxytoluene (BHT) were added as an antioxidant to 500 mg of lung tissue, and the mixture was homogenized in a glass-glass homogenizer (7700 Homogi, Pyrex, Iwaki Glass, Funabashi, Japan). The homogenate was added to 4.5 ml of chloroform-methanol (2:1 vol/vol) and the mixture was centrifuged at 3,000 rpm for 10 min at 4°C. The lower chloroform layer containing lung total lipid was collected. The extraction was repeated three times. After dehydration with anhydrous sodium sulfate, the chloroform layer was concentrated in a rotary evaporator and dried under a nitrogen stream. The lung total lipid obtained was diluted with 100 μl of chloroform-methanol (2:1 vol/vol), and a 30-μl portion was subjected to the CL-HPLC assay. The CL-HPLC system consists of normal-phase HPLC and a hydroperoxide-specific chemiluminescence detector. The HPLC column (JASCO Fine pak SIL NH2–5) was placed in a column oven (860-CO, JASCO, Tokyo, Japan; 40°C). The column mobile phase was isopropanol-methanol-distilled water (26:9:5, vol/vol/vol) and the flow rate was 1.1 ml/min using a JASCO 880-PU pump. The column eluate was mixed with a chemiluminescence reagent at a postcolumn mixing joint. The chemiluminescence reagent was prepared by dissolving 7 μg/ml of cytochrome c (from horse heart, type VI, Sigma Chemical, St. Louis, MO) and 2 μg/ml of luminol (3-aminophthalhydrazide, Wako Pure Chemical, Tokyo, Japan) in 50 mM borate buffer (pH 10.0). The flow rate of the chemiluminescence reagent was 1.0 ml/min (880-PU pump, JASCO). The generated chemiluminescence was measured by a chemiluminescence detector (CLD-100, Tohoku Electronic, Sendai, Japan) and quantified by an integrator (Sie Chromatocorder 12, JASCO). A calibration curve was prepared by using authentic PCOOH produced by photooxidation of egg yolk phosphatidylcholine that had previously been purified. The concentration of hydroperoxide was expressed as picomoles of hydroperoxide-oxygen. For the assessment of oxidative stress in the lung tissue, PCOOH levels were measured by using a modification of the chemiluminescence-high-performance liquid chromatography (CL-HPLC) method of Miyazawa et al. (20, 21) described below.

Thirty-eight rats were divided into eight groups. Twenty-four animals were exposed to hypoxia [inspired oxygen fraction (FIo2) = 0.1]. At 1 (n = 6), 3 (n = 6), 7 (n = 4), 14 (n = 4), and 21 (n = 4) days after the initiation of hypoxic exposure, the rats were killed and their lungs were collected as described below. Fourteen rats were maintained in room air (normoxia), and their lungs were collected on days 1 (n = 6), 7 (n = 4), and 21 (n = 4) of the study.

The lungs were opened via a median sternotomy, and the lungs were removed en bloc and quickly frozen in liquid nitrogen. All frozen tissues were stored at −80°C until extraction of total lipid.

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initiation of the treatment, the rats in the six treatment groups were kept in normobaric hypoxia ($F_{I\text{O}_2} = 0.1$) with water or NAC continued ad libitum. At 1 (water, $n = 6$; NAC, $n = 6$), 7 (water, $n = 4$; NAC, $n = 3$), and 21 days (water, $n = 4$; NAC, $n = 4$) of hypoxic exposure, lungs were isolated and subjected to the measurement of PCOOH levels as described in *Measurement of lung PCOOH levels*.

Assay of activities of XO and xanthine dehydrogenase in the lung tissues. XO and xanthine dehydrogenase (XD) activities were assayed by the fluorometric method of Akaike et al. (1) described below.

Twenty-four rats were divided into six groups. Eighteen animals were exposed to hypoxia ($F_{I\text{O}_2} = 0.1$). At 1 (water, $n = 6$), 3 ($n = 3$), 7 ($n = 3$), 14 ($n = 3$), and 21 ($n = 3$) days after the initiation of hypoxic exposure, they were killed and their lungs were collected as described above. Six rats were maintained in room air, and their lungs were collected (normoxia).

At the day of lung sampling, the rats were anesthetized with pentobarbital sodium (100 mg/kg ip) and ventilated with room air via a tracheal cannula (15G Luer stub adapter, Clauren, Inc.) using a small animal respirator (model SN-480–7, Shinano) at 55 breaths/min with 8 cmH$_2$O inspiratory pressure and 3 cmH$_2$O positive end-expiratory pressure. Next, a midsternotomy was performed, 100 units of heparin were injected into the right ventricle, and the pulmonary artery was cannulated through an incision in the right ventricle and perfused with 0.01 M phosphate-buffered 0.15 M NaCl (PBS; pH 7.4, 37°C, 20 cmH$_2$O pressure), containing 2 mM $p$-aminophenylmethylanesulfonyl fluoride (APMSF; Wako Pure Chemical) and 10 mM dithiothreitol (DTT; Wako Pure Chemical) to wash out residual blood, allowing the fluid to drain through an incision in the left ventricle. Then the left lung was removed, quickly frozen in liquid nitrogen, and stored at −80°C until sample treatment.

The left lung was homogenized (Polytron homogenizer; Kinematica GmbH, Lucerne, Switzerland) with four volumes of ice-cold 50 mM sodium phosphate buffer pH 7.6 containing 0.1 mM EDTA (Wako Pure Chemical), 1 mM APMSF, 1 mM DTT, 10 μg/ml leupeptin (Wako Pure Chemical), 10 μg/ml trypsin inhibitor (Type 1-S from soybean, Sigma Chemical) and 0.32 M sucrose (Wako Pure Chemical). The homogenates were centrifuged at 10,000 g for 30 min at 4°C, and the supernatants were dialyzed for 18 h against 5 liters of PBS at 0°C to remove the low molecular-weight compounds before determination of XO enzyme activity.

Samples were assayed for their XO activity using pterin (Sigma Chemical) as the substrate in a spectrofluorometer (model 650–40; Hitachi, Tokyo, Japan) with excitation at 345 nm and emission at 390 nm. The volume of the assay mixture was 1.0 ml in 50 mM sodium phosphate buffer pH 7.6 containing 0.1 mM EDTA (Wako Pure Chemical), 2 mM APMSF, 1 mM DTT, 10 μg/ml leupeptin (Wako Pure Chemical), 10 μg/ml trypsin inhibitor (Type 1-S from soybean, Sigma Chemical) and 0.32 M sucrose (Wako Pure Chemical). The homogenates were centrifuged at 10,000 g for 30 min at 4°C, and the supernatants were dialyzed for 18 h against 5 liters of PBS at 4°C to remove the low molecular-weight compounds before determination of XO enzyme activity.

Effects of allopurinol treatment on lung PCOOH levels. Twenty-six rats were divided into five groups. The normoxia group was kept under normoxic conditions ($n = 6$). Two groups received water, and the other two groups received allopurinol (50 mg/kg) via gavage every 12 h. Two hours after the second treatment, the rats in the four treatment groups were kept in normobaric hypoxia ($F_{I\text{O}_2} = 0.1$). Water or allopurinol was continued as described above. At 1 (water, $n = 6$; allopurinol, $n = 6$) and 7 days (water, $n = 4$; allopurinol, $n = 4$) of hypoxic exposure, lungs were removed and subjected to measurement of PCOOH levels as described in *Measurement of lung PCOOH levels*.

Effects of allopurinol treatment on pulmonary hypertension, right ventricular hypertrophy, and pulmonary artery media wall thickening induced by chronic hypoxia. Twenty-five rats were divided into two groups: housed in normoxia ($n = 12$) or exposed to hypoxia ($n = 13$). Each group was again divided into two subgroups for treatment with water (normoxia, $n = 6$; hypoxia, $n = 7$) or allopurinol (50 mg/kg; normoxia, $n = 6$; hypoxia, $n = 6$) via gavage every 12 h. Two hours after the second treatment, they were housed for 3 wk under normoxic conditions or in normobaric hypoxia (10% oxygen) with water or allopurinol continued as described above.

At 3 wk after normoxic or hypoxic exposure, mean pulmonary arterial pressure, RV/(LV + S), hematocrit, and pulmonary arterial media wall thickness were measured as described in *Effects of NAC treatment on pulmonary hypertension, right ventricular hypertrophy, and pulmonary artery media wall thickness induced by chronic hypoxia*.

Effects of allopurinol treatment during the initial 3 days of hypoxic exposure on right ventricular hypertrophy and pulmonary artery media thickening. Twenty-four rats were divided into four groups as in the previous allopurinol study. The rats received water or allopurinol (50 mg/kg) via gavage every 12 h. Two hours after the second treatment, they were housed for 3 wk under normoxic conditions or in normobaric hypoxia (10% oxygen) with water or allopurinol administration continued as described above only during the initial 3 days.

At 3 wk after normoxic or hypoxic exposure, RV/(LV + S) and pulmonary arterial media wall thickness were measured as described in *Effects of NAC treatment on pulmonary hypertension, right ventricular hypertrophy, and pulmonary artery media wall thickness induced by chronic hypoxia*.

**RESULTS**

Effects of allopurinol treatment on pulmonary hypertension, right ventricular hypertrophy, and pulmonary arterial media wall thickness induced by chronic hypoxia. Three weeks of hypoxic exposure caused pulmonary hypertension, right ventricular hypertrophy, and polycythemia in rats. Chronic treatment with NAC significantly reduced pulmonary hypertension and right ventricular hypertrophy induced by chronic hypoxia but had no effect on the hematocrit in normoxic or chronically hypoxic rats. Mean pulmonary arterial pressure and RV/(LV + S) in the NAC-treated normoxic rats were not different from those in the water-treated normoxic rats (Table 1).

The lung histology of chronically hypoxia-exposed rats showed an increase in pulmonary arterial media thickness (Fig. 1, A and B). In NAC-treated hypoxic rats, the thickening of pulmonary arterial media was less than that in water-treated hypoxic rats (Fig. 1C). The statistical analysis of lung morphology also revealed that chronic hypoxic exposure induced an in-
hypoxic exposure, NAC treatment reduced the increase in PCOOH levels in the NAC-treated rat lungs tended to be lower than those in the water-treated rat lungs (hypoxia + NAC, 123.4 ± 6.2 pmol/g; hypoxia + water, 231.2 ± 65.4 pmol/g) (Fig. 3).

Assay of activities of XO and XD in the lung tissues. XO activities in the hypoxia-exposed rat lungs were elevated at day 1 of hypoxic exposure (0.458 ± 0.024 nmol isoxanthopterin·min⁻¹·ml⁻¹ of lung homogenate), reached a maximum at day 3 (0.532 ± 0.049), and were maintained at higher levels compared with those in the normoxic rat lungs (0.339 ± 0.019) from the 7th to the 21st day (hypoxia 7 days, 0.433 ± 0.032; 14 days, 0.459 ± 0.006; 21 days, 0.480 ± 0.045) (Fig. 4A). XD activities in the lung tissue began to increase at 3 days after the initiation of hypoxic exposure (normoxia, 1.111 ± 0.051; hypoxia 3 days, 1.368 ± 0.025 nmol isoxanthopterin·min⁻¹·ml⁻¹ of lung homogenate) and then tended to increase more gradually until day 21 (Hypoxia 7 days, 1.418 ± 0.017; 14 days, 1.533 ± 0.089; 21 days, 1.662 ± 0.050) (Fig. 4B). The time course of XO + XD total activities in the lung tissue was similar to that of XD activities (Fig. 4C). The XO-to-XD ratio increased only at day 1 of the hypoxic exposure (normoxia, 0.309 ± 0.022; hypoxia 1 day, 0.401 ± 0.034) (Fig. 4D).

Fig. 1. Muscular pulmonary artery in the rat lung of normoxia + water group (A), in the lung from a rat exposed to normobaric hypoxia (B), and in the lung from a hypoxic rat treated with N-acetylcysteine (NAC) (C) at 3 wk of normoxic or hypoxic exposure. The insides of the pulmonary arteries are filled with barium-gelatin mixture. Note the marked increase in the media wall thickness of the water-treated chronically hypoxic rat and significantly less media wall thickness of the NAC-treated hypoxic rat lung when compared with that of the water-treated hypoxic rat. Elastica-Masson’s stain was used.
Effects of allopurinol treatment on lung PCOOH levels. At days 1 and 7 of hypoxic exposure, treatment with allopurinol reduced the PCOOH levels in the hypoxia-exposed rat lungs (hypoxia 1 day + water vs. hypoxia 1 day + allopurinol: 226.9 ± 20.4 vs. 145.9 ± 7.6 pmol/g, P < 0.05; hypoxia 7 days + water vs. hypoxia 7 days + allopurinol: 293.6 ± 52.1 vs. 134.3 ± 6.1 pmol/g, P < 0.05) (Fig. 5).

Effects of allopurinol treatment on pulmonary hypertension, right ventricular hypertrophy, and pulmonary artery media wall thickness. Three-week hypoxic exposure also caused pulmonary hypertension, right ventricular hypertrophy, and polycythemia when the rats received water via gavage twice a day. However, oral administration of allopurinol significantly decreased hypoxia-induced pulmonary hypertension and right ventricular hypertrophy, whereas allopurinol itself had no effect on these parameters in the normoxic rats. Treatment with allopurinol did not affect the hematocrit in normoxic and chronically hypoxic rats (Table 3).
The lung histology of rats revealed that treatment with allopurinol for 3 wk reduced the hypoxia-induced increase in the media thickness of the pulmonary arteries in the size range of 50–200 μm in external diameter (Table 4).

**Effects of allopurinol treatment during the initial 3 days of hypoxic exposure on hypoxia-induced right ventricular hypertrophy and pulmonary artery media thickening.** Allopurinol treatment during the first 3 days also significantly inhibited hypoxia-induced right ventricular hypertrophy (normoxia + water, 0.23 ± 0.01; normoxia + allopurinol, 0.24 ± 0.01; hypoxia + water, 0.41 ± 0.01; hypoxia + allopurinol, 0.33 ± 0.01) (Fig. 6A) and media wall thickening of the pulmonary arterioles in the size range of 50–200 μm external diameter (Fig. 6B).

**DISCUSSION**

The principal findings of our study are as follows: 1) the antioxidant NAC reduced pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular media thickening caused by 3 wk of normobaric hypoxic exposure; 2) lung tissue levels of PCOOH, a primary peroxidation product of phosphatidylcholine, increased from day 1 to day 7 of hypoxic exposure (and reached a maximum at day 7); 3) treatment with NAC inhibited the increase in PCOOH levels in the lungs from the hypoxia-exposed rats; 4) XO activity in the rat lungs was elevated from day 1 through day 21 of the hypoxic exposure (the maximal values occurred at the third day); 5) treatment with a XO inhibitor, allopurinol, reduced the increase in PCOOH levels in hypoxia-exposed rat lungs and attenuated pulmonary hypertension, right ventricular hypertrophy, and pulmonary vascular media thickening in the rats exposed to hypoxia for 3 wk; and 6) allopurinol treatment only for the first 3 days of a 3-wk hypoxic exposure period inhibited hypoxia-induced right ventricular hypertrophy and pulmonary vascular media thickening. Taken together, these findings suggest that generation of oxidative stress contributes to the development of pulmonary hypertension and pulmonary vascular thickening induced by chronic hypoxia and that lung XO activation during the early phase of chronic hypoxic exposure is involved in the production of reactive oxygen species.

Hypoxia can cause an increase in rat plasma glutathione disulfide level in vivo (4) and increased formation of lipid peroxidative products in cultured bovine pulmonary endothelial cells in vitro (2), suggesting that hypoxic exposure may promote the generation of oxidative stress in vivo. Hypoxic exposure has also been shown to increase the production of PAF in plasma (3) or in bronchoalveolar lavage fluid (24), and reactive oxygen species stimulate the synthesis of PAF by bovine pulmonary arterial endothelium (16). PAF induces the oxidative burst in macrophages (10) and plays a role in the development of pulmonary vascular remodeling induced by hypoxia (22). It is of interest that hypoxia induces generation of intracellular free radicals (7) and causes pulmonary artery remodeling and pulmonary hypertension (14, 15). On the basis of these observations, we hypothesized that oxidative stress might contribute to hypoxia-induced pulmonary hypertension and vascular remodeling.

We found that chronic administration of NAC reduced the hypoxia-induced cardiopulmonary alterations and that treatment with NAC had no effect on the hypoxia-related hemoconcentration, which suggests that the reduction of pulmonary hypertension by NAC was not due simply to an alteration of blood viscosity.

The effect of NAC administration on pulmonary arterial pressure was less than that on pulmonary arterial media thickening. The reason for that is unclear. The pulmonary vascular remodeling associated with development of pulmonary hypertension is histologically characterized not only by increased wall thickness of the muscular pulmonary arteries but also by abnormal extension of muscle into peripheral arteries, where it is not normally present, and reduction in the arterial tissue density (25). We speculate that one reason for the smaller effect of NAC treatment on the development of pulmonary hypertension could be a smaller reduction of pulmonary hypertension and vascular remodeling.

**Table 3. Effects of allopurinol on hypoxia-induced pulmonary hypertension, right ventricular hypertrophy, and hematocrit at 3 wk of hypoxic exposure**

<table>
<thead>
<tr>
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<th>mPAP, mmHg</th>
<th>RV/(LV + S)</th>
<th>Hct, %</th>
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<tbody>
<tr>
<td>Normoxia + water</td>
<td>16.3 ± 1.0(4)</td>
<td>0.22 ± 0.01(6)</td>
<td>39.6 ± 1.0(6)</td>
</tr>
<tr>
<td>Normoxia + allopurinol</td>
<td>15.3 ± 0.6(4)</td>
<td>0.22 ± 0.00(6)</td>
<td>38.9 ± 1.1(6)</td>
</tr>
<tr>
<td>Hypoxia + water</td>
<td>29.3 ± 7.9(3)</td>
<td>0.41 ± 0.04(7)</td>
<td>55.7 ± 1.9(7)</td>
</tr>
<tr>
<td>Hypoxia + allopurinol</td>
<td>21.8 ± 0.9(3)</td>
<td>0.30 ± 0.01(6)</td>
<td>51.8 ± 3.0(6)</td>
</tr>
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Values are means ± SE. *P < 0.05 vs. normoxia + water values. †P < 0.05 vs. hypoxia + water values. No. of rats is indicated in parentheses.
effect of NAC on muscularization of nonmuscular peripheral arteries.

Because the treatment with NAC was effective, we hypothesized that hypoxic exposure might cause generation of oxidative stress in the lung tissue. Indeed, when we measured PCOOH in the hypoxia-exposed rat lungs using the CL-HPLC assay (20, 21), we found that the lung PCOOH levels increased from the first day through the seventh day of hypoxic exposure. This indicates that hypoxia induces oxidative stress in the lung tissue during early hypoxic exposure.

The lung PCOOH levels of NAC-treated hypoxic rats were significantly lower than those of water-treated hypoxic rats on day 1, and they were maintained at a low level at the 7th and 21st days of the hypoxic exposure. The fact that NAC reduced both the hypoxia-induced increase in PCOOH levels and the development of pulmonary hypertension indicates that the generation of oxidative stress may contribute to the hypoxia-induced pulmonary hypertension.

In this study, we also measured lung tissue XO, which is the final purine catabolizing enzyme contributing to oxidative stress in vivo (8, 18), by catalyzing the oxidation of hypoxanthine to xanthine and xanthine to uric acid and generating the superoxide anion (18). Superoxide anion is metabolized to H$_2$O$_2$ by disproportionation (17) and promotes lipid peroxidation in the presence of transitional metals (9). Elevation of XO activity has been reported in bovine pulmonary artery endothelial cells exposed to hypoxia (23). Cultures of bovine pulmonary artery endothelial cells accumulate a significant amount of hypoxanthine in their medium, a substrate for XO, when exposed to hypoxia (12). Thus XO activation in the hypoxia-exposed lung might generate oxidative stress. We found indeed that lung tissue XO activity increased at 1 day after the initiation of the hypoxic exposure, then reached a maximum at 3 days, and then tended to decline.

Because XO can be reversibly converted to XO through oxidation of sulfhydryl groups or irreversibly through proteolysis (5), we measured both XO and XD activities. The lung XO activity and the combined XO plus XD activities of hypoxic rats were significantly higher than those of normoxic rats at third day of the hypoxic exposure and tended to increase gradually from 3rd to 21st day. However, the XO-to-XD ratio increased only at 1 day of the hypoxic exposure, suggesting that hypoxia activates XO in the lung tissue by inducing the conversion of XD to XO early during hypoxia exposure. Subsequently, total XO and XD activities are increased.

Because hypoxic exposure caused lung XO activation, we investigated the effect of allopurinol, a competitive inhibitor of XO, on the generation of oxidative stress and the development of cardiopulmonary changes. Allopurinol significantly inhibited both the increase in lung

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Table 4. Effect of allopurinol treatment on hypoxia-induced pulmonary arterial media wall thickening

<table>
<thead>
<tr>
<th>Condition</th>
<th>External diameter (μm)</th>
<th>Media Wall Thickness, %</th>
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<tbody>
<tr>
<td></td>
<td>50–100 μm</td>
<td>100–150 μm</td>
</tr>
<tr>
<td>Normoxia + water</td>
<td>2.80 ± 0.12</td>
<td>2.38 ± 0.10</td>
</tr>
<tr>
<td>Normoxia + allopurinol (n = 6)</td>
<td>2.74 ± 0.07</td>
<td>2.44 ± 0.09</td>
</tr>
<tr>
<td>Hypoxia + water (n = 7)</td>
<td>5.78 ± 0.15*</td>
<td>4.85 ± 0.13*</td>
</tr>
<tr>
<td>Hypoxia + allopurinol (n = 6)</td>
<td>2.94 ± 0.10†</td>
<td>2.52 ± 0.11†</td>
</tr>
</tbody>
</table>

Values are shown as mean ± SE; n, no. of rats. *P < 0.05 vs. normoxia + water values; †P < 0.05 vs. hypoxia + water values.

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Fig. 6. Effect of allopurinol treatment for initial 3 days of hypoxic exposure on hypoxia-induced right ventricular hypertrophy (A) and pulmonary artery media wall thickening (B) at 3 wk of hypoxic exposure. Ratio of right ventricular free wall weight divided by left ventricle plus septum weight [(RV/LV + S)/A] and pulmonary artery media wall thickness (%WT; B) are shown. Number of animals is indicated in bars (A). *P < 0.05 different from normoxia + water values; †P < 0.05 different from hypoxia + water values. A: under normoxic conditions, water-treated and first 3-day allopurinol-treated animals did not differ in (RV/LV + S) (normoxia + water vs. normoxia + allopurinol, 0.23 vs. 0.24, not significant), whereas, after chronic hypoxia, (RV/LV + S) of 3-day-allopurinol-treated rats was significantly lower than that of water-treated rats (hypoxia + water vs. hypoxia + allopurinol, 0.41 vs. 0.33, P < 0.05). B: allopurinol treatment for the initial 3 days did not affect %WT of the normoxic rats but significantly reduced the pulmonary arterial wall thickening caused by exposure to chronic hypoxia in size ranges of 50–100, 100–150, and 150–200 μm external diameter.
PCOOH levels and the cardiopulmonary alterations induced by chronic hypoxic exposure. This indicates that XO under hypoxic conditions generates oxidative stress, leading to the development of pulmonary vascular thickening and hypertension, and further that there are apparently two phases during chronic hypoxia. The first induction phase, up to day 7, is characterized by XO activation and generation of oxidative stress, whereas the second phase may be an adaptation phase with further pulmonary vascular remodeling. We hypothesized that inhibition or decrease of the oxidative stress during the first induction phase might result in reduction of the cardiovascular alterations occurring in the subsequent adaptation phase. Indeed, the inhibition of XO only during the initial 3 days of the hypoxic exposure was sufficient to reduce the degree of right ventricular hypertrophy and pulmonary vascular thickening that usually develops during 3 wk of chronic hypoxic exposure. Our interpretation of these findings is that the oxidative stress generated via XO activation during the induction phase of chronic hypoxia plays a role in the development of pulmonary hypertension.

Our data do not address the cell sources of hypoxia-related oxidative stress and the sites of XO activation, but we speculate that pulmonary arterial endothelial cell XO may contribute to the generation of oxidative stress in the hypoxia-exposed rat lungs, because hypoxia causes accumulation of lipid peroxidation products (2) and activation of XO (23) in cultured pulmonary arterial endothelial cells.

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REFERENCES