Contribution of xanthine oxidase-derived superoxide to chronic hypoxic pulmonary hypertension in neonatal rats

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From birth, we examined the effects of ROS scavengers (U74389G) on pulmonary vascular remodeling (23), known collectively as vascular remodeling. Evidence suggests that abnormally decreased pulmonary arterial conduit, exaggerated responses to constrictors (3, 14), and reduced compliance, all of which are believed to contribute to a chronic and progressive form of PHT that is refractory to current therapies (13).

A major pathological role for increased generation of reactive oxygen species (ROS) in the pathogenesis of experimental PHT is evidenced by antioxidant intervention studies performed in fetal lambs (24, 39), in hyperoxia-exposed neonatal rats (30), and in hypoxia- (25, 38, 41) or monocrotaline-exposed (9) adult rodents. A putative role for oxidative stress is also supported by observational studies in adult humans with idiopathic PHT (7, 10). The injured pulmonary vasculature is known to be a major source of ROS, particularly superoxide anion (O2•−). Recent studies in experimental models of PHT have indicated that pulmonary vascular-derived ROS may predominantly originate from enzymatic sources. Major enzymatic sources described in adult rats and mice chronically exposed to hypoxia have included xanthine oxidase (XO) (25) and reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase (42), respectively. Furthermore, both NAD(P)H oxidase and uncoupled endothelial nitric oxide synthase (eNOS) have been described as major vascular sources of ROS in fetal lambs with ductal ligation-induced PHT (24, 36).

Under normal conditions, the enzyme xanthine oxidoreductase (XOR) exists primarily in its dehydrogenase form, serving as the rate-limiting step in purine degradation to uric acid. Under conditions of tissue hypoxia (53), ATP is broken down to the purine hypoxanthine, a substrate for XOR, and reversible oxidation or irreversible proteolytic cleavage (17, 54), which converts XOR to an oxidase form that can produce large quantities of ROS, including O2•− and its dismutation product, hydrogen peroxide (H2O2). In adults, increased vascular O2•− production during chronic exposure to hypoxia has been attributed in major part to XO, which has been found to adversely impact endothelial function by impairing nitric oxide (NO) signaling (26) and to directly contribute to experimental pulmonary vascular remodeling (25).

In neonates, PHT complicates a wide variety of hypoxemia-associated clinical syndromes (12), including meconium aspiration, perinatal asphyxia, congenital sepsis, and idiopathic respiratory distress syndrome (18, 64, 65). Human neonates with severe hypoxic respiratory failure have raised XO-derived uric acid levels in urine (44). Furthermore, Allopurinol treatment was reported to decrease mortality in premature infants with severe respiratory distress (6). To our knowledge, however, no previous studies have examined the specific role of xanthine oxidase-derived superoxide to chronic hypoxic pulmonary hypertension in neonatal rats. Am J Physiol Lung Cell Mol Physiol 294: L233–L245, 2008. First published December 14, 2007; doi:10.1152/ajplung.00166.2007.
of oxidants in general, or of XO-derived oxidants, specifically, in the pathogenesis of chronic hypoxic PHT in neonates.

We have recently described a neonatal rat model of PHT secondary to chronic exposure to moderate hypoxia (13% O2) from birth, which leads to increased oxidative stress in the lung (33). Herein, we confirmed in this model, using either of two structurally dissimilar ROS scavengers in vivo, a 21-amino- steroid, U74389G, or a piperidine nitroxide, Tempol, that ROS are critical to pulmonary vascular remodeling. We went on to examine the role of XO as a potentially major source of ROS leading to inhibited arterial relaxation and vascular remodeling in neonatal chronic hypoxic PHT. An additional goal of this study was to compare the effects of a XO inhibitor, Allopurinol, to the ROS scavengers U74389G and Tempol on somatic growth and markers of lung growth, given the concern that effective “broad spectrum” antioxidant interventions may also inhibit normal processes for which low physiological levels of intracellular ROS are required, such as cellular proliferation (30, 31, 61).

MATERIALS AND METHODS

Materials. Plexiglas animal exposure chambers and automated O2 controllers (OxyCycler model 84XOVO) were purchased from BioSpherix (Redfield, NY). 5-Bromo-2’-deoxyuridine (Brdu), in situ Brdu immunostaining kits, and a mouse monoclonal antibody against eNOS were from BD Biosciences (Mississauga, ON, Canada). Amplex Red (N-acetyl-3,7-dihydroxyphenoxazone) XO activity assay kits and dihydroethidium (DHE) were from Molecular Probes (Eugene, OR). Tempol (4-hydroxy-2,2,6,6-tetramethyl-1-piperidinylxyl) and diphenyleneiodonium (DPI) were from Alexis Probes (Eugene, OR). Avidin-biotin-peroxidase complex immunohistochemistry kits, 3,3’-diaminobenzidine staining kits, and normal goat serum were from Vector Laboratories (Burlingame, CA). Acids, alcohols, organic solvents, paraformaldehyde, Permount, and Superfrost/Plus microscope slides were from Fisher Scientific (Whitey, ON, Canada). Mouse monoclonal antibodies against α-smooth muscle actin and XOR (EC 1.1.1.204) were from Neomarkers (Fremont, CA). A rabbit polyclonal antibody against nitrotyrosine was from Upstate Biotechnology (Lake Placid, NY). A rabbit polyclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) and goat antimouse and rabbit IgG-biotin secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). A goat anti-rabbit IgG-peroxidase antibody was from Cell Signaling Technology (Beverly, MA). Avidin-biotin-peroxidase complex immunohistochemistry kits, 3,3’-diaminobenzidine staining kits, and normal goat serum were from Vector Laboratories (Burlingame, CA). Tissue-Tek OCT was from Sakura Finetek (Torrance, CA). U74389G, U46619, and 8-isoprostane affinity purification columns and enzyme immunoassay kits were purchased from Cayman Chemical (Ann Arbor, MI). Allopurinol, Oxypurinol (the active metabolite of Allopurinol), polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), catalase (PEG-CAT), 3-nitrotyrosine, sodium nitroprusside (SNP), a rabbit polyclonal antibody against α-catenin, and all other chemicals and reagents were from Sigma (Oakville, ON, Canada).

Animal exposures and interventions. All procedures involving animals were performed in accordance with the standards of the Canadian Council on Animal Care and were approved by the Animal Care Committees of the Sunnybrook and Hospital for Sick Children Research Institutes. Each litter, maintained at n = 10–12 pups to control for nutritional effects, was nursed in either normoxia (21% O2) or hypoxia (13% O2) from birth for up to 4, 7, or 14 days, as previously described in detail (33). This Pio2 was chosen as the lowest that did not lead to excess mortality by 14 days (33). Pups received either Allopurinol (5 μg/g body wt of 10 mg/ml suspended in 0.9% saline vehicle = 50 mg/kg), U74389G (5 μg/g body wt of 2 mg/ml solution in CS4 vehicle (20 mM citric acid monohydrate, 3.2 mM sodium citrate dihydrate, 77 mM NaCl, pH 3.0) = 10 mg/kg), Tempol (5 μg/g body wt of 20 mg/ml solution in 0.9% saline vehicle = 100 mg/kg), or vehicle alone by daily intraperitoneal injection. The doses of the above compounds used were similar to those previously reported to be effective in vivo by our group and others (16, 25, 30). At the end of each exposure period, pups were either killed by pentobarbital overdose or exsanguinated after anesthesia. Some pups received 20 mg/kg Brdu intraperitoneally 2 h before they were killed.

Morphometric studies. Four animals from each group (2 from each of 2 separate litters) were anesthetized with ketamine (80 mg/kg ip) and xylazine (20 mg/kg ip). After opening of the thoracic cavity and cannulation of the trachea, the pulmonary veins were divided. The pulmonary circulation was flushed with 1 × PBS containing 1 U/ml heparin via a needle inserted in the right ventricle to clear the lungs of blood while the lungs were inflated at a constant pressure of 20 cmH2O. The lungs were then perfusion-fixed with ice-cold 4% (wt/vol) paraformaldehyde in 1 × PBS or the pulmonary circulation was perfused with a hot (60°C) saturated barium-10% (wt/vol) gelatin mixture for 5 min at 100 cmH2O pressure, and the lungs were then placed overnight in 4% (wt/vol) paraformaldehyde in 0.1 M sodium citrate dihydrate, 77 mM NaCl, pH 3.0) and stored at 4°C.

In situ detection of ROS. Pulmonary vascular ROS were detected in situ utilizing DHE, a dye that fluoresces upon reaction with ROS [predominantly O2•− but also H2O2 and hydroxyl radicals (50)], using a method recently described by others (22, 24). Briefly, the lungs of two animals per group and time point were snap frozen in Tissue-Tek OCT, as previously described (28), and stored at −80°C. Frozen tissue was cut by cryostat into 20-μm-thick sections, which were mounted on Superfrost/Plus slides and stored at −80°C until analysis. For inhibitor studies, sections were incubated at room temperature for 30 min with the XO inhibitor Oxypurinol (100 μM), the NAD/PH oxidase inhibitor DPI (50 μM), or 1 × PBS alone (control). ROS scavenging was achieved by a 30-min preincubation with either U74389G (100 μM), PEG-SOD (100 U/ml), or PEG-CAT (300 U/ml). Sections were then incubated with 10 μM DHE in 1 × PBS at room temperature in the dark for the 30 min, coverslipped with fluorescent mounting medium (DAKOcytomeytom, Glostrup, Denmark), and immediately imaged (Pixera Penguin 600CL; Pixera, Los Gatos, CA) by an epifluorescent microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) with appropriate filters (excitation 540 nm and emission 580 nm) and using identical exposure times.

XO activity. XO activity in serum, lung tissue homogenates, or XO standards was measured by colorimetric change of Amplex Red at 562 nm in the presence of hypoxanthine, after deduction of background absorbance, using a commercially available plate-based assay kit (Invitrogen) according to the manufacturer’s instructions. XO catalyzes the oxidation of hypoxanthine or xanthine to O2•−, which spontaneously degrades to H2O2. In the presence of horseradish peroxidase, H2O2 reacts with Amplex Red to generate the red oxidation product, resorufin. The lower limit of detection in this assay is 0.1 mU/ml. For lung tissue homogenates, values were normalized to total lung protein, quantified by the Bradford method.

Total lung DNA content. Total DNA was extracted from lung tissue lysates by phenol/chloroform/isooamyl alcohol extraction followed by precipitation in isopropanol as previously described (59). Samples and DNA standards were quantified by fluorescence spectrophotometry.
using a Qubit Fluorometer and Quant-iT dsDNA BR assay kit, according to the manufacturer's instructions (Molecular Probes).

Right ventricular hypertrophy. Right ventricular hypertrophy (RVH) was quantified by measuring the right ventricle (RV) to left ventricle and septum (LV+S) dry weight ratios from the pooled hearts of each litter, as previously described (30).

Organ bath studies. Third-fourth generation lung intralobar intrapulmonary artery ring segments (average of 100-μm-diameter and 2 mm in length) were dissected from pups exposed to either air or hypoxia and mounted in a wire myograph (Myodaq; Danish Myo Technology, Aarhus, Denmark). All vessels were bathed in Krebs-Henseleit buffer (115 mM NaCl, 25 mM NaHCO₃, 1.38 mM NaHPO₄, 2.51 mM KCl, 2.46 mM MgSO₄, 1.91 mM CaCl₂, and 5.56 mM dextrose) bubbled with 21% O₂/73% N₂/6% CO₂ and maintained at 37°C. After a 1-h equilibration period, arteries were precontracted with the thromboxane analog U46619 (1 μM), and the percentage relaxation induced by acetylcholine (endothelium-dependent relaxation) or SNP (endothelium-independent relaxation) (100 μM each) was measured, as previously described (4). Before applying relaxation stimuli, separate batches of vessels from air-exposed pups were treated with hypoxanthine (1 mM)/XO (10 mU/ml) for 10 min to generate O₂⁻⁻⁻⁻.

Lung eNOS expression. eNOS was quantified by Western blot analyses on total lung protein lysates from four animals representing two litters per group, which were prepared as previously described (28). Protein samples (50 μg/lane) were heated to 40°C for 30 min and fractionated by low-temperature SDS-PAGE on Tris-glycine 4–20% gradient gels under nonreducing conditions to resolve both monomeric (~140 kDa) and dimeric (~280 kDa) forms of eNOS. For quantification of total eNOS (~140 kDa), protein samples were boiled for 5 min and run under reducing conditions by SDS-PAGE. PVDF membranes were blocked with 5% (wt/vol) nonfat milk in TBST [20 mM Tris base, 137 mM NaCl, pH 7.6, with 0.1% (vol/vol) Tween 20] overnight at 4°C and then incubated with anti-eNOS (1/1,000 0.25 μg/ml) for 2 h at room temperature. Blots were washed and placed in the appropriate secondary antibody for 1 h at room temperature. After further washing, the protein bands were imaged by enhanced chemiluminescence (Immobilon Western Detection Reagent; Millipore, Mississauga, ON, Canada) and exposed to blue film (CL-XPosure; Pierce Biotechnology, Rockford, IL). Films were then electronically scanned, and the densities of nonsaturated bands with background removed were quantified using ImageJ software (version 1.30; National Institutes of Health, Bethesda, MD). To control for differences in protein loading, two housekeeping proteins were examined: total eNOS blots.
were coincubated with anti-GAPDH (30 kDa; 1/1,000 = 0.2 μg/ml), as previously described (33), and then stripped and rebotted with anti-α-catenin (150 kDa; 1/2,000 = 0.1 μg/ml). Strong consistency in band densities was observed between the two housekeeping proteins, neither of which changed significantly with exposure to hypoxia at the time points examined (data not shown). Changes in total eNOS expression with exposure to hypoxia are presented as a multiple or a fraction of the control (air-exposed) band density (assigned a value of 1) normalized to α-catenin. Relative eNOS uncoupling was quantified by an increase in the monomer:dimer ratio expressed as a multiple of the control (air-exposed) ratio, which was assigned a value of 1.

**Lung 8-isoprostane.** Total (free and esterified) 8-isoprostane was extracted from the homogenized lung tissue of four litters/group (the pooled lungs of 2 animals were used from each litter) using immunosorbent affinity columns (Cayman) according to the manufacturer’s instructions. 8-Isoprostane was quantified in affinity-purified samples and standards using a commercially available enzyme immunoassay kit, as outlined by the manufacturer. Values are expressed as picograms of 8-isoprostane per milligram of total lung protein.

**Data presentation and analysis.** Unless otherwise stated, all values are expressed as means ± SE. All analyses were performed using SigmaStat version 3.0.1 (Systat software, San Jose, CA). Where three or more groups were compared, statistical significance (P < 0.05) was determined by two-way ANOVA followed by pairwise multiple comparisons using the Tukey test. Where two groups were compared, the Student’s t-test or Mann-Whitney Rank Sum test were used.

**RESULTS**

**Chronic hypoxia-induced changes of vascular remodeling were attenuated by the ROS scavengers U74389G or Tempol.** As shown in Fig. 1, A and B, a twofold increase in the RV/LV+S dry weight ratio after 14 days (indicative of severe RVH) was observed in vehicle-treated animals exposed to hypoxia (P < 0.05 compared with air-exposed, vehicle-treated groups). A statistically significant reduction in hypoxia-induced RVH was observed in animals exposed to hypoxia and treated with either U74389G (Fig. 1A) or Tempol (Fig. 1B; P < 0.01 compared with their respective hypoxia-exposed vehicle-treated groups). As shown in Fig. 1C (U74389G) and 1D (Tempol), attenuating effects on RVH were paralleled by a reduction in arterial medial wall thickening, as demonstrated by elastin staining. As shown in Fig. 2, a widely used in vivo marker of oxidative stress (55), 8-isoprostane, was increased in the lungs of vehicle-treated animals exposed to hypoxia for 14 days from birth. This hypoxia-induced increase was almost completely attenuated by treatment with either U74389G or Tempol (P < 0.05 compared with hypoxia-exposed, vehicle-treated group).

**Chronic exposure to hypoxia induced a time-limited increase in XO activity and expression.** XO activity was quantified in both serum (Fig. 3A) and lung tissue (Fig. 3B) after a 4-, 7-, and 14-day exposure to either air or hypoxia in animals that were treated with either vehicle or Allopurinol. Compared with vehicle-treated, air-exposed controls, animals exposed to hypoxia for 4 days, but not for 7 or 14 days, had significantly increased XO activity in both serum and lung tissue (P < 0.001), which was completely attenuated by treatment with Allopurinol. XOR expression in lung tissue was examined by immunohistochemistry (Fig. 3C), which showed that a 4-day period of exposure to hypoxia greatly increased XOR expression in the pulmonary arterial wall and distal airway epithelium. This hypoxia-induced increase was completely attenuated by treatment with Allopurinol. In keeping with our findings on XO activity in both serum and lung, no increases in lung XOR immunoreactivity were found in hypoxia-exposed groups at either 7 or 14 days (data not shown).

**Exposure to hypoxia increased arterial ROS production, the major sources of which changed with time.** Arterial ROS production was examined in situ using DHE fluorescence as a marker (Fig. 4). Greatly increased DHE fluorescence was observed on the arterial walls of hypoxia-exposed, vehicle-treated animals both early (4 days) and late (14 days) in the course of exposure to hypoxia. At both time points, increased fluorescence induced by hypoxia was attenuated by the O2• scavengers PEG-SOD and U74389G (43), but not by the H2O2 scavenger PEG-CAT, indicating that the predominant ROS being detected was O2•. In keeping with our observation of increased XO activity and expression in the lung being limited to the first week of chronic exposure to hypoxia (present at day 4 but not at later time points; Fig. 3), daily injection with Allopurinol or in situ treatment with its active metabolite, Oxyipurinol, completely attenuated increased DHE fluorescence at 4 days, but not at 14 days. In contrast, the NADPH oxidase inhibitor DPI completely attenuated increased DHE fluorescence after a 14-day, but not a 4-day, exposure to hypoxia.

**Hypoxia-induced changes of vascular remodeling and increased oxidative and nitrative stress in the lung were attenuated by Allopurinol.** Compared with air-exposed, vehicle-treated controls, exposure to hypoxia for 14 days led to severe changes ofvascular remodeling, as evidenced by significantly increased RVH (Fig. 5A; P < 0.01), %MWT (Fig. 5B; P < 0.01), and distal arterial muscularization (Fig. 5C; P < 0.01). All of these changes were significantly attenuated (P < 0.01 compared with hypoxia-exposed, vehicle-treated group) by daily treatment with Allopurinol. Increased oxidative stress, as measured by total lung 8-isoprostane content, was evident after a 4-, 7-, and 14-day exposure to hypoxia (Fig. 6A; P < 0.05 compared with vehicle-treated, air-exposed groups). Consistent with our in situ ROS data (Fig. 4), treatment with Allopurinol completely attenuated this hypoxia-induced increase.
Nitrotyrosine in lung tissue, a “footprint” of peroxynitrite-mediated protein nitration (the reaction product of \( \cdot O_2^- \)/\( HNO_2 \)), was examined by immunohistochemistry. As shown in Fig. 6B, a 4-day period of exposure to hypoxia greatly increased immunoreactive nitrotyrosine in the pulmonary arterial wall and distal airway epithelium. This hypoxia-induced increase in nitration was completely attenuated by treatment with Allopurinol.

Relaxation in arteries from hypoxia-exposed animals was attenuated by XO-derived \( \cdot O_2^- \)/\( HNO_2 \). Exposure to hypoxia for between 4 and 6 days (week 1) or 11 and 14 days (week 2) led to significantly decreased endothelium-dependent (acetylcholine-induced) arterial relaxation (Fig. 7A; \( P < 0.01 \) compared with air-exposed controls), whereas endothelium-independent (SNP-induced) relaxation was only reduced at week 1 (Fig. 7B; \( P < 0.01 \) compared with air-exposed controls). Daily treatment of hypoxia-exposed animals with Allopurinol significantly improved endothelium-dependent arterial relaxation during week 2 (\( P < 0.05 \) compared with hypoxia alone). While there was a trend toward an improvement in both endothelium-dependent and -independent relaxation during week 1 with Allopurinol treatment, these differences did not reach statistical significance (\( P > 0.05 \)).

Acetylcholine- but not SNP-induced relaxation was inhibited in vessels from air-exposed animals by exposure to \( \cdot O_2^- \) generated by hypoxanthine/xanthine oxidase (Fig. 7C; \( P < 0.01 \) compared with control). As shown in Fig. 8A, total eNOS expression was not significantly altered by exposure to hypoxia at either day 4 or day 7 time points (\( P > 0.05 \)). In contrast, the ratio of monomeric:dimeric eNOS, as a measure of eNOS coupling, was significantly increased by exposure to hypoxia after 4 days (\( P < 0.05 \)). There was also a trend toward an increased monomeric:dimeric eNOS ratio after 7 days, but this difference did not reach statistical significance (\( P > 0.05 \); Fig. 8B).
Fig. 4. Representative images of in situ ROS detection in pulmonary vessels (luminae delineated by asterisks) from frozen lung tissue sections using dihydroethidium (DHE) oxidation (red fluorescence) under identical imaging conditions as a marker. Animals were exposed to 21% O2 (air) or 13% O2 (hypoxia) and treated with either 0.9% saline (vehicle) or Allopurinol from birth for either 4 days or 14 days. Bar lengths = 50 μm. At both time points, vascular wall DHE fluorescence was greatly increased by exposure to hypoxia. Increased fluorescence induced by hypoxia was completely attenuated by daily treatment with Allopurinol or in situ addition of Oxypurinol (vehicle + Oxypurinol) in lung sections from vehicle-treated pups at day 4 but not at day 14. Increased vascular wall DHE fluorescence induced by hypoxia was also attenuated by in situ addition of superoxide dismutase (PEG-SOD) or by the superoxide scavenger, U74389G, but not by catalase (PEG-CAT). In situ addition of the NAD(P)H oxidase inhibitor, diphenyleneiodonium (DPI), completely attenuated increased vascular wall DHE fluorescence in a section from a pup exposed to hypoxia for 14 days but not for 4 days.
Somatic growth, lung growth, and cellular proliferation in distal lung air spaces were inhibited by ROS scavengers, but not by Allopurinol. Values after 14 days of exposure to air or hypoxia and daily treatment with vehicle or the different interventions are shown in Table 1. Pups exposed to hypoxia had significantly ($P < 0.01$) decreased body weight but no change in lung weight ($P > 0.05$) compared with air-exposed controls. Despite a lack of change in lung weight, total lung DNA content was significantly decreased by chronic exposure to hypoxia for 14 days (6.64 ± 0.16 µg DNA/mg tissue weight vs. 8.42 ± 0.21 in air controls; $P < 0.001$ by t-test, $n = 7–8$ animals representing 2 litters/group). Body weight and lung weight in air-exposed, vehicle-treated animals were both significantly ($P < 0.01$) decreased by treatment with either of the ROS scavengers, U74389G or Tempol, but not by treatment with Allopurinol ($P > 0.05$). Furthermore, decreased body weight observed in hypoxia-exposed pups was reduced further by treatment with either U74389G or Tempol ($P < 0.01$ compared with respective vehicle-treated, hypoxia-exposed groups). Cellular proliferation in the distal lung air spaces, as quantified by counts of BrdU-positive nuclei (Fig. 9A), was significantly decreased by exposure to hypoxia ($P < 0.001$ compared with air-exposed, vehicle-treated groups). None of the interventions had any significant impact in this outcome ($P > 0.05$), although there was a slight increase observed in the numbers of BrdU-positive cells in hypoxia-exposed Allopurinol-treated pups. Compared with air-exposed, vehicle-treated pups, markedly less arterial smooth muscle layer thickening was evident in a hypoxia-exposed pup treated with Allopurinol (hypoxia Allopurinol).

Fig. 5. RV/LV+S weight ratios indicative of right ventricular hypertrophy (RVH; A), percentage (%) arterial medial wall thickness (MWT; B), and percentage (%) of completely muscular distal pulmonary arteries (C) as markers of pulmonary vascular remodeling. Animals were exposed to 21% O$_2$ (air; open bars) or 13% O$_2$ (hypoxia; closed bars) and treated with either 0.9% saline (vehicle) or Allopurinol (50 mg·kg$^{-1}$·day$^{-1}$) from birth until 14 days ($n = 4$ litters or animals/group). *$P < 0.01$, by ANOVA, compared with all other groups. #$P < 0.01$, by ANOVA, compared with air-exposed groups. D: representative immunohistochemistry for α-smooth muscle actin (brown stain) in lung tissue from animals that were exposed to 21% O$_2$ (air) or 13% O$_2$ (hypoxia) and treated with either 0.9% saline (vehicle) or Allopurinol from birth until 14 days. Bar lengths = 50 µm. Compared with a control pup (air), increased thickening of the smooth muscle layer was present on the walls of pulmonary resistance arteries (pa) in a pup exposed to hypoxia and treated with vehicle (hypoxia vehicle). Compared with hypoxia-exposed, vehicle-treated pups, markedly less arterial smooth muscle layer thickening was evident in a hypoxia-exposed pup treated with Allopurinol (hypoxia Allopurinol).
development in response to chronic postnatal exposure to hypoxia, as previously described by others (11, 46) and in air-exposed pups treated with U74389G or Tempol, but not Allopurinol.

**DISCUSSION**

There is an abundant literature that strongly implicates oxidative stress, due to increased production of ROS, as an important initiating and perpetuating factor in human and experimental PHT (7, 10, 38, 41). We have recently reported that oxidative stress was increased in the lungs of neonatal rats with severe PHT secondary to chronic hypoxia (33). Our current findings, employing the same model, were that: 1) ROS scavengers prevented hypoxia-induced oxidative stress and vascular remodeling; 2) chronic hypoxia induced an early (day 4) and time-limited (not present at later time points that were examined) increase in XO activity in both serum and lung tissue, XOR expression in lung and XO-derived O$_2^{-}$ production by pulmonary arteries, all of which were attenuated by Allopurinol; 3) treatment with Allopurinol attenuated oxidative and nitrative stress (after a 4-day exposure to hypoxia) and prevented, to a significant extent, the chronic changes of vascular remodeling; and 4) chronic exposure to hypoxia reduced both endothelium-dependent and -independent pulmonary arterial muscle relaxation, with only the former being partially improved by treatment with Allopurinol. Addition-
ally, changes seen in remodeled pulmonary resistance arteries were largely characterized by medial wall thickening without evidence of severe luminal encroachment, suggesting that chronic PHT in this model is likely to be caused by enhanced and sustained vasoconstriction (contributed to by thickening of the medial smooth muscle layer and endothelial dysfunction) rather than by luminal obstruction (58). This likelihood is supported by our recent findings demonstrating that increased pulmonary vascular resistance that does not respond to “conventional” vasodilators (suggesting a fixed pathological process) can be acutely normalized by inhibition of Rho-kinase, a signaling peptide that modulates Ca²⁺ sensitivity in smooth muscle (51).

Our observation of an increase in expression of XOR in the hypoxia-exposed lung, which was attenuated by Allopurinol, is in keeping with reports by other groups demonstrating hypoxia-induced upregulation of XO activity and XOR expression in vascular endothelium in vitro (34, 62, 63) and in the adult lung in vivo (25). Conversion of XOR to the oxidase form is believed to occur through two mechanisms: direct exposure to hypoxia through an adenosine-dependent process (34) and endothelial binding of circulating XO (26) secreted by other organs, such as the gut (5) and the liver (66). An additional observation that is particularly relevant to the growing and developing neonate was that inhibitory effects on normal somatic growth and lung cell proliferation observed with ROS scavengers did not result from treatment with Allopurinol.

In keeping with our present findings in neonatal rats, excessive production of O₂⁻ has been previously implicated in hypoxia-induced inhibition of both endothelium-dependent (22) and -independent (47) pulmonary arterial relaxation in adult animals. While O₂⁻ is not itself a highly reactive oxidant (57), it reacts very efficiently with NO to produce peroxynitrite anion, a ROS that is a major oxidizing and nitrating agent (2). Peroxynitrite may contribute to PHT through induction of vasoconstriction either directly (3) or via upregulation of other vasoconstrictors, such as endothelin-1 (15) and isoprostanes (32). It remains uncertain whether peroxynitrite has a direct role in the pathogenesis of chronic hypoxic PHT. First, inhibited endothelium-dependent arterial relaxation in hypoxia-exposed vessels was partially reversed by a XO inhibitor. Second, normal vessels exposed to O₂⁻ generated by hypoxanthine/XO in situ had a pattern of inhibited endothelium-dependent relaxation that was similar to vessels from hypoxia-exposed animals. Third, endothelium-independent relaxation in chronically hypoxic animals was inhibited in week 1, but not week 2, mirroring the timing of increased lung and serum XO activity and in situ XO-derived O₂⁻ production. Finally, we found increased nitrotyrosine residues in the hypoxia-exposed pulmonary vasculature, the timing of which coincided with the peak activity of XO in serum and total lung, and which was attenuated by treatment with Allopurinol.

Inhibited endothelium-dependent relaxation in pulmonary arteries from pups exposed to hypoxia appeared to be caused by uncoupling of eNOS rather than by a decrease in expression. When coupled to heat shock protein 90 (HSP90), endoglin, caveolin, and in the presence of the cofactor tetrahydrbiopoterin (BH₄), eNOS activity results in NO production. Enzyme uncoupling induces O₂⁻ release in addition to NO (20, 21). Such a phenomenon has been suggested to play a role in the pathogenesis of experimental
PHT (24, 35, 40) by resulting in reduced NO availability (impaired vasodilatation) and increased peroxynitrite formation leading to vasoconstriction. BH4 and HSP90 may also be oxidized and/or nitrated by peroxynitrite, which may cause, or further contribute to, eNOS uncoupling (36, 37). That increased eNOS monomer:dimer ratio coincided with the timing of increased vascular nitrotyrosine (day 4) suggests that XO-derived peroxynitrite could have contributed to uncoupling. This possibility warrants exploration in future studies. Recent studies have also identified a potentially major role for vascular cell-derived NAD(P)H oxidase in ROS formation (42, 45, 48). While not explored to any extent in the present study, our observations using in situ measurement of vascular ROS suggested that smooth muscle-derived NAD(P)H oxidase may be a major source of O$_2^•$ in the remodeled pulmonary vasculature.

Apart from a well-established role in pulmonary vascular disease, there is now irrefutable evidence that endogenous ROS also act in physiological roles as intracellular second messengers (8, 60, 61) to enable basic cellular processes such as proliferation. This raises the concern that systemically administered broad spectrum antioxidant interventions, designed to prevent oxidant-mediated injury, could also arrest signals for normal growth in the neonate. In keeping with these concerns, we observed that treatment with either of two structurally dissimilar ROS scavengers inhibited normal somatic and lung growth and inhibited lung cell proliferation in the neonatal rat. By targeting a major pathological source of ROS leading to chronic PHT, in the present case by inhibiting the ROS-producing enzyme XO, associated inhibitory effects on cellular proliferation and growth in normal animals (in which XO activity was not increased) did not occur. However, although treatment with Allopurinol limited the anatomical changes of chronic PHT, it did not significantly impact on inhibited distal airway growth and proliferation, suggesting the latter has a distinct pathogenesis in hypoxia-exposed neonatal rats. Nonetheless, these observations highlight the desirability of alternative approaches to antioxidant therapy in oxidant-mediated neonatal disease (31) by targeting a specific enzymatic or cellular (29) source of oxidants or by targeting downstream mediators of ROS-induced injury (27, 29). It should be noted, however, that Allopurinol has been reported to also possess limited ROS scavenging properties (19, 49); there-

Table 1. **Body weight and lung weight at 14 days**

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<thead>
<tr>
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<th>Vehicle</th>
<th>Allopurinol</th>
<th>U74389G</th>
<th>Tempol</th>
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<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
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<tr>
<td>Air-exposed</td>
<td>29.5±1.15</td>
<td>29.1±1.20</td>
<td>24.3±1.07†‡</td>
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<td>Hypoxia-exposed</td>
<td>26.3±1.32*</td>
<td>27.1±1.85*</td>
<td>22.5±1.40*†‡</td>
<td>21.1±1.29*†‡</td>
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<tr>
<td><strong>Lung weight, mg</strong></td>
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<tr>
<td>Air-exposed</td>
<td>479±25</td>
<td>458±26</td>
<td>444±24†</td>
<td>449±22†</td>
</tr>
<tr>
<td>Hypoxia-exposed</td>
<td>454±31</td>
<td>473±42</td>
<td>420±29‡</td>
<td>375±37*‡</td>
</tr>
</tbody>
</table>

Values ($n = 8–10$ animals/group) represent means ± SD. As there were no differences in values between 0.9% saline- and CS4 vehicle-treated groups, data were combined for clarity (vehicle; $n = 20$ animals). *$P < 0.01$, by ANOVA, compared with respective air-exposed group. †$P < 0.01$, by ANOVA, compared with vehicle-treated group. ‡$P < 0.01$, by ANOVA, compared with respective Allopurinol-treated group.
fore, its effects cannot be definitively attributed only to inhibition of XO. Notwithstanding this limitation, our study is the first, to our knowledge, to ascribe a significant role to XO in the pathogenesis of chronic neonatal PHT.

Importantly, we observed that pulmonary vascular XO-derived \( \text{O}_2^\cdot \) and 8-isoprostan e content, limited in the lung by treatment with Allopurinol, was restricted to the first week of chronic exposure to hypoxia. Despite this, Allopurinol treat-

![Fig. 9. Counts of bromodeoxyuridine (BrdU)-positive nuclei per microscopic field as a marker of cellular proliferation in distal air spaces (A) and representative lung sections immunostained for BrdU (positive nuclei are stained brown). Animals were exposed to 21% O\(_2\) (air; open bars) or 13% O\(_2\) (hypoxia; closed bars) and treated with CS4 vehicle, 0.9% saline (saline vehicle), U74389G (10 mg·kg\(^{-1}\)·day\(^{-1}\)), Allopurinol (50 mg·kg\(^{-1}\)·day\(^{-1}\)), or Tempol (100 mg·kg\(^{-1}\)·day\(^{-1}\)) from birth until 14 days (n = 4 animals/group). *P < 0.001, by ANOVA, compared with respective hypoxia-exposed groups. Lung sections from hypoxia-exposed pups, regardless of treatment group, showed a hypoplastic lung phenotype characterized by enlargement of distal air spaces. Distal air space enlargement was also evident in air-exposed pups treated with U74389G or Tempol, but not with Allopurinol. Bar lengths = 100 \( \mu \)m.](http://ajplung.physiology.org/).
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ment caused a similar degree of attenuating effect on RVH after a 2-wk hypoxic exposure as ROS scavengers. These findings are in agreement with Hoshikawa and colleagues (25), who reported in adult rats that treatment with a XO inhibitor limited to the first 3 days of a 3-wk exposure to hypoxia was enough to limit the subsequent development of chronic PHT. We speculate that a long-lasting attenuating effect of early XO inhibition may result from its protective role on eNOS uncoupling, thus allowing for preservation of endothelium-dependent NO production. Together, these findings provide further evidence that prevention of early ROS-induced changes in the pulmonary vasculature can have an important impact on later changes of vascular remodeling in chronic PHT, which may be accounted for largely by XO during exposure to hypoxia.

In conclusion, this study implicates vascular XO-derived O$_2^-$ as a major contributing factor to inhibited arterial relaxation and pulmonary vascular remodeling in a neonatal rat model of chronic hypoxia-induced PHT. Our findings further suggest that targeted antioxidant therapy, in the present case through inhibition of XO, may represent a safer preventative strategy than systemically administered ROS scavengers for neonatal PHT, in cases where hypoxemia is a major contributing factor.

ACKNOWLEDGMENTS

We thank Dr. Mourad Toporsian for technical assistance.

GRANTS

This work was funded by operating grants (R. P. Jankov and J. Belik) from the Canadian Institutes of Health Research (CIHR), by infrastructure grants from the Canada Foundation for Innovation New Opportunities and Ontario Research Funds (R. P. Jankov), and by a Grant-in-Aid from the Physicians’ Services Incorporated Foundation (R. P. Jankov). R. P. Jankov is supported by a Career Development Award through the Canadian Child Health Clinician Scientist Program, a CIHR Strategic Training Initiative.

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