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Arginase inhibition prevents bleomycin-induced pulmonary hypertension, vascular remodeling, and collagen deposition in neonatal rat lungs

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ARGINASES METABOLIZE L-ARGININE TO FORM L-ORNITHINE AND UREA. L-Ornithine is the precursor for the production of polyamines and collagen, which have been implicated in tissue repair and remodeling, respectively. Arginases also regulate nitric oxide (NO) production by controlling substrate l-arginine availability for NO synthases (NOSs) (46). There is accumulating evidence that arginase activity contributes to the development of pulmonary fibrosis and pulmonary hypertension (PHT) (9, 14, 25, 29, 32, 63).

PHT is a common complication of moderate-severe bronchopulmonary dysplasia (BPD), a chronic lung disease affecting extremely premature infants (6). The underlying pathogenesis of BPD is complex and multifactorial (39). Previous studies from our group have established a model of repeated systemic bleomycin injection in neonatal rats that results in lung injury mimicking characteristics typical of BPD. This model is a useful tool to help understand mechanisms contributing to lung development and remodeling (31) but also to study the effects of therapeutic interventions aimed at preventing lung parenchymal and vascular injury (31, 50, 56).

The chemotherapeutic agent bleomycin sulfate causes a pulmonary inflammatory and fibrotic response in rodents when instilled as a single intratracheal dose (27) or by repeated intraperitoneal injection (3). Bleomycin-induced lung injury is characterized by induction of proinflammatory cytokines (18), influx of macrophages and other inflammatory cells (23), “emphysematous” lung morphology, and severe PHT (49, 60). Herein, we report that arginase expression is greatly increased in the lungs of bleomycin-exposed neonatal rats and that treatment with the arginase inhibitor amino-2-borono-6-hexanoic acid prevented the bleomycin-induced development of pulmonary hypertension and deposition of collagen. Arginase inhibition resulted in increased L-arginine and L-arginine bioavailability and increased pulmonary nitric oxide production. Arginase inhibition also normalized the expression of inducible nitric oxide synthase, and reduced bleomycin-induced nitrative stress while having no effect on bleomycin-induced inflammation. Our data suggest that arginase is a promising target for therapeutic interventions in neonates aimed at preventing lung vascular remodeling and pulmonary hypertension.

METHODS

Animal 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 Committee of the Hospital for Sick Children. Commencing on the day after birth, Sprague-Dawley rat pups received 1 mg/kg bleomycin sulfate (0.2 mg/ml in saline; 5 μl/g body wt by 27-gauge needle in the right iliac fossa) or saline (control) and immediately afterward intraperitoneal 3.5 mg/kg ABH (0.65 mg/ml in saline; 5 μl/g body wt) or an
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Lung arginine and NO. Lung L-arginine levels were significantly lower in bleomycin-exposed compared with control rats (P < 0.05; Fig. 1A). Treatment with ABH resulted in significant increases in L-arginine in both control and bleomycin-exposed animals (P < 0.01; Fig. 1A). Similarly, the ratio of L-arginine/L-ornithine (as a marker of arginine availability) (37) was reduced in the lungs of bleomycin-exposed compared with control animals (P < 0.05; Fig. 1B). Treatment with ABH resulted in a significant increase in the L-arginine/L-ornithine ratio in both control and bleomycin-exposed animals (Fig. 1B).

Consistent with the changes in L-arginine concentration and availability, lung NOx content was reduced in lung tissue of bleomycin-exposed animals when compared with controls (P < 0.01). Treatment with ABH resulted in significantly (P < 0.001) increased NOx concentrations in the lungs of both control and bleomycin-exposed animals (Fig. 1C).

RESULTS

Lung arginine and NO. Lung L-arginine levels were significantly lower in bleomycin-exposed compared with control rats (P < 0.05; Fig. 1A). Treatment with ABH resulted in significant increases in L-arginine in both control and bleomycin-exposed animals (P < 0.01; Fig. 1A). Similarly, the ratio of L-arginine/L-ornithine (as a marker of arginine availability) (37) was reduced in the lungs of bleomycin-exposed compared with control animals (P < 0.05; Fig. 1B). Treatment with ABH resulted in a significant increase in the L-arginine/L-ornithine ratio in both control and bleomycin-exposed animals (Fig. 1B).

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Expression of arginases and NOSs. Arginase I and arginase II (Fig. 2A) and NOS2 (Fig. 2B) mRNA expressions were increased significantly in the lungs of bleomycin-exposed animals when compared with controls \((P < 0.001)\). ABH treatment did not affect bleomycin-induced increases in expression of either arginase isoform. In contrast, the bleomycin-induced increase in NOS2 expression was completely normalized by ABH (Fig. 2B), whereas neither bleomycin exposure nor treatment with ABH had any effect on lung expressions of NOS1 or NOS3 mRNA (Fig. 2B). Examination of lung NOS2 protein content by Western blot similarly showed a significant \((P < 0.01)\) increase with bleomycin exposure that was completely abrogated by treatment with ABH (Fig. 2C).

In situ localization of NOS2 and 3-nitrotyrosine. As shown by representative immunohistochemistry (Fig. 3A), increased NOS2 immunoreactivity was localized to arterial walls, respiratory epithelium, and, to some extent, in the alveolar septae, but not macrophages. Immunostaining for a marker of oxidative and nitritative stress, 3-nitrotyrosine, showed a similar distribution of immunoreactivity to NOS2 in the bleomycin-exposed lung that was greatly decreased in lungs of animals treated with ABH (Fig. 3B).

\textit{PHT.} The bleomycin-induced increases in PVR (Fig. 4A), RVH (Fig. 4B), and medial wall thickening (Fig. 4C) in pulmonary resistance arteries, which have been reported previously, were all prevented by treatment with ABH.

\textit{Collagen deposition.} Collagen content was significantly increased in the lungs of bleomycin-exposed animals, particularly surrounding the respiratory bronchioles and respiratory bronchiole-associated pulmonary resistance arteries, when compared with vehicle-treated controls (Fig. 5). Bleomycin-

![Fig. 2. Lung expression of arginases and NO synthases (NOSs). Rat pups received daily ip 0.9% saline vehicle or 1 mg/kg bleomycin sulfate ± 3.5 mg/kg ABH or vehicle from days 1 to 14 of life. mRNA expression of arginase I or arginase II (A) and neuronal NOS (NOS1), inducible NOS (NOS2), or endothelial NOS (NOS3) (B). C: Western blot analyses of lung NOS2 content. Representative immunoblot are shown adjacent to the graph with noncontiguous gel lanes demarcated by black lines; \(n = 3–4\) samples/group. Bars represent means ± SE. *\(P < 0.001\), by ANOVA, compared with the respective control group. #\(P < 0.05\), by ANOVA, compared with all other groups.]

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mediated collagen deposition was completely abrogated by treatment with ABH (Fig. 5).

Inflammation. Expression of cytokines and chemokines previously reported to be increased in the bleomycin-exposed neonatal rat lung (31) were not affected by treatment with ABH (Table 1). Increased CD-68-positive macrophage numbers in the bleomycin-exposed lung were unaffected by treatment with ABH (data not shown).

Furthermore, ABH treatment did not affect animal weight at day 14 nor did it prevent bleomycin-induced alterations in distal airway morphology, characterized by emphysematous lung structure and septal thinning, as demonstrated by unchanged mean chord length and tissue fraction values (data not shown).

DISCUSSION

Arginase has previously been shown to be involved in the development of PHT and lung fibrosis (9, 25, 29, 32, 44, 63). Using repeated intraperitoneal administration of bleomycin in neonatal rats as a model of chronic neonatal lung injury and PHT, we report increased expression of arginase I, arginase II, and NOS2 in the bleomycin-exposed lung. Concentrations of substrate L-arginine and L-arginine bioavailability for NOS were reduced and collagen content increased in lungs of bleomycin-treated animals. Inhibition of arginase in this model resulted in a significant decrease in NOS2 expression and an increase in pulmonary NO production (NO metabolite concentration), a reduction in oxidative/nitrative stress, as well as prevention of both PHT and

Fig. 3. In situ localization of NOS2 and 3-nitrotyrosine. Rat pups received daily ip 0.9% saline vehicle or 1 mg/kg bleomycin sulfate ± 3.5 mg/kg ABH or vehicle from days 1 to 14 of life. Representative immunohistochemistry for NOS2 (A) and 3-nitrotyrosine (B) as an in situ marker of oxidative/nitrative stress. Bar lengths = 100 μm. Inset: vehicle-treated control section pretreated with peroxynitrite as a positive control.
bleomycin-induced collagen deposition (fibrosis). Collectively, these results suggest that decreased lung NO content as well as remodeling were mediated by increased arginase activity.

Arginase. The two isoforms of arginase, arginase I and arginase II, catalyze the hydrolysis of L-arginine to L-ornithine and urea. Arginase I is a cytosolic enzyme and part of the hepatic urea cycle, whereas arginase II is localized to mitochondria. Arginase isoforms are expressed in aorta, carotid, pulmonary artery, and many other blood vessels (9, 10, 13, 38). Arginases have also been detected in vascular smooth muscle cells (SMCs) and endothelial cells (ECs), but the abundance of each isoform is variable and likely reflective of differences between species, vascular beds, the size and function of blood vessels, and culture conditions (4, 9, 24). L-Ornithine, the product of arginase activity, is substrate for ornithine decarboxylase in the synthesis of the polyamine putrescine (54) as well as ornithine aminotransferase to form pyrroline-5-carboxylate, which is further metabolized to L-proline. Proline is required for the synthesis of structural proteins, including collagen (11, 12, 46).

Deposition of extracellular matrix, including collagen, is a characteristic feature of vascular remodeling resulting in PHT.

Fig. 4. Pulmonary hypertension. Rat pups received daily ip 0.9% saline vehicle or 1 mg/kg bleomycin sulfate ± 3.5 mg/kg ABH or vehicle from days 1 to 14 of life. A: pulmonary vascular resistance (PVR) estimated from echocardiography-derived ratio of right ventricular ejection time (RVET) to pulmonary arterial acceleration time (PAAT); n = 6 animals/group. B: Fulton index [right ventricle (RV)/left ventricle + septum (LV+S) weight ratio] as a marker of right ventricular hypertrophy; n = 7–8 animals/group. C: medial wall area of pulmonary resistance arteries. High-power photomicrograph of elastin-stained pulmonary arteries, demonstrating internal and external elastic laminae. Bar length = 50 μm. Graph bars represent means ± SE. *P < 0.001, by ANOVA, compared with all other groups. #P < 0.05, by ANOVA, compared with all other groups.

Fig. 5. Lung collagen. Rat pups received daily ip 0.9% saline vehicle or 1 mg/kg bleomycin sulfate ± 3.5 mg/kg ABH or vehicle from days 1 to 14 of life. Quantification of Picrosirius Red density (red stain in representative photomicrographs; bar length = 250 μm) as a marker of lung collagen. Graph bars represent means ± SE for 4 animals/group. *P < 0.001, by ANOVA, compared with all other groups.
arginase I, which increases the affinity of the enzyme for NOS2 can lead to increased arginine availability for NOS. NOS may also increase substrate availability for NOS2, and NOS3. Arginase competes with NOS for L-arginine, which IL-13 was specifically overexpressed in the lung (5).

Changes in mRNA expression secondary to bleomycin and/or ABH

<table>
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<th>Gene</th>
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<td>2.1 ± 0.2#</td>
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<tr>
<td>TNF-α</td>
<td>2.8 ± 0.6#</td>
<td>0.9 ± 0.2</td>
<td>3.1 ± 0.5#</td>
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Values are means ± SD of 4 samples/group relative to the control (vehicle only treated) group, which was assigned a value of 1. ABH, amino-2-boronono-6-hexanoic acid. *P < 0.01, by ANOVA, compared with control. #P < 0.05, by ANOVA, compared with control.

but also of pulmonary fibrosis (20). Previous studies have established a role for TGF-β1 in bleomycin-induced collagen gene expression (7, 14) and demonstrated effective prevention of pulmonary fibrosis by inhibition of collagen synthesis (27) in adult rats. The finding that arginase inhibition in our model prevented the bleomycin-induced development of PHT and collagen deposition is therefore in keeping with previous observations. The importance of arginase in the development of PHT and the efficacy of arginase inhibition in prevention have also recently been shown in a guinea pig model of lipopolysaccharide-induced chronic obstructive pulmonary disease (COPD) (44). Evidence supporting a role for arginase II in the development of PAH comes from a transgenic mouse model in which IL-13 was specifically overexpressed in the lung (5). Vascular SMCs and ECs isolated from these mice express only arginase II, whereas alveolar macrophages express both arginase isoforms. Deletion of arginase II in these animals decreased medial wall thickening of the pulmonary arteries and reduced the frequency of neovascularization of small pulmonary arteries (5).

NOS, NO, and oxidative stress. The NOSs are a family of enzymes that catalyze the formation of NO and l-citrulline from l-arginine. There are three distinct isoforms: NOS1, NOS2, and NOS3. Arginase competes with NOS for l-arginine under physiological conditions (61), and induction of arginase activity in the pulmonary circulation leads to a decrease in endothelial NO synthesis from NOS3 (48, 63), a fundamental feature of many cardiovascular disorders. The proliferative and fibrotic effects of arginase activity may be further amplified by the competition of arginase I and NOS2 for l-arginine as substrate that restricts the generation of NO, which is an established inhibitor of SMC proliferation and collagen synthesis (17, 30).

This is in accordance with the observations in our current experiments, in which the concentrations of NOx in lung were reduced in bleomycin-treated rats, despite the finding that NOS2 expression was increased significantly. A similar discrepancy between NOS2 expression and NO production can also be observed in arterial injury models, where NOS2 expression is induced (57, 64), but the simultaneous increase in arginase I compromises NO synthesis at the site of injury (1).

Although arginase regulates NO formation by limiting l-arginine availability for NOS, NOS may also increase substrate availability for arginase. For example, synthesis of NO by NOS2 can lead to S-nitrosylation of a cysteine residue on arginase I, which increases the affinity of the enzyme for l-arginine leading to increased arginase activity in ECs (47). It remains unclear whether prevention of the bleomycin-induced increase in arginase activity by pharmacological inhibition exerts a suppressing effect on NOS2 expression by making this regulatory role of NOS2 redundant. The observation that there was no effect of ABH treatment on inflammatory mediators, including IL-1β, IL-6, and TNF-α, argues against downregulation of NOS2 secondary to a change in the underlying inflammatory response(s). An alternative explanation would be that the increased substrate availability for the NOS isoforms following arginase inhibition resulting in increased NO formation led to negative feedback on NOS2 expression.

Oxidative stress is known to contribute to the development of pulmonary fibrosis, in part by the release of reactive oxygen species from inflammatory cells (34, 59). The absence of its substrate l-arginine or its cofactor tetrahydrobiopterin (BH4) can result in uncoupling of NOS, which then contributes to oxidative stress by forming superoxide anion and subsequently peroxynitrite (2, 15, 16). Nitrotyrosine, which is used to quantify peroxynitrite-mediated nitration of protein tyrosine residues in inflammatory lung disorders (53), was increased in the lung of bleomycin-treated rats and was abrogated by arginase inhibition. We can only speculate that this could be explained by ABH leading to normal l-arginine concentrations and availability for NOS that would promote coupling of the enzyme (and subsequent NO rather than superoxide anion formation) and/or by the fact that ABH prevented the bleomycin-induced expression of NOS2. However, the improvement in l-arginine availability following ABH treatment, although statistically significant, was only moderate. Increasing NOx concentrations in the face of decreased NOS2 expression may also suggest that improved l-arginine availability following arginase inhibition resulted in increased NO production from the constitutive NOS isoforms (NOS1 and NOS3). The important role of NOS2 in the development of pulmonary fibrosis, possibly through a regulatory effect of NOS2 on tissue inhibitor of metalloproteinase-2 mediated by peroxynitrite, has just recently been described in a model of chronic allergen exposure in the mouse (40), and antioxidant treatment has recently been shown to have protective effects against bleomycin-induced lung fibrosis in the rat (19). In addition, pharmacological inhibition of NOS2 was recently shown to decrease bleomycin-induced pulmonary fibrosis in mice, which is consistent with our findings (45). However, inhibition of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme that degrades the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), resulted in even greater reduction of collagen deposition than inhibition of NOS with l-NIL, whereas ADMA treatment had no such effect (45). These observations suggest that the antiinfective effect of DDAH inhibition was ADMA independent. The effects of DDAH inhibition on NO formation or oxidative stress were not examined. We did not investigate whether DDAH or ADMA were affected by arginase inhibition in our model. Alternative mechanisms that could be involved in the bleomycin-induced dysregulation of l-arginine metabolism in bleomycin-induced fibrosis include cofactors for NOS such as BH4, as well as amino acid transporters, e.g., the cationic amino acid transporter 2 (41).

As noted above, in a guinea pig model of inhaled lipopolysaccharide-induce lung injury causing COPD-like lung disease, treatment with ABH prevented pulmonary vascular remodel-
ing, which is consistent with our current observations, but also prevented increased lung IL-8 and neutrophil influx (44). This is in contrast to our observation in the neonatal rat, since arginase inhibition did not affect the bleomycin-induced markers of inflammation. This difference in response to arginase inhibition may be due to differences between species, the type of experimental lung injury, and/or differences in the developmental timing of injury. ABH is a boronic acid agonist that specifically inhibits the catalytic activity of arginase and is significantly more potent than the injury, and/or differences in the developmental timing of injury.

In summary, our data show that arginase inhibition with ABH prevented the bleomycin-induced development of PHT and collagen deposition in neonatal rat lungs. Arginase inhibition further resulted in increased L-arginine levels and bioavailability, and increased tissue NOX concentrations, whereas it prevented the bleomycin-induced expression of NOS2 and reduced oxidative stress in lung tissue. Arginase inhibition had no effect on other markers of bleomycin-induced inflammation.

REFERENCES


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