Evidence for Dysregulation of Dimethylarginine Dimethylaminohydrolase I in Chronic Hypoxia–Induced Pulmonary Hypertension

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**Background**—Chronic hypoxia–induced pulmonary hypertension is associated with increased pulmonary expression of nitric oxide synthase (NOS) enzymes. Nevertheless, some reports have indicated decreased pulmonary production of NO in the disease. To address this paradox, we determined pulmonary concentrations of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) in the hypoxia-induced pulmonary hypertension rat model. In addition, we determined whether dysregulation of the ADMA-metabolizing enzyme dimethylarginine dimethylaminohydrolase I (DDAH I) plays a role in this disease.

**Methods and Results**—Adult male rats were exposed for 1 week to either normoxia or hypoxia (10% oxygen). Lung tissues were used for Western blot analysis of endothelial NOS and DDAH I expression, measurement of lung NO and ADMA content, and in vitro assay of DDAH enzyme activity. Western blot analysis revealed a 1.9-fold increase in endothelial NOS protein and a 37% decrease in DDAH I protein in the lungs of hypoxia-exposed rats. Both pulmonary DDAH enzyme activity and NO content were significantly decreased in the hypoxic group (by 37% and 22%, respectively), but pulmonary ADMA concentrations were increased by 2.3-fold compared with the normoxic group.

**Conclusions**—These data demonstrate that the rat chronic hypoxia–induced pulmonary hypertension model is associated with increased pulmonary concentrations of the NOS inhibitor ADMA. Moreover, pulmonary hypertensive rats exhibit reduced pulmonary expression and activity of the ADMA-metabolizing enzyme DDAH I. The decreased DDAH I and increased ADMA concentrations may therefore contribute to pulmonary hypertension via the competitive inhibition of pulmonary NOS enzymes. (*Circulation*. 2003;108:1493-1498.)

**Key Words:** hypertension, pulmonary ■ hypoxia ■ asymmetric dimethylarginine ■ nitric oxide ■ nitric oxide synthase

Pulmonary hypertension is characterized by increased pulmonary blood pressure, vascular remodeling of the small pulmonary arteries, and right ventricular hypertrophy. Endothelial dysfunction is believed to be an early event in the pathophysiology of pulmonary hypertension. However, the role of endothelium-derived nitric oxide (NO) in the disease is controversial. Clinical studies have demonstrated the efficacy of inhaled NO as a short-term treatment for pulmonary hypertension in both infants and adults, suggesting that NO-dependent vasodilatation is reduced in pulmonary hypertensive lungs. Nevertheless, numerous reports from our laboratory and others demonstrate that pulmonary expression of NO synthase (NOS) enzymes is paradoxically increased in chronic hypoxia–induced pulmonary hypertension. Moreover, although some reports have demonstrated increased NO activity and enhanced production of its mediator cGMP, others have reported decreased pulmonary NO production in this disease. Thus, it appears that elevated NOS expression is not always correlated with elevated NO activity in pulmonary hypertension, suggesting the existence of complex regulatory mechanisms.

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The activity of the NOS enzymes can be competitively inhibited by methylated arginines such as N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) and N\textsuperscript{G},N\textsuperscript{G}-dimethyl-L-arginine (ADMA). Both of these inhibitors are naturally occurring, but the concentration of ADMA in the human circulation is approximately 10 times greater than that of L-NMMA. Moreover, elevated plasma ADMA concentrations are associated with multiple pathological conditions, including renal failure, hypertension, and hypercholesterolemia. In ad-
dition, a recent study demonstrated that plasma ADMA concentrations are increased in patients with severe pulmonary hypertension, suggesting that NOS inhibition by ADMA may contribute to the disease.

Elevated concentrations of ADMA may arise as a result of either increased methylation of arginine residues in proteins or decreased metabolism of ADMA. ADMA and L-NMMA are actively metabolized to L-citrulline and methylamylines by the action of the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Pharmacological inhibition of DDAH leads to increased ADMA concentrations and a reduction in NO-mediated vasodilatation, indicating that DDAH activity controls endogenous ADMA concentrations and thus NO activity. Two distinct human DDAH isoforms (DDAH I and II) have been identified. Interestingly, DDAH I was found to predominate in tissues that express the neuronal NOS isoform, whereas DDAH II is the predominant isoform in tissues expressing endothelial NOS (eNOS), suggesting the possibility of isoform-specific regulation of NOS activity.

In the present study, we used the chronic hypoxia–induced rat pulmonary hypertension model to confirm that pulmonary ADMA concentrations are increased in this disease. We also addressed the hypothesis that altered DDAH activity is responsible for the increased ADMA concentrations and investigated the DDAH isoform responsible for this altered activity.

**Methods**

**Chronic Hypoxia Model and Measurement of Pulmonary Arterial Pressure**

Animal studies were performed in accordance with guidelines established by the Animal Research Committee of the University of Virginia. A total of 48 adult male Sprague-Dawley rats (Hilltop, Scottsdale, Pa) with an initial body weight of 300 g were exposed to normoxia or chronic hypoxia, as described. In brief, groups of 4 to 6 rats were placed in either a normobaric Plexiglas chamber maintained at 10% oxygen (hypoxic group) or a similar chamber open to room air (normoxic group) for a period of 1 week. Both groups of animals were kept in the same room and were maintained at 20°C to 24°C with a 12-hour:12-hour light:dark cycle.

After 1 week of hypoxia or normoxia exposure, rats were anesthetized with isoflurane inhalation, and a tracheal cannula was inserted. The animals were artificially respirated with a Harvard rodent ventilator model 683 (Harvard Apparatus) set at a rate of 60 breaths per minute. The chest of the rat was opened, and an 18-gauge catheter filled with heparinized saline was inserted through the wall of the right ventricle and advanced into the pulmonary artery. Pulmonary arterial pressures were recorded with a Datascope 2001A, Duplicate 200-μL aliquots of the lung tissue filtrate processed as above were assayed in duplicate for NO content by use of a kit based on the Griess reaction (Cayman Chemical). The kit enables the determination of DDAH enzyme activity, and the remaining sample was centrifuged for 15 minutes at 100 000g, 4°C. After ultracentrifugation, supernatants were applied to Centricon YM-30 filter columns (molecular weight cutoff, 30 kDa; Millipore Corporation) and centrifuged for 1 to 2 hours at 5000g, 4°C. To remove hemoglobin, the resultant filtrate was stored at −70°C until the determination of lung NO and ADMA content.

**Measurement of Lung NO Content**

Aliquots (40 μL) of the lung tissue filtrate processed as above were assayed in duplicate for NO content by use of a kit based on the Griess reaction (Cayman Chemical). The kit enables the determination of the sum of lung tissue nitrate and nitrite content (NO3), providing an index of total lung NO production. Values obtained for lung NO3 content were corrected for the weight of each sample and expressed as nmol NO3/g tissue.

**Determination of DDAH Enzyme Activity**

Duplicate 200-μL aliquots of crude lung tissue homogenate were incubated with [14C]L-NMMA (0.04 μCi/mL) for 1 hour at 37°C. The reaction was stopped by the addition of 1 mL of Dowex 50X8-400 resin. After centrifugation at 10 000g for 5 minutes, [14C]L-citrulline was determined by liquid scintillation counting of the supernatant.

**High-Performance Liquid Chromatographic Analysis of Lung ADMA Content**

Quantification of ADMA in samples of lung tissue filtrate was performed as described previously. In brief, dimethylarginines were partially purified by extraction with Bond Elut SCX columns. ADMA was separated with an ODS C18 analytical high-performance liquid chromatography (HPLC) column on a Beckman System Gold apparatus. The ADMA concentration of the samples was determined by comparison with a synthetic standard, and extraction efficiency was determined by the addition of 1 μg L-NMMA to each sample before extraction.
Statistical Analysis
Data are presented as mean±SEM. Statistical significance between normoxia- and hypoxia-exposed animals was tested by the Student’s unpaired t test. Linear regression curves and correlation coefficients were calculated according to the least-squares method. Statistical significance was accepted at a value of P<0.05.

Results
Assessment of the Pulmonary Hypertension Model
Exposure to 7 days of hypoxia caused pulmonary hypertension, as demonstrated by the significantly elevated MPAP recorded in chronic hypoxia–exposed compared with normoxia-exposed animals (Table 1). In addition, hypoxia-exposed animals exhibited significantly reduced weight gain, right ventricular and pulmonary hypertrophy, and elevated hematocrit values compared with normoxia-exposed animals (Table).

Pulmonary eNOS, DDAH I, and DDAH II Protein Expression
Western blot analysis of lung homogenates using a monoclonal eNOS antibody revealed that the expression of the 135-kDa eNOS protein was significantly increased in the lungs of hypoxia-exposed animals compared with normoxia-exposed animals (Figure 1A). Densitometry revealed that the lungs of rats exposed to 7 days of hypoxia expressed 1.9-fold more eNOS protein than those of normoxia-exposed rats (n=10 to 14, P<0.001).

Western blot analysis of lung homogenates using a polyclonal DDAH II antibody revealed that DDAH II protein was not detectable in the lungs of either control or chronic hypoxia–exposed rats. In contrast, the antibody successfully detected a 34-kDa band in a positive control sample of purified recombinant DDAH II protein (data not shown).

Pulmonary NOx Content
To determine whether the increased eNOS protein expression observed in the lungs of hypoxia–exposed animals resulted in increased pulmonary NO production, NOx content was measured in lung tissue samples from hypoxia- and normoxia-exposed rats. In contrast to the increased eNOS protein expression, pulmonary NOx content was significantly decreased by 22.4% in hypoxia- versus normoxia-exposed rats (Figure 2A).

Pulmonary DDAH Enzyme Activity
To determine whether the decreased DDAH I protein expression observed in the lungs of hypoxia-exposed animals was correlated with a decrease in enzyme activity, in vitro DDAH enzyme activity was measured in crude lung homogenates from hypoxia- and normoxia-exposed rats. Pulmonary DDAH enzyme activity was found to be significantly lower in hypoxia- versus normoxia-exposed rats (Figure 2B). Moreover, there was a significant (P<0.001) negative correlation between MPAP and pulmonary DDAH enzyme activity in the normoxia- and hypoxia-exposed animals (Figure 3A).

Pulmonary ADMA Content
A decrease in the expression and activity of the enzyme DDAH may be expected to result in decreased metabolism of the NOS inhibitor ADMA, leading to an increase in ADMA concentrations. To determine whether this was the case in the lungs of hypoxia-exposed rats, pulmonary ADMA concentrations were measured by HPLC analysis of samples from normoxia- and hypoxia-exposed rats. As
predicted, pulmonary ADMA content was found to be increased significantly, by 2.3-fold, in hypoxia-exposed versus normoxia-exposed rats (Figure 2C). Moreover, there was a significant ($P<0.05$) negative correlation between pulmonary DDAH activity and pulmonary ADMA content in the normoxia- and hypoxia-exposed animals (Figure 3B).

**Discussion**

The biological paradox in pulmonary hypertension is increased pulmonary blood pressure and a reduction in NO synthesis despite increased expression of the NOS isoforms. It was recently reported that patients with severe pulmonary hypertension have an elevated circulating concentration of the endogenous NOS inhibitor ADMA. In the present study, we demonstrate that the rat chronic hypoxia-induced pulmonary hypertension model is also associated with reduced NO synthesis and increased pulmonary concentrations of ADMA. Therefore provide an animal model in which to study the mechanisms underlying the increased ADMA concentrations observed in patients with severe pulmonary hypertension.

The salient findings of the present investigation using this model are as follows: (1) decreased pulmonary NO production despite increased pulmonary eNOS expression; (2) a significant negative correlation between pulmonary DDAH enzyme activity and plasma ADMA concentrations, indicating that reduced DDAH activity directly influences the concentration of this endogenous NOS inhibitor; (3) a significant negative correlation between pulmonary DDAH enzyme activity and mean pulmonary arterial pressure, strongly suggesting that reduced DDAH activity plays an important role in the development of pulmonary hypertension.

**Figure 2.** A, Effect of hypoxia-induced pulmonary hypertension on lung NO$_x$ content. NO$_x$ content of lung extracts was determined with a colorimetric kit and then normalized for lung wet weight. B, Effect of hypoxia-induced pulmonary hypertension on total lung DDAH enzyme activity. DDAH enzyme activity was determined by in vitro assay of crude lung homogenates and then normalized for lung wet weight. C, Effect of hypoxia-induced pulmonary hypertension on lung ADMA content. ADMA content of lung extracts was determined by HPLC analysis and then normalized for lung wet weight. All data are expressed as mean±SEM of 6 to 12 animals per group. *$P<0.05$, **$P<0.001$ vs normoxic group.

**Figure 3.** Correlation between pulmonary DDAH enzyme activity and (A) MPAP and (B) pulmonary ADMA concentrations in rats exposed to normoxia or hypoxia for 1 week.
role in the development of chronic hypoxia–induced pulmonary hypertension; and (4) a significant inhibition of DDAH I protein expression, indicating that the DDAH I isoform is largely responsible for the decrease in DDAH activity observed in this model.

The decreased pulmonary NO production that arises as a result of increased ADMA concentrations in chronic hypoxia–exposed rats may contribute to pulmonary hypertension via reduced NO-dependent vasodilatation. In keeping with this hypothesis, the administration of L-arginine, the endogenous NOS substrate, to chronic hypoxia–exposed rats has been shown to result in an increase in plasma NO concentrations and a 50% decrease in right ventricular hypertrophy. Moreover, L-arginine infusion was shown to improve oxygenation in infants with persistent pulmonary hypertension of the newborn, indicating a deficiency in NO-dependent vasodilatation. Similarly, eNOS gene transfer to the smooth muscle cells of the pulmonary vasculature in rats with monocrotaline-induced pulmonary hypertension resulted in a significant decrease in both right ventricular hypertrophy and right ventricular systolic pressure. Together, these studies suggest that pulmonary hypertension results from a chronic decrease in NO availability, which may be a result of increased concentrations of the endogenous NOS inhibitor ADMA. The results of the present study therefore indicate that gene transfer of DDAH to the pulmonary vasculature, by reducing pulmonary ADMA concentrations, may represent a novel therapeutic approach to the treatment or prevention of pulmonary hypertension. Moreover, the fact that we found a significant negative correlation between pulmonary DDAH activity and pulmonary arterial pressure strongly indicates that a treatment that increases DDAH expression and/or activity would be likely to have a protective effect in reducing pulmonary pressure.

The findings of the present study are in agreement with a recent report indicating a significant decrease in DDAH activity in the lungs of newborn piglets with pulmonary hypertension induced by hypobaric hypoxia exposure. In contrast, pulmonary expression of the DDAH II and not the DDAH I isoform was significantly decreased in the piglet pulmonary hypertension model. Possible explanations for this difference in the involvement of the 2 DDAH isoforms between the 2 models include species, age, duration of hypoxia exposure, and hypobaric versus normobaric chambers. Indeed, similar differences in pulmonary gene expression between pulmonary hypertension models have been reported previously, even within the same species.

In the present study, DDAH I protein was found to be highly expressed in the rat lung, whereas DDAH II protein expression was undetectable by Western blot analysis. This result is in contrast with a previous report indicating that DDAH II was the major isoform expressed within human lung at the mRNA level. However, it is interesting to note that the correlation between pulmonary mRNA and DDAH protein expression is poor. In the latter study, porcine lung was shown to express both DDAH I and II mRNA and protein. The difference between this study and the present data may reflect species or developmental differences, although differences in antibody affinity cannot be entirely excluded.

Decreased concentrations of vascular DDAH activity were reported recently in a rat model of type II diabetes mellitus and were associated with increased plasma ADMA concentrations. In this model, DDAH I protein expression was unchanged, and the authors speculated that the reduced DDAH enzyme activity observed both in diabetes and in response to high glucose was a result of a reduced expression of the DDAH II isoform, although this was not determined experimentally. In contrast, the present study clearly demonstrates that the decrease in DDAH enzyme activity observed in the lungs of chronic hypoxia–exposed rats is at least in part a result of the corresponding decrease in the protein expression of the DDAH I isoform. These results therefore indicate that, unlike glucose, hypoxia exposure inhibits the expression of the DDAH I isoform. Further in vitro studies involving the exposure of pulmonary cells to hypoxic conditions will be necessary to directly address the mechanism by which hypoxia decreases DDAH I expression.

In summary, the present study demonstrates for the first time that the rat chronic hypoxia–induced pulmonary hypertension model is associated with reduced pulmonary activity of the ADMA-metabolizing enzyme DDAH. Reduced DDAH activity was correlated with increased pulmonary concentrations of the endogenous NOS inhibitor ADMA, a reduction in NO synthesis, and increased pulmonary arterial pressure. In addition, the protein expression of the DDAH I isoform was significantly decreased in the lungs of rats with chronic hypoxia–induced pulmonary hypertension. Thus, DDAH I may play an important role in the pathogenesis of pulmonary hypertension because of the resultant increased concentrations of ADMA and thus inhibition of pulmonary NOS activity. DDAH I may therefore represent a novel therapeutic target for the treatment and prevention of pulmonary hypertension.

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