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Metabolism of Asymmetric Dimethylarginines Is Regulated in the Lung Developmentally and With Pulmonary Hypertension Induced by Hypobaric Hypoxia

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Background—Nitric oxide (NO) plays an important part in lowering pulmonary vascular resistance after birth, and in persistent pulmonary hypertension of the newborn (PPHN), NO-mediated dilation is dysfunctional. The endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA) circulates in plasma, and its concentrations are elevated in certain cardiovascular diseases, including pulmonary hypertension. ADMA is metabolized by the enzyme dimethyl-arginine dimethylaminohydrolase (DDAH), the activity of which regulates ADMA concentrations and provides a mechanism for modulating NO synthase in vivo. We investigated the changes in expression and activity of the 2 isoforms of DDAH in lungs from newborn piglets both during normal development and in PPHN.

Methods and Results—Using Western blotting, we showed that DDAHI expression did not change in the normal developing lung; however, DDAHII increased after birth and reached a peak at 1 day. This was reflected in an increase in total DDAH activity according to an L-citrulline assay. With pulmonary hypertension, no changes in DDAHI expression were observed, but DDAHII expression was markedly decreased compared with age-matched controls. Total DDAH activity was similarly reduced.

Conclusions—These results indicate that each DDAH isoform is differentially regulated during both lung development and PPHN. Suppression of DDAHII isoform expression may be a mechanism underlying PPHN. (***Circulation***. 2003;107:1195-1201.)

Key Words: nitric oxide ■ hypertension, pulmonary ■ asymmetric dimethylarginine

At birth, after the transition to air breathing, pulmonary vascular resistance (PVR) decreases. The adaptations in pulmonary structure and function that optimize the ventilation/perfusion match of the lung immediately after birth continue with development[1] and are triggered by a number of factors related to parturition and the inhalation of oxygen.[2] In disease states such as persistent pulmonary hypertension of the newborn (PPHN), some of these adaptive processes are attenuated.[3]

The vasodilator nitric oxide (NO) is essential to the regulation of PVR in the fetal and neonatal lung. NO is produced from L-arginine by NO synthases (NOS), the expression and activity of which alter during pulmonary development[4,5] and with PPHN.[4,6] Indeed, low levels of NO generation are believed to be a major factor contributing to the high PVR found in utero,[7,8] and inhibition of NOS in the lungs of fetal sheep induces PPHN after birth.[9]

Asymmetric dimethylarginine (ADMA) and N^G-mono-methyl-L-arginine (L-NMMA) are naturally occurring inhibitors of NOS[10] and are found in particularly high concentrations in the fetus and in amniotic fluid (P. Vallance,

unpublished data, 2001). ADMA and L-NMMA but not the biologically inactive stereoisomer SDMA are degraded in vivo by the enzyme dimethylarginine dimethylaminohydrolase (DDAH).[11] Two isoforms of this enzyme have been characterized,[12] and by regulating ADMA and L-NMMA levels, DDAHs may modulate NOS activity.[13,14] Thus, changes in DDAH could contribute to adaptation of vascular resistance to birth and to an altered vascular reactivity observed in disease. In the present study, we sought to characterize expression of DDAH isoforms in developing lung and to test the hypothesis that DDAH activity and expression change with the development of the lung and with PPHN.

Methods

Animals
The cardiac lobe was taken from normal large white piglets (n=57, Royal Veterinary College, London, UK) in the following age groups: fetal (1 week preterm), newborn (at 5 minutes), 1 day old (12 to 24 hours), 3 days old, and juvenile/adult (14 days to 3 months). Animals were killed with an overdose of pentobarbitone (100 mg/kg), and the

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cardiac lobe was either snap-frozen and stored at −70°C until further use or taken fresh for use in the enzyme activity assay. For immunohistochemistry, lung sections were taken from the hilar region of the lung and then fixed and embedded in wax as described previously.[5] Male Sprague-Dawley rats (250 g; n=8) were killed by cervical dislocation and their lungs dissected out for use in the enzyme assay.

Pulmonary Hypertensive Piglet Age Groups

Newborn pigs were placed in a hypobaric chamber for 3 days with a continuous supply of modified cow's milk (n=12). The newborn piglets were delivered normally and placed in the chamber within 20 minutes of birth. The internal temperature was maintained at 29°C and the air pressure at 50.8 kPa. Animals placed in these chambers developed pulmonary hypertension with right ventricular hypertrophy and had a systemic arterial oxygen saturation of $71\pm5\%$ due to right-left shunting through persistent fetal channels.[15] After 3 days in the chamber, the animals were killed with an overdose of pentobarbitone (100 mg/kg), and the lungs were collected. To confirm that any differences between control animals and animals exposed to hypobaric conditions were caused by hypobaric hypoxia and not differences in environment and/or nutrition, we compared DDAH activity in the lungs of pigs that had been removed from their mother at birth, confined in a chamber under normobaric conditions, and fed cow's milk to that of piglets that remained with their mother and received mother's milk.

All animals received care in compliance with the British Home Office Regulations and the Principles of Laboratory Animal Care formulated by the National Society of Medical Research and the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (DHEW publication No. [NIH] 80-23, revised 1996, Office of Science and Health Reports, DRR/NIH, Bethesda, Md).

Northern Blotting

Total RNA was isolated from snap-frozen porcine lung tissue with TriZol reagent (Gibco BRL) according to the manufacturer's instructions. Northern blot analysis was performed with [^{32}P]-labeled human DDAHI and DDAHII cDNA probes as described previously.[12] Briefly, the RNA was separated by electrophoresis with a 1% agarose-formaldehyde gel and capillary blotted onto Hybond N+ membrane (Amersham). Hybridization to the probes was performed with Express Hyb solution (Clontech). Transcripts that hybridized to the probe were detected with a PhosphorImager (Fuji BAS 1000). The total loaded mRNA was normalized to a blot probed with an 18S ribosomal RNA cDNA fragment.

Preparation of Crude Lung Homogenate for Immunoblotting

Frozen lung (cardiac lobe) was homogenized with a polytron grinder in cold buffer solution (buffer 1) containing 50 mmol/L Tris HCl (pH 7.4), EDTA (0.1 mmol/L), EGTA (0.1 mmol/L), and 0.1% 2-mercaptoethanol with the following protease inhibitors: 1 μmol/L pepstatin A, 2 μmol/L leupeptin, and 1 mmol/L PMSF. The tissue was prepared as described previously.[4] Briefly, lung tissue from the following age groups was homogenized: fetal, newborn, 1-day-old, 3-day-old, juvenile/adult, and 3-day-old hypertensive pigs. The homogenate was then centrifuged at 150g for 10 minutes and the supernatant taken and ultracentrifuged at 100 000g for 60 minutes at 4°C. The supernatant was removed and stored at −70°C until further use. The pellet was homogenized further in buffer 1 containing 1 mol/L KCl and then centrifuged at 100 000g for 30 minutes at 4°C. The supernatant was discarded and the pellet rehomogenized in buffer 1 containing 10 mmol/L CHAPS. Samples were centrifuged at 100 000g for 30 minutes and the supernatant stored at −70°C until further use.

Immunoblotting

Proteins of equal concentration[16] were separated by SDS-polyacrylamide gel (12%) and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked for 1 hour with 5% nonfat dry milk in PBS containing 1% Tween (PBST). After blocking, the membranes were then incubated in the primary DDAHI or DDAHII anti-peptide antibody (1:1000) at 4°C overnight. Membranes were washed in PBST for 1 additional hour before incubation for 2 hours in anti-rabbit horseradish peroxidase–conjugated secondary antibody (1:3000). Membranes were washed for 1 more hour in PBST and developed with enhanced chemiluminescence substrate plus (ECL plus, Amersham). Blots were then scanned into a video image capture system (Syngene; Scientific Laboratory Supplies), and densitometry was performed with Genesnap/Gene tools (Syngene; Scientific Laboratory Supplies).

Enzyme Assay

Fresh lung samples from porcine and rat lung were homogenized in ice-cold sodium phosphate buffer (pH 6.5) and the samples centrifuged at 7000g for 5 minutes to obtain a supernatant.[11] DDAH enzyme activity was determined by measuring the formation of [^{14}C]L-citrulline from [^{14}C]L-NMMA.[17] The standard assay mixture contained 200 μmol/L LNMMA, and 100 mmol/L sodium phosphate buffer (pH 6.5) in a total volume of 0.5 mL containing 0.02 μCi [^{14}C]L-NMMA (specific activity 56 μCi · μmol^{-1}; radiolabeled at the 5-C position; final concentration of L-NMMA 100 μmol/L).

After the addition of 50 μL of the lung homogenate to 50 μL of the assay mixture, the reaction was initiated by incubation at 37°C for 1 hour. In all studies, the reaction was terminated by the addition of 1 mL of cation exchange resin (Dowex 50wx8, H+ form). After centrifugation (10 000g for 5 minutes), 200 μL of the supernatant was taken for the determination of [^{14}C]L-citrulline by β-scintillation counting.

One unit of enzyme was determined as the amount of enzyme that catalyzed the formation of 1 mole of [^{14}C]L-citrulline from [^{14}C]L-NMMA per hour at 37°C. Specific activity was expressed as units/milligram of protein. Background activity at 4°C was subtracted.

Immunohistochemistry

Five-micrometer wax sections were processed for light microscopic immunohistochemistry as described previously.[5] The sections were rehydrated and transferred to 100% methanol with 0.3% hydrogen peroxide for 30 minutes to eliminate endogenous peroxidase activity. After this time, the slides were autoclaved in 10 mmol/L citrate buffer (pH 6.2) for 11 minutes. After this, the sections were rinsed, then blocked with blocking serum (DAKO) for 30 minutes before incubation with a primary polyclonal antibody against DDAHI or DDAHII (1:50 dilution) overnight. Control sections were incubated without primary antibody. The sections were incubated with biotinylated secondary rabbit antibody raised in pig (DAKO) for 30 minutes and visualized by the streptavidin-biotinylated horseradish peroxidase complex (Amersham) for 30 minutes followed by incubation with DAB (3-3′-diaminobenzidine) peroxidase substrate (Sigma). The sections were then counterstained with Mayer's hematoxylin (BDH). Four sections from a group of 1- to 3-day-old pigs were taken, and the mean immunostaining score was noted. Each section was scored blind as follows: 0 = no staining; 1 = slightly stained; and 3 = strongly stained.

Data Analysis

Values are expressed as mean±SEM. For each investigation, 3 to 4 experiments were performed in each age group. Comparisons of DDAH mRNA and protein in the different age groups were analyzed by densitometry and standardized to the respective mRNA or protein level detected at 3 days of age because this was an age group that showed adequate levels of gene and protein expression for both isoforms. DDAH activity in porcine lung was standardized to the activity detected in an adult rat lung. Both densitometry and activity across the age groups were compared by 1-way ANOVA followed by Bonferroni test. Unpaired t tests were performed to compare hypertensive age groups with their age-matched controls. A probability value of <0.05 was considered statistically significant.
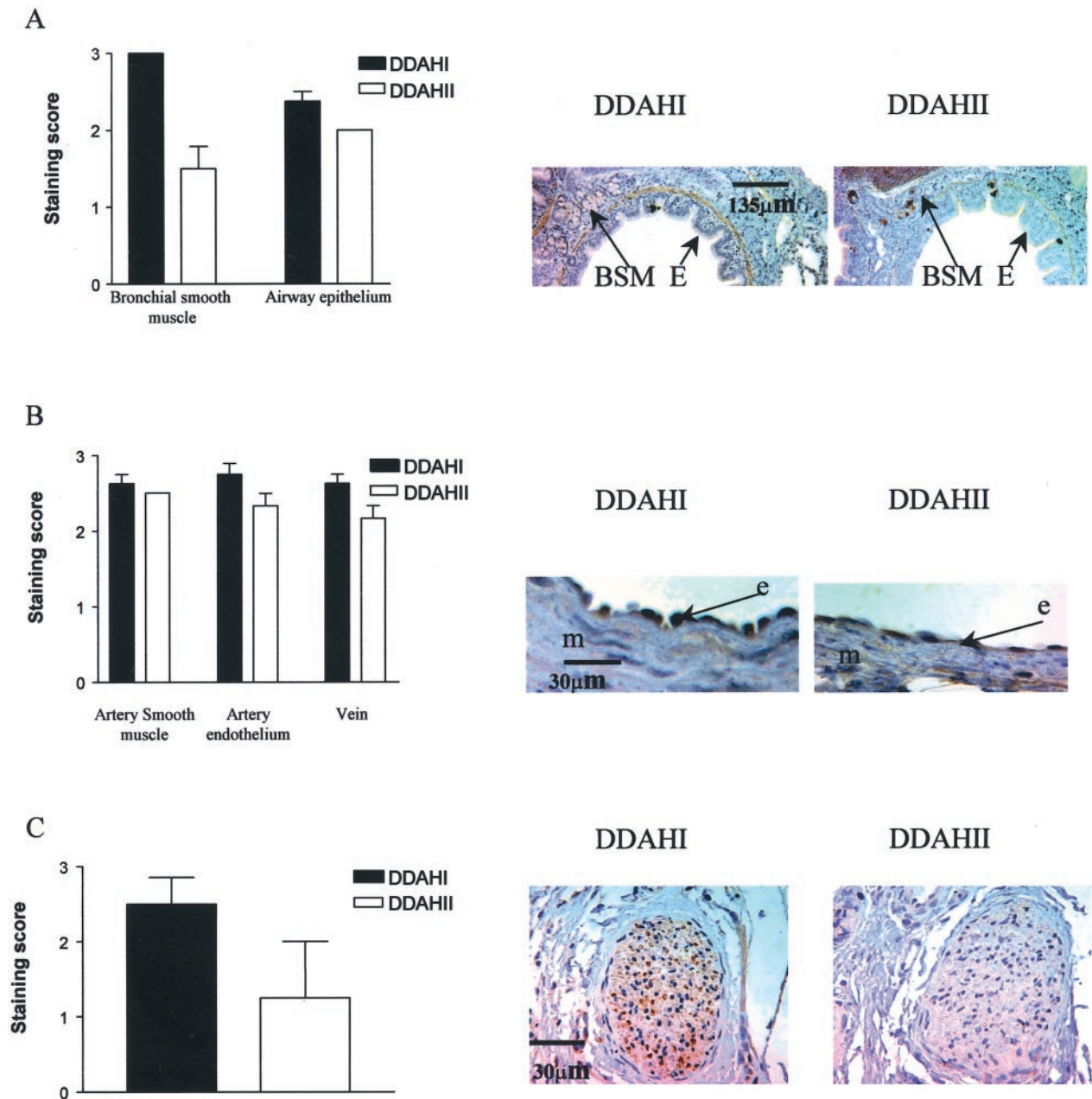
Figure 1. Light micrographs illustrating immunostaining for DDAHI and DDAHII and its corresponding immunostaining score. A, Large airways. BSM indicates bronchial smooth muscle; E, epithelium. Original magnification ×6 objective. B, Conduit arteries and veins. e indicates endothelium; m, media. Original magnification ×40 objective. C, Nerves. Original magnification ×40 objective.

Results

### Immunohistochemical Staining for DDAH Isoforms

Immunoreactivity for DDAHI and DDAHII was shown in both large and small airways and in the pulmonary vasculature. Very little difference was observed in distribution throughout the lung, although DDAHI was expressed more strongly in bronchial smooth muscle and nerves. No differences in the staining score were observed in the media and the endothelium of the pulmonary vasculature, the airway epithelium, or the smaller airways. Immunohistochemical expres-

sion of DDAHI and DDAHII in the airways, the vascular endothelium, and the nerves is shown in Figure 1.

DDAHI Expression

The DDAHI cDNA probe detected a band of mRNA at the expressed size of 4.6 kb. mRNA expression was high in fetal life, decreasing significantly in the newborn and at 1 day of age ($P<0.05$, 1-way ANOVA; Figure 2A). At 3 days of age, mRNA levels were the same as those found in the fetus, but a significant decrease in DDAHI mRNA was observed between 3 days of age and adulthood ($P<0.05$, 1-way ANOVA; Figure 2A).
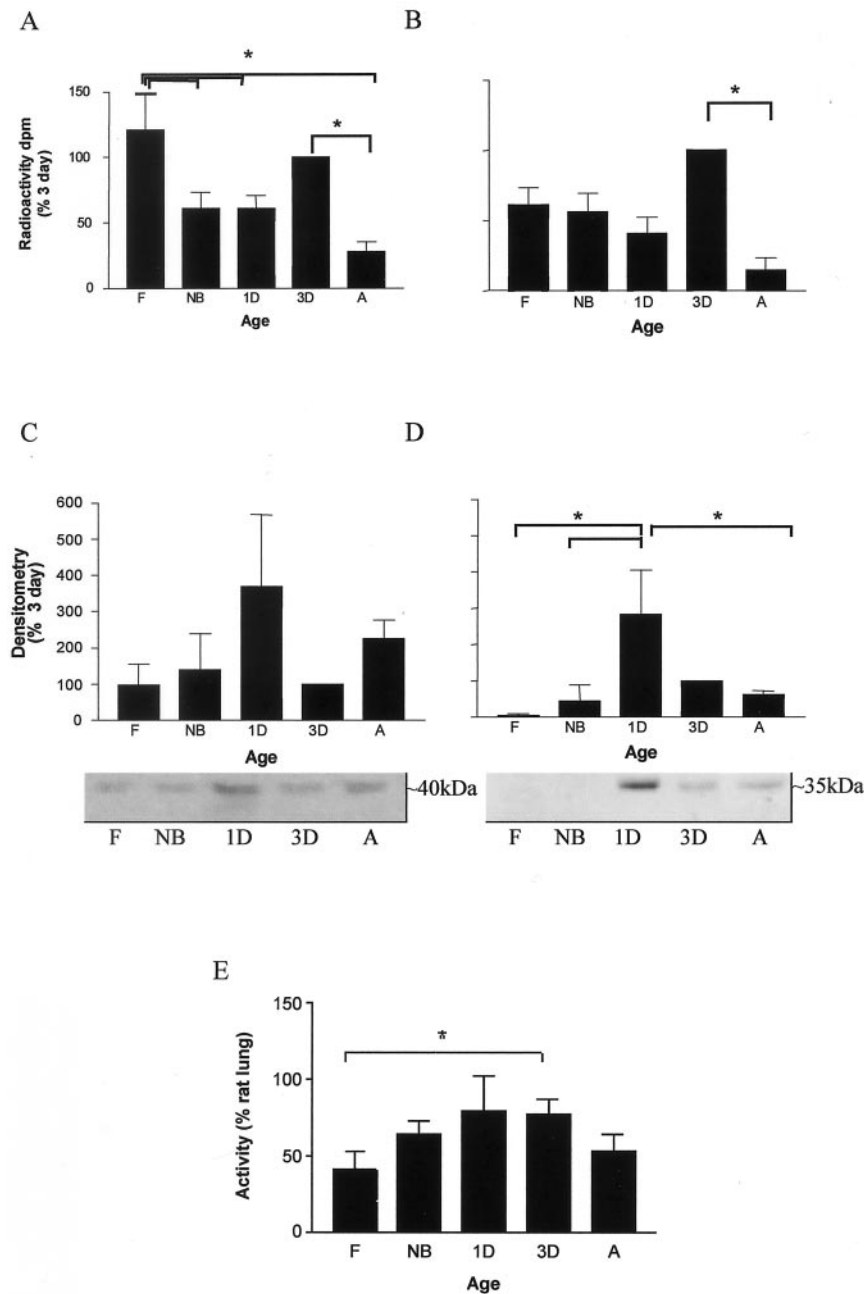
Figure 2. DDAH expression and activity in normal lung development. Taken from fetal, newborn, 1-day-old, 3-day-old, and adult age groups. mRNA of DDAHI (A) and DDAHII (B) shown after PhosphorImager analysis of Northern blots. Measurement of DDAH mRNA was first normalized to 18S and then presented as the percentage of mRNA found in lungs at 3 days of age. DDAHI (C) and DDAHII (D) protein expression is represented as Western blots and a bar chart showing densitometric scanning analysis. Values are expressed as percentage of expression found at 3 days of age. DDAH activity (E) is presented as percentage of activity found in adult rat lung. *$P<0.05$ represents significant difference between age groups. Values are mean±SEM and represent 4 to 6 animals. Lanes: F, fetus; NB, newborn; 1D, 1 day; 3D, 3 day; A, juvenile/adult.

DDAHI antiserum detected an ≈38-kDa band of protein in the soluble fractions of lung homogenates. No expression was found in the particulate fraction of the lung (data not shown). Expression of DDAHI protein did not significantly change with age, although at 1 day of age, protein expression tended to increase (Figure 2C).
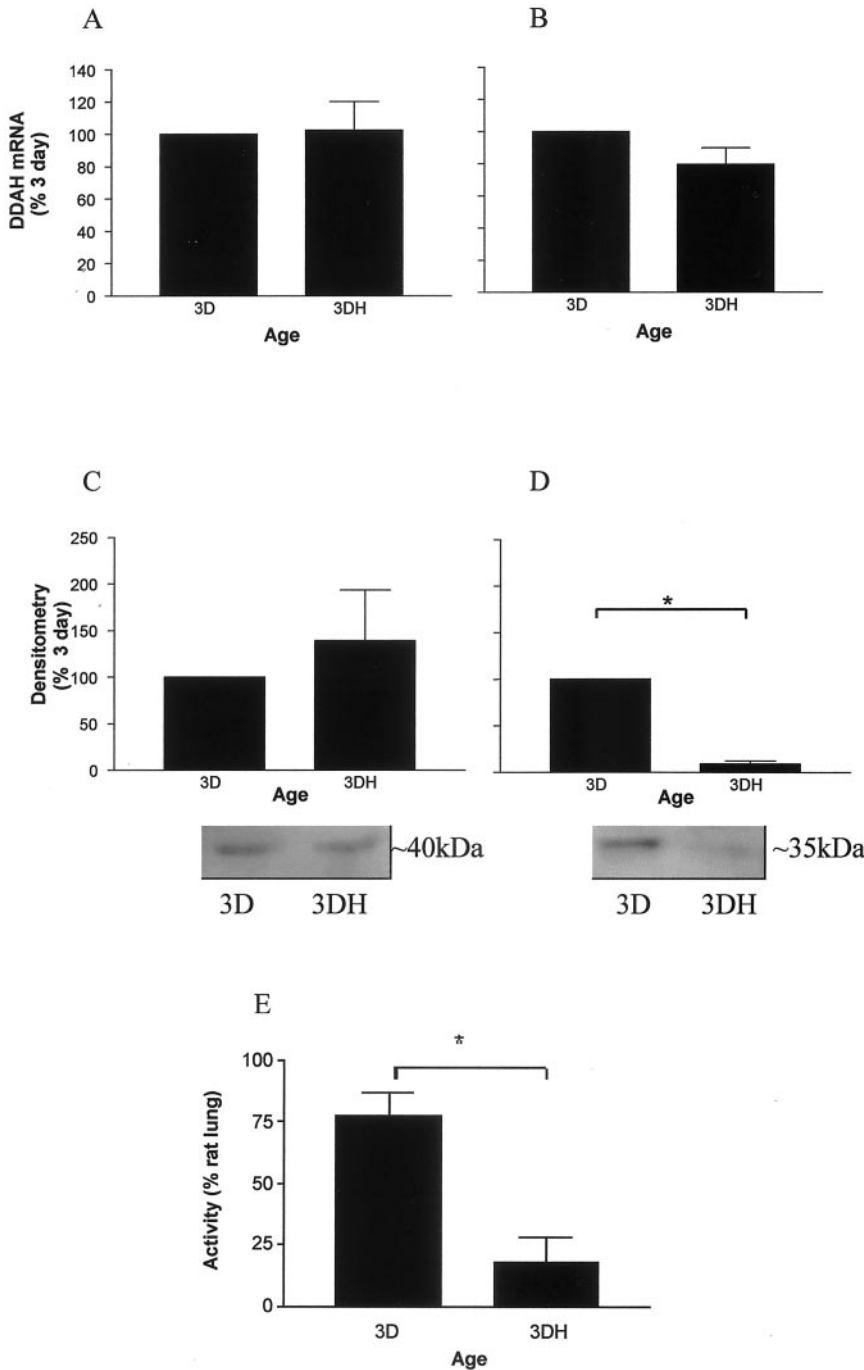
DDAHII Expression

The DDAHII cDNA probe detected a band of mRNA at the expressed size of 2.0 kb. Unlike DDAHI, there was no change immediately after birth, but at 3 days of age, DDAHII mRNA increased significantly ($P<0.05$, 1-way ANOVA; Figure 2B). mRNA tended to decrease between fetal and adult age groups, although this did not reach significance ($P=$NS, 1-way ANOVA; Figure 2B).

DDAHII antiserum detected an ≈35-kDa band in the soluble fraction of the cell. No immunoreactivity was observed in the particulate fraction of the cell.

DDAHII protein was expressed at a low level in samples from both the fetal and newborn age groups, significantly increasing at 1 day of age ($P<0.05$, 1-way ANOVA; Figure 2D). By 3 days of age, protein levels had decreased to those found at birth, and these were maintained with age (Figure 2D).

Persistent Pulmonary Hypertension of the Newborn

The mRNA of both DDAH isoforms in lungs from 3-day-old animals exposed to chronic hypoxia was similar to that found in the normal animal at 3 days of age (Figure 3A and B). Compared with their age-matched controls, protein expres-

Figure 3. DDAH expression and activity in lung taken from hypertensive age groups compared with age-matched controls. Taken from 3-day-old (3D) and 3-day-old hypertensive (3DH) age groups. mRNA of DDAHI (A) and DDAHII (B) shown after PhosphorImager analysis of Northern blots. Measurement of DDAH mRNA was first normalized to 18S and then presented as percentage of mRNA found in lungs at 3 days of age. DDAHI (C) and DDAHII (D) protein expression is represented as Western blots and bar chart showing densitometric scanning analysis. Values are expressed as percentage of expression found at 3 days of age. DDAH activity (E) is presented as percentage of activity found in adult rat lung. *$P<0.05$ represents significant difference between age groups. Values are mean±SEM and represent 4 to 6 animals.

sion of DDAHI did not change in the hypertensive age groups; however, expression of DDAHII significantly decreased with pulmonary hypertension ($P<0.05$, unpaired *t* test; Figure 3D).

DDAH Activity
DDAH activity was lowest in the fetal age group, increasing to a maximum at 1 day of age, reaching significance by 3 days of age ($P<0.05$, 1-way ANOVA; Figure 2E). A significant decrease in activity was observed in the hypertensive age group compared with their age-matched controls ($P<0.05$, unpaired *t* test; Figure 3E). That this decrease in activity results from exposure to hypobaric hypoxia is sup-

ported by the observation that DDAH activity in the lungs of animals that were removed from the mother at birth, confined in a chamber, and fed cow's milk under normobaric conditions was indistinguishable from age-matched controls (36.3 pmol ^{14}C-citrulline per mg^{-1} per h^{-1} versus 34.2 pmol ^{14}C-citrulline per mg^{-1} per h^{-1}, n=2).

Discussion
The results of this study demonstrate the expression of both DDAH isoforms in the lung. DDAHI is more prominent in nerves and bronchial smooth muscle, and both isoforms are expressed in vascular endothelium, smooth muscle, and airway epithelium. There is a substantial increase in DDAH

activity in the whole lung 24 hours after birth, and this would be expected to reduce tissue concentrations of NOS inhibitors and thereby increase NO generation. In animals exposed to hypobaric hypoxia to mimic PPHN, DDAH activity was markedly suppressed. These findings suggest that DDAH isoforms are developmentally regulated in the lung and could contribute to pulmonary vascular adaptation and to the dysfunction in vascular reactivity that occurs with PPHN.

Previous work in our laboratory has shown expression of DDAH mRNA within the lung,[18] and this has been confirmed in the present study, with the additional demonstration of pulmonary expression of DDAH protein and activity. The expression of both DDAH proteins was widespread, with considerable overlap between isoform localization, and reasons for the expression of both isoforms in the same cell type are unknown. DDAH protein was expressed in endothelial cells, smooth muscle cells, nerves, and airways themselves, and consistent with our previous findings, DDAHI predominated in nerves.[18] Previous reports have demonstrated that DDAHI has an expression pattern similar to that of neuronal NOS, whereas DDAHII has an expression pattern similar to that of endothelial NOS (eNOS).[12] The present findings support that observation but indicate that at least in the lung, DDAHI is also highly expressed in vascular tissue.

In addition, we have shown an alteration in DDAH mRNA and protein expression with lung development, although mRNA levels were a very poor marker of protein expression. This finding suggests that distribution of DDAH mRNA in some situations, such as during development, may not be a useful way to study DDAH isoform distribution and activity. The observation that DDAH protein levels change independently of changes in mRNA levels may indicate that DDAH expression can be regulated posttranscriptionally in some situations.

DDAH protein and activity transiently increased at 1 day of age and decreased again by adulthood. This pattern was seen for total activity and for expression of both DDAH isoforms but was most marked for DDAHII. Previous work in this porcine model has shown that eNOS activity is low in the fetal lung,[4] and there is an absence of vasorelaxation to acetylcholine.[19] By 1 day of age, relaxation to acetylcholine appears with a corresponding increase in NOS activity from fetal levels.[4,19] The finding that DDAH activity and expression also increase at this point may suggest that metabolism of endogenous NOS inhibitors facilitates the NO generation and increased NOS activity, but additional studies are required to test this directly. The stimuli that cause the transient posttranscriptional upregulation of DDAH at 1 day of age remain to be determined. In the developing lung of older animals, DDAH activity and expression decreased, whereas NOS activity was maintained, which possibly suggests a smaller role for ADMA metabolism with increasing age. Alternatively, because ADMA and L-NMMA are generated as a consequence of increased protein turnover, it may be that production of methylarginines declines with age, and DDAH expression decreases accordingly.

PPHN has been described as the attenuation of pulmonary development,[20] and in our porcine model of pulmonary hypertension induced by hypobaric hypoxia, it manifests as the maintenance of a fetal state within the lung.[3] In the present study, mRNA levels of both DDAH isoforms were maintained in PPHN. However, protein expression of the DDAHII isoform decreased compared with age-matched controls, as did DDAH activity. We have shown previously that the hypertensive age group has absent relaxant responses to acetylcholine and low eNOS activity[4] in this model. The striking changes in DDAHII expression and activity would be consistent with a rise in ADMA levels and NOS inhibition. Indeed, pharmacological inhibition of DDAH produces vasoconstriction and reduces NO generation.[17] Although we did not measure plasma ADMA levels in the present study, ADMA levels are elevated in adults with pulmonary hypertension,[21] and the present study suggests decreased DDAH expression may contribute to increased plasma ADMA concentrations. Alternatively, reduced DDAH activity may cause a local increase in ADMA without changes in circulating levels. Further studies would be required to test these hypotheses directly. The level of DDAHII expression and overall DDAH activity in the hypertensive lung was similar to that seen in fetal lung, which again suggests that persistence of the fetal state underlies PPHN. The observation that ADMA levels are particularly high in amniotic fluid (P. Vallance, unpublished data, 2001) would be consistent with tonic inhibition of NOS before birth. As DDAHII is induced, the levels of ADMA would fall.

These studies have shown the diverse distributions of DDAHI and DDAHII in the lung, with differential expression of both isoforms occurring with development. Furthermore, they have shown that in our model of pulmonary hypertension, DDAHII expression and activity are substantially reduced. They may explain why NOS activity is reduced in PPHN and why ADMA levels are elevated in individuals with PPHN.

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