

# Voltage-Dependent Anion Channel-2 Interaction with Nitric Oxide Synthase Enhances Pulmonary Artery Endothelial Cell Nitric Oxide Production

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Increased pulmonary artery endothelial cell (PAEC) endothelium-dependent nitric oxide synthase (eNOS) activity mediates perinatal pulmonary vasodilation. Compromised eNOS activity is central to the pathogenesis of persistent pulmonary hypertension of the newborn (PPHN). Voltage-derived anion channel (VDAC)-1 was recently demonstrated to bind eNOS in the systemic circulation. We hypothesized that VDAC isoforms modulate eNOS activity in the pulmonary circulation, and that decreased VDAC expression contributes to PPHN. In PAECs derived from an ovine model of PPHN: (1) there is eNOS activity, but not expression; and (2) VDAC1 and -2 proteins are decreased. Immunocytochemistry, coimmunoprecipitation, and *in situ* proximity ligation assays in human PAECs (hPAECs) demonstrate binding between eNOS and both VDAC1 and -2, which increased upon stimulation with NO agonists. The ability of agonists to increase the eNOS/VDAC interaction was significantly blunted in hypertensive, compared with normotensive, ovine PAECs. Depletion of VDAC2, but not VDAC1, blocked the agonist-induced increase in eNOS activity in hPAECs. Overexpression of VDAC2 in hypertensive PAECs increased eNOS activity. Binding of VDAC2 enhances eNOS activity in the pulmonary circulation, and diminished VDAC2 constrains eNOS in PAECs derived from fetal lambs with chronic intrauterine pulmonary hypertension. We speculate that decreases in VDAC2 may contribute to the limited eNOS activity that characterizes pulmonary hypertension.

**Keywords:** pulmonary hypertension; vasodilation; fetal; protein-protein interactions

At birth, the pulmonary vasculature dilates in response to ventilation, oxygenation, and an increase in shear stress (1–4). Each of these stimuli acts, to a significant degree, through an increase in endothelium-dependent nitric oxide synthase (eNOS) activity and elaboration of nitric oxide (NO) (5). NO, in turn, causes activation of pulmonary artery smooth muscle cell soluble guanylate cyclase (6) and subsequent vasodilation through activation of a calcium-sensitive K<sup>+</sup> channel (7). NO production from pulmonary artery endothelial cells (PAECs) is essential for

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## CLINICAL RELEVANCE

The present data identify a novel interaction of the voltage-dependent anion channel (VDAC)-2 and endothelium-dependent nitric oxide synthase (eNOS) in the pulmonary endothelium that serves to modulate NOS activity in response to physiologic stimuli. This observation likely has implications for both the normal and pathologic pulmonary circulation, as in a model in persistent pulmonary hypertension of the newborn, the VDAC2–eNOS interaction and NOS activity are constrained. Repression of VDAC2 may play an etiologic role in diseases characterized by elevation of pulmonary vascular tone, and VDAC2 may represent a novel therapeutic target for the treatment of pulmonary hypertension.

the successful transition from fetal to air-breathing life (5, 8). In the absence of sufficient NO production, pulmonary vascular resistance remains elevated and blood is shunted away from the lungs, resulting in severe central hypoxemia and a syndrome called persistent pulmonary hypertension of the newborn (PPHN), a significant cause of neonatal morbidity and mortality (9).

Clinical and experimental evidence demonstrates that impaired eNOS activity contributes to the pathogenesis of PPHN. However, the molecular mechanisms leading to insufficient NO production are incompletely understood. In PPHN, constrained NO production may result not only from diminished eNOS expression (10, 11), but also from modulation of eNOS activity by posttranslational modifications, protein-protein interactions, and sequestration into subcellular compartments. For example, myristoylation and palmitoylation enhance eNOS activity by increasing eNOS localization within the caveolae (12). Location of eNOS within the plasma membrane enhances NO production in response to physiologic stimuli (13). Whereas physical interaction with caveolin-1 decreases eNOS activity, interaction with other proteins, such as the molecular chaperone, heat shock protein 90, enhances eNOS activity (14). In a lamb model of PPHN, decreased association of heat shock protein 90 with eNOS is one mechanism leading to attenuated NO production (15). Recent data from our laboratory demonstrate that Rho kinase is an additional factor that constrains eNOS activity in the neonatal pulmonary circulation (16).

Voltage-dependent anion channels (VDACs) are a family of small, pore-forming proteins of the mitochondrial outer membrane and represent the primary route whereby molecules, such as adenosine triphosphate, phosphocreatine, and calcium, traffic between cytosolic and mitochondrial compartments (17, 18). In addition to a role in cellular energy metabolism, VDAC molecules play an essential role in apoptosis (19), and via direct interactions modulate the function of other molecules, including the estrogen receptor- $\alpha$ , and the  $\gamma$ -aminobutyric acid type A receptor (20). Although functional overlap between each of the three VDAC

isoforms likely exists, deletion of VDAC2 results in embryonic lethality (21), whereas deletion of either VDAC1 or -3 results in viable, but distinct, phenotypes (22, 23).

Although VDAC isoforms were initially described to reside exclusively within the mitochondria, recent evidence has shown that, in the case of VDAC1, an alternate splice variant containing a unique N-terminal sequence allows for trafficking through the Golgi into the plasma membrane (24). The observation that VDAC molecules traffic through the cell suggests a broader functional role. In support of this notion, VDAC1 has been identified as a novel binding partner for eNOS in the systemic circulation, with a physical interaction that modulates eNOS activity (25). However, whether physical association between VDAC isoforms and eNOS occurs in the pulmonary circulation, and if dysregulation of this interaction contributes to the pathobiology of pulmonary hypertension, is not known.

In the present report, we demonstrate that eNOS activity is impaired in hypertensive (HTN) compared with normotensive (Norm) fetal PAECs, in the absence of significant alterations in eNOS expression. However, protein levels of VDAC isoforms 1 and 2 were significantly lower in HTN compared with Norm PAECs. Using human PAECs (hPAECs), we demonstrate that both VDAC1 and -2 colocalize with eNOS. Furthermore, by coimmunoprecipitation and *in situ* proximity ligation assays, we demonstrate that both VDAC1 and -2 bind eNOS in hPAECs, and that this interaction is markedly enhanced upon stimulation with NO agonists. A similar pattern of colocalization was observed in the fetal ovine PAECs, and although stimulation with NO agonists increased the interaction between VDAC1 and -2 with eNOS in Norm PAECs, the agonist-induced increase in interaction was blunted in HTN PAECs. Silencing of VDAC1 and -2 in hPAECs demonstrated that VDAC2 is the key modulator of eNOS activity in the pulmonary circulation. Finally, overexpression of VDAC2 in HTN fetal ovine PAECs increased NOS activity upon agonist stimulation.

## MATERIALS AND METHODS

Please see the online supplement for full methods.

### Chronic Intrauterine Pulmonary Hypertension Model

Surgical ligation of the ductus arteriosus of fetal lambs was performed, as previously described as an established model of persistent pulmonary hypertension (26, 27). The procedure was approved by the Animal Care and Use Committee at the University of Minnesota Medical School.

### Cell Culture

Distal ( $\geq 4$ th generation) pulmonary arteries were excised and FPAECs were isolated from late-gestation fetal sheep ( $\sim 141$  d), as previously described (28). hPAECs (Lonza, Basel, Switzerland) were cultured in EGM-2 media (Lonza).

### Quantitative Real-Time PCR

Total RNA was isolated from PAECs, and reverse transcribed. Quantitative real-time PCR was performed using the SYBR Green PCR mastermix on the ABI Real-Time PCR Detection System (Applied Biosystems, Carlsbad, CA). The primer sequences are detailed in the online supplement.

### Protein Isolation and Immunoblotting

Whole-cell lysates were extracted from PAECs and analyzed by SDS-PAGE and Western immunoblot analysis. Band intensities were quantified using a Bio-Rad Image System (Bio-Rad, Foster City, CA).

### RNA Interference

hPAECs were transfected with either VDAC1 or -2 On-Target Plus SMART pool siRNA, or siGenome Non-Targeting siRNA as a control (Dharmacon, Waltham, MA) using Lipofectamine 2,000 (Invitrogen, Carlsbad, CA).

### NOS Activity in Endothelial Cells

NOS activity in intact human and fetal ovine PAECs was determined by measuring the conversion of L-[ $^{14}$ C]Arginine to L-[ $^{14}$ C]Citrulline using a method modified from Schmidt and Mayer (29).

### Immunocytochemistry

hPAECs and fetal ovine PAECs were fixed with 90% ethanol, permeabilized, and incubated with primary antibodies against either VDAC1 and -2 (Abcam, Cambridge, MA) and eNOS (BD-Biosciences, Franklin Lakes, NJ) overnight at 4°C, followed by incubation with goat anti-mouse Alexa 488 and donkey anti-goat Alexa 568 secondary antibodies (Invitrogen/Molecular Probes, Carlsbad, CA).

### Coimmunoprecipitation

Subconfluent hPAECs were lysed and the lysates incubated with either anti-eNOS IgG1 or isotype control (mouse IgG1). Goat anti-mouse-coated Dynabeads (Invitrogen) were then added, and the mixture incubated for 10 minutes at 4°C, and the beads removed by magnetic separation and washed with CHAPS lysis buffer. The washed beads were boiled with 5 $\times$  Laemmli sample buffer and analyzed by immunoblot.

### In Situ Proximity Ligation Assay

Physical interaction between eNOS and VDAC was assessed in PAECs by *in situ* proximity ligation assay (PLA) using the DuoLink kit (OLINK Bioscience, Uppsala, SWE) and antibodies specific for VDAC1 or -2 (Abcam) and eNOS (BD-Biosciences), per the manufacturers' protocol. Images were quantified by measuring the total integrated intensity of the PLA signal versus the total cell area per field using Metamorph Image analysis software (Molecular Devices, Sunnyvale, CA).

### Overexpression of VDAC2

HTN ovine PAECs were transfected with either control-green fluorescent probe (GFP) or VDAC2-GFP plasmids (OriGene, Rockville, MD) by electroporation.

### Statistical Analysis

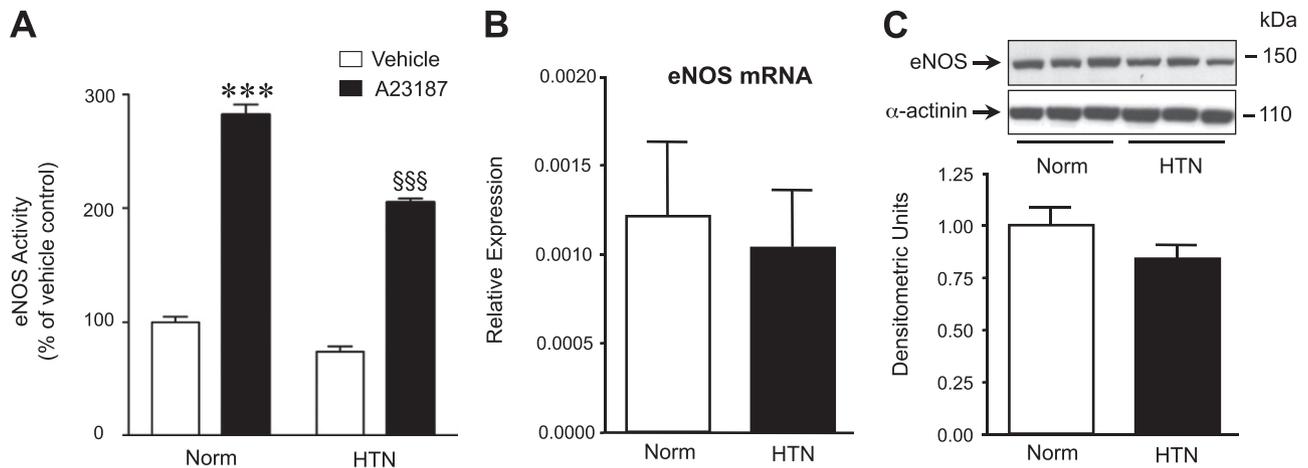
Results are presented as means ( $\pm$ SE). Statistical significance between two groups was tested with Student's *t* test, and between multiple groups by either one or two-way ANOVA, as appropriate, followed by Bonferroni's *post hoc* analysis. A *P* value of less than 0.05 was considered to be statistically significant.

## RESULTS

### Chronic Intrauterine Hypertension Decreases eNOS Activity but Not Expression

To determine the effect of chronic intrauterine hypertension on endothelial NO production, monolayers of PAECs derived from either sham-operated (Norm) or HTN fetal lambs were treated with the calcium-ionophore, A23187, a known NO agonist. Basal NOS activity was lower in HTN versus Norm PAECs ( $P < 0.01$ ). A23187 stimulation increased NOS activity in both Norm ( $***P < 0.001$  versus vehicle) and HTN PAECs ( $§§§P < 0.001$  versus vehicle and versus A23187-stimulated Norm) (Figure 1A); however, the percent increase in NOS activity over baseline induced by A23187 was significantly less in HTN ( $105.1 \pm 3.22\%$ ) as compared with Norm ( $182.2 \pm 9.1\%$ ) PAECs ( $P = 0.0013$ ).

We next determined whether this attenuated response in the HTN cells was due to decreased expression of eNOS. Quantitative real-time PCR and Western immunoblot were used to determine eNOS levels in PAECs derived from Norm and HTN fetal sheep. There was no significant difference in either eNOS gene (Figure 1B) or protein (Figure 1C) expression between the two groups. This observation suggests that the diminished NOS activity in



**Figure 1.** Chronic intrauterine hypertension decreases nitric oxide synthase (NOS) activity in pulmonary artery endothelial cells (PAECs) without changing eNOS expression. (A) PAECs isolated from normotensive (Norm) and hypertensive (HTN) fetal sheep were treated with either vehicle or the NO agonist, A23187 (2  $\mu$ M), and NOS activity was determined by conversion of L-arginine to L-citrulline at 30 minutes (\*\*\*)  $P < 0.001$  in Norm A23187-stimulated versus vehicle Norm, and  $SSS P < 0.001$  in A23187-stimulated HTN versus vehicle HTN, and versus A23187-stimulated Norm PAECs;  $n = 3$ ). Data expressed as percent increase in NOS activity from baseline in Norm and HTN PAECs in response to A23187 treatment. (B) Quantitative RT-PCR (qRT-PCR) was performed and the absolute copy number of eNOS was normalized to 18S rRNA to determine relative expression. There was no difference in eNOS expression between Norm and HTN PAEC. (C) *Top panel:* Western immunoblot to determine the level of eNOS protein expression in Norm versus HTN fetal sheep. *Bottom panel:* Summary of densitometry data with eNOS protein expression normalized to  $\alpha$ -actinin ( $n = 3$  PAEC isolates from three separate animals).

PAECs from lambs with chronic intrauterine hypertension results from decreases in enzymatic activity rather than changes in expression.

#### Chronic Intrauterine Hypertension Decreases Expression of VDAC1 and -2

Recent evidence suggests that the VDAC1 can physically bind eNOS and enhance its activity in systemic endothelial cells (25). To determine if abnormalities in VDAC expression contribute to the diminished NO activity observed in our model of PPHN, we next measured the expression of the three main VDAC isoforms in PAECs derived from Norm and HTN fetal sheep. By quantitative real-time PCR (qRT-PCR), VDAC2 was found to be the most abundant isoform in Norm PAECs (Figure 2B), with a level of expression approximately 50-fold greater than either VDAC1 (Figure 2A) or VDAC3 (data not shown). Although the gene expression of these less abundant isoforms, VDAC1 and -3, did not differ significantly between Norm and HTN PAECs (Figure 2A), VDAC2 gene expression was decreased by 58.8 ( $\pm 7$ )% in HTN PAECs as compared with Norm PAECs (Figure 2B;  $*P = 0.038$ ).

VDAC1 protein expression was decreased in the HTN fetal PAECs as compared with Norm control (Figure 2C;  $*P = 0.0254$ ). Moreover, protein levels of the more abundant isoform, VDAC2, demonstrated an even greater decrease in the HTN as compared with Norm PAECs (Figure 2D). Consistent with the gene expression analysis, there was no difference in VDAC3 protein expression between Norm and HTN fetal PAECs (data not shown).

#### VDAC1 and -2 Physically Interact with eNOS in PAECs

In the systemic circulation, VDAC1 modulates NO production by physically interacting with eNOS (25). To determine if VDAC isoforms physically interact with eNOS in the pulmonary circulation, we first performed immunocytochemistry on hPAECs to detect eNOS, in combination with either VDAC1 or -2. hPAECs were immunostained with antibodies against either VDAC1 or -2 (red), resulting in a punctate, perinuclear staining pattern for both

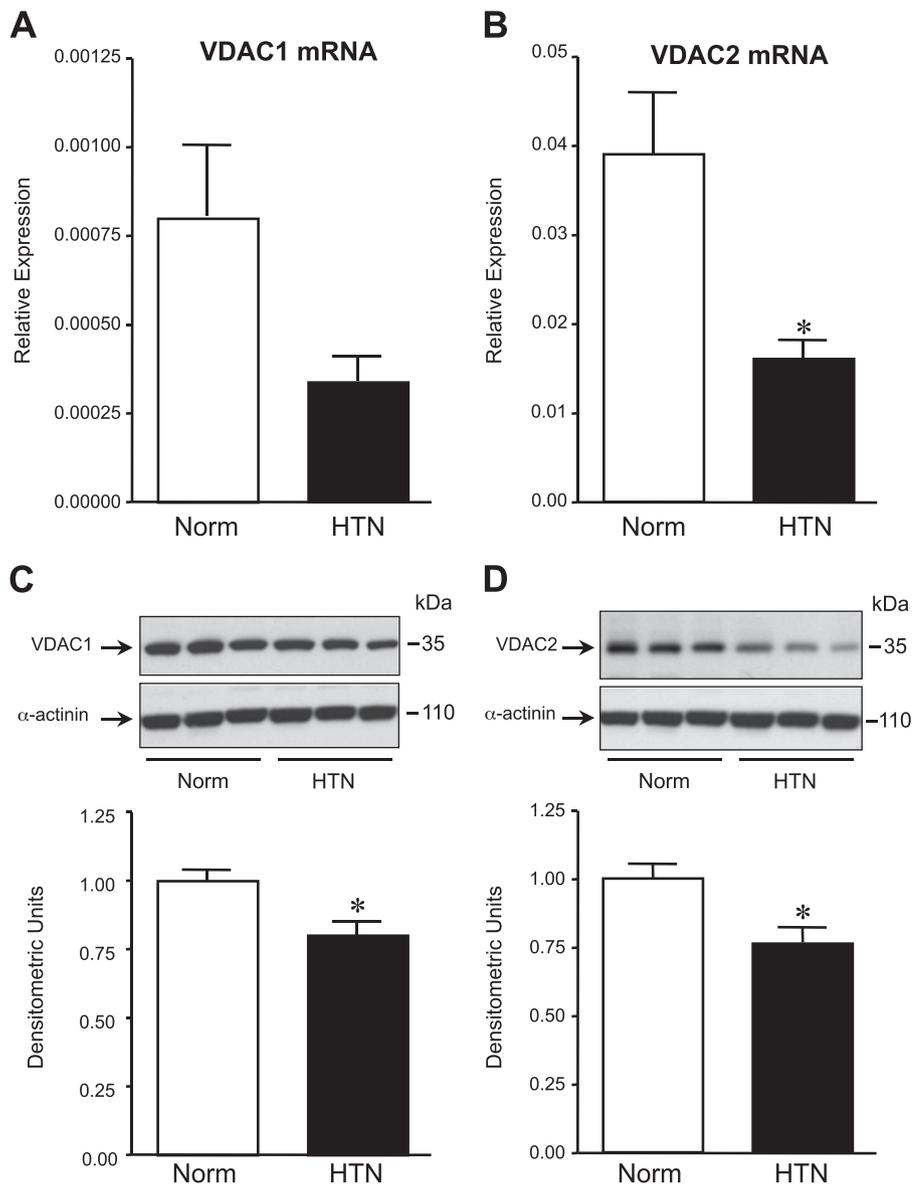
isoforms (Figures 3A and 3B). Incubation of the PAECs with antibodies against eNOS (green) resulted in staining of the plasma membrane and a semilunar, perinuclear pattern consistent with localization within either the Golgi or mitochondria. Composite images demonstrated that both VDAC1 and -2 isoforms colocalize (yellow) with eNOS, with the strongest colocalizing signal noted in a perinuclear pattern.

To demonstrate conclusively whether eNOS was physically interacting with either VDAC isoform, we next performed coimmunoprecipitation experiments and *in situ* PLA in hPAECs. Lysates from hPAECs under basal conditions were immunoprecipitated with an anti-eNOS antibody, and then subsequently immunoblotted with antibodies to eNOS, VDAC1, and VDAC2. The eNOS antibody successfully, and specifically, immunoprecipitated eNOS from hPAEC lysates (Figure 4A, top row). Aggregate densitometric data from multiple coimmunoprecipitations were normalized to immunoprecipitations performed with the isotype control (mouse IgG1) antibody, and summarized in Figure 4B. Relative to the isotype control, the antibody to eNOS coprecipitated with VDAC1 by 151 ( $\pm 31$ )% (Figures 4A and 4B;  $**P < 0.01$ ) and VDAC2 by 166 ( $\pm 12$ )% (Figures 4A and 4B;  $**P < 0.01$ ).

Next, as further confirmation that both VDAC isoforms 1 and 2 physically interact with eNOS, we performed *in situ* PLA, a highly sensitive assay able to detect protein-protein interactions in unmodified cells. Assays were performed on the hPAECs under basal conditions, and also after stimulation with two NO agonists, A23187, and histamine. By this method, physical interaction of VDAC1 and eNOS was confirmed. The interaction between VDAC2 and eNOS was even greater (Figure 4C). Furthermore, stimulation of the hPAECs with either A23187 or histamine resulted in a significant increase in the interaction between both eNOS and VDAC1, as well as eNOS and VDAC2.

#### Chronic Intrauterine Hypertension Decreases the Interaction of eNOS with VDAC Isoforms 1 and 2

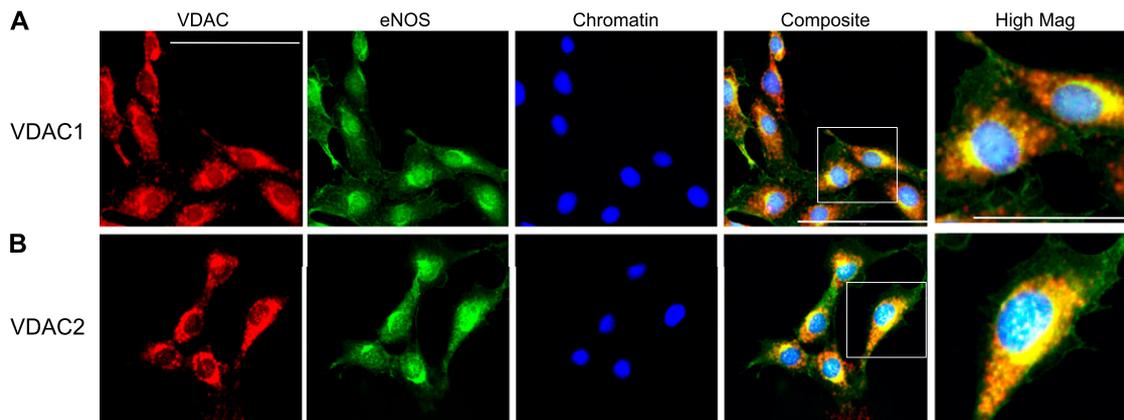
To determine whether chronic intrauterine hypertension modulates the interaction between eNOS and VDAC molecules, we first



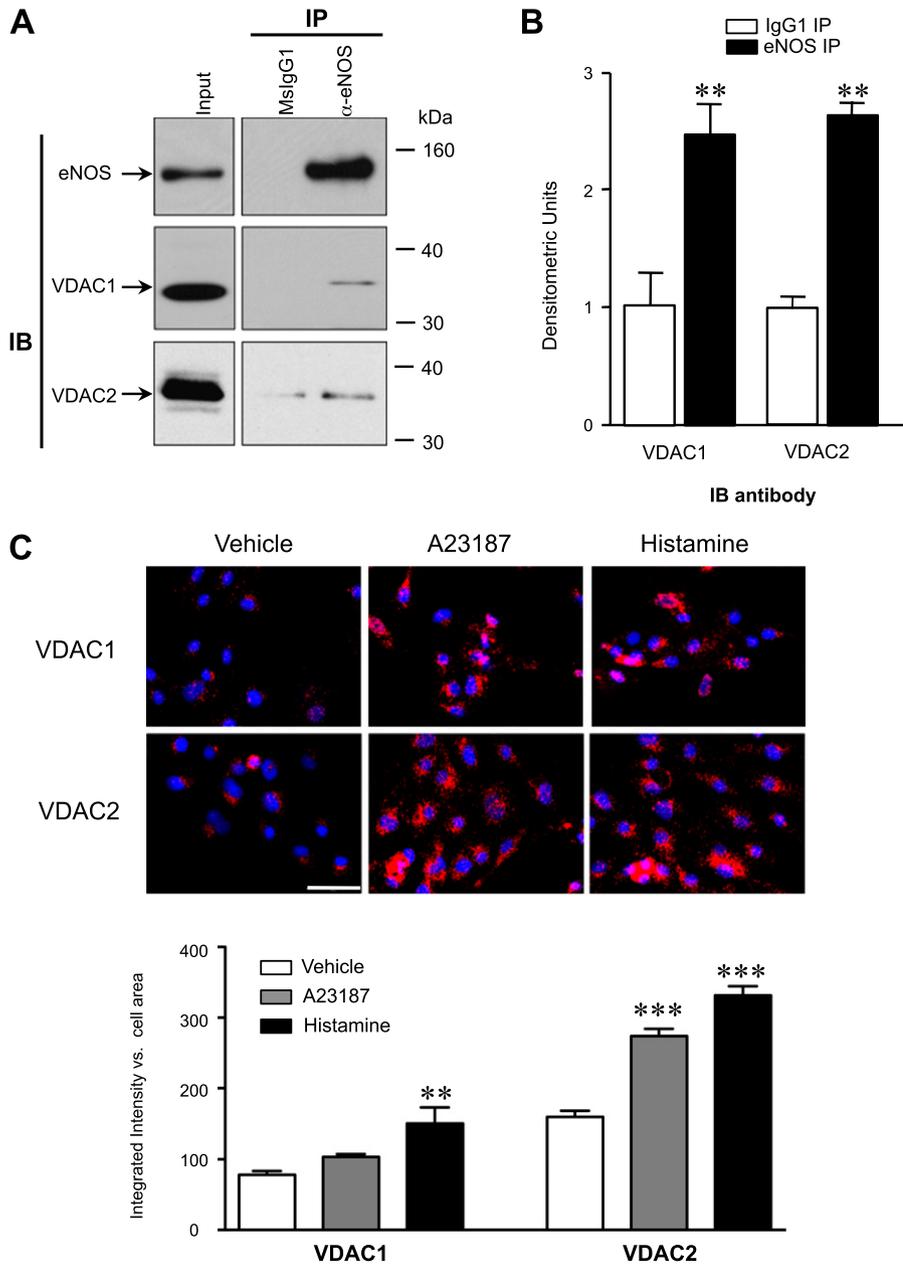
**Figure 2.** Chronic intrauterine hypertension decreases expression of VDAC isoforms 1 and 2. qRT-PCR was performed and the absolute copy number of VDAC1 (A) and VDAC2 (B) was normalized to 18S rRNA to determine relative expression in Norm versus HTN fetal ovine PAECs. Western immunoblot was to determine the level of VDAC1 (C) and VDAC2 (D) protein in Norm versus HTN fetal ovine PAECs, with VDAC1 and -2 protein expression normalized to  $\alpha$ -actinin (\* $P < 0.05$ , with  $n = 5$  and  $n = 3$  PAEC isolates from Norm and HTN animals, respectively).

confirmed that both VDAC1 and -2 colocalize with eNOS in PAECs obtained from Norm and HTN fetal sheep. Similar to the results obtained from the human cells, both VDAC isoforms

1 (Figure 5A) and 2 (Figure 5B) demonstrated significant colocalization with eNOS under basal conditions, with the strongest signal of colocalization occurring in a semilunar pattern consistent



**Figure 3.** eNOS colocalizes with VDAC1 and -2 in human PAECs (hPAECs). Representative immunocytochemistry on hPAECs to detect VDAC1 (A) or VDAC2 (B) and eNOS. Incubation of hPAECs with anti-VDAC antibody (red) and eNOS (green) demonstrate significant colocalization of both VDAC isoforms with eNOS, particularly in a perinuclear distribution. Insets demonstrate higher magnification images. Calibration mark = 100  $\mu$ m in all images.



**Figure 4.** eNOS physically interacts with VDAC1 and -2 in hPAECs, and enhanced interaction occurs with stimulation by NO agonists. (A) Representative coimmunoprecipitations performed with either mouse IgG1 or anti-eNOS as the immunoprecipitating antibody, and immunoblotted with anti-eNOS, -VDAC1, or -VDAC2 antibodies. (B) Summary of densitometric data from replicate coimmunoprecipitations. Data are normalized to immunoprecipitations performed with the isotype control (mouse IgG1) (\*\* $P < 0.01$ ,  $n = 4-7$ ). (C) Representative images from *in situ* proximity ligation assays performed to detect the interaction of eNOS and either VDAC1 or -2 in hPAECs under basal conditions, and after stimulation with either A23187 or histamine. Calibration mark = 50  $\mu\text{m}$ . \*\* $P < 0.01$  versus vehicle; \*\*\* $P < 0.001$  versus vehicle.

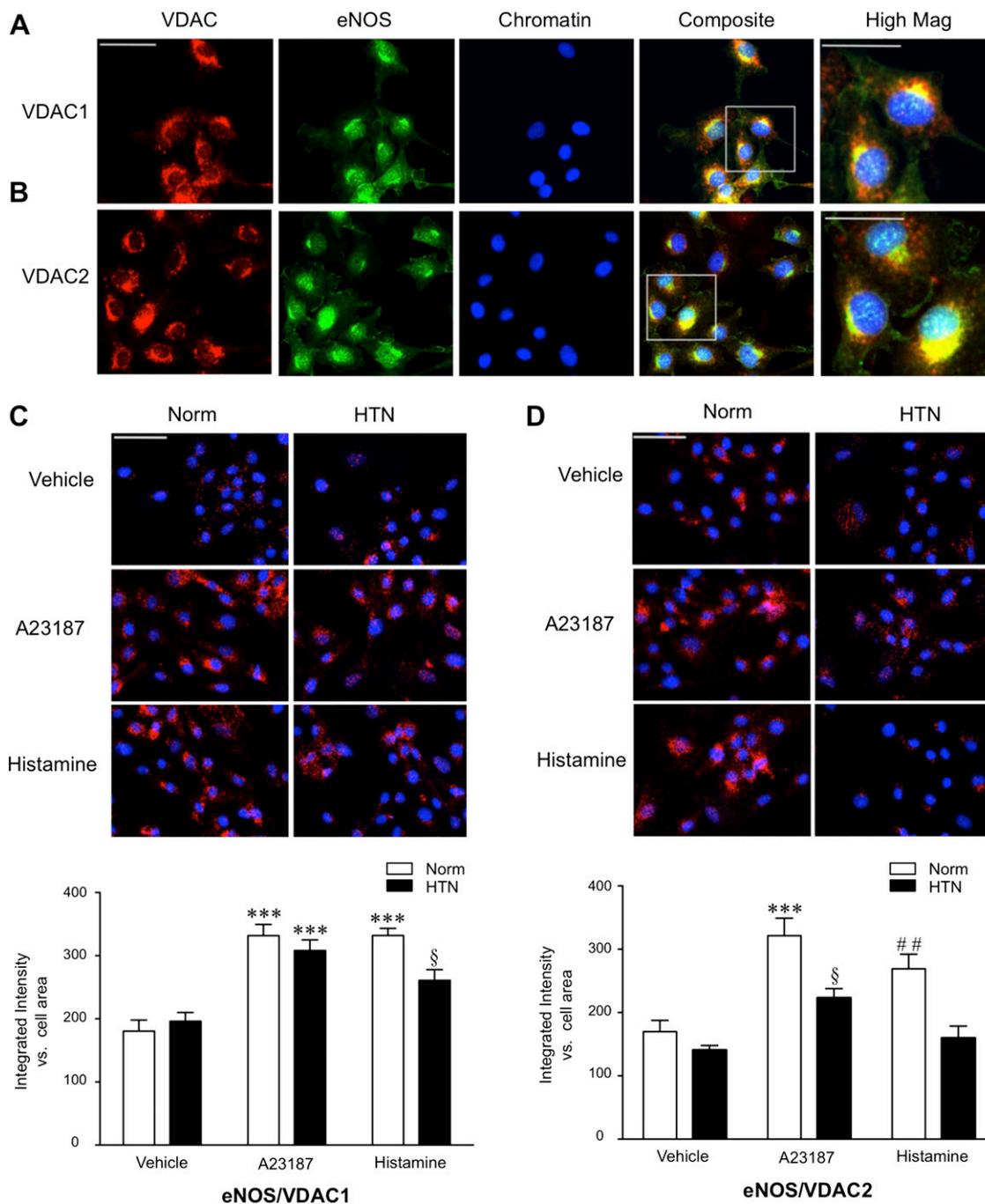
with location within the Golgi. We then quantified the effect of chronic intrauterine hypertension on the amount of eNOS and VDAC interaction by performing *in situ* PLA on Norm and HTN fetal ovine PAECs under basal conditions and after stimulation with either A23187 or histamine. The amount of physical interaction of eNOS and VDAC1 under basal conditions was similar in the Norm and HTN PAECs (Figure 5C). Stimulation with the calcium ionophore, A23187, significantly increased eNOS and VDAC1 interaction in both groups of cells. Although histamine also significantly increased eNOS and VDAC1 interaction as compared with vehicle, in both groups of cells the amount of interaction in the histamine-stimulated HTN cells was significantly less than in Norm cells. The amount of interaction between eNOS and VDAC2 did not differ between Norm and HTN ovine PAECs under control conditions (Figure 5D). However, whereas treatment with either A23187 or histamine significantly increased eNOS-VDAC2 interaction in Norm PAECs, the response was significantly blunted in A23187-treated HTN PAECs. Moreover,

histamine stimulation did not increase eNOS-VDAC2 interaction in the HTN PAECs.

#### Loss of VDAC2 Blunts eNOS Activity

To definitively establish whether either VDAC1 or -2 serves to modulate PAEC eNOS activity in the pulmonary vasculature, we selectively depleted either VDAC1 or -2 in hPAECs using RNA interference. In separate experiments, transfection of hPAECs with either VDAC1 or -2 siRNA reduced the target mRNA by 85.3 ( $\pm 0.3$ )% (Figure 6A; \*\*\* $P < 0.0001$  versus nontargeting control [NTC]) and 88.2 ( $\pm 1.8$ )% (Figure 6B; \*\*\* $P < 0.0001$  versus NTC), respectively, compared with cells transfected with NTC siRNA. At 72 hours after transfection, VDAC1 and -2 protein expression was decreased by 52 ( $\pm 5$ )% and 51 ( $\pm 3$ )%, respectively (Figures 6A and 6B; \*\*\* $P < 0.0001$  versus NTC).

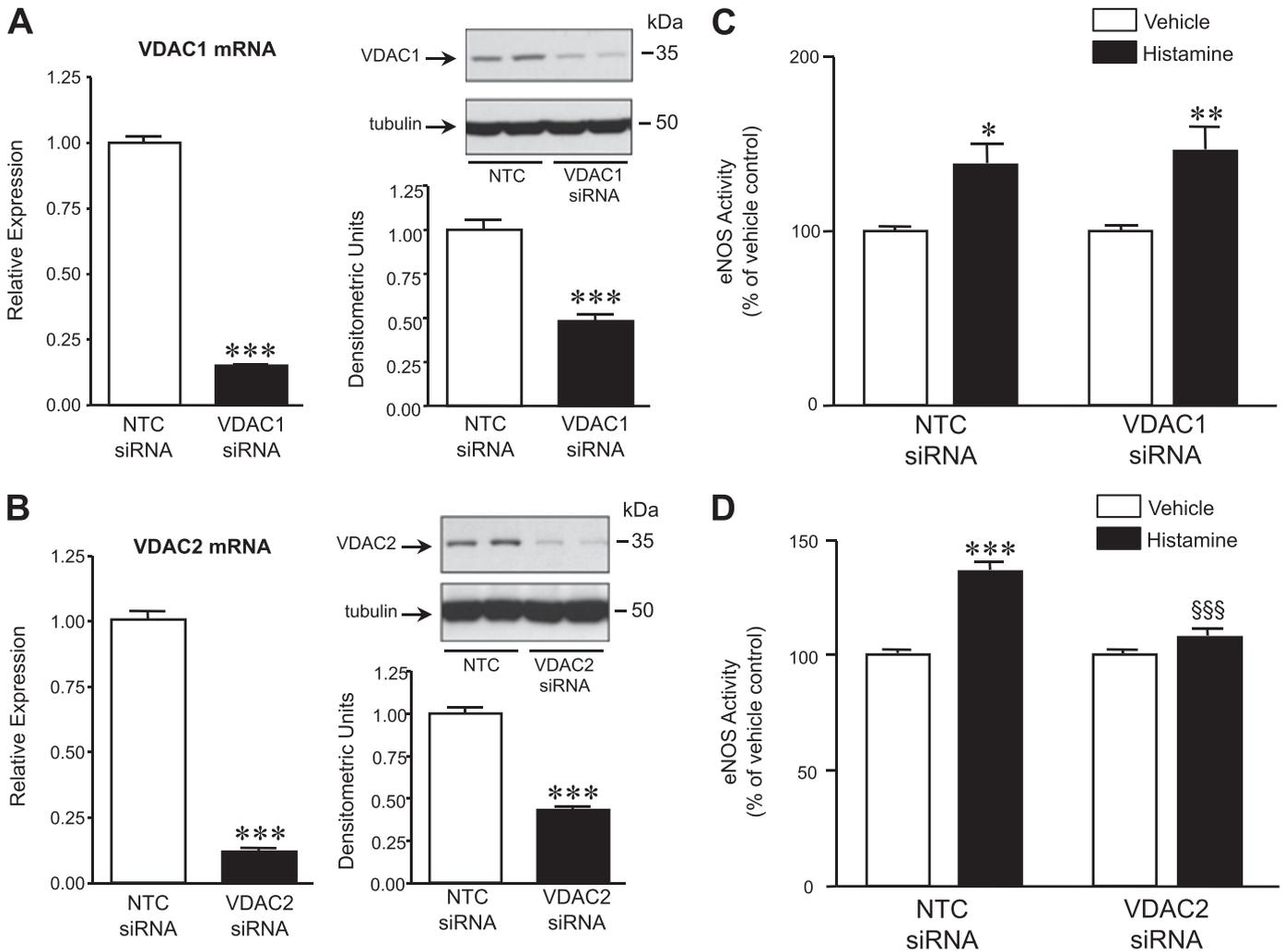
Next, NOS activity was measured in control and VDAC1- and -2-depleted hPAECs. Neither VDAC1 nor -2 siRNA altered



**Figure 5.** Chronic intrauterine hypertension blunts the interaction of eNOS and VDAC isoforms upon stimulation with NO agonists. Representative immunocytochemistry on hPAECs to detect VDAC1 (A) or VDAC2 (B) and eNOS. Incubation of hPAECs with anti-VDAC antibody (red) and eNOS (green) demonstrate significant colocalization of both VDAC isoforms with eNOS. Boxes delineate the image field chosen to depict under higher magnification. Calibration mark = 100  $\mu$ m. (C) Representative images from *in situ* proximity ligation assays performed to detect the interaction of eNOS and VDAC1 in Norm and HTN ovine PAECs under basal conditions, and after stimulation with either A23187 or histamine ( $***P < 0.001$  versus Norm or HTN vehicle control;  $§P < 0.05$  versus HTN vehicle treated and  $P < 0.01$  versus Norm histamine stimulated). Calibration mark = 50  $\mu$ m. (D) Representative images from *in situ* proximity ligation assays performed to detect the interaction of eNOS and VDAC2 in Norm and HTN ovine PAECs under basal conditions, and after stimulation with either A23187 or histamine ( $***P < 0.001$  versus vehicle-stimulated Norm cells;  $§P < 0.05$  versus HTN vehicle-treated cells and  $< 0.01$  versus A23187-stimulated Norm cells;  $##P < 0.01$  versus Norm vehicle-treated cells and histamine-stimulated HTN cells). Calibration mark = 50  $\mu$ m.

the basal level of NO production in the hPAECs. Stimulation with histamine for 30 minutes increased the conversion of [ $^{14}$ C]L-Arginine to [ $^{14}$ C]L-Citrulline in control siRNA-treated hPAECs by 37.5 ( $\pm 12.5$ )% ( $*P < 0.05$ , Figures 6C and 6D). VDAC1 depletion had no effect on the histamine induced increase in NO production, as histamine increased NO production in the VDAC1

depleted cells by 45.6 ( $\pm 13.8$ )% (Figure 6C;  $**P < 0.01$  versus vehicle stimulated;  $P =$  nonsignificant versus NTC histamine stimulated). In contrast, depleting VDAC2 in the hPAECs significantly attenuated the NO response to histamine, as NO production increased by only 8.0 ( $\pm 3.1$ )% over vehicle-stimulated cells (Figure 6D;  $§§§P < 0.001$  versus NTC histamine stimulated).



**Figure 6.** Loss of VDAC2 in hPAECs impairs the histamine-induced increase in NOS activity. (A) qRT-PCR (left) and Western immunoblot (right) to determine VDAC1 gene expression 24 hours after transfection with siRNA. For qPCR, target gene expression was normalized to the levels of 18S rRNA within each sample, and normalized to the average of samples transfected with nontargeting control (NTC) siRNA ( $***P < 0.001$ ,  $n = 3$ ). For WB, summarized densitometric data with expression normalized to tubulin levels within each sample and normalized to the average ratio found in hPAECs transfected with NTC siRNA ( $***P < 0.001$ ,  $n = 12$ ). (B) qRT-PCR (left) and Western immunoblot (right) to determine VDAC2 gene expression 24 hours after transfection with siRNA. For qPCR, target gene expression was normalized to the levels of 18S rRNA within each sample, and normalized to the average of samples transfected with NTC siRNA ( $***P < 0.0001$ ,  $n = 6$ ). For WB, summarized densitometric data are presented with expression normalized to tubulin levels within each sample, and normalized to the average ratio found in hPAECs transfected with NTC siRNA ( $***P < 0.001$ ,  $n = 15$ ). NOS activity was measured in VDAC1- (C) or VDAC2 (D)-depleted hPAECs after treatment with either vehicle (white bars) or histamine (200  $\mu$ M; black bars), and NO activity determined by the conversion of L-arginine to L-citrulline at 30 minutes ( $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$  versus vehicle and  $§§§P < 0.001$  versus histamine-stimulated NTC;  $n = 9$ ).

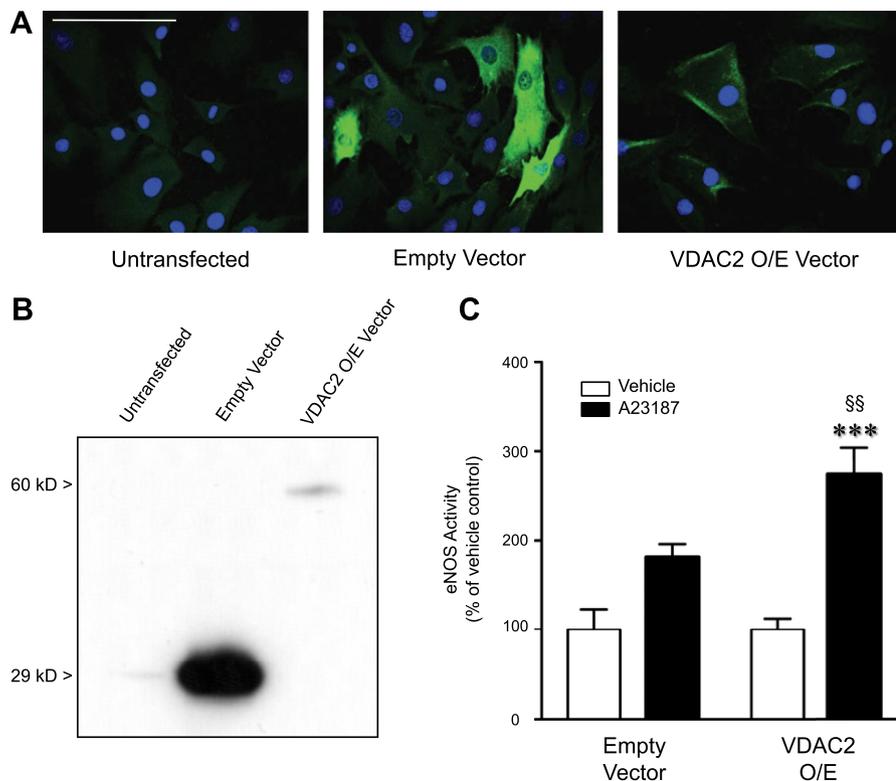
### Overexpression of VDAC2 Enhances eNOS Activity in HTN PAECs

Finally, to demonstrate that the reduced expression of VDAC2 limits NOS activity in the PAECs obtained after chronic intrauterine hypertension, experiments were performed to rescue NOS activity in the HTN PAECs by overexpressing VDAC2. HTN PAECs were transfected with either empty vector or a VDAC2 overexpression vector. Confocal microscopy, to detect GFP, demonstrated a transfection efficiency of roughly 30–50%, with GFP expression detected within the cytoplasm of the control cells, and both on the plasma membrane (Figure 7A) and in a perinuclear pattern (see Figure E2 in the online supplement) in the VDAC2-GFP-transfected cells. By Western immunoblot, to detect GFP, the 29-kD GFP was robustly expressed in vector-transfected cells, and the 60-kD predicted VDAC2 protein containing the C-terminal GFP was specifically detected in the VDAC2-transfected

cells. Finally, by performing NOS activity assays in vector- and VDAC2-transfected cells, we determined whether overexpression of VDAC2 could rescue the impaired NO production observed in the HTN PAECs. NO production was not different in HTN PAEC-transfected with the VDAC2 overexpression vector as compared with cells transfected with the control vector (Figure 7C). However, overexpression of VDAC2 significantly increased NO production in response to A23187, resulting in a 274% increase over vehicle in the VDAC2-transfected cells versus a 181% increase over vehicle in the vector-transfected cells ( $§§P < 0.01$  versus A23187-stimulated vector transfected).

### DISCUSSION

In this study, we demonstrate that chronic intrauterine hypertension blunts NOS activity in PAECs without significantly decreasing eNOS expression. Given the profound clinical implications of



**Figure 7.** Overexpression of VDAC2 increases eNOS activity in HTN PAECs. (A) Representative confocal micrographs after immunostaining to detect green fluorescent probe (GFP) in untransfected, GFP-vector-transfected, and VDAC2-GFP-transfected HTN PAECs. Calibration mark = 100  $\mu$ m. (B) Western immunoblot on protein lysates obtained from untransfected, GFP-vector-transfected, and VDAC2-GFP-transfected HTN PAECs demonstrated strong expression of the 29-kD GFP in the vector-transfected cells, and the presence of the 60-kD predicted GFP-tagged VDAC2 protein in the VDAC2-GFP-transfected cells. (C) NOS activity was measured in HTN PAECs 24 hours after transfection with either empty vector or VDAC2 overexpression vector, and after treatment with either vehicle (white bars), or A23187 (2  $\mu$ M; black bars) ( $***P < 0.001$  versus VDAC2 overexpression PAECs treated with vehicle and  $^{SS}P < 0.01$  versus empty vector PAECs stimulated with A23187;  $n = 3$ ).

impaired NO production in the perinatal pulmonary circulation, we investigated the potential that alternations in protein-protein interactions may account for the constrained NOS activity in HTN PAECs. We report that, in HTN compared with Norm fetal PAECs, protein expression of both VDAC1 and -2 are decreased. We then show that eNOS physically interacts with VDAC isoforms 1 and 2 under basal conditions, and, via physiologic gain-of-function experiments, that agonist-induced increases in NOS activity are associated with enhanced interaction between VDAC isoforms and eNOS. Moreover, chronic intrauterine hypertension significantly compromises the capacity for eNOS to interact with both VDAC1 and -2, with the interaction between eNOS and VDAC2 being most affected. However, although depletion of VDAC2 significantly reduces NO production in stimulated hPAECs, depletion of VDAC1 has no effect, providing evidence that VDAC2 is the main isoform regulating eNOS activity in the pulmonary circulation. Finally, overexpression of VDAC2 increases NOS activity in the HTN PAECs, thus providing further proof of concept that VDAC2 modulates NO production in the pulmonary circulation.

Although the interaction between NOS and VDAC1 has been previously reported in the systemic circulation (25), the present report is the first to demonstrate a role for VDAC isoforms in the pulmonary circulation, and to identify VDAC2 as the primary isoform modulating NOS activity in the pulmonary endothelium. Furthermore, alterations in VDAC expression have not been previously linked with pulmonary hypertension. The observation that, in a model of PPHN, chronic intrauterine pulmonary hypertension (26, 30, 31) decreases the protein expression of VDAC isoforms provides strong support for the notion that NO production is dysregulated in PPHN not only at the transcriptional and translational level, but also via dynamic protein-protein interactions. Although it is not clear how the physical interaction of VDAC and eNOS serves to enhance NOS activity, our immunocytochemical data suggest that this interaction may occur in either the Golgi apparatus or the mitochondria. VDAC2 has 11 putative splice variants, and, as has been recently described for VDAC1, it is possible that one of these alternate splice events

might target the VDAC2 molecule to a specific organelle, such as the Golgi apparatus or the mitochondria.

Increased production of NO from the pulmonary endothelium is necessary for the successful transition from fetal to air-breathing life (5, 8, 32). Previous reports have generally demonstrated that both eNOS activity and expression are decreased in chronic intrauterine pulmonary hypertension (10, 11). However, one report in a lamb model of PPHN found that eNOS expression in the PAECs of control and HTN animals were not different (33), a finding consistent with those of the present report. To some degree, these seemingly disparate findings might be explained on the basis of variations in the duration of intrauterine pulmonary hypertension, or the anatomical site from which the PAECs are derived. In this study, we did not demonstrate a significant reduction in eNOS expression; however, there was a trend toward a decrease in both gene and protein expression. Given the small sample size, it is impossible to rule out a type II statistical error. Irrespective of whether eNOS expression is decreased, it is clear that the ability of eNOS activity to increase in response to physiologic stimuli is blunted in this model of perinatal pulmonary hypertension. Basal NOS activity was decreased in HTN compared with Norm PAECs, and the capacity for agonists to induce an increase in NOS activity was impaired. However, the degree of interaction between eNOS and VDAC2 was not different between the Norm and HTN cells at baseline, but was significantly limited in response to stimulation with NO agonists. Whereas depletion of VDAC2 by RNA interference in hPAECs prevented the histamine-stimulated increase in NOS activity, basal NOS activity was unaffected. Furthermore, while overexpression of VDAC2 in the HTN PAECs did not change basal NOS activity, it significantly increased NOS activity in response to A23187. Taken together, these data suggest that physical interaction with VDAC2 serves as a distinct mechanism to increase NO production in the pulmonary circulation in response to stimulation, and that alternative pathways predominate to control basal NO activity.

To our knowledge, the present article is the first to provide quantitative information surrounding the relative expression of the VDAC isoforms in the pulmonary circulation. It is interesting to note that among the three isoforms of VDAC, chronic intrauterine hypertension causes the greatest decrease in the most abundantly expressed isoform in ovine PAECs: VDAC2. The strategy employed in this study allows us to conclude that, although both VDAC1 and -2 physically interact with eNOS, only VDAC2 silencing in PAECs decreased NO activity. It is possible that VDAC2 is able to effectively compensate when VDAC1 is silenced, but that VDAC1 is insufficient to compensate for loss of VDAC2.

In considering the significance of these studies, it is important to recognize the limitations of our study. First, the work was performed exclusively in isolated PAECs. Although a well accepted model of perinatal pulmonary hypertension was used to derive HTN PAECs, it is likely that greater complexity exists in a more fully integrated model. To this end, experiments using isolated vascular segments, coculture experiments with both endothelial and vascular smooth muscle cells, and further determination of the role of VDAC2 in modulating NO production in whole animals would be important studies to pursue. Second, given the extensive homology between the VDAC isoforms 1 and 2, and the paucity of previous published studies in sheep models, it is impossible to exclude the possibility of some degree of cross-reactivity between the two antibodies used in these studies. Thus, the experiments to selectively deplete each isoform individually using siRNA were essential to determine that VDAC2 specifically modulates NOS activity in the PAECs.

In summary, we have demonstrated a novel interaction between VDAC2 and eNOS in the pulmonary circulation, and found that this interaction is essential for increasing NOS activity in response to physiologic stimuli. Furthermore, we demonstrate that chronic intrauterine hypertension impairs eNOS activity, decreases VDAC2 expression, and limits the interaction of VDAC2 and eNOS in response to stimulation with NO agonists. Whether the binding of eNOS to VDAC facilitates eNOS trafficking or induces a conformational change in a manner that enhances its activity remains unknown. We speculate that repression of VDAC2 expression plays an etiologic role in pulmonary hypertension, and perhaps more generally in diseases wherein vascular tone is elevated. Furthermore, VDAC2 may represent a novel therapeutic target for the treatment of pulmonary hypertension and, perhaps, essential hypertension.

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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