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L-citrulline treatment alters the structure of the pulmonary circulation in hypoxic newborn pigs

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Abstract

BACKGROUND: Dysregulated nitric oxide (NO) signaling contributes to chronic hypoxia (CH)-induced pulmonary hypertension (PH). NO signaling is improved and pulmonary vascular resistance (PVR) is reduced in CH piglets treated with the L-arginine-NO precursor, L-citrulline. We hypothesized that L-citrulline might cause structural changes in the pulmonary circulation that would contribute to the reduction in PVR and that the L-citrulline-induced structural changes would be accompanied by alterations in VEGF signaling.

METHODS: We evaluated small pulmonary arterial (PA) wall thickness, lung capillary density, and protein abundances of VEGF, VEGFR2, and phospho (p)-VEGFR2 in PA and peripheral lung samples of piglets raised in the lab in CH (10–12% O₂) from day of life (DOL) 2 until DOL 11–12 or raised in room air (normoxia) by the vendor and studied on arrival to the lab on DOL 11–12. Some CH piglets were treated with oral L-citrulline (1–1.5 g/kg/d) starting on the third day of hypoxia.

RESULTS: PA wall thickness was 32% less and lung capillary formation was nearly doubled in L-citrulline treated than untreated CH piglets. Both of these L-citrulline-induced structural changes in the pulmonary circulation were accompanied by altered amounts of VEGF protein but not by altered amounts of either VEGFR2 or p-VEGFR2 proteins.

CONCLUSIONS: Alterations in the structure of the pulmonary circulation in CH piglets by L-citrulline are unlikely to be mediated by overall VEGF signaling. Nonetheless, L-citrulline-induced structural changes should reduce PVR and thereby contribute to the amelioration of CH-induced PH.

Keywords

arginine; nitric oxide; neonatal pulmonary hypertension

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INTRODUCTION

Chronic alveolar hypoxia leads to the development of pulmonary hypertension (PH). In the setting of chronic cardiopulmonary disorders accompanied by intermittent or persistent episodes of hypoxia, the development of PH is associated with increased morbidity and mortality¹⁻⁴. The role that impaired nitric oxide (NO) signaling plays in the pathobiology of PH in humans is now well established^{5,6}. Discovering ways to manipulate the NO signaling pathway as therapies for PH continues to be of interest.

Pulmonary vascular remodeling contributes to chronic hypoxia (CH)-induced PH in part by causing thickening of the walls of resistance level arteries⁷. NO has been shown to reduce the adverse remodeling of pulmonary resistance vessels⁸. NO, via modulating vascular endothelial growth factor (VEGF, also referred to as VEGFA), has pro-angiogenic properties that can lead to capillary formation, particularly in the developing lungs of newborns⁹. Increases in lung capillary formation could reduce pulmonary vascular resistance, PVR, by increasing the total cross-sectional area of the pulmonary vascular bed.

We have shown that the L-arginine-NO-precursor, L-citrulline, increases pulmonary vascular NO production, reduces PVR, and inhibits the development of CH-induced PH in newborn piglets^{10,11}. To further evaluate potential mechanisms by which L-citrulline treatment ameliorates PH, we performed studies to determine whether L-citrulline treatment reduces pulmonary vascular wall thickening and/or alters lung capillary formation in lungs of piglets with CH-induced PH. Because of the known interrelationship between NO and VEGF, we also performed studies to determine whether any L-citrulline-induced structural changes in the pulmonary circulation were accompanied by alterations in VEGF signaling.

Materials and Methods

Ethics statement

Use of animals conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and was approved by the Institutional Animal Care and Use Committees of Vanderbilt University Medical Center and the University of Utah Health which are both fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Use.

Lung and small pulmonary artery samples

All samples of lung and small pulmonary arteries (SPAs) used for these studies were previously collected and stored for future use. The piglets from which the lungs and SPAs were collected had been raised under normoxic or hypoxic conditions until day of life 11–12 as previously described¹¹. Most of the normoxic piglets were studied on the day of arrival from the vendor, on day of life 11–12. Piglets in the hypoxic groups were obtained on day of life 2 and raised in a normobaric hypoxic environment until day of life 11–12. For the hypoxic environment, oxygen content was regulated at 10–12% O₂ and CO₂ was maintained at 3–6 Torr by absorption with soda lime. Some hypoxic piglets were treated with oral L-citrulline, 1–1.5 g/kg/d, starting on the third day of hypoxia and continued for an additional 7 days of hypoxia¹¹. We used the highest dose of L-citrulline that we have previously

evaluated for efficacy in ameliorating chronic hypoxia-induced pulmonary hypertension¹¹. On day of life 11–12, piglets were given intravenous pentobarbital (50–100 mg/kg) and intravenous heparin (1,000 IU/kg). Piglets were exsanguinated and their lungs used for sample collection.

Some samples were pieces of lung tissue that were dissected from the lung periphery, frozen in liquid nitrogen, and stored at -80°C until used for these studies. Other samples were SPAs (300 μm diameter) dissected from lungs of all groups, then frozen in liquid nitrogen, and stored at -80°C until used for these studies. Other samples were sections from lungs that had been perfused with normal saline to remove all blood from the pulmonary circulation, then perfused with 10% neutral buffered formalin. Next, the lungs were fixed by instillation of the formalin into the airway, pulmonary artery, and left atrium at pressures of 25 cm H_2O . After 24 hours of fixation, pieces of lung were embedded and stored in paraffin blocks until used for these studies.

Morphometry studies

To assess pulmonary vascular remodeling, lung sections from normoxic $n=3$, untreated chronically hypoxic, $n=3$, and L-citrulline-treated chronically hypoxic, $n=4$, piglets were deparaffinized in Xylene solution and hydrated in ethanol solutions. Slides were stained with 1:1 Gill's Hematoxylin and counterstained with Eosin. SPAs were categorized by external diameter (10–30 μm , 31–50 μm , 51–100 μm , and 100–300 μm), using a Nikon 90i fluorescent microscope with high resolution CCD camera (Nikon Instruments Inc., Melville NY). Wall thickness was measured in a total of 76 vessels for each external diameter category from lung sections of normoxic piglets, 76 vessels for each external diameter category from lung sections of untreated chronically hypoxic newborn piglets, and 101 vessels for each external diameter category from lung sections of L-citrulline treated chronically hypoxic piglets. For each vessel, the percent wall thickness was calculated ($(2 \times \text{wall thickness} / \text{external diameter}) \times 100$).

Stereology for capillary surface density

PECAM1 (CD-31) immunostaining was used for endothelial detection for quantitative histological analysis of capillary surface density. Lung slides from normoxic, $n=4$, untreated chronically hypoxic, $n=4$, and L-citrulline treated chronically hypoxic, $n=4$, piglets were incubated with 1:1000 to 1:2000 dilutions of anti-PECAM1 primary antibody (custom-made rabbit, polyclonal, anti-sheep antibody, antigenic sequence RHYGFNEDVGNHAMKP, Absea, Beijing, China;¹²) diluted in 0.5% casein solution. Antigen detection was performed by incubation in 1:500 dilution of biotinylated anti-rabbit IgG (Cat# BA-1100, Vector Laboratories), rinsing with 0.01 M phosphate-buffered saline (PBS), followed by a 1:500 dilution of streptavidin-horseradish peroxidase conjugate [tyramide signal amplification (TSA) kit, Cat# NEL700; Perkin Elmer, Boston, MA]. Signal amplification was performed by incubating the tissue sections in a 1:100 dilution of biotinyl tyramide (TSA kit NEL700) and then relinked by streptavidin-horseradish peroxidase conjugate. Negative immunostaining controls were omission of the primary antibody, omission of the secondary

antibody, and substitution of the primary antibody with a species-matched, irrelevant antibody.

We used previously described techniques to measure capillary formation^{12,13}. Briefly, capillaries and epithelial cells (the reference space for image analysis) were individually located using designed base (systematic, uniform, random,¹⁴) sampling of slides for areas of assessment. Randomly overlaid grids were automatically applied using a computer-assisted true-color imaging system (Bioquant Life Science, Nashville, TN). Locations at which the grid intersection points met with capillaries (identified by PECAM1 immunostaining and characteristic structural findings) were then individually used for capillary quantification. Likewise, locations at which the grid intersection points met with epithelial cells (identified by counterstaining and characteristic structural findings) were individually used for quantification of epithelium. Tissue sections were analyzed without knowledge of the piglet group from which the tissue was taken. Data are also expressed as capillary to epithelial ratio to ensure appropriate comparison to a baseline of tissue surface area.

Immunostaining for VEGFR2

Lung sections from normoxic n=4, untreated chronically hypoxic, n=4, and L-citrulline treated chronically hypoxic, n=4, piglets were deparaffinized in xylene solution and gradually hydrated in ethanol solutions ranging from 100% to 80%. VEGFR2 antigens were exposed by using an antigen unmasking method to improve signal intensity (Cat# H-3300, Citrate Antigen Unmasking solution, Vector Laboratories). Next, the sections were treated with 3% hydrogen peroxide to block endogenous peroxidases as well as with an avidin/biotin kit to block endogenous avidin and biotin (Cat# SP-2001, Vector Laboratories), respectively. Subsequently, the tissue sections were incubated overnight at 4°C in the presence of anti-VEGFR2 antibody (Cat# ab39256, rabbit polyclonal, Abcam, Cambridge, MA) diluted in universal blocking solution of 0.5% Casein, with dilutions ranging from 1:500 to 1:1000 optimized for each piglet lung. Following incubation with primary antibody, slides were washed with PBS. Antigen detection used a 1:500 dilution of anti-rabbit-horseradish peroxidase conjugate (PI-1000, Vector Laboratories, Inc.) before diaminobenzidine (DAB) visualization. Slides were counterstained with 1:1 Gill's hematoxylin for ~ 7 seconds. Slides were then serially dehydrated and mounted.

Immunoblot analysis of VEGF, VEGFR2, and phosphorylated VEGFR2 (P-VEGFR2)

Peripheral lung tissue from normoxic, n=5, untreated chronically hypoxic, n=5, and L-citrulline treated chronically hypoxic, n=9, piglets were prepared by homogenizing 100 mg of lung tissue in 500 mL of homogenization buffer. Samples were centrifuged at 10,000 RPM and the supernatant collected. Protein concentrations were measured by the Bradford protein assay. Supernatants were applied to tris-glycine pre-cast 4–20% (for VEGF) or 4–12% (for VEGFR2 and P-VEGFR2) gels (Invitrogen, Carlsbad, CA) so that equal amounts of protein were loaded. Electrophoresis was carried out and the proteins were transferred from the gel to a polyvinylidene difluoride membrane (Cat# LC2005, 0.45 µm pore size, Invitrogen, Carlsbad, CA). To block non-specific protein binding, membranes were incubated with 5% non-fat dried milk in phosphate-buffered saline with 0.1% Tween (PBST), then washed with 1% milk in PBST.

For VEGF detection, membranes were incubated with the primary antibody (1:250, Cat# sc-152, rabbit monoclonal, Santa Cruz Biotechnology, Dallas, TX) diluted in PBS containing 0.1% Tween-20 and 1% non-fat dried milk (carrier buffer). Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000, Cat# 7074S, goat anti-rabbit, Cell Signaling, Danvers, MA). Bands were visualized by chemiluminescence using PerkinElmer Western Lighting Plus ECL in conjunction with an automated detection system (iBright CL1000, Invitrogen, Carlsbad, CA). The membranes were re-probed for β -actin using a 1:50,000 dilution of primary antibody (Cat# A1978, mouse monoclonal, Sigma-Aldrich, St. Louis, MO) and 1:5000 dilution of horseradish peroxidase-conjugated secondary antibody (Cat# 7076S, horse anti-mouse, Cell Signaling, Danvers, MA).

For VEGFR2 detection, membranes were cut horizontally at 62 kDa and separated. The top portion was incubated with the VEGFR2 primary antibody (1:250, Cat# ab39256, rabbit polyclonal, Abcam, Cambridge, MA) diluted in PBS containing 0.1% Tween-20 and 1% non-fat dried milk (carrier buffer), then incubated with a horseradish peroxidase-conjugated secondary antibody (1:2500, Cat# 7074S, goat anti-rabbit, Cell Signaling, Danvers, MA). The lower portion of the membrane was incubated alongside the upper membrane in 1% milk in PBST. Membranes were then incubated with antibody for β -actin (Cat# A1978, mouse monoclonal, Sigma-Aldrich, St. Louis, MO), rinsed three times with 1% milk in PBST, then incubated with a horseradish peroxidase-conjugated secondary antibody (Cat# 7076S, horse anti-mouse, Cell Signaling, Danvers, MA). Following incubation with the secondary antibodies, the upper and lower portions were again washed with PBST and exposed (iBright CL1000, Invitrogen by Thermo Fischer Scientific, Waltham, MA).

For the phosphorylated VEGFR2 (p-VEGFR2) detection, membranes were cut horizontally at 62 kDa. The top portion was incubated with the Phospho-VEGFR2 primary antibody (1:250, Cat# orb256644, rabbit polyclonal, Biorbyt, San Francisco, CA) diluted in PBS containing 0.1% Tween-20 and 1% non-fat dried milk (carrier buffer). Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000, Cat# 7074S, goat anti-rabbit, Cell Signaling, Danvers, MA). The lower portion was incubated alongside the upper membrane in 1% milk in PBST. Membranes were incubated with antibody for β -actin (Cat# A1978, mouse monoclonal, Sigma-Aldrich, St. Louis, MO), then incubated with a horseradish peroxidase-conjugated secondary antibody (Cat# 7076S, horse anti-mouse, Cell Signaling, Danvers, MA). Following incubation with the secondary antibodies, both portions were washed with PBST and exposed (iBright CL1000, Invitrogen by Thermo Fischer Scientific, Waltham, MA). The upper portion was stripped at room temperature using stripping buffer (Restore Western Blot Stripping Buffer, Cat# 21059, Thermo Fischer Scientific, Waltham, MA), washed with PBST, and blocked with 5% non-fat dried milk in PBST. The membrane was re-probed for VEGFR2 as described above.

Similar methods were used for immunoblot analysis of VEGF, VEGFR2, and p-VEGFR2 in samples of SPAs from each group (normoxic, n=5; untreated chronic hypoxic, n=5; and L-citrulline treated chronic hypoxic, n=9).

Statistical Analysis—Data in the Table are presented as mean and SD. Groups were compared by one-way ANOVA with Student Newman Keul's post hoc comparison test. P values less than 0.05 were considered significant.

Results

Figure 1 shows representative sections of slides used to assess vascular wall thickness for different sizes of SPAs from lungs of normoxic piglets (A and B), untreated CH piglets (C and D), and L-citrulline treated CH piglets (E and F). As shown in the Table and illustrated in Figure 1, vascular wall thickness was significantly greater for all size ranges of SPAs that we evaluated from lungs of untreated CH piglets when compared to those from lungs of normoxic piglets. Although remaining greater than vessels in lungs of normoxic piglets, values for vascular wall thickness in all size ranges of SPAs that we evaluated from lungs of L-citrulline treated CH piglets were reduced when compared to those from lungs of the untreated group of CH piglets (Table, Figure 1).

To quantify capillary surface density in peripheral lung tissue, we used stereological analysis of slides stained for PECAM-1, an endothelial marker. Representative PECAM-1 immunostained tissue sections of peripheral lung tissue from each of the three groups are shown in Figure 2 A–C. Stereological analysis is summarized in Figures 2 D–F. Epithelial surface density did not differ between any of the groups (Figure 2 E). Therefore, capillary surface density findings were the same regardless of whether or not the data were expressed relative to epithelial surface density (Figures 2 D and F). Specifically, capillary surface density was similar in lung tissue from normoxic piglets and untreated CH piglets (Figures 2 D and F). However, the capillary surface density for lung tissue from the L-citrulline treated group was nearly doubled when compared to that for both the normoxic animals and the untreated group of CH piglets (Figures 2 D and F).

Immunohistochemical localization of VEGFR2 protein in representative lung tissue sections from all three groups of piglets is shown in Figure 3. For lung tissue from normoxic, control (Figures 3 A and D), untreated chronically hypoxic (Figures 3 B and E), and L-citrulline treated chronically hypoxic piglets (Figures 3 C and F), immunostaining for VEGFR2 was found in capillary endothelial cells and alveolar epithelial cells (Figures 3 A–C) and was also found in pulmonary arterial endothelial cells and smooth muscle cells (Figures 3 D–F).

We next performed studies to evaluate for possible alterations in VEGF signaling in SPAs of untreated and L-citrulline treated groups of CH piglets. To do this, we used SPAs from all three groups and performed immunoblot analysis for VEGF-A (hereafter referred to as VEGF), VEGFR-2, the major mediator of the mitogenic, angiogenic enhancing effects of VEGF¹⁵, and for p-VEGFR2^{16,17}, which reflects activation of VEGFR-2. We found that VEGF protein levels were less in SPAs from both the untreated and L-citrulline treated groups of CH piglets than in those from the normoxic group (Figure 4). Moreover, VEGF protein abundance was lower in SPAs from the L-citrulline treated than in the untreated group of CH piglets (Figure 4). VEGFR2 protein levels did not differ between SPAs from any of the 3 groups (Figures 5 A and B). Likewise, values for p-VEGFR2 did not differ between SPAs from untreated and L-citrulline treated groups of CH piglets (Figures 5 A and

C). However, similar to findings for VEGF, p-VEGFR2 protein levels were lower in SPAs from both the untreated and L-citrulline treated groups of CH piglets than those from the normoxic group (Figures 5 A and C).

Because of our interest in capillaries, we performed immunoblot analysis for VEGF, VEGFR-2, and for p-VEGFR2 using samples of peripheral lung tissue, which is comprised primarily of alveolar epithelial cells and capillaries. We found that VEGF protein abundance was less in peripheral lung tissue from the untreated group of CH piglets than in peripheral lung tissue from either the normoxic piglets or the L-citrulline treated group of CH piglets (Figure 6). Notably, VEGF protein abundance was similar for lungs from L-citrulline treated CH animals and normoxic animals (Figure 6). However, VEGFR2 protein abundance did not differ between peripheral lung tissue from any of the 3 groups (Figure 7 A–C). Moreover, although protein abundance for p-VEGFR2 was greater in the peripheral lung tissue from the untreated group of CH piglets than in lung tissue from the normoxic group, p-VEGFR2 levels did not differ between lung tissue from the L-citrulline treated group of CH piglets and the other two groups (Figure 7 A–C).

Discussion

Findings in this study add to our previous findings^{10,11} and provide new information regarding the impact of L-citrulline therapy on lungs of piglets with CH-induced PH. One new finding is that, in comparison to untreated CH piglets, the vascular wall thickness of SPAs ~300 microns diameter was reduced in piglets who received L-citrulline treatment during CH. Another new finding is that capillary formation was much greater in lungs of piglets treated with L-citrulline during CH than in lungs of untreated CH piglets. These findings reflect important structural alterations of the pulmonary circulation that could contribute to the ability of L-citrulline to ameliorate CH-induced PH in newborn piglets.

Consistent with previous findings in our laboratory, exposure to CH increased the wall thickness of SPAs⁷. The contribution from CH-induced pulmonary vascular wall thickening to elevations in PVR is well described^{18,19}. It follows that the ability to inhibit/reverse wall thickening could contribute to the reduction in PVR found in newborn piglets treated with L-citrulline during CH.

We have not previously evaluated the effect of CH on lung capillary formation. Unlike the impact on vascular wall thickness, our findings do not suggest that a change in capillary formation occurred with CH and thereby contributed to PH. This finding contrasts with findings in experimental animal models of bronchopulmonary dysplasia (BPD) in which impairments in angiogenesis are found and may contribute to the development of PH associated with BPD. However, either premature animals, such as lambs²⁰, with lungs at the saccular stage of development, or newborn rodents born at term with lungs at the saccular stage of development are used in the BPD models²¹. Our use of piglets at the more mature alveolar stage of development to create CH-induced PH may explain the lack of impact from CH on capillary formation. However, the increase in capillary formation that we found in lungs of L-citrulline treated CH piglets would increase the overall cross-sectional area of the pulmonary circulation and could thereby reduce PVR. This finding is of interest because the

impact from PH treatments on lung capillary development, especially in the lungs of developing animals, is currently little explored ²².

We previously showed that the L-arginine-NO precursor, L-citrulline, increases NO production in the pulmonary circulation of newborn piglets with CH-induced PH ^{10,11} and in PAECs cultured under hypoxic conditions ²³. The antimitogenic, antiproliferative, and antihypertrophic properties ^{24,25} of NO could underlie L-citrulline-induced reductions in pulmonary vascular wall thickness. NO's vasodilatory, pressure reducing effects could also help ameliorate vascular wall thickening. This is because hypoxia-induced vascular wall thickening could be due in part to an adaptive response to increased pressure ¹⁸. Another possibility is that the pro-angiogenic properties of NO ²⁶ could mediate the L-citrulline-induced increase in lung capillary formation. That is, it seems likely that NO production mediates the structural changes in the pulmonary circulation that we found in untreated vs L-citrulline treated lungs from CH piglets.

It merits comment that in addition to augmenting the endogenous production of NO, L-citrulline supplementation reduces the generation of the reactive oxygen species (ROS), superoxide ¹¹. This contrasts to the situation where administering inhaled NO provides exogenous NO without concomitantly reducing ROS. This issue is important because the bioavailability of NO can be limited due to inactivation by the interaction with ROS ²⁷. Thus, the ability of inhaled NO to deliver bioavailable NO is problematic in situations complicated by the presence of ROS in airways and lung tissue. In comparison, L-citrulline-induced increases in NO produced by pulmonary arterial endothelial cells should be readily bioavailable to adjacent pulmonary arterial smooth muscle cells. It is possible that NO derived via L-citrulline supplementation will enhance lung capillary formation in clinical and experimental situations where inhaled NO has little to no effect.

NO is known to modulate VEGF protein amounts in a number of cell types ^{28,29}. We were therefore not surprised to find that VEGF protein abundances were lower in SPAs and peripheral lung samples from CH vs normoxic piglets. These hypoxia-induced reductions in VEGF protein parallel our previous findings of lower amounts of NO produced by lungs ³⁰ and SPAs ¹¹ from CH vs normoxic piglets. In comparison, only some of the differences in VEGF protein amounts in lungs and SPAs from L-citrulline treated vs untreated CH animals parallel our previous findings of L-citrulline-induced increases in NO production ^{10,11,23}. Specifically, whereas VEGF protein was greater in peripheral lung tissue from L-citrulline than untreated CH piglets; VEGF protein was less in SPAs of L-citrulline treated than untreated CH piglets.

Differences in bio-mechanical stresses experienced by different compartments of the pulmonary circulation might help explain the VEGF protein findings. Elevations in stretch and fluid shear stress have been shown to increase VEGF protein ³¹⁻³³. Capillaries in the lungs from L-citrulline treated piglets might have experienced an increase in shear stress from NO-induced elevations in flow ³⁴, which, in turn, would increase VEGF protein. In contrast, L-citrulline-induced increases in NO production would dilate resistance level pulmonary arteries, reducing pressure/stretch, which could decrease VEGF protein. Taken together, our findings suggest that the impact of treatment with L-citrulline on VEGF protein

is not limited to the direct interplay between NO and VEGF, but is likely to be influenced by the effect of biomechanical stresses on NO and VEGF, which differ along the longitudinal axis of the pulmonary circulation.

VEGF signals through the receptors, VEGFR1 and VEGFR2. The precise function of VEGFR1 is still debated due, at least in part, to the fact that its kinase activity is only weakly induced upon binding to ligands such as VEGF¹⁵. We evaluated VEGFR2 because there is broad agreement that VEGFR2 is the major mediator of the mitogenic, angiogenic enhancing effects of VEGF¹⁵. The localization of VEGFR2 was unchanged by L-citrulline treatment and did not differ from that reported previously by members of our group in premature lamb lungs¹³. We also evaluated the amount of phospho (p)-VEGFR2, which reflects the activation state of VEGFR2^{16,17}. If p-VEGFR2 findings reflect overall VEGF signaling, then our findings show that, concurrent with lower VEGF protein amounts, a reduction in overall VEGF signaling occurred in SPAs of CH vs normoxic piglets. By comparison, p-VEGFR2 findings in peripheral lung tissue from CH vs normoxic piglets suggest that despite a reduction in VEGF protein amounts, overall VEGF signaling was increased in lung tissue consisting largely of alveolar epithelial cells and capillaries.

The role of altered VEGF signaling and PH remains controversial. This is in part due to the complexity of VEGF biology. In fact, both increases and decreases in VEGF signaling have been found in other animal models of PH^{35–38}. The ability to correlate decreases vs increases in VEGF protein and PH may depend in part on which vessels along the longitudinal axis of the pulmonary circulation are involved, the age and species of the animals studied, and differences in a variety of experimental conditions and techniques. Indeed, unlike our findings with SPA (pulmonary arteries 300 microns diameter), other investigators found that the concentration of VEGF was greater but the amount of VEGFR2 was less in pulmonary arteries from piglets that were raised in CH versus normoxia for 14 days³⁹. However, these other studies were performed with pulmonary arteries that were dissected from the hilum ranging down to 100 microns in diameter, i.e. composed of pulmonary arteries that were much larger than used in our study. In addition, whereas we used an immunoblot technique, enzyme immunoassay was used to assess VEGF in the other study. Thus, experimental differences can contribute to different conclusions regarding the role of VEGF signaling in PH and likely explain why findings from investigators using a similar model of chronic hypoxia-induced PH in newborn piglets differ from ours.

Whether changes in overall VEGF signaling might contribute to the amelioration of PH in L-citrulline treated CH piglets also merits comment. Despite differences in VEGF protein amounts, L-citrulline treatment did not alter VEGFR2 nor p-VEGFR2 in either type of lung tissue that we studied. Thus, VEGF signaling does not appear to mediate the ability of L-citrulline to ameliorate CH-induced PH.

Limitations of our study design should be mentioned. We did not evaluate the effect of L-citrulline treatment on capillary formation or VEGF signaling in normoxic, control piglets. Nor did we perform studies to determine whether there are differences between male and female piglets. In addition, nutrition for the normoxic, control piglets was provided by the sow; whereas the hypoxic piglets received artificial sow milk replacer. Hence, it is possible,

but will require further evaluation, that sex or nutrition, factors that are known to influence PH and capillary formation, had an impact on our findings.

In summary, we found that changes in VEGF protein amounts accompanied L-citrulline-induced alterations in both pulmonary vascular wall thickness and capillary formation in lungs of piglets with CH-induced PH. Since phospho-VEGFR2 protein amounts remained similar, the possibility that overall VEGF signaling might directly cause the L-citrulline-induced pulmonary vascular structural changes seems unlikely. Nonetheless, our finding that the structure of the pulmonary circulation was altered by L-citrulline in a fashion that should reduce PVR adds to our previous studies and continues to provide evidence that L-citrulline treatment may be a useful therapy to ameliorate PH in infants suffering from conditions associated with intermittent or persistent episodes of hypoxia.

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Figure 1.

Representative sections of slides used to assess vascular wall thickness for different sizes of small pulmonary arteries in lungs from normoxic, control piglets (A and B), untreated chronically hypoxic piglets (C and D), and L-citrulline treated chronically hypoxic piglets (E and F).

Figure 2. A-C. Representative PECAM-1 immunostained slides used to assess capillary surface density in lungs from normoxic, control piglets (A), untreated chronically hypoxic piglets (B), and L-citrulline treated chronically hypoxic piglets (C).

For all three groups of lungs, immunostaining for PECAM-1 was used as an endothelial marker to assist with capillary identification (arrows in each slide point to capillaries).

Figure 2. D-F. Stereological evaluation of capillary surface density (D), epithelial surface density (E), and capillary surface density relative to epithelial surface density (F) in lung tissue from normoxic, control piglets, untreated chronically hypoxic piglets, and piglets treated orally with L-citrulline during exposure to chronic hypoxia.

Stereological analysis of PECAM-1 immunostained slides showed that there was no difference in capillary surface density between normoxic, control piglets and untreated chronically hypoxic piglets (D); whereas capillary surface density in the L-citrulline treated group of chronically hypoxic piglets was significantly greater compared to both of the other groups of piglets (D). There was no difference in epithelial surface density between any of the three groups (E). Thus, even when expressed relative to epithelial surface density (F), capillary surface density was greater in lungs from the L-citrulline treated group of chronically hypoxic piglets than lungs from both normoxic control and untreated chronically hypoxic groups of piglets (F). *different from normoxic control; ** different from untreated

chronic hypoxia. Data were compared by one-way ANOVA with Student Newman Keul's post hoc comparison test; $P < 0.05$.

Figure 3. Immunohistochemical localization of VEGFR2 protein in representative lung samples from normoxic, control piglets (A and D), untreated chronically hypoxic piglets (B and E), and L-citrulline treated chronically hypoxic piglets (C and F).

For all three groups, peripheral lung samples (Figures A-C) show that immunostaining for VEGFR2 protein can be found in the endothelium of capillaries (arrows) and in alveolar epithelial cells (forked arrows). In addition, for all three groups, lung samples containing small pulmonary arteries (Figures D-F) show that immunostaining for VEGFR2 protein can be found in pulmonary vascular endothelial cells (arrows) and smooth muscle cells (arrowheads).

Figure 4. VEGF protein abundance in small pulmonary arteries from normoxic, control piglets, untreated chronically hypoxic piglets, and piglets treated orally with L-citrulline during exposure to chronic hypoxia.

VEGF protein abundance was less in small pulmonary arteries from L-citrulline treated chronically hypoxic piglets compared to both other groups of piglets. VEGF protein abundance was also less in small pulmonary arteries from the untreated chronically hypoxic piglets than in normoxic control piglets. *different from normoxic control; ** different from untreated chronic hypoxia. Data were compared by one-way ANOVA with Student Newman-Keul's post hoc comparison test; $P < 0.05$.

Figure 5. VEGFR2 and p-VEGFR2 protein abundance in small pulmonary arteries from normoxic, control piglets, untreated chronically hypoxic piglets, and piglets treated orally with L-citrulline during exposure to chronic hypoxia.

VEGFR2 protein abundance did not differ between small pulmonary arteries from any of the 3 groups of piglets (A and B). P-VEGFR2 levels did not differ between small pulmonary arteries from untreated and L-citrulline treated piglets but were lower in small pulmonary arteries from both groups of chronically hypoxic piglets than from normoxic control piglets (A and C). *different from normoxic control. Data were compared by one-way ANOVA with Student Newman Keul's post hoc comparison test; $P < 0.05$.

Figure 6. VEGF protein abundance in peripheral lung tissue from normoxic, control piglets, untreated chronically hypoxic piglets, and piglets treated orally with L-citrulline during exposure to chronic hypoxia.

VEGF protein abundance was less in peripheral lung tissue from piglets raised in chronic hypoxia compared to both other groups of piglets. VEGF protein abundance was similar in lungs from L-citrulline treated chronically hypoxic piglets and normoxic control piglets.

*different from normoxic control; ** different from L-citrulline treated chronic hypoxia. Data were compared by one-way ANOVA with Student Newman Keul's post hoc comparison test; $P < 0.05$.

Figure 7. VEGFR2 protein abundance in peripheral lung tissue from normoxic, control piglets, untreated chronically hypoxic piglets, and piglets treated orally with L-citrulline during exposure to chronic hypoxia.

VEGFR2 protein abundance did not differ between peripheral lung tissue from any of the 3 groups of piglets (A and B). P-VEGFR2 protein abundance was greater for peripheral lung tissue from the untreated group of chronically hypoxic piglets than in lung tissue from the normoxic, control piglets (A and C). P-VEGFR2 levels did not differ between lung tissue from the L-citrulline treated group of piglets and the other 2 groups of piglets (A and C).

*different from normoxic control. Data were compared by one-way ANOVA with Student Newman Keul's post hoc comparison test; $P < 0.05$.

Table.

Measurements of vascular wall thickness for four different size groups of small pulmonary arteries from lungs of normoxic control piglets, untreated chronically hypoxic piglets, and L-citrulline-treated chronically hypoxic piglets

	Wall thickness for 10–30 μ m diameter vessels (% external diameter)	Wall thickness for 31–50 μ m diameter vessels (% external diameter)	Wall thickness for 51–100 μ m diameter vessels (% external diameter)	Wall thickness for 100–300 μ m diameter vessels (% external diameter)
Normoxic, Control n=76 vessels from 3 lungs	12.7 $\pm$ 4.6	9.0 $\pm$ 2.0	8.0 $\pm$ 2.2	6.0 $\pm$ 3.0
Untreated chronically hypoxic N=76 vessels from 3 lungs	37.4 * $\pm$ 16	27.0 * $\pm$ 8.3	19.8 * $\pm$ 4.0	16.3 * $\pm$ 3.3
L-citrulline-treated chronically hypoxic N=101 vessels from 4 lungs	25 * $\pm$ 11.6	17.0 * $\pm$ 4.5	15.0 * $\pm$ 3.6	12.4 * $\pm$ 2.6

All values are mean + SD.

*
p<0.05 different from control

p<0.05 different from chronically hypoxic, ANOVA with Student Newman Keul's post hoc comparison test.