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Impaired cerebral angiogenesis in the fetal lamb model of persistent pulmonary hypertension

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ABSTRACT

Background: Persistent pulmonary hypertension of the newborn (PPHN) is associated with increased risk of neuro-developmental impairments. Whether relative fetal hypoxia during evolution of PPHN renders the fetal brain vulnerable to perinatal brain injury remains unclear. We hypothesized that in utero ductal constriction, which induces PPHN also impairs cerebral angiogenesis.
Methods: Fetal lambs with PPHN induced by prenatal ligation of the ductus arteriosus were compared to gestation matched twin controls. Freshly collected or fixed brain specimens were analyzed by immunohistochemistry, Western blot analysis, and RT-PCR.
Results: Cortical capillary density was decreased in PPHN lambs compared to controls (Glut-1, isolectin B-4 and factor VIII, $n=6$, $p<0.05$). Hypoxia inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) protein levels were decreased in cortical cell lysates of PPHN lambs. PPHN increased angiopoetin-1 (Ang-1) and tyrosine-protein kinase receptor (Tie-2) protein expression while angiopoetin-2 (Ang-2) protein levels were decreased ($n=6$, $p<0.05$). PPHN did not change mRNA levels of these proteins significantly ($n=6$).
Conclusions: PPHN decreased cortical capillary density in fetal lamb brain. PPHN decreased the expression of proteins involved in angiogenesis. These findings suggest that PPHN is associated with impaired cortical angiogenesis.

1. Introduction

Persistent pulmonary hypertension of the newborn (PPHN) predisposes infants to long-term neurodevelopmental impairment (Konduri et al., 2007a; Berti et al., 2010). Up to 25% of infants that survive PPHN have neuro-developmental impairments, including moderate-severe cerebral palsy and Bayley Mental and Psychomotor developmental indices below 70, despite advances in intensive care support (Konduri et al., 2007a). The onset of PPHN occurs during a time of robust cerebral angiogenesis in the newborn and this process is highly sensitive to changes in blood flow and oxygen levels (Dore-Duffy and LaManna, 2007; Trollmann and Gassmann, 2009). The clinical course of infants with PPHN suggests that their adaptation to systemic hypoxemia is impaired. It is unknown if the fetal brain undergoes appropriate vascular remodeling in the presence of PPHN and alterations in oxygen supply in utero. Our study investigated the adaptive mechanisms of cerebrovascular remodeling to PPHN in the fetal lamb brain.

PPHN occurs when the pulmonary vascular resistance fails to decrease at birth (Konduri and Kim, 2009). The affected infant fails to establish adequate oxygenation during postnatal life and may develop multi-organ dysfunction (Lapointe and Barrington, 2011). Hypoxemia in these infants is due to right to left extra-pulmonary shunting of blood as a result of supra-systemic pulmonary artery pressures. In utero pulmonary hypertension from ductal constriction leads to impaired development of pulmonary vasculature and decreased blood flow from right ventricle to the aortic arch, which may lead to altered cerebral blood flow. Previous studies suggested that altered autoregulation of cerebral blood flow in PPHN may cause hypoxic brain injury and contribute to cognitive deficits in

Abbreviations: CNS, central nervous system; PPHN, persistent pulmonary hypertension in the newborn; PDA, patent ductus arteriosus; Glut-1, glucose transporter-1; HIF-1, hypoxic inducible factor-1; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; Ang-1, angiopoetin-1; Ang-2, angiopoetin-2; Tie-2, tyrosine-protein kinase receptor.

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this condition (Berti et al., 2010). Studies that evaluated school-aged outcomes in PPHN survivors showed that these outcomes were not significantly improved, despite advances in neonatal intensive care, suggesting that prenatal factors may also predispose these infants to postnatal impairments (Rosenberg et al., 2010). Hypoxemia and decreased cerebral blood flow in utero during evolution of PPHN may confer vulnerability to neurodevelopmental impairment.

The cerebral cortex undergoes vascular remodeling in order to preserve oxygen and nutrient supply needed to support neuronal function and metabolic needs. Understanding the mechanisms that facilitate this adaptation may help understand fetal brain adaptation to impaired oxygen states. Our group has shown that fetal lambs exposed to in utero PPHN are relatively hypoxemic compared to controls at birth (Konduri et al., 2003) and that maladaptation to changes in PaO_2 in PPHN in the lung occurs (Teng et al., 2011, 2009). Sheep have been used extensively to investigate many aspects of CNS homeostasis (Chen et al., 2012; van den Heuij et al., 2014). The development of the ovine brain at birth is similar to that of the human infant with respect to completion of neurogenesis, cerebral sulcation, and detection of the cortical component of somatosensory evoked potentials (Back et al., 2006; Bernhard et al., 1967). For these reasons, the sheep model is ideal to test the hypothesis that cerebral angiogenesis is impaired in the lamb cerebral cortex, as it is in the lung (Teng et al., 2011, 2009).

Microvascular remodeling occurs in the cerebral cortex in order to preserve tissue oxygen and energy supply for neuronal function in hypoxic states (Benderro and Lamanna, 2011). In the rodent model of chronic hypoxia, exposure to mild hypoxia results in systemic and central nervous system adaptations that allow acclimatization to changes in the microenvironment (Dore-Duffy and LaManna, 2007; Mironov et al., 1994; Stewart et al., 1997; Harik et al., 1996). The first structural changes to chronic hypoxia are seen at 4–7 days and continue for up to 3 weeks (Dore-Duffy and LaManna, 2007; Harik et al., 1996). The structural changes not only include increased capillary density, but also capillary length and diameter (Mironov et al., 1994; Stewart et al., 1997). Angiogenesis is a highly complex and coordinated process requiring multiple angiogenic and regulatory factors, receptors, and intracellular signaling pathways (Benderro and Lamanna, 2011). There appears to be at least two major pathways and likely a number of other redundant pathways responsible for brain angiogenesis: hypoxia-inducible factor-1 (HIF-1) dependent upregulation of vascular endothelial growth factor (VEGF) and HIF-1 independent, cyclooxygenase-2 (COX-2) dependent process with upregulation of angiopoietin-2 (Ang-2) (Benderro and Lamanna, 2011). Brain vascular remodeling in response to changes to oxygen availability depends on the balance between these two angiogenic pathways. We hypothesized that dysregulation of these intersecting pathways contribute to impaired cerebral angiogenesis in PPHN.

2. Materials and methods

2.1. Creation of PPHN model

This study was approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC) and conformed to the current guidelines of NIH Care and Use of Laboratory Animals. PPHN was induced by fetal ductus arteriosus constriction at 128 ± 2 days gestation (term gestation ≈ 145 days) in twin gestation sheep as we described previously (Konduri et al., 2003, 2007b). Control fetal lambs received sham operation. After 8 days of ductal constriction, fetal lambs were delivered by Cesarean section, euthanized, weighed, gender determined, and the brain was quickly removed and weighed. One half of the brain was removed and frozen for protein analysis. The parietal lobe was sectioned, formalin-fixed, and paraffin-embedded for immunohistochemistry.

2.2. Immunohistochemistry and determination of capillary density

Brain samples from normotensive and hypertensive lambs from six separate experiments were fixed in 10% neutral-buffered formalin, processed, and embedded in paraffin. Coronal serial sections (5 μm) were obtained and mounted on positively charged glass slides (Fisher). For isolectin B4, slides were hydrated with deionized water, antigen retrieval with citrate buffer (pH 6, Dako, Carpinteria, CA, s1699) at 90 °C for 20 min was performed, and then slides were cooled in citrate buffer for 20 min. All slides were stained using a Dako Autostainer Plus using programmed steps for peroxidase blocking and incubation with biotinylated primary antibody (1:200, Vector, Burlingame, CA, b-1205) for 60 min in room temperature. Following incubation with antibody overnight at 4 °C, slides were rinsed in TBST (Thermo Scientific, Fremont, CA, TA-999-TT), then Dako tertiary with Streptavidin HRP (1:300, Dako, P039701-2) and DAB application (Dako, K346811-2) was performed and the slides were counterstained with hematoxylin (Dako, s33093-2) followed by 0.1% Ammonium hydroxide solution. For Glut-1 and factor VIII a Leica Bond Max Immunostainer was used. Both antibodies required antigen retrieval using Leica HI antigen retrieval reagent (AR9961), with Glut-1 needing 10 min and factor VIII, 20 min. Both antibodies were detected and visualized using Bond Polymer Refine Detection System (DS9800) with the addition of a DAB enhancer (AR9436), using the MOD F protocol/software from Leica. An optimal concentration of 1:900 was determined for Glut-1 (Thermo-Fisher, MS-10637) and 1:100 for factor VIII (Biocare, CP039). All slides were counter-stained with hematoxylin and coverslipped using a synthetic mounting media. Omission of the primary antibody served as negative control. Quantification analysis was performed by a researcher blinded to the treatment groups using a computer-assisted method with Visiomorph™ (Visiopharm, Denmark).

2.3. Quantitative analysis of image data

Microscope slides of the histological staining were scanned at 40×, 20×, 10×, and 5× magnification using a Nanozoomer HT system (Hamamatsu, Japan) in the Pediatric BioBank & Analytical Tissue Core at the Children's Research Institute at the Medical College of Wisconsin. The resulting digital images were used for quantitative analysis using commercial image post-processing software (Microimager and Visiomorph, Visiopharm, Denmark). Brown, DAB-positive pixels, blue (Hematoxylin) pixels, and white (background) pixels were classified by the software user using linear Bayesian Classification. Counts and area of DAB-positive events (<5 μm and >800 μm were excluded) and area of hematoxylin-positivity were determined and exported to an Excel worksheet, where the number of DAB-positive events (=micro vessels or capillaries) per square millimeter tissue was calculated as count DAB/sum of areas DAB and Hematoxylin.

2.4. Preparation of whole cell lysate

Frozen brain cortex samples from normotensive and hypertensive lambs from six separate experiments were dissected and homogenized in ice-cold lysis buffer (modified RIPA) containing 1% protease inhibitor cocktail (Roche, Nutley, NJ) and centrifuged to remove cell debris. Total protein concentrations of the homogenates were determined with a biccinchoninic acid protein assay (BCA, Pierce, Rockford, IL).

2.5. Western blot detection and quantification of protein

An aliquot of protein (30 μg) was fractionated by SDS-PAGE electrophoresis and transferred onto PVDF membranes (0.2 micron, Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with primary antibodies: HIF-1α (mouse monoclonal, 1:250, Invitrogen, Camarillo, CA), VEGF (mouse monoclonal, 1:500, Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 (rabbit polyclonal, Cayman Chemical, Ann Arbor, MI), Ang-1 (goat polyclonal, 1:500, Santa Cruz Biotechnology), Ang-2 (rabbit polyclonal, 1:500, abcam, Cambridge, MA), and Tie-2 (rabbit polyclonal, 1:200, Santa Cruz Biotechnology). Membranes were then blotted with HRP-conjugated secondary antibody (1:10,000) appropriate for each primary antibody. The membranes were exposed to Hyperfilm ECL (Phenix, Candler, NC) after treatment with Super-Signal West Pico (Pierce). Band intensity was analyzed with NIH ImageJ (US NIH, Bethesda, Maryland, USA, http://imagej.nih.gov). The final values represent an average of the densitometric values obtained from two to three different immunoblots. The densitometric values were presented as a ratio to the densitometric values of β-actin used as a housekeeping protein.

2.6. Quantification of mRNA abundance

RNA was extracted from frozen tissue from normotensive and hypertensive lambs from six separate experiments using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad). cDNA was synthesized from the extracted RNA using the iScript cDNA synthesis kit (Bio-Rad). The PCR primers were designed for sheep using Primer3 as previously described (Rozen and Skaletsky, 2000) and shown in Table 1. Real-time RT-PCR was performed using the iQ5 multicolor real-time PCR detection system (Bio-Rad). The PCR cycle was started at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and then 58 °C for 1 min. Melting temperatures were monitored