Developmental acceleration of bradykinin-dependent relaxation by prenatal chronic hypoxia impedes normal development after birth.


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Running Head: Chronic hypoxia and impaired development of vasorelaxation

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Abstract

Bradykinin-induced activation of the pulmonary endothelium triggers nitric oxide (NO) production and other signals that cause vasorelaxation, including stimulation of large-conductance K⁺ (BKCa) channels in myocytes that hyperpolarize the plasma membrane and decrease intracellular Ca²⁺. Intrauterine chronic hypoxia (CH) may reduce vasorelaxation in the fetal transition, and contribute to pulmonary hypertension of the newborn. Thus, we examined the effects of maturation and CH on the role of BKCa channels during bradykinin-induced vasorelaxation by examining endothelial Ca²⁺ signals, wire-myography and Western Immunoblot on pulmonary arteries (PA) isolated from near term fetal (~ 140 days gestation) and newborn, 10 to 20 days old, sheep that lived in normoxia at 700 m or in CH at high altitude (3,801 m) for >100 days. CH enhanced bradykinin-induced relaxation of fetal vessels but decreased relaxation in newborns. Endothelial Ca²⁺ responses decreased with maturation but increased with CH. Bradykinin-dependent relaxation was sensitive to 100 μM LNAME or 10 μM ODQ, supporting roles for endothelial nitric oxide synthase and soluble guanylate cyclase activation. Indomethacin blocked relaxation in CH vessels suggesting upregulation of PLA₂ pathways. BKCa channel inhibition with 1 mM TEA reduced bradykinin-induced vasorelaxation in the normoxic newborn and fetal CH vessels. Maturation reduced whole-cell BKCa α₁ subunit expression but increased β₁ expression. These results suggest that CH amplifies the contribution of BKCa channels to bradykinin-induced vasorelaxation in fetal sheep, but stunts further development of this vasodilatory pathway in newborns. This involves complex changes in multiple components of the bradykinin-signaling axes.
Introduction

The regulation of smooth muscle tone in pulmonary arteries during development is a delicate balance of vasoconstrictive and vasorelaxant pathways. Endothelial cells play a crucial role in determining the overall level of vasorelaxation (39, 67), and endothelium-dependent relaxation is partially mediated through bradykinin stimulation (31). Bradykinin is a potent vasodilator that is important in the fetal pulmonary circulation as well as during inflammation and whose relationship to pulmonary hypertension has been explored (5, 31, 83).

Endothelial bradykinin receptor activation induces vasorelaxation through modulation of several different intracellular signaling pathways that are largely dependent on a rise of endothelial intracellular Ca^{2+} (67). The most widely studied pathway is bradykinin-induced activation of endothelial nitric oxide synthase (eNOS), an enzyme that generates nitric oxide (NO) (64). NO acts on nearby smooth muscle cells to cause downstream stimulation of soluble guanylate cyclase (sGC) pathways that leads to vasorelaxation (3, 45). Previous studies have shown that the regulation of vessel relaxation through various receptor signaling systems is altered during pre- and post-natal development as well as following prenatal chronic hypoxia, which imposes a significant stress on the fetus (9, 10, 35). Evidence suggests that acetylcholine (ACh)-dependent endothelium-mediated relaxation of the pulmonary vasculature is reduced in the fetus relative to the adult (57). Prenatal chronic hypoxia further suppresses ACh-induced relaxation in the fetus, but this is not due to changes in eNOS expression (91). The suppression of ACh-dependent relaxation maintains high pulmonary vascular
resistance (PVR) that restricts blood flow, which is important because the lung is not yet required for gas exchange. However, during the transition at birth from fetus to newborn, the pulmonary vessels dilate rapidly, increasing blood flow to the alveoli, and allow for proper gas exchange in the newborn lung.

Chronic hypoxia is a known risk factor in the development of pulmonary hypertension. It can significantly enhance vasoconstriction as well as reduce vasodilatory capacity. Subsequently, these effects elevate pulmonary pressure, which can result in pulmonary hypertension. The risk of pulmonary hypertension is especially prominent among newborns exposed to chronic hypoxia in utero due to pregnancy at high-altitude, placental insufficiency, smoking, maternal anemia, or other causes.

Persistent pulmonary hypertension of the newborn (PPHN) due to hypoxia or other etiologies is an incapacitating disease that can lead failure of the ductus arteriosus to close, resulting in severe systemic hypoxia. Unfortunately, there are few treatment options and no cures. Numerous studies have indicated that endothelium-derived relaxing factors, especially those associated with NO, are crucial in the modifications associated with loss of vasodilatory capacity and development of pulmonary hypertension.

Our knowledge regarding the influence of prenatal chronic hypoxia on endothelium-dependent relaxation is limited. The available evidence indicates that there is enhanced eNOS expression, reduced sGC, and CO mediated relaxation but enhanced BKCa function. In fetal lamb eNOS expression is unchanged but there is enhanced PKG but reduced cGMP function. The diversity in the dysfunctions and compensations associated with high altitude gestation
have led us to design a series of studies to test how prenatal chronic hypoxia affects early postnatal bradykinin-induced vasorelaxation. We tested the specific hypothesis that prenatal chronic hypoxia impairs the normal development of relaxation through eNOS-dependent pathways. This hypothesis was tested in studies on arteries isolated from term fetal and newborn sheep housed at either low or high altitude, allowing for direct comparative analyses.

Methods

Experimental animals

Experimental procedures were performed on sheep arteries because the developmental progression of their lungs and the extent to which prenatal chronic hypoxia affects them are comparable to humans (67). These studies were performed within the regulations of the Animal Welfare Act, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, “The Guiding Principles in the Care and Use of Animals” approved by the Council of the American Physiological Society, and the Animal Care and Use Committee of Loma Linda University (LLU). The tissue preparation, wire myography and imaging experimental procedures and protocols are based on previously published methodology (10, 35, 37). Sheep were obtained from Nebeker Ranch (Lancaster, CA; at an elevation of 720 m), and normoxic animals were brought to LLU (353 m; arterial PaO₂ = 95 ± 5 Torr) for experimental study. Animals in the chronic hypoxic experimental group were acclimatized to high-altitude (3,801 m, PaO₂ = 60 ± 5 Torr) at the Barcroft Laboratory, White Mountain Research Station (Bishop, CA) for ~110 days (35, 37). The hypoxic ewes were then transported to LLU for experimental study. To maintain hypoxic conditions in pregnant sheep, a tracheal
catheter was placed in the ewe shortly after arrival. The tracheal catheter allowed N\textsubscript{2} to reach the animal at a rate adjusted to maintain PaO\textsubscript{2} at \( \sim 60 \) Torr, equivalent to the PaO\textsubscript{2} at the White Mountain Research Station \((47)\). This PaO\textsubscript{2} was maintained until the time of the experimental study. With regards to the newborn animals, ewes in the normoxic and chronic hypoxic groups were allowed to give birth naturally, and were transported to Loma Linda University Animal Care when the lambs were 8 to 17 days of age, as described previously \((10)\). After arrival and until study, which ranged from 1 to 2 days later, hypoxic animals were kept in a chamber supplied with 14 to 16\% oxygen that simulated the altitude at the Barcroft Laboratory. Both groups of lambs were studied between 10 and 20 days after birth.

For the fetal studies, within 1 to 5 days of arriving at LLU, pregnant sheep were sacrificed with an overdose of Euthasol (pentobarbital sodium 100 mg/Kg and phenytoin sodium 10 mg/Kg; Virbac, Ft. Worth, TX), the proprietary euthanasia solution. Lungs were removed from the fetuses and used immediately for contractility and imaging experiments. Care was taken during arterial isolation and wire mounting to ensure that the endothelium was not disrupted.

**Tissue preparation**

As detailed previously, 4th to 5th branch order pulmonary arteries with internal diameters of about 500-700 \( \mu \)m were isolated from full-term fetal \((138-141 \) days) or newborn \((10 – 21 \) days old) sheep from the different experimental groups \((10, 35, 37)\). The parenchyma was removed carefully from the pulmonary arteries for contractility studies. The arteries were then cut into 5 mm long rings in ice-cold phosphate-free balanced salt solution (BSS) of the following composition (mM): 126 NaCl; 5 KCl; 10
HEPES; 1 MgCl$_2$; 2 CaCl$_2$; 10 glucose; pH 7.4 (adjusted with NaOH). Imaging studies and the contraction studies in which arteries were treated with the NO donor N-Nitrosoproline (ProliNO), which releases NO in a 1:2 ProliNO:NO molar ratio with a dissociation half-life of ~2 seconds at physiological pH and temperature (76), were performed in BSS. All other contraction studies were performed with a modified Krebs-Henseleit (K-H) solution containing in mM: 120 NaCl; 4.8 KCl; 1.2 K$_2$HPO$_4$; 25 NaHCO$_3$; 1.2 MgCl$_2$; 2.5 CaCl$_2$; 10 glucose. To depolarize the arteries, NaCl was omitted from the BSS or K-H solutions and replaced with equimolar KCl.

**Contraction Studies**

Wire-mounted pulmonary arterial rings were suspended in organ baths (Radnoti Glass Instruments, Inc. Monrovia, CA) containing 5 or 10 ml of modified K-H solution or BSS maintained at 37ºC. Arteries in modified K-H were aerated with 95% O$_2$-5% CO$_2$ (pH=7.4) while unaerated HEPES-buffered BSS was used for the ProliNO studies to prevent sparge-induced loss of free NO into the atmosphere. Each ring was suspended between two tungsten wires passed through the lumen. One wire was anchored to the glass hook at the bottom of the organ chamber, while the other was connected to a tissue hook attached to a low compliance force transducer (Radnoti Glass Instruments Inc.) that measured isometric force (10, 35, 37). The transducers were connected to an analog-to-digital data interface (Powerlab 16/30 A/D Instruments, Colorado Springs, CO; or MP100, Biopac, Goleta, CA) attached to a computer. Changes in tension were recorded using Chart 5.5 or 7.0 (AD Instruments), or AcqKnowledge 3.9 (Biopac Systems, Inc.), and the obtained data was stored on digital media for later analysis.
At the beginning of each experiment, vessels were equilibrated without tension for a minimum of thirty minutes. Tension was then applied to approximately 0.75 grams and allowed to stabilize, as previously described (10, 35, 37). To allow for comparative evaluation of smooth muscle contraction and relaxation, isolated pulmonary arterial rings bathed in modified K-H were stimulated with 125 mM KCl (high-K) to cause membrane depolarization and activate L-type Ca\(^{2+}\) channels (Ca_L) (35). In some experiments, the tension was normalized to a control response obtained with high-K (%TK\(_{\text{max}}\)). To evaluate dose-response relaxation characteristics, bradykinin was used to relax the arteries following pre-contraction with 10 μM phenylephrine (PE). In these experiments, the tension was normalized to the maximum 10 μM PE pre-contracted tension (%T\(_{10\,\mu\text{M PE}}\)) as denoted on each figure panel.

**Confocal Microscopy Studies**

\([\text{Ca}^{2+}]_i\) was measured in pulmonary arterial endothelial cells *in-situ*, with the Ca\(^{2+}\) sensitive dye Fluo-4 AM (Invitrogen, Carlsbad, CA) using a Zeiss 710 NLO laser scanning confocal imaging workstation (Thornwood, NY) with an inverted microscope (Zeiss Axio Observer) using procedures based on those previously described (35, 37). Fluo-4 AM was dissolved in DMSO and added from a 1 mM stock solution to the arterial suspension at a final Fluo-4 concentration of 10 μM, along with 0.1% Pluronic® F-127 for 1 hour at room temperature in the dark in BSS. Arterial segments then were washed with BSS for 30 min to allow for dye esterification and cut into linear strips. The arterial segments were pinned to Sylgard® (Ellsworth Adhesives, Germantown, WI) and placed in an open-bath imaging chamber (Warner Instruments, Hamden, CT) mounted on the confocal imaging stage. Cells were illuminated at 488 nm with a krypton argon laser,
and the emitted light was collected using a photomultiplier tube (PMT) with a band-limited spectral grating of range 493 to 622 nm with full-frame images made every 700 ms. To ensure that the endothelial intracellular Ca\textsuperscript{2+} concentration was recorded, the pinhole was adjusted to provide an imaging depth of 5.4 μm, and the sample was focused above the internal elastic lamina layer, which has significant autofluorescence when excited at 488 nm in this preparation. This depth is substantially deeper than an individual endothelial cell based on morphological examination of fixed and live preparations (data not shown) and accounts for sample ruffling, thus allowing for the examination of many more endothelial cells than otherwise would have been achieved.

Images were acquired at a 12-bit sampling depth. Recordings were made using an immersion 40X Plan Apochromat, 1.0 NA objective mounted on an inverterscope objective inverter (LSM Tech, Wellsville, PA), which allowed for imaging in an upright configuration. To prevent arterial movement during recordings, arteries were pre-treated for one hour with a cocktail including 10 μM Y27632 to inhibit Rho kinase, 10 μM Cytochalasin D to inhibit actin polymerization, and 10 μM W-7 to inhibit Myosin Light Chain Kinase (10, 43, 56, 88). The concentrations of these drugs were chosen based on the outcomes of wire myography studies where the influence of the drugs on vessel reactivity were examined individually. Regions of interest were detected automatically post-hoc using the LC Pro plug-in for ImageJ (22). For presentation purposes, the fractional fluorescence intensity was automatically calculated using LC Pro.

Background was subtracted from measurements using the equation:

\[ \frac{F}{F_0} = \frac{F}{F_0} - \text{baseline} \]
Where baseline is the intensity from a region of interest with no cells, F is the fluorescence intensity for the region of interest, and F₀ is the fluorescence intensity during a period when there was no Ca²⁺ activity as determined automatically. False positives were excised from the final data set by their relationship to the timing of the application of bradykinin and by visual analysis of the data.

**Western Immunoblot Studies**

To generate whole-cell lysates, pulmonary arteries from fetal and newborn sheep were harvested, cleaned and rapid-frozen in liquid nitrogen and stored at -80°C. Tissues subsequently were homogenized using glass-on-glass in a RIPA extraction buffer containing 10 mM DTT and a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO #M1745). Samples were centrifuged at 5000 G for 20 minutes; supernatants were collected and analyzed using SDS-PAGE along with reference control samples from pregnant adult sheep middle cerebral arteries (MCAs). Separated proteins were transferred to nitrocellulose membranes at 200 mA for 90 minutes in Towbin buffer (25 mM Tris, 192 mM Glycine, and 20% Methanol). Membranes were blocked using 5% milk in Tris-buffered saline at pH 7.45 (M-TBS) for 1 hour at room temperature with continuous shaking. Primary antibodies were incubated for 12 hours at 4°C using the following dilutions for BK-α 300:1 (Alomone, Jerusalem, Israel. #APC-021) and BK-β1 300:1 (Alomone #APC-036) (44). For visualization, membranes were incubated for 90 minutes with a secondary antibody conjugated to DyLight 800 (Pierce Chemical, Rockford, IL #46422). Subsequently, membranes were stripped and reprobed using antibodies against β-Actin (Sigma-Aldrich #A2228 Monoclonal Anti-β-Actin produced in mouse, clone AC-74) as a loading control. Anti-β-Actin was diluted using a factor of
1:5000, and incubated for 90 minutes in a milk-TBS buffer with 0.1% Tween-20.

Membranes were imaged on LI-COR Bioscience’s Odyssey system and individual protein bands were quantified using LI-COR Image Studio® software. Protein abundance was normalized using β-Actin as a loading control, and expressed as a relative abundance compared with an arbitrary reference standard (pooled whole-cell lysate obtained from pregnant adult sheep middle cerebral arteries).

**Chemicals and drugs**

Most reagents and chemicals were purchased from Sigma-Aldrich, while bradykinin, Y-27632, and W-7 were purchased from Tocris (Minneapolis, MN).

**Statistical Methods and Sampling**

All time-series recordings were graphed with IGOR pro 6.0 (Wavemetrics, Lake Oswego, OR), and summarized data presented as mean ± S.E.M. Statistical analyses were made using GraphPad Prism 5.0 (La Jolla, CA). Data was evaluated for normality prior to any comparative statistical analysis. The specific test used is denoted in the figure legend or table. Dose-response curves were fitted in Prism 5.0 using the Hill equation, with non-linear curve fit analysis (10, 35). A total of 605 arterial segments from 61 sheep were tested for contractility. The Ca$^{2+}$ imaging studies arteries were performed on tissues from 6 fetal normoxic, 4 fetal hypoxic, 3 newborn normoxic, and 4 newborn hypoxic sheep. Western blot analysis was performed on tissues from 9 fetal normoxic animals and from 6 animals in each of the other three groups. P<0.05 was accepted as statistically significant, unless otherwise noted.

**Results**

**Endothelial cells of fetal sheep pulmonary arteries**
The first set of studies imaged the endothelium of intact, isolated, ovine pulmonary arteries to demonstrate that our arterial isolation techniques do not disrupt the endothelial layer. These images are important, as subsequent figures show the findings of studies designed to evaluate functional responses of the endothelium in pulmonary arteries from fetal and newborn sheep housed at low or high altitude. Representative images are presented in Figure 1, illustrating that the endothelial cells have an ellipsoid shape, which is presumed to be along the direction of blood flow, and that the cells are closely associated with one another. No quantitative measurements of endothelial cell shape or structure were performed in the present studies.

**Prenatal chronic hypoxia and bradykinin – mediated relaxation**

The first series of functional studies was designed to determine the extent to which bradykinin-relaxes pulmonary arteries and the extent to which this is altered by prenatal chronic hypoxia. These studies were based on the general premise that prenatal chronic hypoxia impedes relaxation (20, 21, 87). This proposition was investigated by examining dose-dependent bradykinin-mediated relaxation of 10 μM phenylephrine pre-contracted vessels. Dose-response curves of the data from these studies and summary results are shown in Figure 2. Figures 2A and B show that prenatal chronic hypoxia enhances the bradykinin-induced vasorelaxant response in arteries from the fetus, but that relaxation is blunted in vessels from the chronic hypoxic newborn. Figure 2C and Table 1 illustrate that the potency is enhanced by postnatal maturity, but unaffected by high altitude chronic hypoxia. Figure 2D and Table 2 show that continued life at high altitude impairs the normal developmental increase in
maximum response to bradykinin, in that the relaxation is equivalent to vessels from the hypoxic fetus.

Prenatal chronic hypoxia and endothelial Ca\(^{2+}\) signals

Bradykinin-induced relaxation is well regarded as being related to Ca\(^{2+}\) dependent signaling in endothelial cells (78). Because of the influence of development and chronic hypoxia on bradykinin induced relaxation of pulmonary vessels presented in Figure 2, we examined the potential that prenatal chronic hypoxia enhances bradykinin-induced Ca\(^{2+}\) signals in fetal endothelial cells but restricts them in the newborn. Figure 3 shows Ca\(^{2+}\) responses in fetal endothelial cells recorded before and during 1 μM bradykinin administration in the presence of extracellular Ca\(^{2+}\). The data in Figure 3A shows in situ fluorescence images of endothelial cells in the arterial wall isolated from a prenatal chronic hypoxic fetal sheep for various time points as shown for the fluorescence intensity tracing in Figure 3B. Bradykinin rapidly increased cytosolic Ca\(^{2+}\) in the pulmonary arterial endothelial cells, and these signals decayed slowly back to baseline. Qualitatively, the increase in Ca\(^{2+}\) was similar for all experimental groups examined, and thus other representative images are not provided.

The magnitude and kinetics of these fluorescence Ca\(^{2+}\) signals were then quantified. Figures 4 provides summaries of the quantitative analysis for various aspects of the Ca\(^{2+}\) responses. These data demonstrate that the amplitude (Figure 4A) increases modestly after birth in arterial endothelial cells from control animals. However, prenatal chronic hypoxia enhances the amplitude of the Ca\(^{2+}\) signal in the fetus but not the newborn. The area under the curve (Figure 4B), event duration (Figure 4C) as well as decay (Figure 4D) all decreased following birth but are increased somewhat by
chronic hypoxia in the newborn. The duration of Ca2+ rise (attack, Figure 4E) became shorter after birth and following prenatal chronic hypoxia in cells from the fetus. When compared to the vasorelaxant influences of bradykinin, the data suggest postnatal maturation enhances Ca2+ sensitivity, while chronic hypoxia augments Ca2+ signals in neonatal endothelial cells, but suppresses postnatal sensitivity.

**Prenatal chronic hypoxia and eNOS dependent relaxation**

Given the uncoupling between endothelial Ca2+ signals and vasorelaxation following prenatal chronic hypoxia and postnatal maturation, the next series of studies tested the hypothesis that altered bradykinin-induced eNOS activation mediates this detachment. To evaluate this process, nitric oxide synthesis was inhibited with 100 µM L-NG-Nitroarginine Methyl Ester (LNAME) (71). Figure 5 shows that LNAME successfully reduced bradykinin-induced vasorelaxation in 10 µM phenylephrine pre-contracted hypoxic fetus as well as newborn normoxic and hypoxic vessels. In addition to the increased sensitivity of fetal vessels to LNAME following hypoxia, hypoxic fetal vessels displayed greater reductions in overall vasorelaxation. The summary data in Table 1 show that the potency of bradykinin-induced relaxation was unaffected by LNAME. Table 2 demonstrates that the maximum response was not affected by eNOS inhibition in normoxic fetal vessels, but was significantly reduced in the newborn and the hypoxic fetus. Before birth, prenatal chronic hypoxia increased the role for eNOS, but the change in maximum response was virtually identical to that of hypoxic newborns. Overall, these findings suggest that prenatal chronic hypoxia accelerates development of eNOS dependent vasorelaxation in the fetus possibly involving sGC, PKG or other
downstream components of the pathway, but this acceleration restrains the normal
development of pulmonary vascular relaxation to bradykinin.

Prenatal chronic hypoxia and prostacyclin-dependent vasorelaxation

Prostacyclin (PGI$_2$) is released by the endothelium and is another significant
signaling arm activated by endothelial bradykinin stimulation (11). To test the role of
PGI$_2$ in bradykinin-induced vasorelaxation, 10 μM indomethacin (INDO) was added to
inhibit cyclooxygenase (COX) dependent PGI$_2$ production. At this concentration, INDO
inhibits the activity of both COX 1 and 2 (61, 66). Figure 6 shows dose-dependent
relaxation of 10 μM phenylephrine pre-contracted vessels to bradykinin in the presence
of 10 μM INDO. As illustrated in Figures 6A and 6B, COX inhibition attenuated the
maximal vasorelaxation response in arteries from fetuses following prenatal chronic
hypoxia, without effect on arteries from normoxic fetuses. Figure 6C shows that the
relaxation to bradykinin in normoxic newborn arteries was largely unaffected by COX
inhibition although phenylephrine vasoreactivity was enhanced. This compares to the
hypoxic newborn data shown in Figure 6D that were far more sensitive to INDO. Table 1
shows that COX inhibition failed to alter potency in all groups, but Table 2 shows that
normoxic arteries were resistant to INDO while prenatal chronic hypoxia unmasked
PGI$_2$-dependent relaxation in arteries from both fetus and newborn.

Prenatal chronic hypoxia and sGC relaxation

Soluble guanylate cyclase (sGC) is activated by NO in smooth muscle
downstream of eNOS stimulation (4). Such activation leads to cGMP formation with
subsequent vasorelaxation. To establish a more complete picture of the impact of
prenatal chronic hypoxia on the role of sGC, we inhibited this enzyme with 10 μM 1H-
[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) prior to endothelial stimulation with bradykinin (29). Figure 7 shows the dose-dependent vasorelaxation to bradykinin in the presence and absence of ODQ. Figures 7B-D show that sGC inhibition suppresses bradykinin-mediated relaxation in the newborn and the hypoxic fetus, while the meager relaxation in normoxic fetus was relatively unaffected (Figure 7A). As summarized in Table 1, ODQ treatment did not affect potency in arteries from fetal or newborn normoxic or hypoxic sheep. However, Table 2 shows maximum response was reduced in all groups except for the normoxic fetus.

The results of the LNAME and ODQ studies illustrate that both eNOS and sGC are important to bradykinin-dependent relaxation. There is potential that chronic hypoxia accelerates their development *in utero*, but impedes normal development of these or other pathway components after birth. Nonetheless, previous evidence indicates eNOS expression is unaffected by prenatal chronic hypoxia in our fetal sheep (91). Because of these findings, we performed a series of experiments to examine the direct impact of NO on vessel reactivity. In particular, we evaluated the potential for NO to directly stimulate sGC activity by addition of the NO donor ProliNO (76). Figure 8 shows results from 10 μM 5-HT pre-constricted vessels relaxed with ProliNO in the presence and absence of the sGC-inhibitor ODQ, where 5-HT was used because it induces robust contraction (35). Overall, ProliNO effectively relaxed 5-HT pre-contracted vessels from normoxic and prenatal chronic hypoxic fetal and newborn sheep (Figure 8A). sGC inhibition effectively reduced the potency in all groups, and reduced the maximum response of ProliNO-induced relaxation in the hypoxic fetus.

Large conductance Ca^{2+} activated K^{+} channels and bradykinin relaxation
K⁺ channels are critical for establishing myocyte resting membrane potential and are frequent targets of endogenous and therapeutic vasodilators (52). One primary target of NO-dependent signaling is the large-conductance Ca²⁺-activated K⁺ (BKCa) channel (79). The BKCa channel is stimulated by ryanodine receptor-dependent Ca²⁺ sparks and independently modulated by protein kinases A and G downstream of sGC and cGMP (3, 74, 77, 79). To establish the role BKCa channels play in bradykinin-mediated vasorelaxation, arteries were treated with 1 mM tetraethylammonium (TEA), a concentration that blocks roughly one-half of the current due to BKCa channels (44, 46). Figure 10 shows the dose-dependent relaxation of phenylephrine pre-contracted vessels to bradykinin in the presence and absence of 1 mM TEA. BKCa channel inhibition with TEA had no significant effect on bradykinin-induced vasorelaxation in normoxic fetal and hypoxic newborn vessels as shown in Figures 9A and 9D, respectively. However, Figure 9B shows that 1 mM TEA impaired bradykinin-mediated vasorelaxation at 1 µM bradykinin in arteries from prenatal chronic hypoxic fetuses, while inhibition of the BKCa channel had no effect on bradykinin-induced relaxation in arteries from hypoxic newborns (Figure 9D). BKCa channel inhibition weakened bradykinin-induced relaxation in the normoxic newborn (Figure 10C), indicating a substantial developmental increase in the role of the BKCa channel in bradykinin relaxation. Table 1 shows that 1 mM TEA failed to affect the potency of bradykinin-induced relaxation in any of the groups. Table 2 shows that BKCa channel inhibition reduced the maximum response in the prenatal chronic hypoxic fetus and in the normoxic newborn. Although chronic prenatal hypoxia promoted BKCa related relaxation...
in the fetus, it suppressed the normal development of BK$_{Ca}$-mediated relaxation in the newborn.

**BK$_{Ca}$ Channel Protein Expression**

It has been well established that the BK$_{Ca}$ channel is a crucial regulator of myogenic tone and blood pressure, and is especially important to O$_2$-induced pulmonary arterial relaxation at birth (12, 70, 86). The BK$_{Ca}$ channel is made up of four, pore-forming α subunits to which accessory β subunits can attach (51, 63). Four classes of β subunits have been identified to date, although there may be more (62, 84). These accessory β subunits can regulate voltage and Ca$^{2+}$ sensitivity to specialize BK$_{Ca}$ channel opening behavior, with the β1 subunit being the most common β subunit in vascular smooth muscle cells (13, 63). In its absence, mouse arterial tone and blood pressure have been shown to increase due to a reduction in BK$_{Ca}$ Ca$^{2+}$ sensitivity and coupling to Ca$^{2+}$ sparks (69). Furthermore, other studies have shown that the activity of the BK$_{Ca}$ channel is developmentally regulated, and is differentially affected by O$_2$ tension (73). To determine whether or not the increase in relaxation seen in fetal arteries following prenatal chronic hypoxia was mediated by a change in BK$_{Ca}$ channel expression, Western Immunoblot analysis was conducted for the α and β1 subunits. Figure 10 shows the results of this analysis. As shown in Figure 10B maturation of the fetus to the newborn significantly decreased α subunit expression under normoxic conditions. However, α subunit expression did not decrease further following prenatal chronic hypoxia. Figure 10C and 10D shows that maturation significantly increased the β1 subunit expression as well as the ratio of β1 to α subunit expression regardless of oxygenation status.
Discussion

These studies are the first to examine the influence of prenatal chronic hypoxia on bradykinin-mediated pulmonary arterial relaxation of both the fetus and newborn, and to elucidate the roles of important underlying signaling pathways. A schematic summary of these findings and relationship to our previous work is presented in Figure 11 [37]. In the pulmonary arterial endothelium, we examined the importance of Ca^{2+} elevations, eNOS, and COX signaling. In smooth muscle, we evaluated subsequent activation of sGC and downstream influences on BK_{Ca} channels. Our studies demonstrate that prenatal chronic hypoxia accentuates relaxation in the fetal pulmonary vasculature through enhanced pathway development, but this elevation leads to impediments following birth that ultimately impair pulmonary vascular relaxation in the newborn.

Prenatal chronic hypoxia enhanced bradykinin-mediated pulmonary arterial relaxation in the fetus. This was a surprise because chronic hypoxia generally increases pulmonary arterial pressure and depresses pulmonary arterial vasodilatory capacity, as was observed in the newborn (Figure 2D) and previous studies [40]. The findings suggest that fetal sheep adapt in utero to compensate for eventual birth in a high-altitude, low-oxygen environment. Based on this line of reasoning, the resultant increase in blood flow at birth in high-altitude adapted fetuses with heightened bradykinin-mediated arterial relaxation would enhance O_{2} diffusion from the alveoli into the bloodstream. O_{2} uptake would be improved when the newborn breathes, allowing the animal to thrive during the fetal to newborn transition.
This theoretical cause-and-effect relationship was not seen in high altitude newborn sheep two weeks after birth. Rather, the normal course of the development of pulmonary vasorelaxation was restricted (Figure 2D). This loss could potentially contribute to the elevation in pulmonary pressures and increases in pulmonary vascular resistance that we and others have documented in lambs born at high-altitude (10, 40). Furthermore, it is possible that the prenatal acceleration of vasorelaxation responses to bradykinin is redistributing blood flow from developing vital organs to the lung, resulting in blood flow distribution issues. Such an alteration of distribution following chronic hypoxia previously was reported in studies on our fetal sheep (47). These studies showed that blood flow to the fetal brain and heart was maintained despite a significant decline in cardiac output and reduction of flow throughout the body. This preservation in perfusion is indicative of the autoregulatory nature of blood flow to brain and heart. If chronic prenatal hypoxia also decreases fetal pulmonary vascular resistance and reallocates blood flow to the lung these effects could, jointly, place additional stress on other vital organ systems that potentially compromises their function and growth.

The improvement in bradykinin potency following birth could be due to various modifications in the bradykinin signaling pathway. Based on classical pharmacological principles there could be changes in receptor density, ligand affinity, or receptor coupling. Changes in bradykinin receptor expression likely underlie the enhancements and reductions in the maximal relaxation response, but not the effects on potency. The modifications in potency more likely reflect improved receptor affinity or coupling of the receptor to intracellular signaling pathways. Receptor glycosylation can influence receptor occupancy and ligand binding affinity (53). Another possibility is that changes
in the expression of the angiotensin type 2 receptor (AT2) can accentuate the potency of bradykinin (7, 92). Indeed, in the mouse lung AT2 mRNA receptor expression increases following antenatal hypoxia, while AT1 expression remains stable (34). This mirrors the protein expression changes with ontogeny, where AT2R expression increases from the neonatal mouse to the adult, while AT1R expression is steady (25).

Another alternative that influences receptor activation is that there could be alterations in tyrosine or serine/threonine phosphorylation, which are important to bradykinin receptor desensitization (53). Ontogeny also could lead to modifications in cell signaling pathways downstream of bradykinin receptor activation. The reduction in Ca²⁺ signaling we observed supports this premise and illustrates that ontogeny enhances Ca²⁺ sensitization of eNOS. However, there also may be changes in MAPK, JAK/STAT, or other signaling systems such as changes in receptor acylation, which influences G protein receptor coupling (53). Although it is quite plausible that one or more of these options are involved in the changes in bradykinin potency it was beyond the scope of the present studies to evaluate this in depth. Even still, such changes are important as they likely facilitate vessel relaxation and are therefore important during the transition to breathing air.

NO signaling is well regarded as being vital to pulmonary vasorelaxation (17, 38, 64, 67). Thus, the finding that eNOS was important to bradykinin-induced vasorelaxation was expected. Although the data are not wholly conclusive, the prospect that eNOS inhibition has greater effect on the arteries of fetal prenatal chronic hypoxic sheep than on the arteries from normoxic fetal sheep is intriguing. These enhancements in eNOS-dependent vasorelaxation are potentially related to the increased endothelial
Ca\textsuperscript{2+} signaling observed in prenatal chronic hypoxic vessels and concomitant enhancement in Ca\textsuperscript{2+} dependent activation of eNOS (36, 64).

The discovery that bradykinin Ca\textsuperscript{2+} signals in the endothelium were suppressed following birth although relaxation was enhanced is provocative. These data suggest that after birth eNOS is more readily activated by Ca\textsuperscript{2+}. The improvement of Ca\textsuperscript{2+}-eNOS coupling can be due to a number of processes (17, 36, 82). Potentially amino acid residues that sensitize eNOS to calmodulin as well as Ca\textsuperscript{2+} are phosphorylated (36).

Binding partners may have greater interaction or there may be changes in the abundances of co factors such as L-Arginine, flavin adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin that facilitate NO production either directly or indirectly (23, 64, 82). These changes could also include alterations in spatial –temporal coupling based on the characteristics of the Ca\textsuperscript{2+} signals and eNOS cellular distribution (22, 64). Indeed, there was a faster rate of Ca\textsuperscript{2+} rise following chronic hypoxia that paralleled the changes due to ontogeny. In addition, following birth the decay of Ca\textsuperscript{2+} was more rapid, an effect that was blunted by hypoxia. These findings coupled with the multifaceted changes in the Ca\textsuperscript{2+} spike amplitude and area under the curve suggest there are complex changes in release, influx, sequestration, as well as extrusion. This includes modifications in the expression or activation of InsP\textsubscript{3} receptors, or Ca\textsuperscript{2+} permeable ion channels at the plasma membrane, extrusion of Ca\textsuperscript{2+} from the cytosol by plasma membrane or endoplasmic reticulum Ca\textsuperscript{2+} ATPases, or even storage of Ca\textsuperscript{2+} in the endoplasmic reticulum (32, 33). Resolving the developmental mechanisms that govern increased bradykinin-mediated relaxation through eNOS-Ca\textsuperscript{2+}
coupling and the influence of chronic hypoxia on the developmental process are important, but require additional experimentation.

The finding that sGC and eNOS antagonism caused equivalent inhibition of bradykinin-mediated relaxation provides added evidence that a substantial portion of the bradykinin relaxation is through coupling of eNOS and sGC. Further, the inhibitory studies suggest that the fidelity of coupling between eNOS and sGC is not modified by either maturation or chronic hypoxia. Nonetheless, ProliNO greatly intensified and normalized the extent of relaxation across all groups (Figure 8). Interestingly, sGC inhibition shifted the potency of ProliNO as opposed to reducing the maximal relaxation to bradykinin shown in Figure 7. Although unlikely, such differences in how sGC inhibition influences bradykinin versus ProliNO relaxation may reflect non-specific effects of ODQ that attenuate the bradykinin-induced vasodilation pathway at points upstream of sGC activation (6, 29, 80, 85). The differences in the actions of ODQ also suggest that while a major portion of bradykinin-induced relaxation is through cGMP generation, its mechanism of action may also involve NO-degradation through phosphodiesterases or through NO-independent pathways (6, 85). The ProliNO data further illustrate that bradykinin does not maximally activate relaxation pathways and there is substantial NO-sensitive and sGC-insensitive reserve. This sGC-insensitive reserve likely includes protein nitrosylation, which is a key mediator of NO dependent signaling (55).

Although prostacyclin signaling pathways were not fully evaluated, our indomethacin studies were revealing. The fetal normoxic studies that show a lack of a role for COX-dependent vasorelaxation were expected, and follow from earlier studies
that illustrate prostacyclins have little effect on pulmonary vascular tone until after birth (54). The failure of indomethacin to substantially reduce bradykinin-dependent relaxation in newborns was interesting and compares with previous studies. In studies performed in newborn sheep as well as goats, direct stimulation with PGI$_2$ agonists and precursors elicits pulmonary vascular vasorelaxation (15, 28). One explanation is that bradykinin does not fully activate resident PGI$_2$ synthesis pathways. The finding that chronic hypoxia augmented indomethacin-sensitive bradykinin-induced relaxation was also unexpected as previous work in piglets showed that PGI$_2$ production is suppressed (20, 21). Resolving whether greater indomethacin-sensitivity is due to improved activation of dormant PGI$_2$ synthesis pathways or whether there are phenotypic changes that increase the expression of key synthesis enzymes, or pathway elements in the arterial myocytes that govern prostacyclin-dependent vasorelaxation is worthy of future investigation (20, 21, 28).

The suppression in the NOS-independent component to bradykinin-induced vasodilation following hypoxia in the newborn is intriguing. Restriction of prostacyclin-dependent signaling is unlikely because our indomethacin data suggest that PLA$_2$ dependent mechanisms are upregulated following chronic hypoxia. However, chronic hypoxia may suppress the function of other NOS-independent pathways important to vasodilation not described in this report including activation of stored forms of NO (16), epoxyeicosatrienoic acids (14) as well as myoendothelial gap junctions (24, 50). Resolving which, if either, of these or other potential ionic or non-ionic pathways are suppressed by chronic hypoxia will require additional studies.
Bradykinin-induced relaxation in porcine pulmonary resistance arteries is dependent on the activity of multiple K⁺ channels. In particular, BKCa channels in vascular smooth muscle are important to fetal bradykinin-induced pulmonary arterial relaxation. Previous work has also shown that BKCa channels are important to oxygen-dependent relaxation in fetal ovine pulmonary arteries. Our findings are consistent with these reports, and suggest that prenatal chronic hypoxia augments the role for BKCa channels in fetal sheep, which may provide a distinct advantage for the lamb during birth. Nonetheless, whatever temporary advantage this increased role for BKCa channels may provide during the fetal transition is followed quickly by impaired development of normal BKCa related vasorelaxation two weeks after birth.

Impediments in BKCa function are often caused by chronic hypoxia-induced channel dysfunction or changes in subunit expression. In the present study, we inhibited BKCa channel activity in myography studies and conducted Western Immunooblots to investigate the possibility that BKCa channel expression parallels the changes in BKCa mediated relaxation. Such a correlation would indicate that the augmented role for BKCa channels in the fetus and the attenuation of normal BKCa relaxation in the newborn are brought on by hypoxia-induced changes in channel expression, as we have observed in the uterine arteries of pregnant ewes. However, the tension and expression studies did not agree with one another. The lack of change in α or β1 subunit expression following chronic hypoxia in the fetus or newborn was surprising. We expected to see increases in α and β1 subunit expression in the prenatal chronic hypoxic fetus to explain the observed increase in vasorelaxation.
Indeed, previous studies have shown such a correlation between BK$_{\text{Ca}}$ channel function and expression, where BK$_{\text{Ca}}$ channels in cerebral arteries and aorta of spontaneously hypertensive rat arteries undergo a decrease in Ca$^{2+}$ sensitivity due to a disproportionate expression of α and β1 subunits, either by downregulation of the β1 subunit or upregulation of the α subunit (2, 58). Furthermore, a study on fetal and adult ovine pulmonary arterial smooth muscle cells showed that short-term hypoxia in cultured in vitro pulmonary arterial smooth muscle cells increased expression of both the α and β1 subunits (72). The discrepancies between our results and those of previous studies point to a number of possible explanations. The implication is that sustained, intrauterine low-O$_2$ conditions have a unique effect on the function and expression of the BK$_{\text{Ca}}$ channel compared to the effects seen following induced, short-term, hypoxia. However, we have yet to explore activation of whole-cell or transient BK$_{\text{Ca}}$ currents by membrane voltage or the coupling to Ca$^{2+}$ sparks, which are vital to how BK$_{\text{Ca}}$-induced membrane hyperpolarization induces vessel relaxation (12, 37).

The observed increase in BK$_{\text{Ca}}$ channel activity following prenatal chronic hypoxia in the fetus may also be explained by changes in expression or post-translational modifications that increase BK$_{\text{Ca}}$ channel function. Studies also illustrate that single-nucleotide polymorphisms within either the α or β1 subunits can confer a gain or loss of function (8, 19). It follows that “gain of function” alternative splice variants of the α and β1 subunits could be activated during hypoxic stress, thereby conferring an increase in Ca$^{2+}$ sensitivity and vasorelaxation. Secondarily, the location of the subunits is also vital to channel function. For example, in addition to the plasma membrane, BK$_{\text{Ca}}$ channels are also expressed in the mitochondrial membrane of neurons and this can
influence cell excitability (75, 90). Unfortunately, our Western Immunoblot analysis only measured whole-cell protein content and did not provide information regarding subunit cellular location. Thus, it is possible that chronic prenatal hypoxia could modify channel targeting and the proportion of the channels retained in the sarcoplasmic-endoplasmic reticulum versus those channels transported to the mitochondrial and plasma membranes. An alternative explanation for our findings is that of chronic prenatal hypoxia-induced changes in the expression of BK_{Ca} channel γ subunits, which interact with β1 subunits. In a key study performed in rat cerebral arterial myocytes, the γ subunit was found to be important to vasorelaxation, where it facilitated the coupling of BK_{Ca} channels to Ca^{2+} sparks by shifting voltage dependence and Ca^{2+} sensitivity, assisting in membrane hyperpolarization and concomitant vasorelaxation (18). Thus, one potential explanation for the mismatch between data from tension studies and α and β1 subunit expression data could be an upregulation in the γ subunit, which improves BK_{Ca} channel function. Ultimately, however, resolving this and other potential explanations will require further investigation.

Although the data suggest BK_{Ca} dependent dilation of pulmonary arteries is mediated through the NO-cGMP-PKG signaling axis, PKG also causes vasodilation through other mechanisms. In addition to activating K^{+} channels, hyperpolarizing the plasma membrane, and reducing voltage-dependent Ca^{2+} influx, PKG also functionally decreases the sensitivity of contraction to increases in the cytosolic Ca^{2+} concentration. In particular, PKG can dilate vessels by promoting de-phosphorylation of the myosin light chain kinase. This is achieved by phosphorylation and activation of the myosin light chain phosphatase (26, 27, 81). Further to this, PKG inhibits RhoA activity, which is a
key modulator of the Rho Kinase pathway (27, 49). This is directly relevant to the current studies, as we have previously shown that Rho Kinase accounts for roughly half of the arterial tension due to serotonin or membrane depolarization in pulmonary arteries of sheep (10, 68). Future studies are therefore needed to discriminate the interaction between PKG and various pathways.

**Perspective**

The present series of studies illustrate that bradykinin-dependent vasorelaxation is mediated through a combination of eNOS-sensitive and insensitive pathways. From a methodological standpoint, our results demonstrate the importance of conducting whole-animal studies in addition to *In Vitro* experimentation. Sustained stress, such as prenatal chronic hypoxia, can result in complex changes via the interaction of numerous systems not represented by inducing short-term stress in isolated cells. Terrestrial prenatal chronic hypoxia due to living at moderately high altitude influenced the development of eNOS dependent pathways, causing adaptations that improve pulmonary blood flow and blood oxygenation in the fetus and newborn infant. Presumably, these adaptations allow newborn lambs to survive birth in the rarified environment. Unfortunately, the increased blood flow that results from these adaptations may lessen blood flow necessary for the proper development of other vital organs. Based on our data, prenatal chronic hypoxia blunts normal development of bradykinin-mediated vasodilatory pathways, resulting in lessened responsiveness. In turn, this could exacerbate pulmonary pressures and reduce the ability of newborn hypoxic lambs to thrive. On the whole, our results demonstrate that prenatal chronic hypoxia takes a significant toll on proper pre- and postnatal development of the bradykinin-activated,
eNOS—BK$_{Ca}$ vasodilatory pathway in sheep. The dramatic effects of hypoxia observed here provide valuable insights into the mechanisms contributing to pulmonary hypertension in the newborn, and demonstrate the potential for targeting the eNOS—BK$_{Ca}$ signaling axis in afflicted newborns.

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Contributions in order of involvement
Experimental Design SMW, LDL, WJP, RBT, ABB, MST, MF. Data collection QB,
SMW, MR, AB, RBT, RHW. Data analysis and interpretation CBJ, SMW, RBT, CW,
QB, AB, MR, WJP, LDL, ABB, MF, MST, RHW. Figure and manuscript preparation
CBJ, SMW, CW, RBT, QB, AB, MR, LDL, WJP, ABB, RBT, MF, MST, RHW.
References


33. **Goyal R, Creel KD, Chavis E, Smith GD, Longo LD, and Wilson SM.** Maturation of intracellular calcium homeostasis in sheep pulmonary arterial smooth


40. Herrera EA, Pulgar VM, Riquelme RA, Sanhueza EM, Reyes RV, Ebensperger G, Parer JT, Valdez EA, Giussani DA, Blanco CE, Hanson MA, and


60. Llanos AJ, Ebensperger G, Herrera EA, Reyes RV, Pulgar VM, Seron-Ferre M, Diaz M, Parer JT, Giussani DA, Moraga FA, and Riquelme RA. Fetal and


79. **Saqueton CB, Miller RB, Porter VA, Milla CE, and Cornfield DN.** NO causes perinatal pulmonary vasodilation through K+-channel activation and intracellular Ca2+


Figure Legends

Figure 1. Representative micrographs of endothelium in fetal pulmonary arteries. Average Fluo-4 fluorescence signals in endothelial cells of pulmonary arterial segments isolated from (A) normoxic fetal sheep, (B) prenatal chronic hypoxic fetal sheep, (C) normoxic newborn sheep, and (D) prenatal chronic hypoxic newborn sheep. The panels represent the average intensity from 100 frames for normoxic and 120 frames for hypoxic animals at 1.28 frames per second. Image brightness was adjusted for display purposes. Images were made with a 40X water immersion Apochromat objective. Scale bar is 20 μm.

Figure 2. Chronic prenatal hypoxia enhances neonatal bradykinin-induced pulmonary arterial relaxation but suppresses postnatal vascular relaxation. Dose-response curves of pulmonary arterial rings exposed to 10 pM to 1 μM bradykinin in an additive manner normalized to %T_{10 μM PE} for normoxic (filled circles, solid line) and prenatal chronic hypoxic (open circles, dashed line) fetal (A) and newborn (B) sheep, and their respective (C) potency and (D) maximum response values. Lines show resultant fits with a Hill equation to the dose-response relationships, and markers show mean ± SEM. The data were analyzed by two-way analysis of variance with a Bonferroni post-test analysis for each dose. * (P<0.05), *** (P<0.001) denotes statistical significance between normoxic and hypoxic groups, while † † † (P<0.001) denotes difference between fetal and newborn groups.

Figure 3. Chronic prenatal hypoxia increases bradykinin-mediated cytosolic Ca^{2+} responses. (A) Representative images corresponding in time to the trace of fractional
Fluo-4 fluorescence (B) for four pulmonary arterial myocytes from a prenatal chronic hypoxia fetus in the presence of extracellular Ca^{2+}. Lower case letters in panels A and B denote the times at which the images occurred. The colored circles correspond to the trace of the same color in panel B. The brightness of the images of panel A were adjusted for display purposes. Images were made with a 40X water immersion Apochromat objective at 1.28 frames per second. Scale bar is 20 μm.

**Figure 4. Chronic hypoxia augments Ca^{2+} signals in neonates, but suppresses sensitivity postnatally.** (A) Amplitude of the fractional fluorescence, (B) area under the curve, (C) duration, (D) decay, and (E) attack of the Ca^{2+} event for regions of interest that were automatically detected during the fluorescence study described for Figure 3. Bars indicate mean ± S.E.M. ** (P<0.01), *** (P<0.001) denotes statistically significant effect of chronic prenatal hypoxia based on Kruskal-Wallis nonparametric one way analysis of variance with a Dunn’s Multiple Comparison Test, while † † † (P<0.001) denotes effect of maturation.

**Figure 5. eNOS inhibition reduces bradykinin-induced pulmonary arterial relaxation.** Dose-response curves of pulmonary arterial rings exposed to 10 pM to 1 μM bradykinin in an additive manner normalized to %T_{10 \mu M \text{PE}} for (A) normoxic fetal sheep, (B) prenatal chronic hypoxic fetal sheep, (C) normoxic newborn sheep, and (D) prenatal chronic hypoxic newborn sheep in the presence of DMSO (filled circles, solid line) for vehicle control and 100 μM LNAME (open circles, dotted line) for eNOS inhibition. Lines show resultant fits to the dose-response relationships with a Hill equation, and markers show mean ± SEM. The data were analyzed by two-way analysis of variance with a
Bonferroni post-test analysis for each dose. Statistical significance is noted relative to DMSO control * (P<0.05), ** (P<0.01), *** (P<0.001).

**Figure 6. COX inhibition of prostacyclin synthesis suppresses bradykinin-induced relaxation.** Dose-response curves of pulmonary arterial rings exposed to 10 pM to 1 μM of bradykinin in an additive manner normalized to %T_{10 \mu M \text{PE}} for (A) normoxic fetal sheep, (B) prenatal chronic hypoxic fetal sheep, (C) normoxic newborn sheep, and (D) prenatal chronic hypoxic newborn sheep in the presence of DMSO (filled circles, solid line) for vehicle control and 10 μM indomethacin (open circles, dotted line) to inhibit PGI2 production. Lines show resultant fits to the dose-response relationships with a Hill equation, and markers show mean ± SEM. The data were analyzed by two-way analysis of variance with a Bonferroni post-test analysis for each dose. Statistical significance is noted relative to DMSO control * (P<0.05), ** (P<0.01), *** (P<0.001).

**Figure 7. sGC inhibition suppresses bradykinin-mediated relaxation.** Dose-response curves of pulmonary arterial rings exposed to 10 pM to 1 μM of bradykinin in an additive manner normalized to %T_{10 \mu M \text{PE}} for (A) normoxic fetal sheep, (B) prenatal chronic hypoxic fetal sheep, (C) normoxic newborn sheep, and (D) prenatal chronic hypoxic newborn sheep in the presence of DMSO (filled circles, solid line) for vehicle control and 10 μM ODQ to inhibit sGC (open circles, dotted line). Lines show resultant fits to the dose-response relationships with a Hill equation, and markers show mean ± SEM. The data were analyzed by two-way analysis of variance with a Bonferroni post-test analysis for each dose. Statistical significance is noted relative to DMSO control * (P<0.05), ** (P<0.01), *** (P<0.001).
Figure 8. NO-dependent vasorelaxation is independent of age and chronic hypoxia. ProliNO-induced isometric tension values normalized to %T$_{10 \mu M 5-HT}$ for (A) normoxic (filled circles, solid line) and prenatal chronic hypoxic (open circles, dashed line) fetal and newborn sheep, (B) normoxic and (C) prenatal chronic hypoxic sheep in the absence (squares, dashed line) of ODQ, in the presence of 10 μM ODQ (inverted triangles, dotted line), and in the presence of NaOH (filled circles, solid line) as a vehicle- and time-matched control. Lines show resultant fits with a Hill equation to the dose-response relationships, and markers show mean ± SEM. The data were analyzed by two-way analysis of variance with a Bonferroni post-test analysis for each dose. Statistical significance is noted between groups (A) or relative to the presence of ProliNO (B and C) * (P<0.05), ** (P<0.01), *** (P<0.001).

Figure 9. Prenatal chronic hypoxia promotes importance of BK$_{Ca}$ potassium channels to bradykinin-mediated arterial relaxation in the fetus, but stunts normal relaxation in the newborn. Dose-response curves of pulmonary arterial rings exposed to 10 pM to 1 μM of bradykinin in an additive manner normalized to %T$_{10 \mu M PE}$ for (A) normoxic fetal sheep, (B) prenatal chronic hypoxic fetal sheep, (C) normoxic newborn sheep, and (D) prenatal chronic hypoxic newborn sheep in the presence of DMSO (filled circles, solid line) for vehicle control and in the presence of 1 mM TEA (open circles, dotted line) to inhibit BK$_{Ca}$ channels. Lines show resultant fits to the dose-response relationships with a Hill equation, and markers show mean ± SEM. The data were analyzed by two-way analysis of variance with a Bonferroni post-test analysis for each dose. Statistical significance is noted relative to DMSO control (**=p<0.01).
Figure 10. Maturation alters BK_{Ca} α and β1 subunit expression. Western blot analysis of pulmonary arteries from fetal and newborn sheep for A) A representative sample of fetal and newborn normoxic and hypoxic expression of BK_{Ca} α and β1 subunits and β-actin to which channel subunit expression were normalized. B) α subunit expression, C) β1 subunit expression. Protein abundance was normalized to β -actin and expressed as a relative abundance compared with the reference MCA standard, and bars show mean ± SEM. The data were analyzed by two-way analysis of variance with a Bonferroni post-test analysis. * (P<0.05) or ** (P<0.01) indicates statistically significant difference in subunit expression due to maturation.

Figure 11. Influence of early postnatal maturation and intrauterine chronic hypoxia on bradykinin induced vasorelaxation in fetal and newborn pulmonary vessels. The schematic provides an overview of the findings of the current studies. The data indicate that postnatal chronic hypoxia has an interactive influence on the normal development of bradykinin-induced vasorelaxation with development that involves modification to the function of the endothelium (above the hashed line) and smooth muscle (below the hashed line). The influence of developmental age is shown on the left side of each pathway step, while the effect of chronic hypoxia on the fetus (F) and newborn (N) are to the right. These observed changes are codified as functional increases (green circles, “+”), decreases (red circles, “-“) or no changes (open circles, “N”). The pathways are depicted as either a coupled sequence (straight lines) or through a multistep process (dashed lines), where intermediate pathway steps were not evaluated. BK_{Ca} – Large Conductance Voltage and Calcium Sensitive Potassium Channel; COX 1/2 – Cyclooxygenases 1 and 2; eNOS – Endothelial Nitric Oxide
Synthase; NO – Nitric Oxide; RyR – Ryanodine Receptor (see Ref 27); sGC – Soluble Guanylate Cyclase.
Figure 2
Figure 4

(A) Amplitude (F/F₀)

(B) AUC (F*/F₀)

(C) Duration (s)

(D) Decay (s)

(E) Attack (s)

- **: p < 0.01
- ***: p < 0.001
- †††: p < 0.0001

- Fetus vs. Newborn
- Normoxia vs. Hypoxia

- Black bars: Normoxia
- White bars: Hypoxia
Figure 5

A. Normoxia

B. Hypoxia

C. Normoxia

D. Hypoxia

**Figure 5**
Figure 8

A. %T 10 μM 5HT

B. Normoxia

C. Hypoxia

D. Normoxia

E. Hypoxia

Figure 8
Figure 9

**A. Normoxia**

**B. Hypoxia**

**C. Normoxia**

**D. Hypoxia**

- Fetus
- Newborn

*Control* vs. *TEA*
Figure 10

A

B

C

Figure 10
### Table 1

**Chronic hypoxia, maturation and potency of bradykinin-induced relaxation**

| Condition | Normoxic | | | | Hypoxic | | | |
|-----------|----------| | | |----------| | | |
|           | Mean ± SEM | N | Mean ± SEM | N | Mean ± SEM | N | Mean ± SEM | N |
| Control   | -6.86 ± 0.21 | 14 | -7.89 ± 0.16* | 6 | -6.95 ± 0.15 | 14 | -7.91 ± 0.12* | 16 |
| LNAME     | -6.93 ± 1.09 | 5 | -8.43 ± 0.26 | 4 | -6.83 ± 0.53 | 6 | -7.61 ± 0.43 | 7 |
| INDO      | -6.38 ± 0.78 | 5 | -7.89 ± 0.15 | 4 | -6.87 ± 0.74 | 3 | -7.04 ± 0.38 | 4 |
| ODQ       | -6.89 ± 0.84 | 4 | -7.89 ± 0.36 | 3 | -6.51 ± 0.51 | 3 | -7.86 ± 0.67 | 4 |
| TEA       | -6.44 ± 0.90 | 5 | -7.65 ± 0.24 | 4 | -7.05 ± 0.32 | 3 | -7.68 ± 0.23 | 4 |

Values are Log concentration for the IC<sub>50</sub> for bradykinin induced relaxation for data presented graphically in Figures 2, 5-7 and 9. N value reflects the number of animals studied for each condition in each group. Using a 95% Confidence Interval based in the log(inhibitor) vs. response curve fit to the data: **”** denotes a significant leftward shift in the IC<sub>50</sub> compared to fetus based on the control traces shown in Figure 2.

### Table 2

**Chronic hypoxia, maturation and maximum response of bradykinin-induced relaxation**

| Condition | Normoxic | | | | Hypoxic | | | |
|-----------|----------| | | |----------| | | |
|           | Mean ± SEM | N | Mean ± SEM | N | Mean ± SEM | N | Mean ± SEM | N |
| Control   | 83 ± 2 | 14 | 11 ± 4* | 6 | 47 ± 3‡ | 14 | 47 ± 2‡ | 16 |
| LNAME     | 91 ± 5 | 5 | 42 ± 4† | 4 | 79 ± 5† | 6 | 76 ± 3†,‡ | 7 |
| INDO      | 85 ± 10 | 5 | 19 ± 5 | 4 | 76 ± 12† | 3 | 66 ± 7†,‡ | 4 |
| ODQ       | 91 ± 5 | 4 | 44 ± 6† | 3 | 79 ± 8† | 3 | 73 ± 5†,‡ | 4 |
| TEA       | 78 ± 14 | 5 | 36 ± 5† | 4 | 73 ± 5† | 3 | 40 ± 5 | 4 |

Values are % of the T<sub>Kmax</sub> for phenylephrine induced contraction for data presented graphically in Figures 2, 5-7 and 9. N value reflects the number of animals studied for each condition in each group. Using a 95% Confidence Interval based in the log(inhibitor) vs. response curve fit to the data: **”** denotes significant increase in maximum response as compared to fetus, “†” significant depression in maximum response as compared to control, “‡” significant difference in maximum response as compared to normoxic counterpart.