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# Role of Protein Kinase G in Regulating Vascular Tone in Hypoxic Ovine Cerebral Arteries

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Role of Protein Kinase G in Regulating Vascular Tone
in Hypoxic Ovine Cerebral Arteries

by

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A Dissertation submitted in partial satisfaction of
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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

 

 

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# ACKNOWLEDGEMENTS

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## ABBREVIATIONS

| | |
|---|---|
| 5-HT | 5-Hydroxy tryptamine (serotonin) |
| 5HT2a | 5-HT receptor 2a |
| 8-pCPT-cGMP | 8-(p-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate |
| AEBSF | 4-(2-Aminoethyl)benzenesulfonyl |
| AH | Adult hypoxic |
| AN | Adult normoxic |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| ATPase | Adenosine 5'-triphosphatase |
| BK | Big potassium channel |
| BKα | BK channel alpha protein |
| BKβ | BK channel beta protein |
| BPDE | Bovine phosphodiesterase |
| $Ca^{+2}$ | Calcium +2 |
| $CaCl_2$ | Calcium chloride |
| cAMP | Cyclic adenosine monophosphate |
| CArG | DNA receptor binding motif (not an acronym) |
| cGMP | Cyclic guanosine monophosphate |
| $CO_2$ | Carbon dioxide |
| CREB | Cyclic-AMP response element binding protein |
| CRP4 | Cysteine-rich protein 4 |
| DTT | Dithiothreotol |

| | |
|---|---|
| $EC_{50}$ | half maximal effective concentration |
| EDTA | Ethylene diamine tetra-acetate |
| EGTA | Ethylene glycol tetraacetic acid |
| eNOS | Endothelial nitric oxide synthase |
| FH | Fetal hypoxic |
| FN | Fetal normoxic |
| GATA6 | A zinc finger transcription factor (not an acronym) |
| GPCR | G-Protein coupled receptor |
| GRK | GPCR receptor kinase |
| GTP | Guanosine triphosphate |
| HEPES | Hydroxyethyl-1-piperazineethanesulfonic acid |
| IBTX | Iberiotoxin |
| $IP_3$ | Inositol triphosphate |
| $K^+$ | Potassium ion |
| Ka | Binding affinity association constant |
| KCl | Potassium chloride |
| kDa | Kilodalton |
| Kg | Kilogram |
| LLU | Loma Linda University |
| L-NAME | Nitro-L-arginine methyl ester |
| L-NNA | NG-nitro-L-arginine |
| LTH | Long-term Hypoxia |
| M | Molar |

| | |
|---|---|
| m | meter |
| mA | milliamp |
| MCA | Middle cerebral artery |
| MGSO$_4$ | Magnesium sulfate |
| MLC$_{20}$ | Myosin light chain - 20 kDa |
| mm | millimeter |
| mmHG | millimeters of mercury (unit of pressure) |
| M-TBS | Milk-TBS buffer |
| NaHO$_3$ | Sodium bicarbonate |
| nM | Nano molar |
| NO | Nitric Oxide |
| PAGE | Polyacrylamide gel electrophoresis |
| PaO$_2$ | Arterial O$_2$ pressure |
| pD2 | potency, negative log of EC$_{50}$ |
| PDE | Phosphodiesterase |
| PFA | Paraformaldehyde |
| pKa | Negative log of receptor binding affinity (-log Ka) |
| PKA | Protein kinase A |
| pKa | Negative log of receptor affinity (-log Ka) |
| PKC | Calcium-dependent protein kinase |
| PKG | Protein kinase G |
| PKG-I | Protein kinase G isoform I |
| PKI | pKa of an inhibitor |

| | |
|---|---|
| PMCA | Plasma membrane Calcium ATPase |
| Po | Ion channel open probability |
| PPA | Protein proximity analyzer (software) |
| PPI | Protein proximity index |
| PRKG | Protein kinase G gene |
| Q1,2,3,4 | Quadrant 1, 2, 3, 4 |
| RA | Receptor agonist |
| RAB11a | Ras-associated binding protein 11a |
| RIPA | Radio immunoprecipitation assay buffer |
| RNA | Ribose nucleic acid |
| RT | Total receptor concentration |
| SDS | Sodium dodecyl sulfate |
| SER922,1151,1154 | Serine 922,1151,1154 residue |
| sGC | Soluble guanylyl cyclase |
| SRF | Serum response factor |
| STREX | BKα alternate splice exon insert |
| TBS | Tris-buffered saline |
| TIFF | Tagged Image File Format |
| μM | Micro molar |
| VSM | Vascular smooth muscle |
| VSMC | Vascular smooth muscle cell |
| xG | Unit of G-force |
| ZERO | BKα isoform lacking STREX insert |

ABSTRACT OF THE DISSERTATION

Role of Protein Kinase G in Regulating Vascular Tone
in Hypoxic Ovine Cerebral Arteries

by

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Nitric oxide (NO) and its precursors have been used clinically to induce

vasorelaxation long before the specific mechanism of how this operates was understood.

With the discovery in 1977 of the influence of NO on guanylate cyclase, and the

subsequent elucidation of the NO pathway, the role of cyclic guanidine monophosphate

(cGMP)-dependent protein kinase (PKG) has been shown to be the main mediator of NO-

induced vasorelaxation. Less well understood is the behavior of this pathway in

conditions of long term hypoxia (LTH). It was later shown that LTH attenuates the ability

of PKG to mediate vasorelaxation. How this operates is the focus of this investigation.

Here we tested the hypothesis that LTH attenuates NO-induced vasorelaxation by

reducing the ability of PKG to phosphorylate its target proteins that mediate vascular

relaxation and contraction. A prominent target of PKG involved with vasorelaxation is

the BK channel alpha protein. To examine the influence of LTH on PKG-induced

vasorelaxation, and to demonstrate the relative influence of the BK channel in these

conditions, we used carotid and middle cerebral arteries from normoxic and chronically

hypoxic fetal and adult sheep. These arteries were denuded of endothelium and used in

concentration-response relations using 5-HT as a contractant and measured contractile

force. To measure the relative influence of the BK channel in these arteries, we used the selective BK channel blocker iberiotoxin. From this we demonstrated that while hypoxia affects both BK and non-BK channel mechanisms, the influence of LTH on the BK channel is dominant in the loss of PKG-mediated vasorelaxation. We also examined whether hypoxia attenuates PKG expression and specific activity, as well as the BK channel alpha and beta proteins. Using Western blotting and enzyme kinase activity assays, we learned that LTH has a modest influence on BK channel proteins expression, and no influence on PKG expression or activity. We next examined whether LTH influences protein organization, which could potentially control PKG-mediated vasorelaxation in normoxic and hypoxic conditions. Using confocal microscopy, we learned that LTH strongly alters protein organization, and specifically the proximal association of three proteins associated with vasorelaxation, namely PKG, BK-alpha and BK-beta.

**CHAPTER ONE**

**INTRODUCTION**

**Vasorelaxation**

The human body is amazingly well-engineered to survive and to adapt in response to a wide range of changes in environmental conditions, including temperature, available nutrients, ambient oxygen, and more. For example, a rapid decrease in available oxygen or a rise in temperature triggers an acute hemodynamic shift that favors survival of critical organs (brain, heart, kidneys etc.) at the expense of more hypoxia-tolerant tissues (skeletal muscle, adipose, etc.). This is possible because vascular tissues are richly invested with smooth muscle that is able to contract or relax under a well-regulated network of sensors and effectors.

*Contraction of Vascular Smooth Muscle*

Contraction of vascular smooth muscle is important for maintaining vascular tone and regulating peripheral vascular resistance, essential for controlling cardiac output and normal blood pressure.  Intrinsic effectors of vasoconstriction include angiotensin (5), endothelin (30), epinephrine, norepinephrine, and many more. Release of serotonin (5-hydroxytryptamine) from platelets at the site of a tissue injury induces a powerful constriction of arterial smooth muscle, effective in reducing an acute loss of blood (2). At the cellular level, vasoconstriction is mediated by a release of calcium which is the main signal for activating the contractile apparatus (12).

### *Relaxation of Vascular Smooth Muscle*

Relaxation of vascular smooth muscle is dominantly under control of vascular endothelium and mediated by the nitric oxide (NO) pathway (33). NO-induced vasorelaxation is important for enlarging vessel luminal diameter to increase blood flow and delivery of oxygen and nutrients in response to metabolic demand. Vascular tone is thus a dynamic balancing act of vasoconstriction and vasorelaxation.

The main effector of the NO pathway is the cGMP dependent protein kinase (PKG) which phosphorylates a repertoire of proteins to induce vasorelaxation by decreasing calcium release or reducing calcium sensitivity of the contractile apparatus (3). However, it was observed more than a decade ago that vascular smooth muscle (VSM) exhibits a peculiar phenomenon: that nitric oxide (NO) is relatively ineffective as a vasodilator in chronically hypoxic animals, including humans (12, 28, 46). This is a clinically important issue because strategies to increase oxygen perfusion during conditions of acute hypoxia or ischemic injury may fail in critical situations, particularly in individuals with a history of exposure to chronic hypoxia. The mechanisms underlying this loss of function are not completely understood. The foregoing investigation examines one aspect of this phenomenon: that is the role of cyclic GMP-dependent protein kinase (PKG) as a mediator of the NO signaling pathway, and its apparent loss of function secondary to chronic hypoxia.

Given that literally hundreds of known cellular proteins are involved with the physiology of VSM contraction, the scope of this investigation has necessarily included multiple likely candidates to explain the loss of PKG-mediated vasorelaxation under hypoxic conditions. This has included each step of the NO signaling pathway, receptor

affinity and occupancy, changes in ion channels, and the abundance and specific activity of PKG itself. It appears that no single candidate protein accounts for the observed hypoxic attenuation of PKG-mediated vasorelaxation. Rather several proteins are cooperatively influential in regulating vascular tone and are sensitive to chronic hypoxia. In particular, the large-conductance calcium-activated potassium channel (BK) is an important mediator of vasorelaxation: it has several known PKG serine/threonine kinase targets that influence its conductance (50), and it has been shown to have a reduced potassium conductance under hypoxic conditions (27). The role of PKG in mediating the changes in the BK channel in response to chronic hypoxia is not established in cerebral arteries. That was the focus of this investigation.

To identify a specific role of PKG in the hypoxia-induced shift in VSM tone, this study focused on the BK channel and the influence of PKG on this ion channel under normoxic and hypoxic conditions. The main hypothesis is that chronic hypoxia attenuates the ability of PKG to activate BK channels in cerebral arteries. Whereas PKG has previously been shown to promote vasorelaxation in normoxic ovine carotid arteries, and that hypoxia attenuates this phenomenon (42), this proposed study investigates the effects of hypoxia on the influence of PKG specifically by action on the BK channel in cerebral arteries. In this study 5-hydroxytryptamine (5-HT, aka serotonin) was used as a VSM contractant. PKG was manipulated (activated) by use of the phosphodiesterase-resistant 8-(p-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (aka 8pCPT-cGMP). The BK channel was manipulated (inhibited) by the channel-selective inhibitor iberiotoxin.

# Vascular Smooth Muscle

## *Vascular Smooth Muscle Phenotypes*

Long-term changes in the responsiveness of cerebral arteries due to maturation or chronic stress typically involves a change the relative mix of VSM phenotypes. Four separate VSM phenotypes are recognized, which includes 1) proliferative, 2) migratory, 3) secretory, and 4) contractile (21). Traditionally, the contractile phenotype has been recognized as the mature endpoint of phenotypic progression. It is now understood that this is reversible; the contractile phenotype has been shown to revert to a synthetic phenotype under various conditions (31) including hypoxic stress or multiple passages in organ culture (20). This reversion is characterized by a downregulation of the expression of contractile genes and their respective proteins. Typically, in a given artery, and within a range of stressors and/or maturity, all four phenotypes are represented, albeit of varied proportions.

## *Cerebral Vascular Arteries as Conduits*

In the average adult human, the brain represents about 2% of the total body weight. Yet despite its relatively small size, the brain consumes about 20% of the oxygen, and therefore total calories of the entire body (4). Equally remarkable, this high rate of metabolism is fairly constant despite strongly variable mental and motor activity (33). Cerebral arteries serve an obvious function, that of a conduit to transport oxygen, nutrients and signaling molecules to the brain. Less obvious is the regulatory role in maintaining a delicate homeostasis between the fairly constant metabolic demand of the brain, and strongly variable conditions of somatic hydrostatic pressure and available

oxygen. Middle cerebral arteries (MCAs) are anatomically situated between the much larger carotid arteries, and the smaller pial resistance arteries/arterioles. As such they have much in common with the carotids structurally and anatomically, and respond similarly to systemic influences. Compared with carotid arteries, MCAs are an order of magnitude smaller diameter, have fewer layers of VSM, are invested with substantially less adventitial material, and are typically more compliant. Consideration of cerebral arteries as a passive conduit is a useful abstraction for purposes of contrasting their basic transport function with their dynamic adaptive properties. As arteries become less compliant and less responsive to stressors, due to passage of time, hypoxia or other pathologies, the passive transport model more closely approximates their actual function.

### *Cerebral Vascular Arteries as Regulators of Vascular Tone*

Middle cerebral arteries are anatomically closer to the neural tissues they service, compared with carotids, and would be expected to be more responsive to metabolic demand. But unlike the downstream pial arteries, MCAs lack direct retrograde neuronal connectivity that couple local oxygen demand with rapid changes in flow volume (8). Vasorelaxation in MCAs is largely mediated by endothelial release of NO, and is sensitive to pressure and flow dynamics, as well as global systemic influences (38). By contrast, regulation of pial resistance arteries is dominated by an endothelial-mediated but neuronally-coupled sensitivity to metabolic events proximally downstream (8). Responses to contractile and vasorelaxant stimuli of both MCAs and pial arteries is rapid, typically on the order of seconds to minutes.

**The Nitric Oxide (NO) Pathway**

Regulation of cerebral vascular circulation is very dynamic, and is driven by metabolic demand (27). This is accomplished in part by the "neurovascular unit", of which the endothelium plays a major role (36). Oxygen perfusion in the brain is enhanced locally by vasorelaxation which increases the luminal volume and leads to increased transport of oxygen and nutrients. Vasorelaxation is likewise controlled in part by the endothelium which in response to a stimulus activates the NO signaling pathway (11) (Figure 1). This pathway is well-studied. Briefly, NO is synthesized from arginine in vascular endothelium by action of endothelial nitric oxide synthase (eNOS). The NO thus generated is freely permeable into vascular smooth muscle (VSM) and has a half-life on the order of a few seconds (24), which makes it an ideal paracrine signal; this effect is very localized. NO in turn activates soluble guanylate cyclase (sGC) which in turn converts guanine triphosphate (GTP) into cyclic guanine monophosphate (cGMP). The half-life of cGMP is variable depending on the abundance and activity of endogenous phosphodiesterase (PDE). Inhibition of PDE is known to enhance/prolong the effects of cGMP and increase vasorelaxation (28, 35). Cyclic GMP itself has multiple signaling receptors within VSM including prominently PKG (40), which in turn has many cellular kinase targets (32). The role of PKG in VSM is generally to promote vasorelaxation (29) by adding a phosphate group to multiple cellular proteins involved with contraction/relaxation, and to promote and maintain the contractile VSM phenotype in cerebral arteries (19).
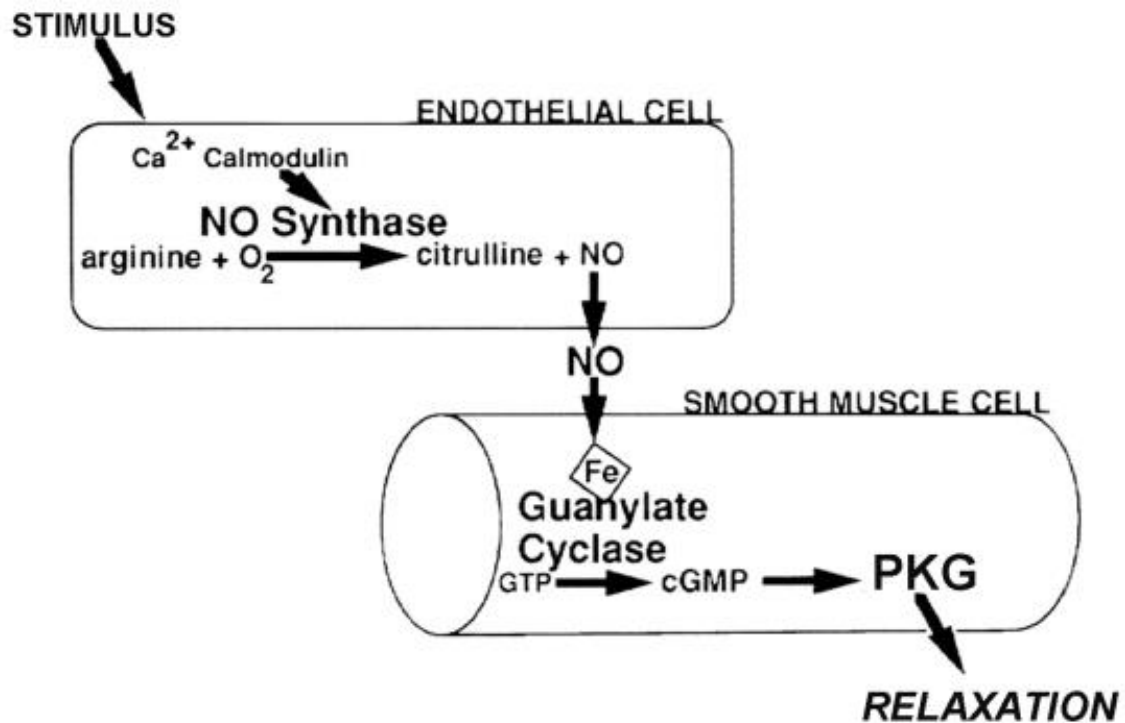
**Figure 1. The Nitric oxide pathway**. In response to a stimulus, the vascular endothelium activates nitric oxide synthase which converts arginine to nitric oxide (NO). NO is freely permeable across the plasma membrane. NO diffuses into nearby vascular smooth muscle cells (VSMCs) where it activates soluble guanylate cyclase (sGC) which in turn converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). cGMP binds to the regulatory domain of cGMP-dependent protein kinase (PKG) which induces a conformational shift in the dimeric PKG protein that exposes its catalytic domain, which effectively activates its kinase activity. Activated PKG phosphorylates a repertoire of proteins within the VSMC that promotes vasorelaxation.

## Cyclic GMP-dependent Protein kinase (PKG)

Cyclic GMP-dependent Protein kinase (PKG) is a serine/threonine kinase that is expressed in vascular smooth muscle (16) and has multiple target proteins (32). PKG-I exists in two alternately spliced isoforms PKG-Iα and PKG-Iβ. PKG-Iα is 10-fold more sensitive to activation by cGMP compared with PKG-Iβ (41). PKG has three functional domains: 1) N-terminal domain has a protein recognition/docking and auto-inhibitory domain, 2) a regulatory domain that binds to cGMP, and 3) a catalytic domain that includes a substrate recognition motif (26). Monomeric PKG is auto-inhibited by its N-terminal tail which effectively occupies/occludes the catalytic domain. Dimerization of PKG displaces the auto-inhibitory tail and exposes the two cGMP regulatory domains. Binding of cGMP induces a conformational change in PKG that allows recognition and binding to its substrate and activation of its serine/threonine kinase domain. This process is dynamic and reversible. PKG itself is subject to activation by V-src, a cellular tyrosine kinase (16). Activity of cGMP-activated PKG is elevated 4-fold when phosphorylated by V-src. PKG is sensitized to cGMP by action of calcium-activated protein kinase (PKC) on PKG Threonine T58, although this phosphorylation is not alone sufficient to activate PKG (10). These suggest multiple regulatory opportunities for activation and sensitization of PKG. However, this investigation has shown that changes in specific activity of PKG under hypoxic versus normoxic conditions is not significant in middle cerebral arteries.

### PKG Activates Proteins That Regulate Vascular Tone

Monomeric PKG is ubiquitous in vascular smooth muscle. It is freely soluble in the cytoplasm, but could be described as "sticky" when activated by cGMP. That is, binding of cGMP to the regulatory domain of PKG exposes its protein recognition domain, that causes it to rapidly (trans) autosphorylate and dimerize into its enzymatically active configuration. PKG then effectively associates with its kinase substrate proteins (1, 3). PKG substrates that affect vascular tone include the BK channel alpha subunit (which promotes hyperpolarization of the VSM), myosin light chain phosphatase (which de-activates MLC20-induced VSM contraction) (44), and many more (32).

### PKG and its Downstream Effectors Are Implicated in Hypoxia-Associated Loss of Vasorelaxation

Earlier studies have shown that the NO pathway mediates vasorelaxation and that hypoxia diminishes the effectiveness of this pathway (42). These investigations have established that 1) endothelial eNOS abundance and specific activity remain largely unaffected by chronic hypoxia, 2) sGC abundance and specific activity are likewise mostly unaffected and 3) intracellular cGMP levels increase due to a hypoxia-mediated suppression of PDE (28). This seeming paradox, the loss of vasorelaxation accompanied by an increase in cGMP, resolves when it is regarded that the depression of PDE represents an incomplete attempt to compensate for hypoxia-related stress in a feed-forward paradigm. This immediately implicates PKG and its downstream mediators as having a mechanistic role in the hypoxia-mediated loss of vasorelaxation. This investigation has demonstrated that in carotid and middle cerebral arteries, the abundance

and specific activity of PKG is only moderately affected by chronic hypoxia. This places

the focus directly on PKG substrates, their abundance and localization, and their

susceptibility to phosphorylation by PKG. Prominent among these is the BK channel,

which is a known PKG substrate that plays a major role in VSM relaxation. This

investigation has thus focused on the role of PKG and the BK channel proteins.


## The BK Channel

The BK (big potassium) channel is bound to the plasma membrane in VSM and

selectively conducts $K^+$ ions outwardly. It consists of a homotetramer of four ~125 kDa

alpha proteins, and associates with anywhere from zero to four beta subunits which serve

to stabilize and regulate the BK channel activity (25) and increase its calcium sensitivity

(17). Conductance of $K^+$ is controlled mostly by intracellular $[Ca^{2+}]$, but is also voltage-

and stretch-sensitive (7, 34). Calcium sensitivity is conferred by the intracellular C-

terminal RCK (Regulator of Conductance of $K^+$) domain which contains four $Ca^{2+}$

binding motifs (the "calcium bowl"), which upon association with $Ca^{2+}$ confers a shift in

its structural conformation that increases its "open" probability (Po) by several fold. PKG

has been shown to influence BK channel $Ca^{2+}$ sensitivity (18). There are several

serine/threonine PKG targets within the C-terminal domain that affect channel $Ca^{2+}$

sensitivity (45, 46), (15). The regulation of BK channel sensitivity by PKG is complex

and involves a concomitant influence of PKA and PKC serine/threonine targets near the

PKG phosphorylation site (45). These affect the ability of PKG to up-regulate BK

channel conductance. The role of hypoxia in this process is not well understood. It is a

premise of this investigation that hypoxia may influence target proteins rather than

altering PKG itself, which would thereby alter the ability of PKG to promote

vasorelaxation. Hypoxia-driven alterations in the phospho-status of the BK channel

proteins or their local distribution would conceivably alter the ability of PKG to interact

with these proteins. Given that the BK channel has a dominant role in promoting

vasorelaxation (6), it is of interest to know the relative influence of BK- versus non-BK

effects on vasorelaxation. One arm of this study partitioned these effects, expressed as a

fraction. It is also apparent that the sheer abundance and distribution of the BK channel

proteins could potentially determine BK channel activity. Recent studies suggest that the

abundance and localization of the beta-1 subunit is a major determinant of BK channel

activity in some tissues (17). This investigation measured the abundance of BK channel

alpha and beta proteins in cerebral arteries by Western blotting. Colocalization of alpha

with beta subunits was measured by confocal microscopy.

**Protocols**

*Protocol 1 - Functional:  Contractile Force*

The first protocol measures contractile force as a **functional** outcome in each of

the eight experimental groups. This is accomplished by suspending artery segments in an

isotonic Krebs solution and measuring force using an isometric force transducer. 5-HT is

used as a contractant and the resultant force is normalized as a percentage of the

maximum $K^+$ induced contraction. Artery segments were either 1) untreated (control), 2)

treated with 30 μM 8-pCPT-cGMP, 3) 100 nM iberiotoxin, or 4) both 8-pCPT and

iberiotoxin. The independent effects of hypoxia and age/maturity is directly calculated

using an algebraic model.

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### *Protocol 2 - Structural:  Abundance of BK Channel Proteins*

The second protocol uses Western blotting to measure the **structural** abundance of the two main BK channel proteins, BK-I alpha and BK-beta1 in cerebral arteries. Three other forms of BK-beta exist; beta1 is the primary subunit that stabilizes and modulates the BK-I alpha subunit in VSM, although minor expression of beta-2 and beta-4 are reportedly present in cerebral arteries (22). The mechanisms whereby chronic hypoxia is able to influence BK channel activity are not well understood, but includes alternate splicing (STREX vs. ZERO isoforms (39), (37)), and may involve RNA editing, transcription, translation and cellular trafficking. The sheer abundance of the BK-alpha subunit perhaps represents an upper limit on the functional capacity of the BK ion channel. Given the recent understanding of the role of the BK-beta1 subunit to regulate and modify BK channel activity (17), the abundance of this protein is of high interest. As many as zero to four BK-beta1 subunits are known to associate with the BK-alpha tetramer. A strict stoichiometry between alpha and beta subunits is not essential for channel conductance; but a higher ratio of beta to alpha subunits in the membrane complex correlates with increased calcium sensitivity and channel activity (43). Thus the *ratio* of the two BK subunit types is also of interest. Western blotting is considered to be semi-quantitative (9, 13, 14, 23), but is sufficient to address the experimental question, especially since it is the *relative differences* in protein abundance and ratios that are of interest.

### *Protocol 3 - Relational:  Colocalization of PKG with Channel Proteins*

The third protocol examines cellular protein-protein **relationships by** use of confocal microscopy using dual fluorescent-labeled markers. The purpose for this was to correlate structural and functional relationships that cannot be explained by mere abundance of PKG or its substrates. Independently, each of the three possible pairs of these three proteins were imaged, then analyzed using Colocalizer Pro® to quantify the degree of colocalization. Additionally, these same images were further analyzed by calculation of a protein proximity index,

Taken together, these three protocols represent a functional, structural, and relational measure to quantify the effect of chronic hypoxia on the ability of PKG to influence BK channel activity in fetal and adult cerebral arteries. Each protocol of this investigation is represented graphically in Figure 2.
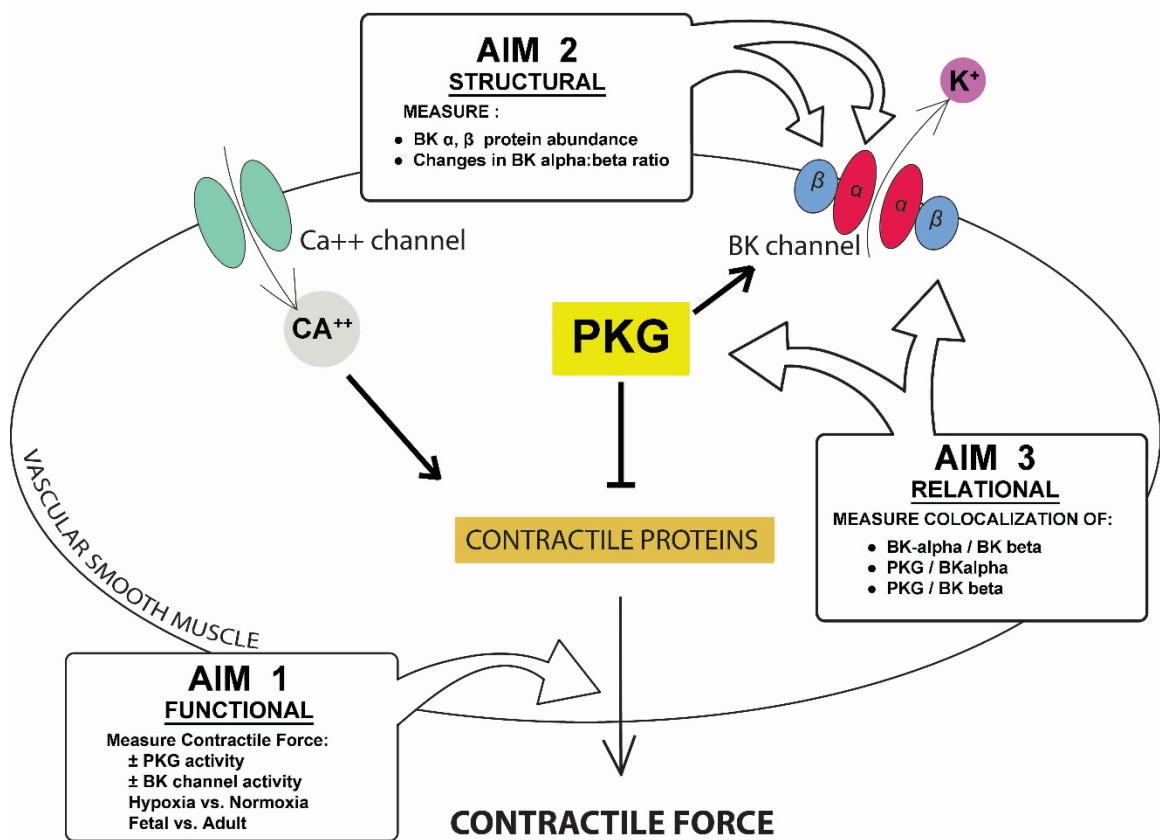
**Figure 2. Experimental design**. Each aim of this investigation is represented by each corresponding protocol. **Aim 1** examines a measurable functional endpoint, contractile force under conditions of normoxia and hypoxia, and in fetal and adult arteries. **Aim 2** examines the structural abundance of key proteins, PKG and BK channel alpha and beta isoforms using Western blotting analysis. **Aim 3** examines the structural relationships of these same proteins, using confocal microscopy the measure colocalization.

## The Purpose for this Study

The main hypothesis of this study is that **chronic hypoxia alters the ability of PKG to influence the BK potassium channel** and thereby diminishes its ability to induce vasorelaxation in cerebral arteries. Given that PKG is pleiotropic and has multiple target proteins, many of which are involved with regulation of vascular tone, it was necessary to separately identify the BK- versus the non-BK influences of PKG under hypoxic and normoxic conditions. To accomplish this, the BK channel was selectively inhibited by use of iberiotoxin. Effects attributable to the BK channel therefore appeared as functional *differences* between inhibited and uninhibited arteries. Non-BK channel effects of PKG are also subject to the influence of chronic hypoxia, which was likewise measured in BK-inhibited normoxic and hypoxic arteries. Each of the experiments was performed in both fetal and adult cerebral arteries. This defines three orthogonally independent axes: 1) BK vs. non-BK, 2) hypoxic vs. normoxic, and 3) fetal vs. adult; and thus eight separate experimental groups.

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# CHAPTER TWO

# HYPOXIC DEPRESSION OF PKG-MEDIATED INHIBITION OF

# SEROTONERGIC CONTRACTION

# IN OVINE CAROTID ARTERIES

## Abstract

Chronic hypoxia attenuates soluble guanylate cyclase-induced vasorelaxation in serotonin (5-HT)-contracted ovine carotid arteries. Because protein kinase G (PKG) mediates many effects of soluble guanylate cyclase activation through phosphorylation of multiple kinase targets in vascular smooth muscle, we tested the hypothesis that chronic hypoxia reduces the ability of PKG to phosphorylate its target proteins, which attenuates the ability of PKG to induce vasorelaxation. We also tested the hypothesis that hypoxia attenuates PKG expression and/or activity. Arteries from normoxic and chronically hypoxic (altitude of 3,820 m for 110 days) fetal and adult sheep were denuded of endothelium and equilibrated with 95% $O_2$-5% $CO_2$ in the presence of nitro-L-arginine methyl ester (L-NAME) and NG-nitro-L-arginine (L-NNA) to inhibit residual endothelial nitric oxide synthase. Concentration-response relations for 5-HT were determined in the presence of prazosin to minimize activation of α-adrenergic receptors. The PKG activator 8-(p-chlorophenylthio) guanosine 3',5'-cyclic monophosphate (8-pCTP-cGMP) reduced agonist binding affinity of the 5-HT receptor in a concentration-dependent manner that was attenuated by hypoxia. Expression and activity of PKG-I was not significantly affected by chronic hypoxia in either fetal or adult arteries, although PKG-I abundance was greater in fetal arteries. Pretreatment with the large conductance calcium-sensitive potassium channel (BK) inhibitor iberiotoxin attenuated the vasorelaxation induced by 8-

pCPT-cGMP in normoxic but not chronically hypoxic arteries. These results support the hypothesis that hypoxia attenuates the vasorelaxant effects of PKG through suppression of the ability of PKG to activate large conductance calcium-sensitive potassium channels in arterial smooth muscle. The results also reveal that this hypoxic effect is greater in fetal than adult arteries and that chronic maternal hypoxia can profoundly affect fetal vascular function.

## Introduction

Chronic hypoxia is a common stressor in many clinical pathologies and is particularly devastating during pregnancy (17, 28). Maternal hypoxia induces a broad variety of effects in both mother and fetus, including numerous changes in vascular structure and function (26, 46). Prominent among these functional changes is a depressed capacity for vasorelaxation evident in both the pulmonary (50) and systemic (33) circulations. These effects are explained in part by hypoxic depression of endothelial nitric oxide (NO) release (35) and efficacy (45). In turn, chronic hypoxia also modestly depresses the ability of NO to activate soluble guanylate cyclase and stimulate the synthesis of cGMP (9, 46). More importantly, chronic hypoxia can attenuate the ability of cGMP to promote vasorelaxation in multiple vascular beds (36).

For vasorelaxation, the primary target of cGMP is cGMP-dependent protein kinase (PKG), a serine-threonine kinase expressed as two isoforms (Iα and Iβ) in mammalian vascular smooth muscle (47). When bound to cGMP both isoforms of PKG are enzymatically active as homodimers that phosphorylate serine-threonine residues in a broad variety of target proteins (2, 7, 39). At the plasmalemma, PKG can phosphorylate

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the α-subunit of the large conductance calcium-sensitive potassium (BK) channel and thereby increase its sensitivity to cytosolic $Ca^{2+}$ and its opening probability (38, 40). PKG-mediated phosphorylation also activates plasma membrane $Ca^{2+}$-ATPase (48) and inhibits the plasmalemmal L-type calcium channel (16); both of these effects reduce cytosolic $Ca^{2+}$ concentration and promote vasorelaxation. Inside the smooth muscle cell, PKG-mediated phosphorylation can stimulate the sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA) calcium pump (8) and attenuate sarcoplasmic reticulum (SR) $Ca^{2+}$ release through the inositol 1,4,5-trisphosphate (IP3) receptor (19). PKG-mediated phosphorylation of transcription factors such as cAMP response element binding protein (CREB) and serum response factor (SRF) influence transcription of multiple genes involved in regulation of smooth muscle phenotype (6, 37).

Our main hypothesis is that chronic hypoxia alters the capacity for PKG-mediated vasorelaxation through direct effects on PKG and/or the proteins it phosphorylates. To test this hypothesis, we examined the effects of chronic hypoxia on PKG abundance and activity in ovine carotid arteries (20). Because effects of chronic hypoxia differ in fetal and adult arteries (45), we examined both fetal and adult carotid arteries using a model designed to assess the effects of maternal hypoxia on fetal oxygenation. This model is clinically relevant to fetal hypoxemia resulting from placental insufficiency, maternal smoking, maternal pulmonary insufficiency, etc. (26). To minimize confounding effects of endothelial and extravascular stimulation of PKG, the vascular endothelium was mechanically removed, any residual endothelial NO synthase was inhibited, and adrenergic receptors were selectively antagonized so that the results uniquely represent the effects of PKG in smooth muscle pharmacomechanical coupling following hypoxic

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acclimatization. In an effort to identify the categories of protein targets whose phosphorylation by PKG may change with hypoxia, we compared multiple endpoints between normoxic and hypoxic arteries. These endpoints included the ability of PKG to attenuate 5-HT-induced contractile force, to alter agonist affinity, and to influence BK channel function. Serotonin was used as the contractile agonist in these experiments owing to its ubiquitous potency (43), well-characterized endothelium-independent 5-HT2A receptor-mediated mechanism of contraction (42), and the potential for regulation of the binding affinity of this receptor by phosphorylation (1). The BK channel was specifically examined because of its role in membrane potential homeostasis, high $K^+$ currents, $Ca^{2+}$ sensitivity, and known PKG phosphorylation sites. Together, these experiments provided a unique insight into the age-dependent effects of chronic hypoxia on PKG function in vascular smooth muscle.

## Materials and Methods

All procedures in this study were approved by the animal Research Committee of Loma Linda University and adhere strictly to the policies and practices according to the National Institutes of Health Guide governing the care and use of laboratory animals.

### *Tissue Harvest and Preparation*

All common carotid arteries used in this study were harvested using sterile techniques from normoxic and chronically hypoxic fetal (139–142 days' gestation) and young (18–24 mo. old) nulliparous adult sheep. In normoxic animals maintained at sea level arterial oxygen tensions (PaO$_2$) averaged $23 \pm 1$ and $102 \pm 2$ Torr in fetal and adult

sheep, respectively (18). Corresponding arterial blood pressures averaged $44 \pm 2$ and $81 \pm 3$ mmHg, respectively. Chronically hypoxic sheep were maintained for ~110 days at high altitude (altitude 3,820 m, Barcroft Laboratory, White Mountain Research Station, Bishop, CA). For pregnant ewes, the period at altitude corresponded to the final 110 days of gestation, where term averages 143 days. At altitude, fetal and adult $PaO_2$ values averaged $19 \pm 1$ and $64 \pm 2$ Torr, respectively. Corresponding blood pressures in these animals averaged $52 \pm 1$ and $88 \pm 4$ mmHg, respectively. Pregnant ewes were anesthetized with 30 mg/kg pentobarbital, intubated, and then placed on 1.5–2.0% halothane. The anesthetized fetus was then exteriorized through a midline vertical laparotomy and euthanized by rapid removal of the heart and exsanguination. Non-pregnant adult animals were euthanized by intravenous administration of 100 mg/kg pentobarbital. Harvested carotid arteries were placed in Krebs buffer solution containing (in mM) 122 NaCl, 25.6 $NaHCO_3$, 5.17 KCl, 2.49 $MgSO_4$, 1.60 $CaCl_2$, 2.56 dextrose, 0.027 EGTA, and 0.114 ascorbic acid, bubbled with 95% $O_2$-5% $CO_2$. Arteries were debrided of loose extracellular and connective tissue and then denuded of endothelium by passage of a stainless steel rod through the lumen. The vascular endothelium was systematically removed to minimize the potentially confounding effects of endothelial NO release on cGMP synthesis and PKG activation.

### *Determination of Concentration-Response Relations for 5-HT*

Denuded carotid arteries were cut into segments 1 mm to 2 mm in length, mounted on tungsten wire loops, and suspended from a force transducer in a sodium-replete Krebs buffer solution (pH 7.4) at 38°C (the ovine core temperature) and bubbled with 95% $O_2$

and 5% $CO_2$. To inhibit endogenous nitric oxide (NO) production, 10 $\mu$M nitro-L-arginine methyl ester (L-NAME) and 10 $\mu$M NG-nitro-L-arginine (L-NNA) were added to the Krebs buffer solution. To selectively inhibit activation of $\alpha$1-adrenergic receptors by 5-HT, 1.0 $\mu$M prazosin and 0.2 $\mu$M cocaine were added. In preliminary experiments, both endothelium-intact and endothelium-denuded segments were studied. Because endothelium removal had no effect on average contractile efficacy, but reduced its variability, all further experiments were performed in endothelium-denuded segments. The denuded segments were equilibrated for 30 min and then stretched to a baseline tension of 1.5 g, which corresponds with an optimal stretch ratio of ~1.8 times the unstressed diameter. Contractile force was measured directly using an isometric force transducer (Kent Scientific, Torrington, CT) and recorded directly by computer. Concentration-response curves were obtained by cumulative addition of half-log concentrations of 5-HT across a range of $10^{-10}$ to $10^{-4}$ M. In preliminary experiments, norepinephrine was also used as the contractile agonist, but it was significantly less potent and less efficacious than serotonin in producing contractions; all further experiments were performed using 5-HT. $EC_{50}$ values (molar concentration at which the contractile response was half the maximal induced contraction) were expressed as pD2 values ($-\log EC_{50}$). All contractile responses were normalized to the maximum force produced by exposure to a potassium-Krebs solution containing (in mM) 5.17 NaCl, 25.6 $NaHCO_3$, 122 KCl, 2.49 $MgSO_4$, 1.60 $CaCl_2$, 2.56 dextrose, and 0.027 EGTA. To test the contribution of large conductance calcium-sensitive potassium (BK) channels to 5-HT contractions, concentration-response experiments were also carried out in the presence of the selective BK channel blocker iberiotoxin at a concentration of 100 nM (dissolved in

Krebs), a concentration that previous work has shown to be optimal (41). Iberiotoxin was added to the baths, in both the presence and absence of 30 µM (4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP), 30 min before commencement of the concentration-response sequences.

### *Measurement of Agonist Binding Affinity and Receptor Occupancy*

The 5-HT-receptor dissociation constants were determined via the Furchgott method of partial irreversible blockade (13) with the alkylating agent phenoxybenzamine (50–150 nM), as originally described in detail by our laboratory (15, 42). Briefly, pairs of artery segments were untreated or pretreated with the PKG activator 8-pCPT-cGMP across a range of concentrations from 0 to 30 µM to determine the influence of PKG. The final concentration of phenoxybenzamine used in each artery was chosen to achieve a 50% decrease in 5-HT efficacy with 30 min incubation, followed by a washout. Pairs of adjacent phenoxybenzamine-treated and -untreated artery segments were then assayed to obtain concentration-response curves across a range of $10^{-10}$ to $10^{-4}$ M 5-HT.  Equiactive concentrations of 5-HT in treated [A'] and untreated [A] arteries were plotted as a double reciprocal plot of 1/[A] versus 1/[A'] from which the regression line was calculated; the dissociation constant was taken as the ratio $K_a$ = slope – (1/intercept). Using the apparent $K_a$ for the 5-HT2A receptor complex, fractional receptor occupancy was calculated from the equation: [RA]/[RT] = [A]/([A] + Ka), where [RA] was the concentration of the receptor-agonist complex, and [RT] was the total receptor concentration (31).

### *Western Blot Analysis of PKG Abundance*

Common carotid artery segments were homogenized using glass-on-glass mortars

and pestles in 50 mM HEPES (pH 7.4), 2 mM magnesium acetate, 2 mM DTT, 1 mM

EDTA, 520 μM AEBSF, 7.5 μM pepstatin-A, 7 μM E-64, 20 μM bestatin, 100 μM

leupeptin, and 0.4 μM aprotinin. Supernatants were collected after centrifugation at

100,000 g for 1h at 4°C and analyzed for protein content using the Bradford assay, as

described previously (4, 34), and then divided for separate analysis of PKG abundance by

Western blot and PKG activity. Samples for PKG abundance were separated by SDS-

PAGE and then transferred to nitrocellulose membranes for immunodetection. Primary

antibodies for total PKG were obtained from Stressgen (1:1,000; catalog no.  KAS-

PK005). This antibody detected a PKG-I epitope common to both the α- and β-isoforms.

Blots were visualized using the chemiluminescent substrate Supersignal West Dura

(catalog no. 37071, Thermo Scientific), and images were then captured on an Alpha

Innotech Fluorchem (Cell Biosciences, Santa Clara, CA).  Relative abundances of total

PKG were normalized to standards prepared from arteries harvested from adult normoxic

non-pregnant ewes.


### *Measurement of PKG-I Activity*

Samples of supernatants from the homogenates prepared for measurements of PKG

abundance (described in Western blot analysis of PKG abundance) were cleared for 30

min at 30°C after which 5 mM NaF and 1 μM PKI (a PKA inhibitor, Sigma-Aldrich

P6061) were added. After 30 min of incubation, sample aliquots were added to reaction

buffer in 96-well plates to yield final concentrations of 200 μM ATP with 300–400

cpm/pmol [λ-$^{32}$P]ATP (NEN), 200 μM PKG substrate BPDEtide (BMLP112-0001, Enzo Life Sciences), and 10 μM 8-pCPT-cGMP (Sigma-Aldrich, C5438) in homogenizing buffer. Timed reactions were terminated by addition of phosphoric acid. Phosphorylated BPDEtide and unreacted [λ-$^{32}$P]ATP were separated through phosphocellulose paper using a Millipore 96-well filtration plate. Filters were washed with phosphoric acid, allowed to dry, added to vials with scintillation cocktail, and assayed using a λ-scintillation counter. Counts were converted into moles $^{32}$P using a calibration curve counted in parallel with the samples and normalized to sample protein content to obtain activity in units of pmol $^{32}$P transferred per minute per milligram of protein. Each assay included blanks with: 1) no added 8-pCPT-cGMP; 2) no added BPDEtide substrate; and 3) neither 8-pCPT-cGMP nor BPDEtide to enable correction for total nonspecific background.

### *Data Analysis and Statistics*

Significant differences in contractile variables were determined using Behrens-Fischer tests for two-group comparisons and ANOVA followed by Duncan's post hoc simultaneous comparisons of more than two groups. Analyses using the SPSS software routinely confirmed homogeneity of variance and normal data distributions (SPSS v19). Determinations of pKa values were based on double reciprocal plots from which slopes were determined by linear regression as described by Furchgott (13, 31). Contractile forces were normalized relative to the contraction as measured upon exposure to isotonic 120 mM potassium-Krebs in the same artery segment. The relative influence of PKG stimulation on the BK potassium channel compared with all other downstream targets

was estimated with an algebraic model where a = maximum response to 5-HT in control arteries (untreated), b = total maximum response to 5-HT in the presence of PKG stimulation by 8-pCPT-cGMP, c = maximum response to 5-HT in presence of iberiotoxin, and d = maximum response to 5-HT in the presence of iberiotoxin and with pretreatment using 8-pCPT-cGMP. The total effect of PKG activation was defined as (a - b), and the effect of PKG activation independent of BK channel activity was defined as (c - d). The BK channel-dependent effect of PKG activation was calculated as (a - b) - (c - d). Maximum efficacy of 5-HT was calculated using SPSS (version 19) with a nonlinear regression model using the four-parameter Hill equation $y = (e + [(f - e)/\{1 + 10^{[([agonist] - g) \times h]}\}])$, where "e" represents the calculated $E_{max}$.

## Results

A total of 57 sheep were used in this study of which 27 were term fetal lambs and 30 were adult sheep. From these animals we harvested a total of 233 carotid artery segments, including 113 fetal segments and 120 adult segments. Throughout the text, "n" represents the number of animals, and not the number of segments used in each experiment. Unless stated otherwise, statistical significance implies $P < 0.05$. All values are given as means ± SE.

### *Effects of Hypoxia, Age, and 8-pCPT-cGMP on 5-HT Concentration-Response Relations*

Maturation significantly reduced maximal responses to 5-HT (efficacy) in normoxic animals (Fig. 1, left). Chronic hypoxia increased maximal responses to 5-HT in

both fetal and adult arteries and eliminated age-related differences in efficacy (Fig. 1, right). Pretreatment with 8-pCPT-cGMP, a cell-permeant and phosphodiesterase-resistant PKG activator, significantly attenuated 5-HT efficacy in normoxic fetal and adult arteries in a concentration-dependent manner. In hypoxic arteries, 8-pCPT-cGMP had no significant effects on 5-HT efficacy at any of the concentrations tested. The effects of 8-pCPT-cGMP on 5-HT efficacy were similar in endothelium-intact fetal (control: $131 \pm 7$; 8-pCPTcGMP: $123 \pm 5$) and endothelium-denuded fetal (control: $138 \pm 5$; 8-pCPT-cGMP: $129 \pm 5$) artery pairs (n = 7). Similarly the effects of 8-pCPT-cGMP on 5-HT efficacy were the approximately the same in endothelium-intact adult (control: $126 \pm 9$; 8-pCPT-cGMP: $110 \pm 16$) and endothelium-denuded adult (control: $133 \pm 4$; 8-pCPT-cGMP: $124 \pm 3$) artery pairs (n $\pm$ 5).

### *Effects of Hypoxia, Age, and 8-pCPT-cGMP on 5-HT Agonist Potency and Binding Affinity*

Values for 5-HT potency (pD2 = -log of $EC_{50}$) were significantly greater in fetal ($6.5 \pm 0.2$, n $\pm$ 7) than adult ($5.5 \pm 0.3$, n = 6) normoxic arteries (Fig. 2, top). Potency values were not significantly different in normoxic and hypoxic arteries for either age group. Pretreatment with 8-pCPT-cGMP attenuated 5-HT potency in normoxic fetal arteries only at 30 μM and was without effect in normoxic adult arteries at any concentration tested. Pretreatment with 8-pCPT-cGMP attenuated 5-HT potency at 10 and 30 μM in hypoxic fetal arteries and at 3, 10, and 30 μM in hypoxic adult arteries. Chronic hypoxia significantly enhanced the depressant effects of 8-pCPT-cGMP on 5-HT potency. Values for 5-HT binding affinity (pKa) were significantly greater in fetal (6.14

± 0.48, n = 7) than adult (5.49 ± 0.27, n = 6) normoxic arteries (Fig. 2, bottom). Binding affinity values were not significantly different in normoxic and hypoxia arteries for either age group. Pretreatment with 8-pCPT-cGMP attenuated pKa values at 10 and 30 μM in both fetal and adult normoxic arteries. Pretreatment with 8-pCPT-cGMP attenuated pKa values only at 30 μM in hypoxic adult arteries and was without effect in hypoxic fetal arteries. Chronic hypoxia significantly attenuated the depressant effects of 8-pCPT cGMP on 5-HT binding affinity.

### *Effects of Hypoxia, Age, and 8-pCPT-cGMP on 5-HT Occupancy-Response Relations*

To correct for group differences in 5-HT binding affinity, the concentration-response relations were converted to occupancy-response relations using the Furchgott method (13). This correction altered the shapes of the concentration-response relations but still revealed a significant depressant effect of 8-pCPT-cGMP on occupancy-response relations in both fetal and adult normoxic arteries (Fig. 3, left). Equally important, this depressant effect of 8-pCPT-cGMP on occupancy-response relations was not evident in either fetal or adult hypoxic arteries (Fig. 3, right). These results revealed that 8-pCPT-cGMP attenuated 5-HTinduced contractility at a step downstream from ligand-receptor binding and that this effect of 8-pCPT-cGMP was absent in chronically hypoxic fetal and adult arteries.

*Effects of Hypoxia, Age, and 8-pCPT-cGMP on PKG Abundance and Specific*

*Activity*

When expressed relative to abundances in normoxic adult carotid arteries, the relative abundance of total PKG was significantly greater in fetal compared with adult arteries in both normoxic and hypoxic groups (Fig. 4). In tissue homogenates, total PKG activity was significantly greater in fetal than adult arteries at 5.5 and 8.0 min of reaction in normoxic than in hypoxic homogenates for either age group. When total PKG activity was normalized relative to total PKG abundance to estimate specific activity, the resulting values were similar in all groups (Fig. 5, inset).

*Interactive Effects of 8-pCPT-cGMP and Iberiotoxin on 5-HT-Induced*

*Contraction*

In all arteries, the magnitude of decrease in 5-HT efficacy produced by 8-pCPT-cGMP was significant in both the presence and absence of iberiotoxin, although the magnitudes of these effects varied considerably with both age (Fig. 6, top vs. bottom) and hypoxia (Fig. 6, left vs. right). Iberiotoxin attenuated the magnitude of 5-HTinduced contraction in all arteries, indicating a strong BK channel influence on vasorelaxation. Analysis of these results with our algebraic model quantified the relative contributions of BK-dependent and BK-independent components of PKG action on 5-HT efficacy. In both fetal and adult arteries, the total magnitude of PKG-mediated inhibition of 5-HT efficacy was reduced by hypoxia, and the BK-dependent component was virtually eliminated (Fig. 7). In contrast, the BK-independent component of PKG-mediated inhibition of 5-HT efficacy was unchanged by hypoxia in both age groups.
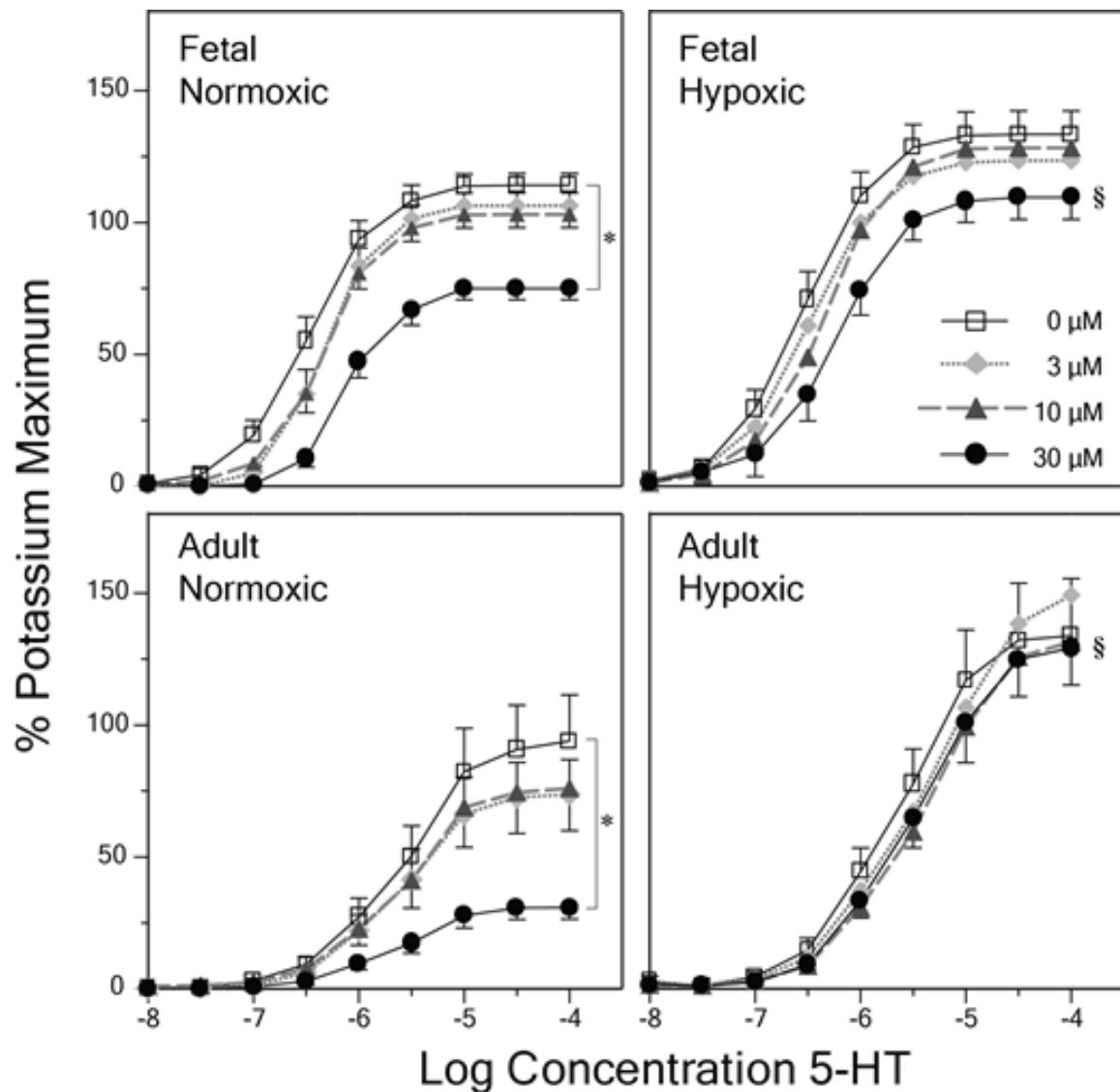
**Figure 1.** Effects of 8-(p-chlorophenylthio)-guanosine 3',5'-cyclic monophosphate (8-pCPT-cGMP) and hypoxia on 5-HT concentration-response relations. Maximum contractile responses to graded concentrations of 5-HT did not vary significantly with either age or hypoxia in untreated arteries (0 μM 8-pCPTcGMP). Pretreatment with 8-pCPT-cGMP attenuated 5-HT efficacy in a concentration-dependent manner but only in normoxic arteries. *Significantly ($P < 0.05$) less than corresponding untreated controls. In arteries pretreated with 30 μM 8-pCPTcGMP, 5-HT efficacy was greater in hypoxic than normoxic arteries for both the fetus and adult. §Hypoxic values significantly different ($P < 0.05$) than equivalently treated normoxic values in corresponding age groups. Error bars indicate means ± SE for n ≥ 6 for all groups.
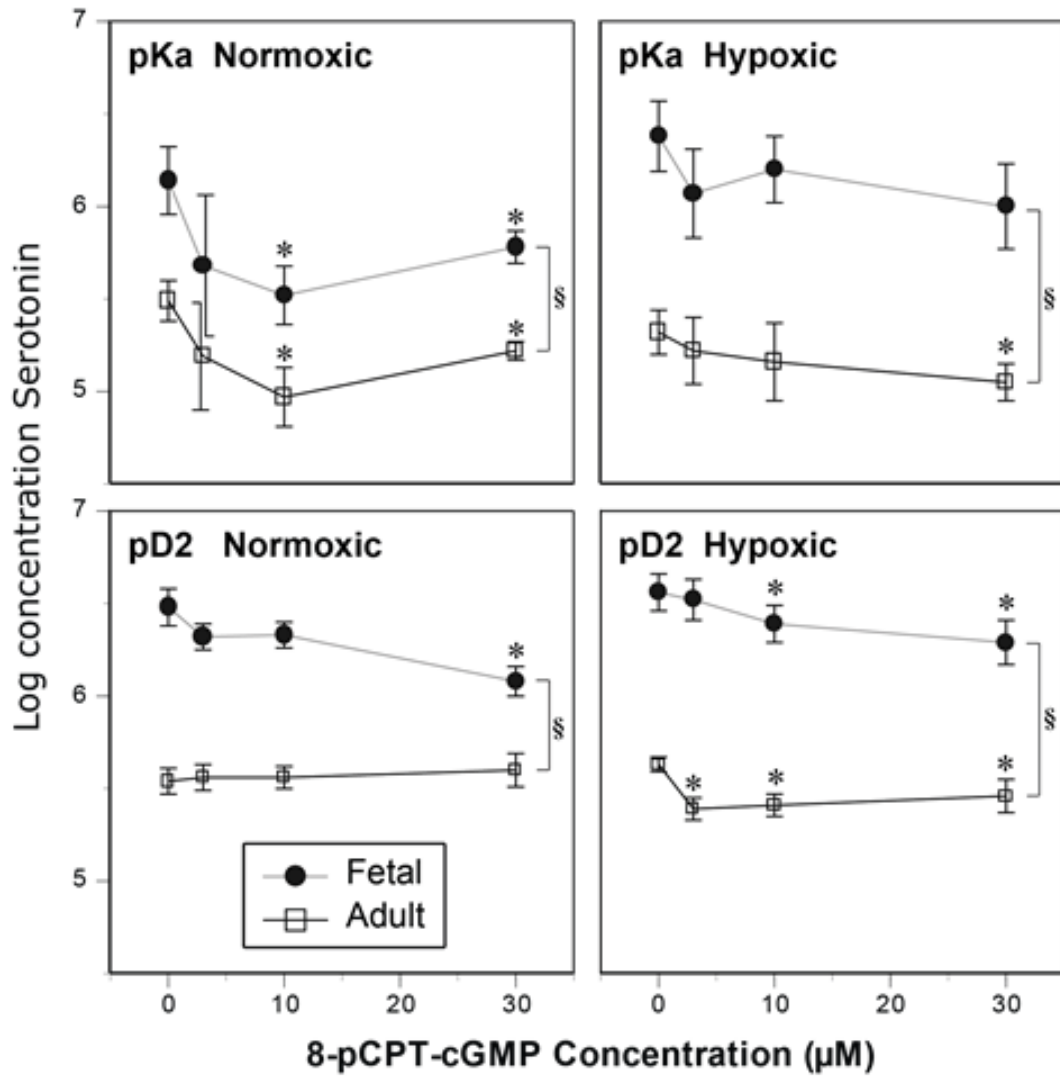
Fig. 2. Effects of 8-pCPT-cGMP and hypoxia on agonist affinity and potency for 5-HT. In untreated control arteries (0 μM 8-pCPT-cGMP), agonist potencies ($pD2 = -\log EC_{50}$) were significantly greater in fetal than adult arteries for both normoxic and hypoxic groups. Normoxic (*top left*) and hypoxic (*top right*) potencies in untreated controls were not significantly different in either fetal or adult arteries. Treatment with 8-pCPTcGMP significantly depressed pD2 in normoxic fetal arteries at 30 μM (*top left*), in hypoxic fetal arteries at 10 and 30 μM (*top right*), and in hypoxic adult arteries at all concentrations (*top right*). In untreated control arteries, agonist affinity (pKa) was significantly greater in fetal than adult arteries for both normoxic and hypoxic groups (*bottom left* and *right*). Normoxic (*bottom left*) and hypoxic (*bottom right*) affinities in untreated controls were not significantly different in either fetal or adult arteries. Treatment with 8-pCPT-cGMP significantly depressed pKa in normoxic fetal and adult arteries at 10 and 30 μM and also in hypoxic adult arteries at 30 μM. §Significant differences ($P < 0.05$) between untreated control fetal and adult arteries. *Values significantly different ($P < 0.05$) from corresponding untreated controls. Error bars indicate means ± SE for $n \geq 6$ for all groups.

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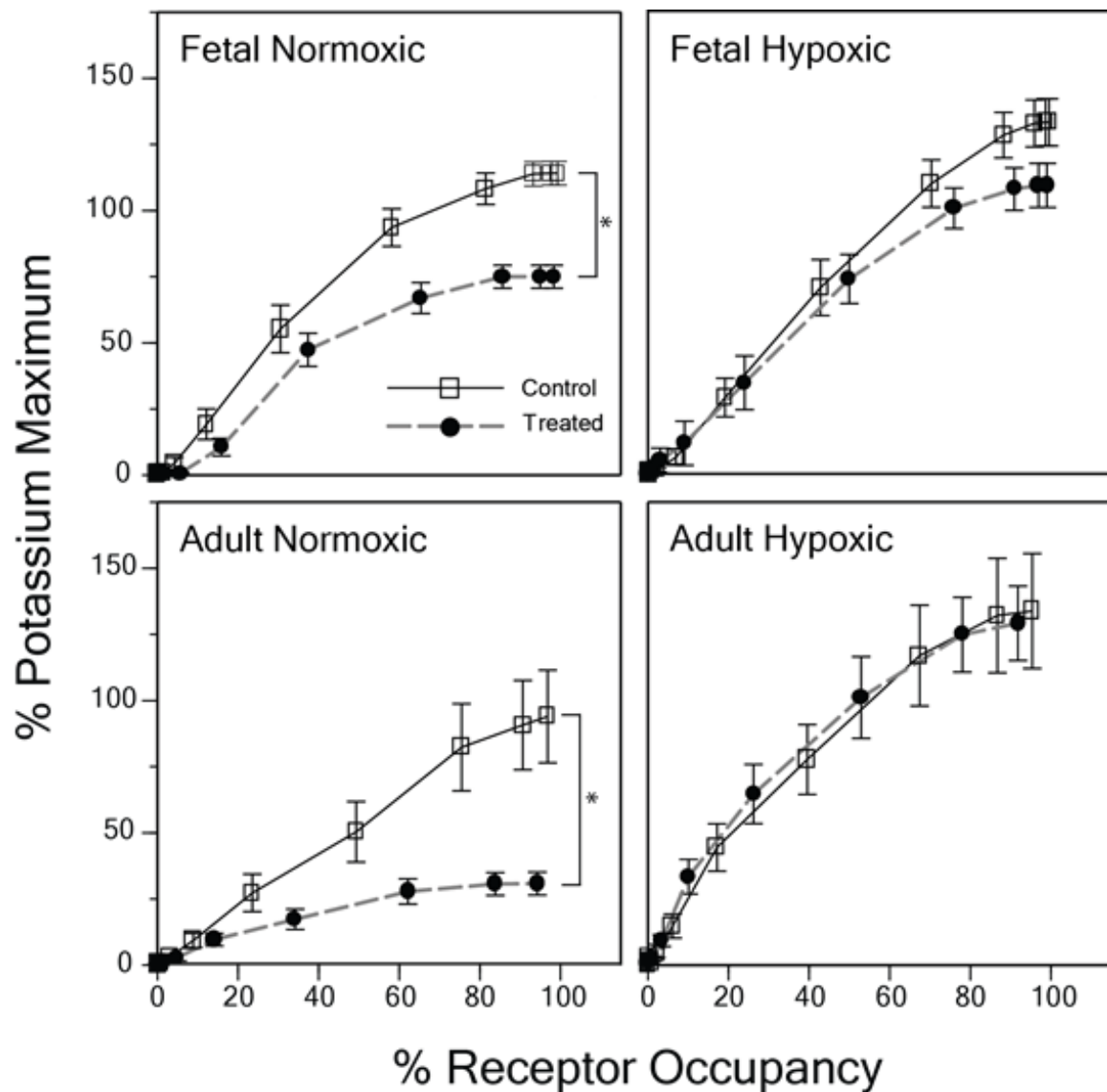
**Fig. 3. Effects of 8-pCPT-cGMP and hypoxia on occupancy-response relations for 5-HT**. The 5-HT concentration-response relations shown in Fig. 1 were converted to occupancy-response relations using values of affinity (pKa) shown in Fig. 2 to correct for differences in agonist binding affinity. The resulting occupancy-response relations revealed that compared with untreated control arteries, pretreatment with 30 μM 8-pCPT-cGMP significantly reduced maximum efficacy for 5-HT in normoxic (*left*) but not hypoxic (*right*) arteries from both age groups. *Significant differences ($P < 0.05$, repeated measures ANOVA) between untreated (0 μM 8-pCPT-cGMP) and treated (30 μM 8-pCPT-cGMP) arteries. Error bars indicate means ± SE for $n \geq 6$ for all groups.
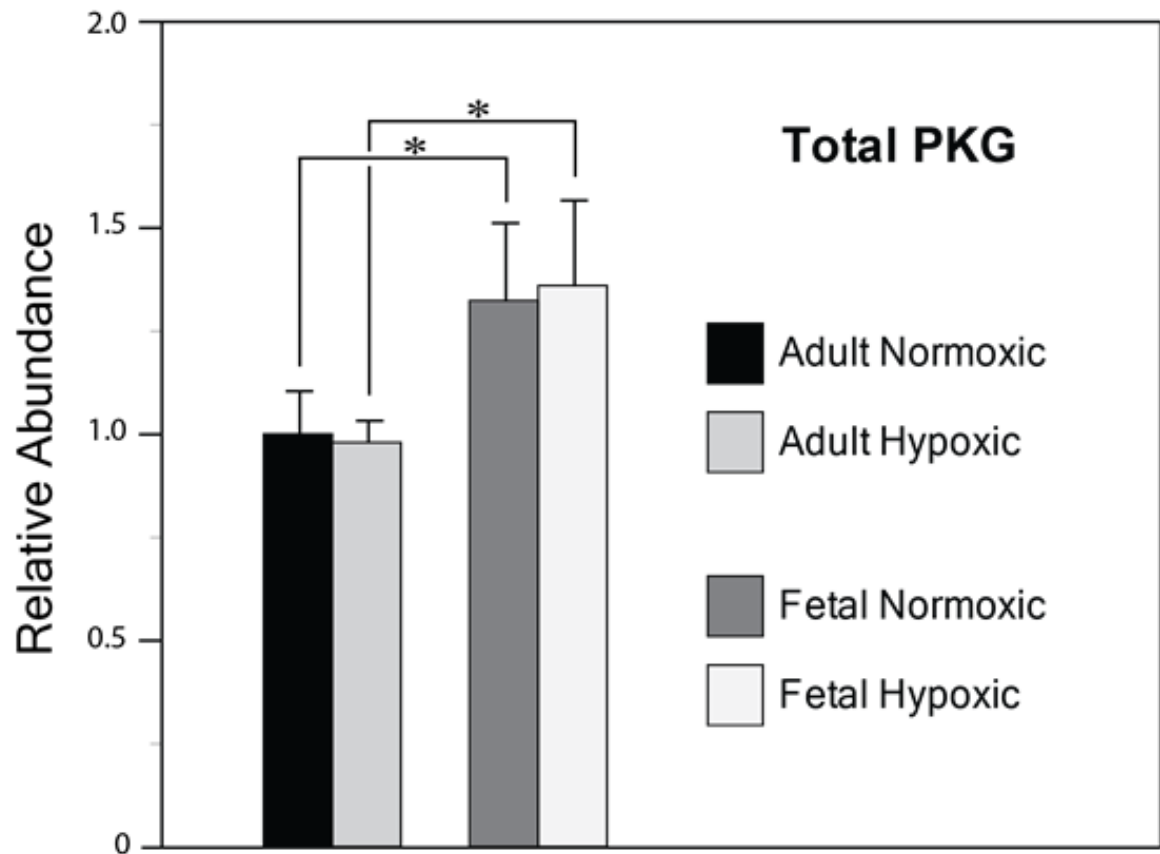
**Fig. 4.** Effects of hypoxia on the abundance of protein kinase G (PKG) isoforms. The abundance of total PKG was determined by Western blot analysis using an antibody against a PKG-I epitope common to both the α and β isoforms. All abundances were calculated relative to known amounts of a standard pool prepared from normoxic adult arteries. *Relative abundance of total PKG was significantly greater in fetal than adult arteries and was not affected by hypoxia. Error bars indicate means ± SE for $n \geq 6$.
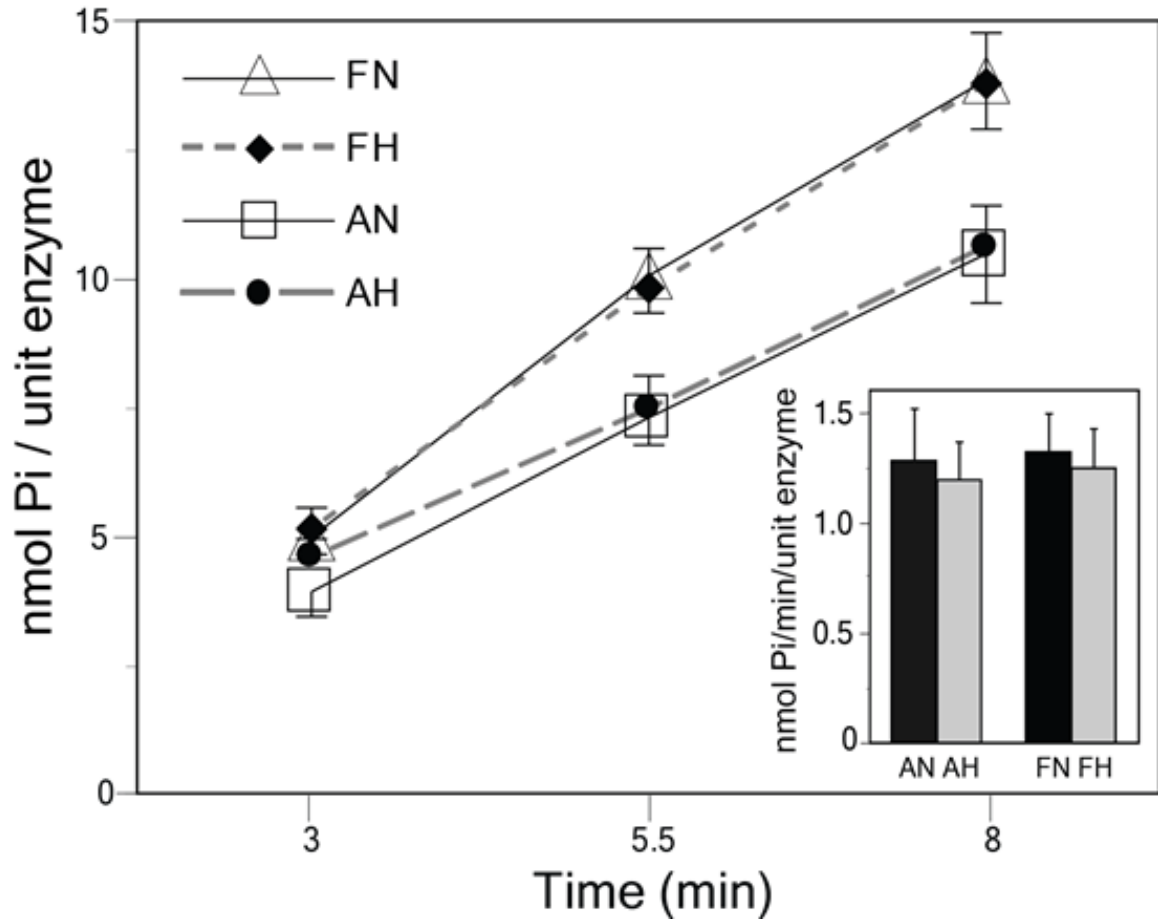
**Figure 5.** Effects of hypoxia on PKG activity. Using a peptide substrate derived from bovine phosphodiesterase (abbreviated as BPDE in the figure), whole artery PKG activity (line graph, nmol ^{32}P-labeled BPDE/ relative unit PKG) was not significantly affected by hypoxia in either fetal or adult homogenates. Total PKG activity was greater in fetal compared with adult artery tissue homogenates, which reflects the modestly greater total PKG abundance in fetal arteries. When whole artery PKG activity was normalized relative to PKG abundance (Fig. 4), estimates of specific activity (*inset*, nmol ^{32}P-labeled BPDE/min/relative unit PKG) did not vary significantly with hypoxia. Error bars indicate means $\pm$ SE for $n \geq 5$.
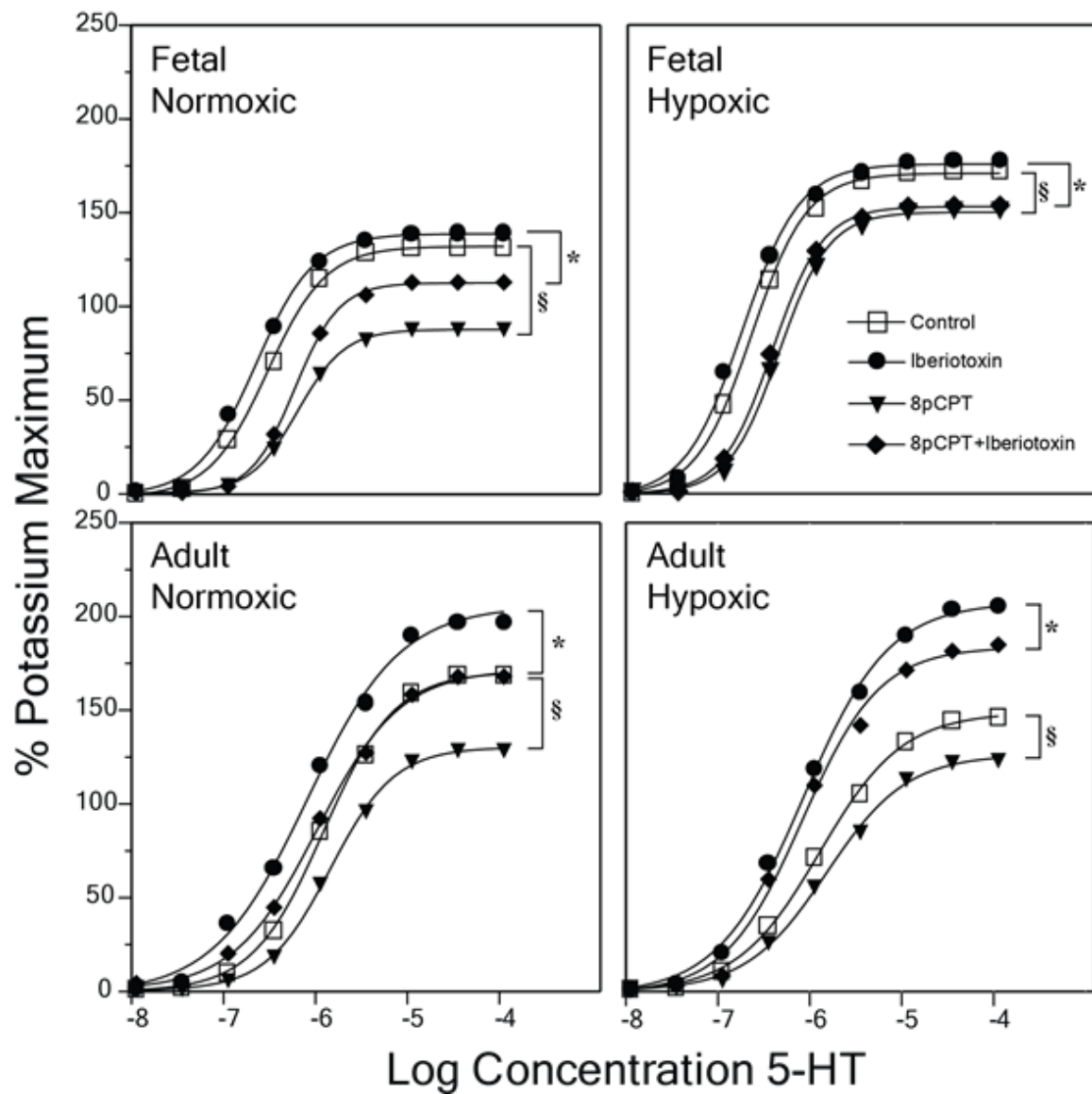
Fig. 6. Interactive effects of 8-pCPT-cGMP and iberiotoxin on 5-HT concentration-response relations. The relative contributions of PKG and BK channels to contractile responses to 5-HT were identified by addition of 30 μM 8-pCPT-cGMP, a PKG activator (▼), 100 nM iberiotoxin, a BK channel blocker (●), or both (♦), respectively, in matched sets of adjacent segments from the same artery. §Total effect of PKG activation on 5-HT contraction was defined as the difference in maximum response between control and 8-pCPT-cGMP-treated arteries. *Effect of PKG activation independent of BK channels was defined as the difference in maximum response between arteries treated with iberiotoxin alone and both iberiotoxin and 8-pCPT-cGMP. Error bars indicate means ± SE for $n \geq 8$.
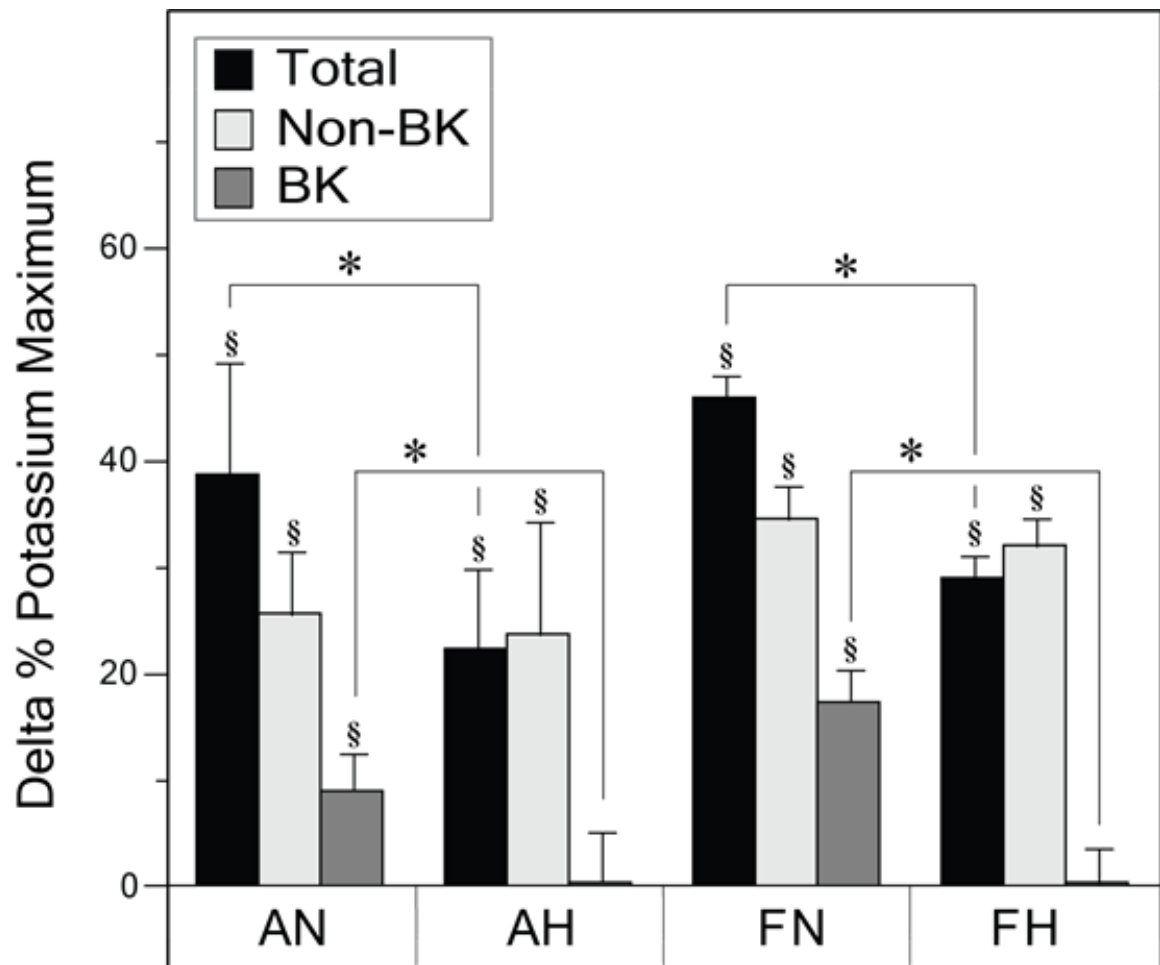
**Figure 7.** Estimates of the relative magnitudes of BK-dependent and BK-independent components of PKG activation on 5-HT-induced contractions. The total effects of PKG activation on the maximum contractile response to 5-HT were partitioned into BK-independent and BK-dependent components via calculation of differences in efficacy as defined in Fig. 6. Hypoxia virtually eliminated the BK-dependent component of PKG-mediated inhibition of 5-HT efficacy in both fetal and adult arteries. *Hypoxia attenuated total magnitude of effect of PKG on contractility in both fetal and adult arteries but had no significant effect on the BK-independent component. §All components of the PKG effect were significantly greater than zero except for the BK-dependent component in hypoxic arteries. Error bars indicate means ± SE for $n \geq 6$.

## Discussion

This study of the effects of chronic hypoxia on the vasorelaxant efficacy of PKG in endothelium-denuded and NO synthase-inhibited arteries offers five main observations: 1) the ability of PKG to attenuate 5-HT-induced contractions was dramatically reduced in hypoxic compared with normoxic arteries, and this effect was greater in adult than fetal carotids; 2) PKG activation attenuated ligand binding affinity for 5-HT, and this effect was significantly reduced by chronic hypoxia in both fetal and adult arteries; 3) receptor occupancy-response relations, which corrected for differences in binding affinity, also revealed that the ability of PKG to attenuate 5-HT-induced contractions was virtually eliminated by chronic hypoxia in both fetal and adult arteries; 4) the catalytic activity of total PKG was similar in fetal and adult arteries and was not affected by chronic hypoxia; and 5) the ability of PKG activation to reduce 5-HT-induced contractions was attenuated by pretreatment with the BK channel blocker iberiotoxin in normoxic but not hypoxic arteries from both fetus and adult. Given that these observations were made in endothelium-denuded arteries treated with L-NAME to inhibit NO synthase activity and with prazosin to inhibit activation of adrenergic receptors, this study focused on the ability of smooth muscle PKG to influence serotonergic contraction. Specifically, these observations support the hypothesis that chronic hypoxia acts directly on vascular smooth muscle to attenuate cGMP-induced vasorelaxation through reduced ability of PKG to activate BK channels.  Equally important, the results demonstrate that these effects of hypoxia on PKG-mediated inhibition of serotonergic contractions are markedly different in fetal and adult arteries.

### *Effects of Hypoxia and 8-pCPT-cGMP on 5-HT Efficacy and pKa*

Chronic hypoxia is well established as a major modulator of vascular structure, contractility, and vasorelaxant efficacy in many artery types (3, 25, 42). A key finding among previous studies is that chronic hypoxia can attenuate endothelial NO release through reductions in endothelial NO synthase-specific activity (35). In parallel, chronic hypoxia can also reduce relaxation responses to NO in part through reduced soluble guanylate cyclase activity, and in part through attenuation of the ability of 8-pCPT-cGMP, a PKG activator, to promote vasorelaxation (46). These results support the hypothesis that chronic hypoxia depresses cGMP-dependent protein kinase activity. Consistent with these results, chronic hypoxia markedly attenuated the ability of 8-pCPT-cGMP to inhibit contractile responses to 5-HT (Fig. 1). As previously reported, this effect of hypoxia was greater in adult than in fetal arteries (46). For a majority of G protein-coupled receptors, including those that mediate contractile responses to 5-HT, agonist binding affinity is subject to regulation through phosphorylation by multiple serine-threonine kinases including PKC, PKA, and specific GPCR receptor kinases (1, 5). This evidence suggests that PKG could attenuate 5-HT-induced contraction through desensitization of the 5-HT-2a receptors that mediate contractile responses to 5-HT in ovine common carotids (42). To explore this hypothesis and its corollary that hypoxia attenuates this effect of PKG, our experimental approach examined the effects of the PKG activator 8-pCPT-cGMP on 5-HT binding affinity (pKa) and potency (pD2) in normoxic and hypoxic arteries (Fig. 2). Treatment with 8-pCPT-cGMP significantly depressed pKa at multiple concentrations in normoxic fetal and adult arteries, but at only

30 μM in hypoxic adult arteries, and was without effect in hypoxic fetal arteries. Conversely, in normoxic arteries 8-pCPT-cGMP significantly attenuated pD2 only at 30 μM in fetal carotids, but in hypoxic arteries it significantly attenuated pD2 at multiple concentrations in both fetal and adult arteries. Together, these results demonstrate that 8-pCPT-cGMP significantly modulates interaction between

5-HT and its receptor through mechanisms that are sensitive to hypoxia. Given that pKa is determined primarily by interactions between a ligand and its receptor, but pD2 also includes influences of receptor density, the results are consistent with previous reports that hypoxia alters 5-HT receptor density (3). The small sizes of these effects, however, suggest that influences on binding affinity and potency alone cannot explain the ability of hypoxia to ablate PKG-mediated attenuation of serotonergic contractions (Fig. 1). To correct for any effects of 8-pCPT-cGMP and hypoxia on 5-HT binding affinity and potency, the concentration-response relations shown in Fig. 1 were converted into occupancy-response relations using the Furchgott transformation (13). This conversion eliminated the influence of changes in binding affinity and revealed changes in coupling between activated receptors and the contractile apparatus. As shown in Fig. 3, 8-pCPT-cGMP still significantly attenuated 5-HT-induced contractility in normoxic, but not hypoxic, arteries. These results demonstrate that 8-pCPT-cGMP significantly depressed coupling between the 5-HT-2a receptor and the contractile apparatus in both fetal and adult arteries through mechanisms that were highly sensitive to the effects of chronic hypoxia.

### *Effects of Hypoxia on PKG Abundance and Specific Activity*

Downstream from receptor activation, a key determinant of the ability of 8-pCPT-cGMP to alter 5-HT-induced contraction is the abundance of PKG. In turn, it is possible that hypoxic reductions in PKG abundance could explain hypoxic ablation of the ability of 8-pCPT-cGMP to reduce 5-HT-induced contractions. To test this hypothesis, the experimental approach included abundance measurements of total PKG, which included contributions from both the Iα and Iβ isoforms (11). These measurements indicated that neither postnatal age nor hypoxia had any significant effect on the abundance of total PKG (Fig. 4). These findings strongly demonstrated that hypoxic reductions in PKG abundance could not explain hypoxic ablation of the ability of 8-pCPT-cGMP to reduce 5-HTinduced contractions in adult arteries; another mechanism must be involved. Another key determinant of the ability of 8-pCPTcGMP to alter 5-HT-induced contraction is the kinase activity of the PKG enzyme. Correspondingly, inhibition of PKG kinase activity could potentially explain hypoxic ablation of the ability of 8-pCPT-cGMP to reduce 5-HT-induced contractions. To test this hypothesis, our experimental approach included measurements of PKG-dependent kinase activity in artery homogenates. Consistent with the observed age-related differences in PKG abundance (Fig. 4), total tissue activity was greater in fetal than adult arteries. However, hypoxia had no significant effect on total PKG activity in either fetal or adult artery homogenates (Fig. 5). Normalization of total tissue activities relative to PKG abundance further revealed that specific kinase activity for PKG varied with neither postnatal age nor hypoxia (Fig. 5, inset). Together, these findings demonstrated that changes in neither PKG abundance nor specific kinase activity could explain hypoxic inhibition of 8-pCPTcGMP- induced

reductions in 5-HT-induced contractions. Equally important, these results suggest that effects of hypoxia on protein targets of PKG may explain hypoxic inhibition of PKG-induced attenuation of 5-HT-induced contractions.

### *Interactive Effects of Hypoxia, 8-pCPT-cGMP, and Iberiotoxin on 5-HT Efficacy*

Among the many protein targets of PKG, one of the most prominent in regards to smooth muscle contractility is the BK channel (11, 29). Phosphorylation of S1072 by PKG directly promotes activation of the BK channel (12) and thereby hyperpolarizes the plasmalemma and promotes vasorelaxation (24, 47). These characteristics raise the possibility that PKG may attenuate 5-HT-induced contractions by activating the BK channel. In addition, it is possible that hypoxia in some way interferes with the ability of PKG to promote activation of the BK channel and thereby ablates the effects of PKG activation of 5-HT-induced contractility. To test this hypothesis, the experimental approach evaluated the effects of BK channel blockade on the ability of 8-pCPTcGMP to inhibit 5-HT-induced contractions. Blockade of BK channel currents relied on the effects of iberiotoxin, a well-defined and highly selective blocker of the BK channel (14). Previous studies from our laboratory have shown that 5-HT increases BK channel activity in sheep arteries, and that iberiotoxin at 100 nM can block this contribution (41). To quantify contributions of BK channel activation to PKG-mediated inhibition of 5-HT efficacy, we repeated the 5-HT concentration-response experiments as shown in Fig. 1, in the presence of iberiotoxin. With this approach, the total effect of PKG was defined by the difference in 5-HT efficacy measured in the presence and absence of 30 M 8-pCPT-

cGMP (Fig. 6). Similarly, the BK-independent effect of PKG was defined by the difference in 5-HT efficacy measured in the presence of iberiotoxin either with or without 30 $\mu$M 8-pCPT-cGMP. In turn, the BK-dependent component of PKG-mediated inhibition of 5-HT efficacy was the simple paired difference between the total component and the BK-dependent component of PKG-mediated inhibition. To simplify comparisons among these components, their average values were plotted in Fig. 7. As shown in Figs. 1, 6, and 7, hypoxia reduced total PKG-mediated inhibition of 5-HT efficacy in both fetal and adult arteries. The BK-independent component of PKG-mediated inhibition (Fig. 7, light gray) was significantly greater than the BK-dependent component in both fetal and adult arteries (Fig. 7, dark gray). More importantly, BK-independent component was not significantly different in normoxic and hypoxic arteries of either age group. This finding suggests that BK-independent mechanisms of PKG-mediated vasorelaxation, such as inhibition of calcium sequestration and release (8, 19), calcium influx (16), calcium extrusion (23), and myofilament calcium desensitization (30) are resistant to chronic hypoxia. In contrast, the BK-dependent component of PKG-mediated vasorelaxation was greater in fetal than adult arteries and was virtually ablated by chronic hypoxia in both age groups. These results extend previous findings in cultured pulmonary artery smooth muscle (36) and strongly suggest that hypoxic attenuation of PKG-mediated inhibition of 5-HT efficacy is attributable to suppression of the BK-dependent component of PKG-mediated vasorelaxation.

*Overview*

The mechanisms whereby hypoxia suppresses PKG-mediated activation of BK channels remain uncertain.  These effects could be explained by hypoxic inhibition of BK channel expression, but support for this mechanism is not evident in the literature. Hypoxia has been shown to upregulate expression of the β regulatory subunit of the BK channel (49), but how this effect might help mediate hypoxic suppression of PKG-mediated activation of BK channels remains to be demonstrated.  Similarly, the effects of chronic hypoxia on expression of the BK α-subunit also remain unreported. An alternate mechanism could involve a generalized hypoxic inhibition of BK activation (32), perhaps through depression of calcium sensitivity of the channel (10, 22). Hypoxic upregulation of generalized phosphatase activity could explain such an effect, but support for this mechanism is lacking. Another interesting possibility is that hypoxia alters the organization and distribution of PKG within the smooth muscle cell and thereby limits access of the kinase to its primary targets. Certainly, the mechanisms that mediate hypoxic inhibition of PKG-mediated activation of BK channels are worthy of future investigation (Fig. 8). Another worthy topic is how PKG inhibits agonist-binding affinity, and how this effect can be modulated by hypoxia; none of these mechanisms are known. What the present study does show is that hypoxia has little effect on PKG abundance or catalytic activity. Instead, hypoxia influences vascular smooth muscle to modulate the targets of PKG, including most prominently the BK channel. This finding raises new questions regarding possible effects of hypoxia on other PKG targets, what duration and severity of hypoxia are necessary to elicit these effects, and what other pathophysiological perturbations might produce similar effects.  The model used in the

present study was designed to assess the effects of maternal hypoxia on fetal oxygenation. Even though basal oxygen tensions under both normoxic and hypoxic conditions were markedly different in fetuses compared with non-pregnant adults, it is striking that the ability of hypoxic acclimatization to ablate PKG-mediated vasodilation of serotonergic contractions was similar in both adult and fetal sheep. This finding strongly suggests that a common mechanism of response to chronic hypoxia was operating in both adults and fetuses despite major differences in their ambient oxygen tensions. Although it is not certain that chronic hypoxia can ablate PKG-mediated inhibition of contractions induced by agonists other than 5-HT, it is conceivable that other calcium-dependent (27) and calcium-independent (21) signal transduction pathways might have a similar effect on BK channel function. Many contractile agonists utilize common intracellular IP3-dependent and calcium-dependent signal transduction pathways with potential to activate BK channels (44); it seems probable that other contractile agonists might reveal similar effects of chronic hypoxia (32). Conversely, preliminary results from our laboratory suggest that the effects of chronic hypoxia on the ability of PKG to attenuate tone may be specific for the agonist used to initiate contraction, particularly in fetal arteries. To fully evaluate how chronic hypoxia differentially influences the PKG-sensitive and agonist-specific components of contraction will require many additional studies. Whether the observed loss of PKG function represents a pathophysiological consequence or a biologically advantageous adaptation remains an open question. What does seem clear is that chronic hypoxia depresses multiple components of the NO-cGMP-PKG vasorelaxation pathway including endothelium-dependent vasodilation (45), inhibition of endothelial NO release (35), and

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inhibition of smooth muscle soluble guanylate cyclase activity (46). Because the patterns of PKG-dependent effects were similar in endothelium-intact and endothelium-denuded arteries, the present results further demonstrate that chronic hypoxia also depresses the ability of smooth muscle PKG to promote vasodilatation. The consequences of this pattern of effects remain uncertain but seem likely to enhance vasoconstrictor responses, which could help overcome typical hypoxic depression of contractility (32) but might also reduce the efficiency of flow-metabolism coupling and blood flow autoregulation. Such changes could increase vulnerability to, and could compromise recovery from, ischemic insults. For example, this pattern of increased vulnerability could be particularly important for the neurovascular unit, where the fine balance between metabolism and perfusion determines cell fate following a major insult. Alternatively, increased contractility may help maintain peripheral vascular resistance and arterial pressure at altitude. Overall, these effects of chronic hypoxia on the NO-cGMP-PKG vasorelaxation pathway seem likely to be compensatory given that pregnant sheep and their fetuses thrive at high altitude.
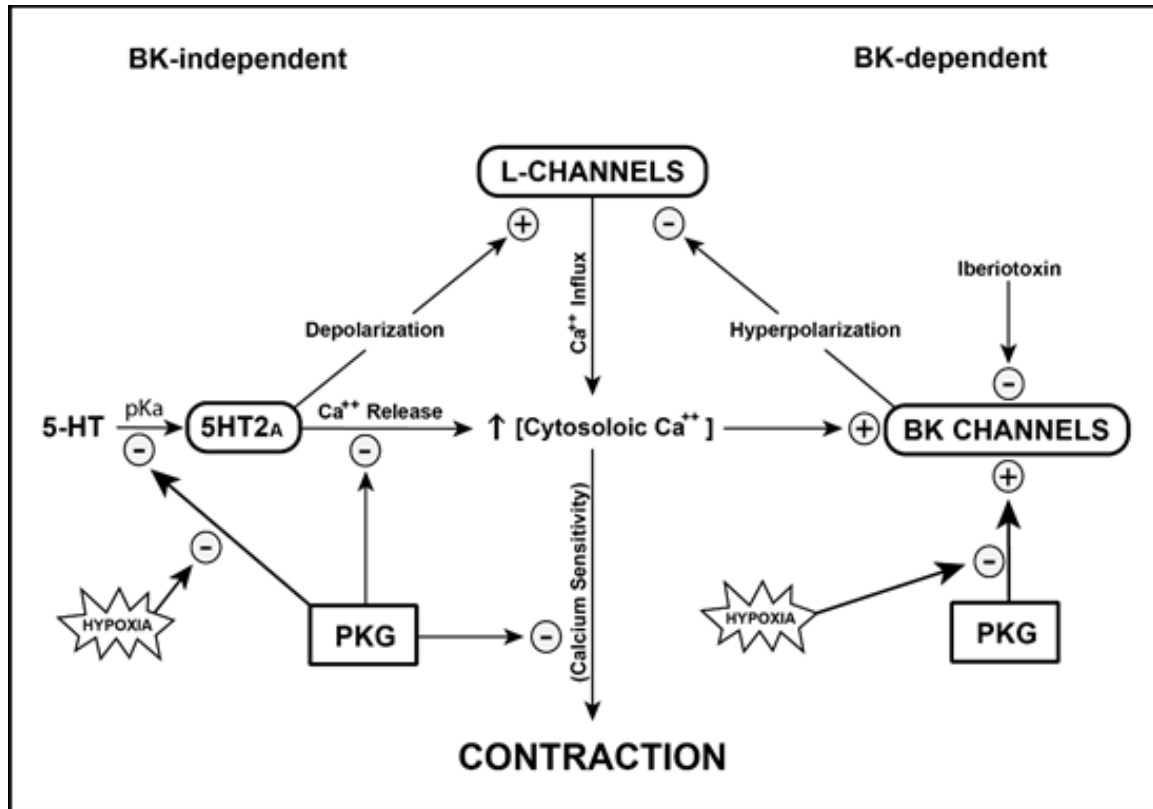
Fig. 8. Schematic representation of the interactive effects of PKG and chronic hypoxia on 5-HT induced contractions. Serotonin binds the 5-HT2A receptor to mobilize intracellular calcium and promote depolarization, which facilitates calcium influx and promotes contraction. Left side indicates large conductance calcium-sensitive potassium channel (BK)-independent effects of PKG, which include inhibition of agonist binding affinity. Right side indicates BK-dependent effects of PKG, which include direct or indirect activation of BK channels, with subsequent membrane hyperpolarization, attenuation of calcium influx, and reduced contraction. Chronic hypoxia inhibits both the effects of PKG on agonist binding affinity and the ability of PKG to activate BK channels. Hypoxia may also have other PKG-independent effects including stimulation of myofilament calcium sensitivity and inhibition of BK activation.

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### *Perspectives and Significance*

From a clinical perspective, these results predict that vascular reactivity to both endogenous and exogenous NO should be significantly depressed in both neonates and adults adapted to chronic hypoxia due to attenuation of the ability of PKG to activate BK channels. The results also suggest, however, that a BK-independent component of PKG-mediated vasorelaxation persists following hypoxic acclimatization, in which case increased activation of soluble guanylate cyclase or inhibition of phosphodiesterase might yield increased inhibition of vasoconstriction through BK-independent pathways (Figs. 7 and 8). Alternatively, hypoxia might also alter BK function, perhaps through PKC-mediated phosphorylation of the BK channel, which can block the effects of subsequent phosphorylation by PKG (51, 52). Therapeutically, such an effect of PKC might be reversed by PKC inhibitors. Another alternate approach might be to drive the BK channels more directly through caffeine-induced activation of ryanodine receptors with subsequent release of subsarcolemmal calcium and BK channel activation (44). Identification of which of these approaches might be most efficacious in clinical settings awaits future experimentation.

## Grants

## Disclosures

## Author Contributions

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# CHAPTER THREE

# CHRONIC HYPOXIA ATTENUATES THE ABILITY OF PROTEIN KINASE G TO INFLUENCE BK POTASSIUM CHANNEL ACTIVITY IN CEREBRAL ARTERIES

## Abstract

Long-term hypoxia (LTH) attenuates nitric oxide (NO) induced vasorelaxation in ovine middle cerebral arteries. Because cGMP-dependent protein kinase (PKG) is an important mediator of NO signaling in vascular smooth muscle, we tested the hypothesis that LTH diminishes the ability of PKG to phosphorylate its target proteins and cause vasorelaxation.  Prominent among proteins that regulate vascular tone is the large conductance calcium-sensitive potassium (BK) channel, which is a substrate for PKG and is responsive to phosphorylation on multiple serine/threonine residues.  Given the influence of these proteins, we also examined whether LTH attenuates PKG and BK channel protein abundance and PKG kinase activity.  Middle cerebral arteries were harvested from normoxic and hypoxic (altitude of 3,820 m for 110 days) fetal and adult sheep.  These arteries were denuded and equilibrated with 95% $O_2$-5% $CO_2$ in the presence of nitro-L-arginine methyl ester (L-NAME) to inhibit potential confounding influences of events upstream from PKG.  Expression and activity of PKG-I were not significantly affected by chronic hypoxia in either fetal or adult arteries.  Pretreatment with the BK inhibitor iberiotoxin attenuated vasorelaxation induced by 8-pCPT-cGMP in normoxic but not LTH arteries. The spatial proximities of PKG with BK channel alpha- and beta-proteins were examined by use of confocal microscopy, which revealed a strong dissociation of PKG with these proteins after LTH.  These results support the hypothesis

that hypoxia reduces the ability of PKG to attenuate vasoconstriction in part through

suppression of the ability of PKG to associate with and thereby activate BK channels in

arterial smooth muscle.

**Introduction**

LONG-TERM HYPOXIA (LTH) triggers adaptive responses in the developing

fetus that can enhance long term risk for many pathologies (35). In particular, maternal

and perinatal hypoxia induce changes in vascular structure and reactivity that manifest as

reduced vascular compliance and capacity for vasorelaxation (32). Correspondingly,

Nitric oxide (NO) and the NO pathway, which potently influence vascular tone and

vasorelaxation, appear quite vulnerable to modulation by LTH (20). Hypoxia can

compromise multiple components of this pathway including vascular endothelial function

and eNOS activity, vascular soluble guanylate cyclase (sGC) abundance and activity, and

cGMP-dependent protein kinase (PKG) activity (33). Given that PKG is a prominent

endpoint effector of the NO pathway, this investigation focused on the influence of LTH

and the ability of activated PKG to modify its target proteins and regulate vascular tone.


*Acute and Chronic Influence of PKG on Cerebral Vascular Smooth Muscle*

Given that vascular tone is largely a product of cytosolic calcium concentration

($[Ca2+]i$) and myofilament calcium sensitivity, acute regulation of tone by PKG involves

both terms of this equation (28). PKG influences $[Ca2+]i$ by inhibiting release from the

sarcoplasmic reticulum (SR); by stimulating extrusion via the plasma membrane Ca2+

ATPase transport protein (PMCA) and the Na+/ Ca2+ exchanger (15); and by stimulating

sequestration via the sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) and the SR

(9). Also, PKG-mediated phosphorylation of the large-conductance BK potassium

channel alpha protein (BKα) increases its Ca2+ sensitivity (21), (46) and thereby

indirectly inhibits Ca2+ entry by promoting hyperpolarization and attenuating voltage-

gated Ca2+ channel activity. PKG also acutely influences myofilament Ca2+ sensitivity at numerous regulatory points, including activation of myosin light-chain phosphatase (MLCP) (29), phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (23), the 20 kDa heat-shock protein-20 (HSP20) (6), and more. The BK channel is of particular interest as it is a dominant mediator of acute PKG-induced vasorelaxation (4), and is also vulnerable to the stress of LTH (38). Long-term influences of PKG involve its ability to promote and maintain the contractile vascular smooth muscle (VSM) phenotype, which is likewise vulnerable to LTH in this capacity. PKG or PKA action at ser133 on the cAMP response element-binding protein (CREB) promotes expression of VSM contractile proteins (7, 34). PKG also directly and indirectly regulates phenotype by influencing contractile gene expression via serum response factor (SRF) and myocardin (40). It has been shown that high PKG activity correlates with increased myocardin expression (45), and that myocardin promotes SRF binding to CArG promoter sequences. Also, PKG phosphorylation of ser104 on cysteine-rich protein-4 (CRP4) promotes cooperativity of GATA6 and SRF which likewise leads to increased SRF binding to CArG (43). In turn, SRF binding to CArG domains activates a repertoire of VSMC genes that characterize the contractile phenotype (43). More empirically, it has been shown that activation of PKG correlates with expression of VSM contractile proteins (24), and that hypoxia-induced attenuation of PKG leads to repression of VSMC-specific genes (44), while overexpression of PKG reverses this. This implies a critical role for PKG in establishing and maintaining the contractile VSM phenotype.

It is apparent that PKG plays a prominent role in acute and long-term establishment and maintenance of the contractile VSM phenotype via multiple pathways that are

vulnerable to the influence of LTH. Altered structure-function relations under hypoxic stress represent an adaptive homeostasis that favors near-term survival. And yet, mechanisms underlying the disruptive effects of LTH on the role of PKG in cerebral VSM remain largely unexplored. The observed loss of contractile function may represent a lower density of contractile proteins and therefore lower contractant efficacy; or a shift within the subpopulation of contractile VSMs to a synthetic phenotype; or it could represent, not exclusively, a reorganization or inactivation of the existing contractile apparatus. The main hypothesis is that hypoxia alters the ability of PKG to influence its target proteins, and the BK channel in particular, due in part to a structural re-organization of effector proteins. A corollary to this is that maturation from fetus to adult influences the response to hypoxic stress. To test these ideas, it is of interest to quantify the relative influence of the BK channel in cerebral VSM under normoxic and hypoxic conditions. To accomplish this we used the selective BK channel inhibitor iberiotoxin to distinguish BK- vs. non-BK channel effects (8, 17, 36). In order to study vasorelaxation, we used 5-hydroxytryptamine (5-HT, aka serotonin) as a contractant, against which the effects of relaxation could be contrasted. Use of 5-HT required that we validate the effect of PKG and LTH independently on the 5-HT receptor (5-HTR) pathway; to this end we examined the influence of PKG and hypoxia on 5-HTR affinity and receptor occupancy. In this study we used middle cerebral arteries from fetal and adult sheep to examine two main endpoints: a functional endpoint, the 5-HT concentration-response; and a structure-relational endpoint, which was the organization among vasorelaxant proteins (PKG, BKα, BKβ1) as measured with confocal microscopy.

**Materials and Methods**

All procedures in this study were approved by the Animal Research Committee of Loma Linda University and adhered strictly to the policies and practices set forth by the National Institutes of Health Guide governing the care and use of laboratory animals. A total of 50 sheep were used in this study of which 24 were term fetal lambs, including 13 that were from high altitude, and 11 that were from low altitude; and 26 adult sheep of which 13 were from high altitude and 13 from low altitude. From these animals we harvested a total of 272 middle cerebral artery segments, including 132 fetal segments and 140 adult segments. A total of 104 segments were used for functional studies to measure contractile force, and 168 were used for organ culture and confocal imaging. Throughout the text, "n" represents the number of animals, and not the number of segments used in each experiment. Statistical significance implies $P \leq 0.05$. All values are given as means $\pm$ SE.

*Tissue Harvest and Preparation*

All middle cerebral arteries used in this study were harvested from normoxic and chronically hypoxic term fetal (139–142 days gestation, where full term is 145) and young (18–24 mo. old) nulliparous adult sheep. Normoxic animals were obtained (Nebeker Ranch, Lancaster, CA; 720 m altitude) and brought to the LLU animal care facility (353 m altitude), where arterial oxygen tensions (PaO2) averaged $23 \pm 1$ Torr and $102 \pm 2$ Torr in fetal and adult sheep respectively. Chronically hypoxic sheep were maintained for ~110 days at high altitude (altitude 3,820 m, Barcroft Laboratory, White Mountain Research Station, Bishop, CA). For pregnant ewes, the period at altitude

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corresponded to the final 110 days of gestation. At high altitude, PaO2 values averaged

$19 \pm 1$ and $64 \pm 2$ Torr for fetal and adult sheep respectively. Sheep brought to our animal

care facility from high altitude were maintained at low PaO2 by placement of a tracheal

catheter that was ventilated with an N2-enhanced mixture of breathing gas to maintain an

arterial PaO2 of ~60 Torr. For hypoxic study animals, arterial blood was obtained and

monitored intermittently several times a day, and the breathing gas ratios were adjusted

as necessary. Pregnant ewes were anesthetized with 30 mg/kg pentobarbital, intubated,

and then placed on 1.5–2.0% halothane. The anesthetized fetus was then exteriorized

through a midline vertical laparotomy and euthanized by rapid removal of the heart and

exsanguination. Non-pregnant adult animals were euthanized by intravenous

administration of pentobarbital sodium (100 mg/kg) and phenytoin sodium (10 mg/kg).

Harvested middle cerebral arteries were placed in sodium Krebs buffer solution

containing (in mM) 122 NaCl, 25.6 NaHCO3, 5.17 KCl, 2.49 MgSO4, 1.60 CaCl2, 2.56

dextrose, 0.027 EGTA, and 0.114 ascorbic acid, bubbled with 95% O2-5% CO2. Arteries

were subsequently debrided of loose extravascular and connective tissue, and the

endothelium was substantially disrupted (herein referred to as "denuded") by passage of

an abrasive tungsten wire through the lumen followed by irrigation with Krebs buffer.

Given that residual endothelial activity could potentially confound the results, NG-nitro-

L-arginine (L-NNA) and nitro-L-arginine methyl ester (L-NAME) and was added to the

Krebs buffer to inhibit endothelial NO release and minimize soluble guanylate cyclase

(sGC) activity. Functional studies on these prepared arteries commenced without delay.

### *Measurement of Agonist Binding Affinity and Receptor Occupancy*

The 5-HT-receptor dissociation constants were determined using the Furchgott method of partial irreversible blockade (14) with the alkylating agent phenoxybenzamine (50–150 nM), as previously described (19, 39). Using this method, pairs of artery segments were pretreated with the PKG activator 8-pCPT-cGMP across a range of concentrations from 0 to 30 µM to determine the influence of PKG. The final concentration of phenoxybenzamine was chosen to achieve a 50% decrease in 5-HT efficacy with a 30-minute incubation. Pairs of adjacent phenoxybenzamine-treated and -untreated artery segments were then assayed to obtain concentration-response curves across a double reciprocal plot of 1/[A] versus 1/[A'] range of 10-10 to 10-4 M 5-HT. From these, a dissociation constant for the 5-HT receptor complex, Ka, was taken as the slope – (1/intercept). Using this, a fractional receptor occupancy [RA]/[RT] was calculated using the method described by Parker (31), where [RA]/[RT] = [A]/([A] + Ka). Here [RA] represents the concentration of the receptor-agonist complex, and [RT] is the total receptor concentration.

### *PKG Abundance and Activity*

Middle cerebral artery segments were homogenized using glass-on-glass mortars and pestles in 50 mM HEPES buffer (pH 7.4), containing 2 mM magnesium acetate, 2 mM DTT, 1 mM EDTA, 520 µM 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 7.5 µM pepstatin-A, 7 µM E 64, 20 µM bestatin, 100 µM leupeptin, and 0.4 µM aprotinin. Supernatants were collected after centrifugation at 100,000 xG for 1 h at 4 °C and analyzed for protein content using the Bradford assay, as

previously described (3). Aliquots were then divided for separate analysis of PKG abundance by Western blot and PKG kinase activity. Relative abundances of PKG-α and -β isoforms were determined by use of SDS-PAGE separation and visualized using methods previously described in detail by our laboratory (38). Relative abundances of total PKG were normalized to standards prepared from arteries harvested from adult normoxic non-pregnant ewes. PKG kinase activity was determined using aliquots of supernatants from the above homogenates using methods described previously in detail by our laboratory (38). Briefly, samples were incubated for 30 minutes, added to a homogenizing reaction buffer in 96-well plates containing [$\gamma$-$^{32}$P]ATP (NEN), a PKG substrate BPDEtide (200 μM) (BMLP112-0001, Enzo Life Sciences), and 8-pCPT-cGMP (10 μM). Timed reactions were terminated by addition of phosphoric acid, and then phosphorylated BPDEtide and unreacted [$\gamma$-$^{32}$P]ATP were separated through phosphocellulose filter paper. Washed filters were added to vials with a scintillation cocktail and assayed using a liquid scintillation counter. Counts were converted into picomoles $^{32}$P by use of a calibration curve that was produced concurrently with the samples and normalized to sample protein content to obtain PKG kinase activity, expressed as pmol $^{32}$P transferred per minute per milligram of protein. Each assay included controls using: 1) no added 8-pCPT-cGMP; 2) no added BPDEtide substrate; and 3) neither 8-pCPT-cGMP nor BPDEtide to correct for total nonspecific background.

### *BK Channel Protein Abundance*

Middle cerebral arteries (MCAs) from fetal and newborn sheep were harvested, debrided, rapid-frozen in liquid nitrogen and stored at -80 °C. Tissues were subsequently

homogenized using glass-on-glass in RIPA extraction buffer containing 10 mM DTT and a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO #M1745). Samples were centrifuged at 5000 xG for 20 minutes, then supernatants were collected and analyzed using SDS-PAGE along with reference control samples from pregnant adult sheep 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). 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 re-probed 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 M-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).

### 5-HT Concentration Response

Denuded ovine MCAs were cut into segments about 3 mm in length, mounted on

tungsten wire loops, and suspended from a force transducer in a sodium Krebs buffer solution (pH 7.4) at 38 °C (the ovine core temperature) and bubbled with 95% O2 and 5% CO2. Nitro-L-arginine methyl ester (L-NAME) (10 µM) and NG-nitro-L-arginine (L-NNA) (10 µM) were added to the Krebs buffer solution to inhibit endogenous nitric oxide (NO) production and sGC activation. To selectively inhibit activation of α1-adrenergic receptors by 5-HT, and synaptic uptake of 5-HT, 1.0 µM prazosin and 0.2 µM cocaine were added, respectively. In preliminary experiments, both endothelium-intact and endothelium-denuded segments were studied. Removal of endothelium did not affect contractile efficacy, but reduced its variability; therefore, further experiments were performed using endothelium-denuded segments. The denuded segments were equilibrated for 30 minutes and then stretched to a baseline tension of 0.75 g (fetal) or 1.5 g (adult), which corresponded with an optimal stretch ratio of ~1.8 times the unstressed diameter. Contractile force was measured directly using an isometric force transducer (Kent Scientific, Torrington, CT) and recorded directly by computer in real time using a LabView®-based instrument interface. Concentration-response curves were obtained by cumulative addition of half-log concentrations of 5-HT across a range of 10-10 to 10-4 M. In these experiments 5-HT was the contractant of choice, in part because it was previously validated in our laboratory (37), and preliminary experiments using norepinephrine showed it to be significantly less potent, less efficacious and more variable than 5-HT in producing contractions in cerebral VSM; therefore, all further experiments were performed using 5-HT. $EC_{50}$ values (molar concentration at which the contractile response was half the maximal induced contraction) were expressed as pD2 values (-log $EC_{50}$). All contractile responses were normalized to the maximum force

produced by exposure to a potassium-Krebs solution containing (in mM) 5.17 NaCl, 25.6 NaHCO3, 122 KCl, 2.49 MgSO4, 1.60 CaCl2, 2.56 dextrose, and 0.027 EGTA. To measure the relative contribution of large conductance calcium-sensitive potassium (BK, KCa1.1) channels to 5-HT contractions, concentration-response experiments were carried out in the presence or absence of the selective BK channel blocker iberiotoxin at a concentration (100 nM) that previous work has shown to be optimal. Iberiotoxin was added to the baths, in both the presence and absence of 30 µM 8-(4-chlorophenylthio)-guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP) 20 min before starting the concentration-response measurements.

### *Confocal Imaging Microscopy*

Fetal and adult hypoxic and normoxic middle cerebral arteries were harvested, debrided and sectioned into 3-mm segments. Segments were fixed in 4% paraformaldehyde (PFA) for 24 hours, then transferred to PBS until further processing. Fixed artery segments were then imbedded in paraffin and cut into 5 µm sections and mounted onto glass slides for immunohistochemistry processing and staining. BKα protein was labelled using a primary antibody (Alomone APC-021 rabbit polyclonal, or Santa Cruz cat# SC-33607, goat polyclonal) and then counterstained using a fluorescent tagged secondary antibody, donkey anti-rabbit (Thermo-Fisher Alexa Fluor® 633 cat# A-21070), or donkey anti-goat (Thermo-Fisher cat# A-11055 Alexa Fluor® 488 nm). BKβ1 protein was labelled using a primary antibody (Alomone APC-036) and counterstained using a fluorescent tagged secondary antibody, donkey anti-rabbit (Thermo-Fisher cat# A-21206 488 nm,). PKGα protein was labelled using a primary antibody specific for a

100 aa n-terminal epitope (Enzo Life sciences cat# ADI-KAP-PK005-F, rabbit polyclonal), and counterstained using a fluorescent tagged secondary antibody, donkey anti-rabbit (same as above). Stained tissues were illuminated at 488 nm with a krypton-argon laser, and at 633 nm with a helium-neon laser. The emitted light was collected using a photomultiplier tube with a band-limited spectral grating in the range 500–600 nm for Alexa Fluor 488 images (peak excitation/emission 496/519 nm) and 600-700 for Alexa Fluor 633 images (peak excitation/emission 632/647 nm). Images were acquired using an Olympus FV1000 confocal microscope and using Olympus FluoViewTM imaging software (Olympus Scientific Solutions, Waltham, Massachusetts).

### *Determination of Protein Colocalization - Quadrant Analysis*

Images that were acquired using confocal microscopy were analyzed to determine protein colocalization using Colocalizer Pro® software (CoLocalization Research Software, Switzerland). These TIFF images contained separate plates for each fluorochrome, and thus each separate protein of interest, as well as a merged image which artificially appeared as yellow and represented a spatial colocalization of the two proteins within each separate voxel (146x146x545 nm @488 nm green; 185 x 185 x693 nm @ 633 nm red). Although this colocalization did not prove a functional relationship, it did provide a solid quantitative basis for any potential cooperativity between any two given proteins. Individual red and green pixels from these images were separately quantified and plotted on a two-dimensional grid (Fig. 7a). Background noise intensities were removed using a 12% (31/255 instrument range) threshold on both axes, based on the pixel intensity distribution of control samples and apparent population boundaries. High

and low intensity thresholds were set at 50% (128/256 instrument range) for both red and green axes based on a median population distribution of a set of reference control samples. This 50% high-low threshold was maintained consistently throughout all subsequent analyses. The two-dimensional distribution and the 50% threshold boundaries thus define a four-quadrant grid (figure 7, inset). Given that voxel dimensions for the separate wavelengths are not identical, this presents a volume accessible to the longer wavelength (red) that is not accessible to the shorter wavelength (green). To eliminate this bias, we limited the analysis to only colocalized pixels above the 12% threshold. Pixels where one or both wavelength signal intensities were below the 12% threshold were not considered in the analysis. Thus the sum of all colocalized pixels above the 12% threshold represents a denominator for calculating the relative fraction of colocalized pixels in each representative quadrant for each study group (FN, FH, AN, AH). Quadrant 1 (Q1) represented the fraction of pixels with simultaneous high-red and high-green intensities. Likewise, Quadrants Q2 and Q4 represented low-green/high-red, and high-green/low-red respectively. Quadrant Q3 was low intensity on both axes. Using this approach, separate images were analyzed for each protein pair of interest (BKα with BKβ1, PKG with BKα, and PKG with BKβ1).

### *Determination of Protein Proximity Index (PPI)*

A protein proximity index (PPI) was calculated for each protein pair of interest using the method described earlier by Zinchuk et al (47) together with the "Protein Proximity Analyzer" software ("PPA", kindly provided by the Department of Anesthesiology, David Geffen School of Medicine, UCLA). Each of the two methods

employed (quadrant analysis and PPI analysis) arrived with different algorithms for eliminating noise. Using PPI analysis, ratios of auto-correlation to cross-correlation parameters were taken as a measure of true protein-protein proximity within the limits of each voxel dimension (41). Noise was eliminated by an algorithm that recognized a "slow-decay" surface on a 3-dimensional mesh plot. Calculated PPI values for each protein pair were represented wherein each protein appeared in the denominator separately, in a manner reminiscent of classical Mander's correlation coefficients, e.g. [P1+P2]/P1TOT, and [P1+P2]/P2TOT, where [P1+P2] represented colocalized protein signal within a given quadrant, and P1,2TOT represented the total protein signal for a given wavelength above the 12% threshold. In ideal conditions where statistical noise and pseudo-colocalization were perfectly eliminated, the PPI index would represent the actual ratio of colocalized protein.

### *Data Analysis and Statistics*

Significant differences in contractile variables were determined using Behrens-Fischer tests for two-group comparisons, and ANOVA followed by Duncan's post hoc simultaneous comparisons of more than 2 groups. Analyses using SPSS® software (SPSS v22, IBM Corporation, Armonk, NY) confirmed homogeneity of variance and normal data distributions in all reported experiments. Determinations of pKa values were based on double reciprocal plots from which slopes were determined by linear regression as described by Furchgott (14). Contractile forces were normalized relative to the contraction produced by exposure to isotonic 120 mM potassium-Krebs in the same artery segment. The relative influence of PKG stimulation on the BK potassium channel

compared with all other downstream targets was estimated using an algebraic model

introduced previously (38) where a = maximum response to 5-HT in control arteries

(untreated), b = total maximum response to 5-HT in the presence of PKG stimulation by

8-pCPT-cGMP, c = maximum response to 5-HT in presence of iberiotoxin, and d =

maximum response to 5-HT in the presence of iberiotoxin and with pre-treatment using

8-pCPT-cGMP. The total effect of PKG activation was defined as (a-b), and the effect of

PKG activation independent of BK channel activity was defined as (c-d). The BK

channel-dependent effect of PKG activation was calculated as (a-b) – (c-d). Maximum

efficacy of 5-HT was calculated using SPSS® and GraphPad® (GraphPad Software, Inc.

La Jolla, CA) with a non-linear regression model using the four-parameter Hill equation

$\{e + ( (f - e) / (1 + 10^{(([agonist] - g) \times h)))\}$, where "e" represents the calculated Emax,

and f, g, and h are Hill coefficients.


## Results

### *Effects of Hypoxia, Age, and 8-pCPT-cGMP on 5-HT Concentration-Response Relations*

Hypoxia had a strong effect on the maximal vasorelaxation in response to

treatment with graded concentrations of 8-pCPT-cGMP up to the maximum of 30 µM in

both fetal and adult arteries (Fig. 1, left panels). Hypoxia did not significantly affect the

response to the maximal 5-HT concentration (Emax) in untreated fetal arteries (69.7 ± 3.8

normoxic vs. 77.2 ± 2.9 hypoxic), (Fig. 1a, top panels), but had a modest effect in adult

arteries (69.9 ± 16.8 normoxic vs. 96.7 ± 17.7 hypoxic) (Fig. 1a, lower panels). By

contrast, maturation had a significant effect on Emax among hypoxic (77.2 ± 16.7 fetal

vs. $96.7 \pm 17.7$ adult), but not normoxic ($69.7 \pm 24.1$ fetal vs. $69.8.7 \pm 16.8$ adult) arteries (Fig. 1a, upper vs. lower panels). Loss of 8-pCPT-cGMP-dependent vasorelaxation potential among both hypoxic fetal and adult arteries was the main finding of this experiment. Owing to the robust response to 30 µM 8-pCPT-cGMP, this concentration was used in subsequent experiments throughout.

### *Effects of Hypoxia, Age, and 8-pCPT-cGMP on 5-HT Agonist Potency*

The potency (pD2, or -log $EC_{50}$) of 5-HT was significantly and similarly attenuated by 8-pCPT-cGMP in both fetal and adult arteries (Fig. 1b).  Hypoxia increased the pD2  values for 5-HT in untreated adult but not fetal arteries.  Correspondingly, 8-pCPT-cGMP did not significantly affect 5-HT pD2 values in hypoxic fetal arteries, but significantly decreased these values in hypoxic adult arteries (Fig. 1b).  The 5-HT pD2 values were similar in untreated normoxic fetal and adult arteries, but were significant less in untreated hypoxic fetal than in hypoxic adult arteries ($6.8 \pm 0.3$ fetal vs. $7.2 \pm 0.13$ adult).

### *Effects of Hypoxia, Age, and 8-pCPT-cGMP on 5-HT Receptor Binding*
### *Affinity*

Values for 5-HT binding affinity (pKa) as a function of 8-pCPT-cGMP concentration were significantly altered only in normoxic fetal arteries ($6.32 \pm 0.23$ at 0.0 µM 8-pCPT-cGMP vs $5.82 \pm 0.49$ at 30.0 µM 8-pCPT-cGMP) (Fig. 2a, first panel). Long term hypoxia attenuated this depressant influence of 8-pCPT-cGMP on 5-HT binding

affinity in fetal arteries. Adult arteries were unaffected by hypoxia with regard to any influence of 8-pCPT-cGMP on 5-HT binding affinity.

### *Effects of Hypoxia, Age, and 8-pCPT-cGMP on 5-HT Receptor Occupancy-Response Relations*

To correct for the observed differences in 5-HT receptor binding affinity, particularly with fetal arteries, the concentration-response relations for 5-HT were converted to occupancy-response relations by the method described by Parker (28), and using the experimentally determined pKa values (Fig. 2b). Any differences attributable solely to the effects of hypoxia on receptor affinity were fully compensated by this correction for receptor occupancy. In the converted occupancy-response curves, the effects of 8-pCPT-cGMP on contractile responses persisted in normoxic arteries, but were still attenuated in hypoxic arteries, indicating that the observed changes in the influence of 8-pCPT-cGMP on vasorelaxation involved effects independent of changes in receptor binding affinity, and that hypoxia acted downstream from the 5-HT receptor binding event. The 8-pCPT-cGMP-induced decrease in contractile responses to 5-HT as a function of receptor occupancy were significant in both fetal and adult normoxic arteries ($38.0 \pm 12.1$ and $26.05 \pm 9.7$ respectively) (Fig. 2b left panels); whereas in hypoxic arteries, induced vasorelaxation is not significantly different compared with controls ($-1.4 \pm 10.2$ and $8.9 \pm 7.0$ respectively) (Fig. 2b right panels) at maximum receptor occupancy.

*Effects of Hypoxia, Age, and 8-pCPT-cGMP on PKG Abundance and Specific*

*Activity*

PKG-I exists in two splice-variant isoforms, designated PKG-Iα and PKG-Iβ. Total PKG was determined by Western blot using an antibody that recognizes a common epitope. Relative abundances were further normalized to adult normoxic values (AN=100%) to emphasize similarities and differences in protein expression. Total PKG abundance showed only slight and non-significant variation among the four study groups (FN, FH, AN, AH), as did the PKG-Iα isoform (Fig. 3a). The PKG-Iβ isoform was significantly more abundant in the fetal compared with adult arteries, but was not significantly affected by LTH. Although the fetal PKG-Iβ isoform was of higher abundance compared with adult arteries, the influence of PKG-Iβ on total PKG abundance was minimal, implying that it was of lower over-all abundance compared with PKG-Iα and contributed less to the measure of total PKG expression.

Whole artery PKG kinase activity was unaffected by maturation (fetal vs. adult), nor by LTH. The timed kinetic assay showed no significant differences in kinase activity, expressed as nmol $^{32}$Pi-labeled BPDE/unit PKG (Fig. 3c). The kinetic rates (specific activity) were likewise nearly identical among the four study groups (AN, FH, AN, AH), expressed as nmol $^{32}$Pi-labeled BPDE/minute/unit PKG. Fetal PKG specific activity showed a slight but not significantly higher trend compared with the adults; and the normoxic fetal and adult specific activity appeared slightly but not significantly higher than their hypoxic counterparts. Importantly, any measured differences in PKG abundances as previously measured (above) were not reflected in their functional kinase

activity. Neither PKG abundance nor activity were able to account for hypoxic attenuation of PKG-induced depression of contractile responses to 5-HT.

### *Interactive Effects of 8-pCPT-cGMP And Iberiotoxin on 5-HT-Induced Contraction*

Treatment of arteries with 8-pCPT-cGMP significantly attenuated 5-HT efficacy in all study groups (Fig. 4a, Ctrl vs. 8-pCPT-cGMP). Pretreatment with iberiotoxin alone increased 5-HT efficacy in all arteries; but addition of 8-pCPT-cGMP to iberiotoxin treated arteries demonstrated a persistent but diminished vasorelaxation, indicating a significant component of non-BK channel mediated vasorelaxation (Fig. 5a, Iberiotoxin vs. Iberiotoxin + 8-pCPT-cGMP). The relative contributions of BK- vs. non-BK channel mediated vasorelaxation was quantified using our algebraic model. This showed that in hypoxic arteries the BK-independent component had a small but not significant attenuation. By contrast, the BK-dependent PKG-mediated vasorelaxation was nearly abolished in both fetal and adult arteries (Fig. 4b).

### *Effects of Hypoxia and Age on BK Protein Isoform Abundance*

The abundance of BKα and BKβ1 was measured by Western blot. Across both age groups, ANOVA revealed a significant depression of abundance for BKα that was individually significant by Student's-T in fetal ($1.20 \pm 0.10$ FN vs. $0.67 \pm 0.08$ FH, $p \leq 0.02$) but not adult ($2.46 \pm 0.51$ vs. $1.89 \pm 0.51$, ns) arteries (Figure 5b). The effects of hypoxia on BKβ1 abundance were not significant in either age group.

*Effects of Hypoxia and Age on Protein Colocalization*

Visual inspection of merged confocal images provided insight into the extent and magnitude of protein colocalization, where red or green represented individual proteins of interest, and yellow shades (artificially) represented a spatial convergence of separate red and green pixels (Fig. 6). Normoxic artery images among all four study groups, and for each of the three protein pairs contained noticeably more yellow-shaded pixels within the medial layer compared with their hypoxic counterparts. Representative scattergrams for fetal normoxic and hypoxic middle cerebral arteries demonstrated a substantial loss of protein colocalization secondary to LTH (Fig. 7a). "Colocalization" in this context was defined as a concurrency of two separate proteins within the confines of each voxel interrogated. This defined a linear separation range of 0-583 nm, i.e. the extreme corners of each individual rectangular 146x146x545 nm voxel. Each of the six protein pairs examined showed qualitatively similar scattergrams (not shown). For the protein pair BKα with BKβ (Fig. 7a), the vertical axis (λ633, red) represented BKα, and the horizontal axis (λ488, green) represented BKβ1 across the full resolution (intensities of 0-255) of the analysis software. Quadrant 1 (high BKα, high BKβ1) was nearly depleted of colocalized pixels in the fetal hypoxic panel. Quadrant 1 colocalization expressed as a fraction of the total revealed that LTH significantly affected all three protein pairs (BKα with BKβ1, PKG with BKα, and PKG with BKβ1) in both fetal and adult arteries (Fig. 7b, histogram). Maturity (fetal vs. adult) displayed a modest but not significant effect on colocalization of BKα with BKβ1, and PKG with BKβ1 in normoxic cerebral arteries. Maturity had no significant effect on colocalization of any protein pairs among hypoxic arteries.

A calculated protein proximity index (PPI) using these same images corroborated the results from the above quadrant analysis. A histogram of PPI results (Fig. 8c) likewise showed that LTH significantly depressed colocalization of BKα with BKβ1 as a ratio of total BKβ1 protein [(BKα+BKβ1)/BKβ1] in both fetal and adult arteries (first panel). LTH also depressed the [(PKG+BKα)/PKG] and [(PKG+BKβ1)/PKG] PPI indices in both fetal and adult arteries (panels 3 & 4). LTH had less influence on the PPI index of BKα colocalized with BKβ1 expressed as a ratio to total BKα [(BKα+BKβ1)/BKα] (panel 5). However, maturity had a small but significant influence on the [(BKα+BKβ1)/BKβ1] PPI index as well as the [(PKG+BKα)/BKα] and [(PKG+BKβ1)/BKβ1] indices among LTH but not normoxic arteries (panels 2, 5, 6).

**Discussion**

This study on the effects of long term hypoxia in endothelium-denuded ovine cerebral arteries offered five main observations: 1) the PKG influence on 5-HT receptor affinity was diminished with LTH, but this represented only a minor contribution; 2) activation of PKG to induce vasorelaxation was significantly less effective in both fetal and adult LTH acclimatized cerebral arteries; 3) PKG abundance and specific activity were unaffected by LTH; 4) BK channel protein abundance was modestly downregulated with LTH, BKα more than BKβ1, and fetal more than adult; 5) structural organization of key vascular proteins was strongly affected by LTH and may have accounted partially for the observed functional loss of PKG-mediated vasorelaxation.

The role of vascular endothelium and the NO pathway as a mediator of vasorelaxation has been well studied. This work has consistently shown that the role of

the endothelium as a master regulator of vascular tone is reliant on endogenous PKG, which serves as the main end effector of this pathway. Previous work in carotid arteries (38) has shown that a substantial component of the loss of PKG influence following hypoxic adaptation occurs independently of the endothelium and the NO pathway. Of greater clinical importance are the hypoxic adaptations within cerebral arteries, and cerebrovascular VSM in particular. To this end, we set about to directly examine the role of PKG, as well as those of its target proteins, to identify specific mechanisms responsive to hypoxic stress. As a first approach, completion of 5-HT concentration-response experiments yielded insight into the contractile effects of PKG activation under varying conditions of oxygen availability and developmental maturity.

### *Concentration-Response of 8-pCPT on 5-HT Potency and Efficacy*

Graded hemilog concentrations of 8-pCPT-cGMP yielded corresponding responses to 5-HT in normoxic fetal and adult arteries (Figure 1a). In contrast, the effects of 8-pCPT-cGMP were strongly attenuated in hypoxic arteries. Interestingly, increasing concentrations of 8-pCPT-cGMP also significantly diminished 5-HT potency (pD2) in all but hypoxic fetal arteries (Figure 1b). These changes in pD2 in LTH acclimatized arteries suggested possible changes in binding affinity attributable to PKG. This raised a question about the role of the 5-HT receptor in hypoxic adaptation, its possible interaction with PKG, and whether this could be of sufficient magnitude to account for the observed loss of PKG effectiveness in LTH. To answer this, we more directly examined the influence of PKG on the 5-HT receptor.

### *Influence of PKG on Events Downstream from 5-HT Receptor Binding*

The 5-HT2 receptor is a member of the G-protein coupled receptor (GPCR) super-family (10). Phosphorylation of the 5-HT2 receptor by cyclic nucleotide-dependent kinases and G-protein receptor kinases (GRKs) can promote binding of beta-arrestins that influence receptor behavior including agonist binding affinity and intracellular coupling (18, 27). Furthermore, affinity is biologically regulated, which establishes a rationale for examining effects of PKG on pKa (26). These experiments demonstrated an influence of PKG on 5-HT2 receptor ligand binding affinity, albeit of low magnitude (Fig. 2a). However, the observed efficacy of 5-HT is a product of binding affinity (pKa), membrane receptor density and coupling efficiency. To correct for variations in observed 5-HT2R affinity, we converted 5-HT concentration-response curves to receptor occupancy curves. If loss of PKG-induced vasorelaxation were attributable to alterations in receptor binding affinity, this correction would abolish the effects of 8-pCPT-cGMP on 5-HT-induced contraction. In contrast, the effects of PKG persisted (Fig. 2B), indicating that the effects of hypoxia on PKG-mediated inhibition of vasoconstriction were predominantly downstream from the 5-HT receptor binding event. This raised a more obvious question: could changes in PKG abundance or activity account for the observed loss of its influence in LTH acclimatized arteries?


### *Effects of Hypoxia on PKG Abundance and Specific Activity*

Differentiated vascular smooth muscle expresses PKG predominantly as two splice variant isoforms, PKG-Iα and PKG-Iβ, which exclusively express the first (1α) or second (1β) coding exon of the PRKG gene (30). By Western blot, the abundance of

PKG-Iα was not significantly altered by LTH nor by maturity (Figures 3 a, b). The abundance of PKG-Iβ was significantly greater in fetal compared with adult arteries (Fig. 3b), but was not significantly altered by hypoxia. Furthermore, age-related differences in PKG-Iβ abundance did not significantly influence total PKG expression, indicating that PKG-Iβ was proportionally far less abundant than PKG-Iα. Because only the PKG-Iα isoform typically is associated with regulation of cytosolic calcium and myofilament calcium sensitivity (2, 16), variations in PKG-Iβ abundance probably had little influence on the vasorelaxant effects of 8-pCPT-cGMP. In light of these results, we concluded that changes in overall PKG abundance could not account for the observed loss of PKG-mediated inhibition of contraction in LTH adapted arteries.  Thus, we next examined PKG kinase activity under conditions of normoxia and hypoxia. Given that PKG has multiple phosphorylation sites that influence its catalytic activity (13), it was conceivable that LTH could influence PKG specific activity independent of its abundance. However, measurement of PKG total and specific activity revealed no significant effects of LTH in either fetal or adult arteries (Fig. 3c).  Given that neither PKG abundance nor activity were altered by LTH, the focus of this study next shifted to identification of one or more PKG substrates that might be altered by LTH.

### *Effects of LTH on 8-pCPT-cGMP Mediated Attenuation of 5-HT-Induced Contraction*

Prominent among PKG substrates is the BK channel, which has a major role in mediating vasorelaxation (21). Pretreatment with iberiotoxin, a BK channel antagonist, can identify the influence of the BK channel apart from the collective non-BK channel

components of vasorelaxation (8, 17, 42). We have previously used this principle to develop an algebraic model that can quantify the relative importance of the BK channel in PKG-mediated vasorelaxation (38). When we used this approach with LTH-acclimatized middle cerebral arteries, two main observations emerged. First, PKG activation induced significant vasorelaxation in both normoxic and hypoxic arteries from both fetuses and adults, but the magnitude of this effect was strongly diminished in hypoxic arteries only (Figure 4a and b). Second, iberiotoxin revealed that hypoxia independently downregulated both BK- and non-BK channel-mediated components of vasorelaxation, but had considerably more influence on the BK component. Given the demonstrated importance of the BK channel in mediating vasorelaxation, and its sensitivity to hypoxia, our experimental approach next determined if LTH-mediated decreases in BK channel protein abundances might help explain hypoxic inhibition of PKG-induced attenuation of 5-HT contractions.

### *Effects of Hypoxia on BK Channel Protein Abundances*

Arteries acclimatized to LTH exhibited a consistent but modest attenuation of BKα abundance across both age groups (ANOVA $P < 0.005$) that was particularly strong in fetal arteries ($p < 0.02$), (Fig. 5b left panel). Conversely, BK-β1 abundance was not significantly altered by LTH (Fig. 5b right panel). Whereas the decrease in BKα abundance could contribute to the observed loss of PKG regulatory influence, especially in fetal hypoxic arteries, the magnitude of this decrease was not proportional to the magnitude of the loss of vasorelaxation secondary to hypoxia (Fig. 4b). This suggested that alterations in BK protein expression may influence vascular responses to PKG

activation following LTH acclimatization, but these decreases were probably not the dominant effect of LTH, particularly in adult arteries. Other possible effects of LTH on the vascular influences of PKG independent of PKG activity include changes in its trafficking, subcellular localization, as well as the proximity and affinity for possible substrates. For example, protein kinase C (PKC) has been shown to influence the ability of PKG to recognize, bind and then phosphorylate the BKα protein (46). How LTH modulates the vascular influences of PKG through changes in its trafficking and subcellular organization remain largely unstudied. To gain insight into the possible effects of LTH on cellular protein organization, we next examined the structural-spatial relations of PKG with BK channel proteins using confocal microscopy.

### *Effects of LTH on the Cellular Organization and Confocal Colocalization of Vascular Proteins*

Homogenization of vascular tissue for the purpose of measuring kinase activity disrupts protein organization, scaffolding, and protein-protein relationships that may be essential for in vivo behavior. To explore the importance of physiological conditions that might reflect vascular adaptation to LTH, we measured the structural proximity of three highly interactive proteins that govern vascular tone: PKG, BKα, and BK-β1. These three proteins rely on direct contact and interaction to effect changes in vascular tone. PKG has a substrate recognition domain that mediates substrate binding, and is distinct from its catalytic domain (13). Loss or diminution of direct contact between PKG and its target substrates predict a loss of kinase activity and function. Similarly, direct association of BK-β1 with its BKα counterpart within the plasmalemma increases Ca2+ sensitivity of

the BK channel and increases K+ conductance (25). Loss of direct association of BK-β1 with BKα predicts a diminution of Ca2+ sensitivity and thus attenuation of channel function, hyperpolarization and vasorelaxation. Although PKG does not directly phosphorylate BK-β1, PKG and Rho kinase can both phosphorylate RAB11a, which promotes rapid trafficking of BK-β1 to the plasmalemma and binding to BKα (22).

Our working hypothesis was that protein colocalization represents the potential for protein-protein interaction and therefore function. Correspondingly, any change in structural organization that results in a loss of colocalization should represent a loss of potential for protein-protein interaction and decreased function. To test this idea, we used confocal microscopy to measure protein colocalization and thereby estimate the potential for protein-protein interaction within the limits of voxel resolution (≈146x146x545 nm). Pairs of proteins tagged with 488 nm (green) or 633 nm (red) fluorochromes were visualized in separate confocal images and in merged images wherein coincident red and green pixels appeared yellow (Figure 6). Owing to the functional dependence of the labeled proteins on close proximal relationships, we interpreted loss of colocalization to imply a loss of PKG function in LTH arteries. To this end, the extent of protein colocalization was quantified non-parametrically by use of quadrant analysis (5, 11), and parametrically by determination of the protein proximity index.

### *Quadrant Analysis of Colocalization*

To obtain a non-parametric analysis of colocalization, the experimental approach employed a quadrant analysis of confocal images as previously described (1, 12). Using

this method, individual pixels were assigned to a quadrant based on their separate red (low or high) or green (low or high) signal intensities. Pixels thus grouped were weighted equally; differences in signal intensities within a quadrant were not considered. The power of this analytic technique was most useful in visualizing and quantifying shifts between quadrants and changes in population distributions under varying conditions. For each of the protein pairs examined, the effect of LTH on middle cerebral arteries showed a strong shift from Q1 (high red, high green) to Q3 (low red, low green), indicating a large reduction in colocalization of these proteins in response to hypoxic stress (Figure 7). This was observed for both fetal and adult arteries; each of the Normoxic-Hypoxic contrasts for each of the six protein pairs represented in Figure 7 were strongly significant, suggestive of an important structural reorganization in response to LTH. This finding was consistent with a reduced ability of PKG to depress contractile tone following LTH.

### *Protein Proximity Index Analysis of Colocalization*

To corroborate the above non-parametric quadrant analysis, we also performed a parametric "protein proximity index" (PPI) analysis, using the same images used for the quadrant analysis. This approach enhanced the rigor of the colocalization analysis because the two methods relied on distinctly separate algorithms and underlying assumptions. As such, the PPI method was designed to be more sensitive to the full range of signal intensities and thus constitutes a fully parametric analysis. The calculated protein proximity index values revealed a strong loss of colocalization among eight of the twelve protein ratios examined (Figure 8), and largely corroborated the quadrant analysis.

Interestingly, the four protein ratios that did not exhibit an apparent loss of colocalization all had BKα subunit values in the denominator, suggesting that hypoxic decreases in BKα subunit abundance offset the associated decreases in colocalization expressed in the numerator of the PPI ratios. Conversely, hypoxic changes in the PPI ratio were most robust when the denominator values were associated with proteins, including PKG and BK β1, that did not change in response to LTH. Overall, two quite distinct and independent analytical methods, quadrant and PPI analysis, supported a similar interpretation: LTH strongly attenuated colocalization among three highly interactive proteins that together governed the effects of PKG on vascular tone.

### *Perspectives and Significance*

In summary, this investigation revealed that acclimatization to long-term hypoxia depressed PKG-mediated attenuation of vasoconstriction in cerebral arteries through a modest heterogeneous effect on pKa, and significant effects on BKα abundance and BKα/BK-β1/PKG colocalization. Together, the results suggest that loss of PKG-mediated attenuation of vasoconstriction following acclimatization to LTH involves multiple factors, the most important of which is decreased interaction of PKG with BKα and BK-β1. In aggregate, the results predict a loss of efficacy for therapeutic strategies reliant on the NO pathway for which PKG is the main endpoint effector. These include exogenous NO, its precursors, and phosphodiesterase inhibitors, all of which are commonly prescribed. Hypoxic loss of PKG efficacy could potentially affect individuals acclimatized to chronic hypoxia, either by extended excursion to high altitude, or by predisposing medical conditions that include perinatal hypoxic stress, such as maternal

smoking, placental insufficiency, or lung disease. Treatment and management of these pathologies deserve further investigation, particularly in relation to therapeutic manipulation of the NO/cGMP/PKG pathway.

The analytical utility of confocal microscopy has evolved dramatically during the past decade, and an ever growing armamentarium of new tools has facilitated novel insights into the important relations between protein organization and cell function. In the present study, the use of confocal microscopy helped demonstrate that three key proteins that govern cerebrovascular tone disengage spatially under conditions of LTH, and that this loss of organization correlates strongly with attenuation of their known functions. These findings augment an emerging awareness in vascular biology of regulatory systems that rely on changes in molecular trafficking and dynamic subcellular organization. Although the present results strongly suggested hypoxic changes in vascular protein organization, the findings were limited by the voxel dimensions of the analyzed images, which were an order of magnitude larger than the size of the proteins being studied. Clearly, changes in colocalization alone do not definitively prove changes in direct protein interaction. Such proof will require higher resolution measurements of protein-protein proximities by use of FRET, proximity ligation assays (PLA), or by super-resolution microscopy. Aside from this limitation, this present investigation introduces a new paradigm for hypoxic adaptation: namely that apart from changes in abundance and phospho-status, changes in subcellular protein location, trafficking and temporospatial relationships represent a dominant mechanism whereby chronic hypoxia regulates vascular reactivity, remodeling and structure-function relations.
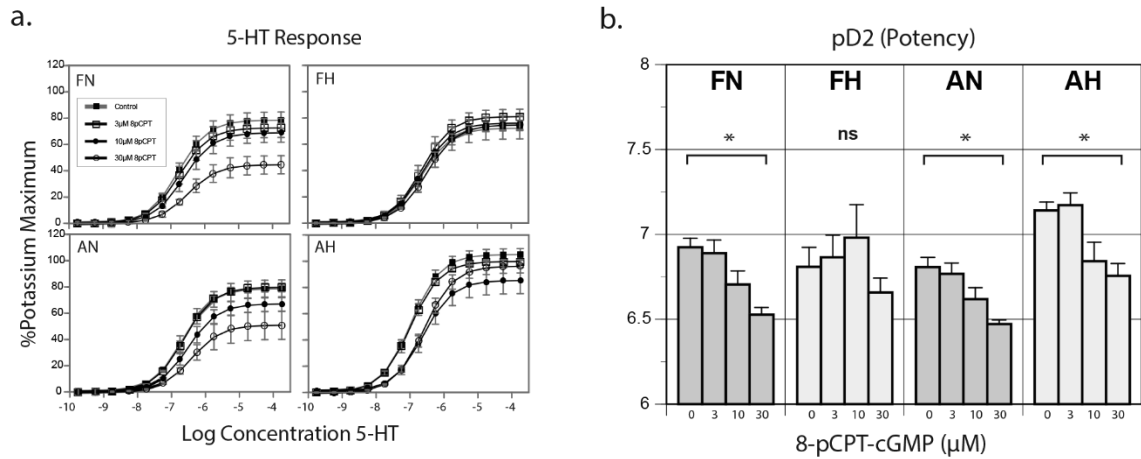
*Figure 1. Effects of 8-pCPT-cGMP and hypoxia on 5-HT potency and concentration-response relations:* **Figure 1a:** Pretreatment with graduated concentrations of 8-pCPT-cGMP determined the optimal concentration for investigating attenuation of vascular tone in ovine middle cerebral arteries. This approach demonstrated that hypoxia attenuated 5-HT-induced contraction in both fetal and adult arteries at the optimal concentration of 30 μM. **Figure 1b**: Adult hypoxic arteries exhibited modestly greater contractile responses and 5-HT potency compared with normoxic arteries. Increasing 8-pCPT-cGMP concentrations significantly attenuated 5-HT potency in all but the fetal hypoxic arteries.
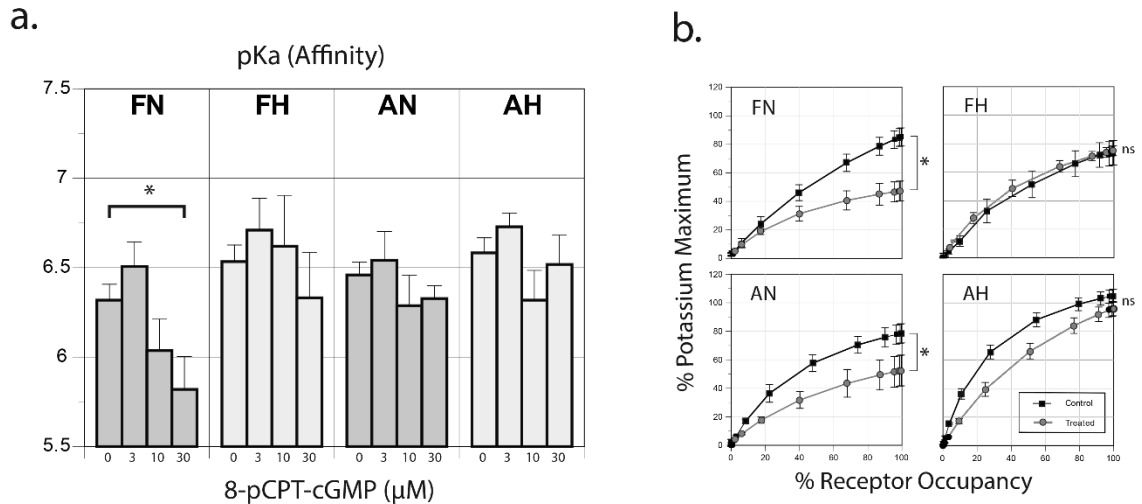
**Figure 2.** *Effects of 8-pCPT-cGMP and hypoxia on occupancy-response relations for 5-HT:* 5-HT receptor affinity was determined for each experimental group using the Furchgott method (pKa, **Figure 2a).** The 5-HT concentration-response relations were converted to occupancy-response relations using pKa values to correct for differences in agonist binding affinity. The resulting occupancy-response relations revealed that, compared with untreated control arteries, pretreatment with 30 µM 8-pCPT-cGMP significantly reduced maximum efficacy for 5-HT in normoxic (**Figure 2b, left panels**) but not hypoxic (**Figure 2b, right panels**) arteries from both age groups. *Significant differences ($P < 0.05$, repeated measures anova) between untreated and treated (30 µM 8-pCPT-cGMP) arteries. Error bars indicate means $\pm$ SE for $n \geq 5$ for all groups.
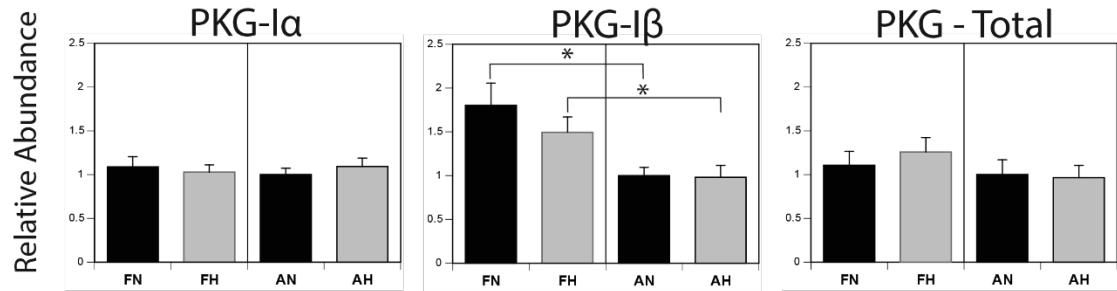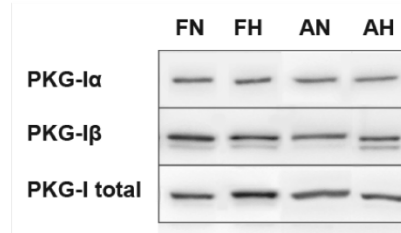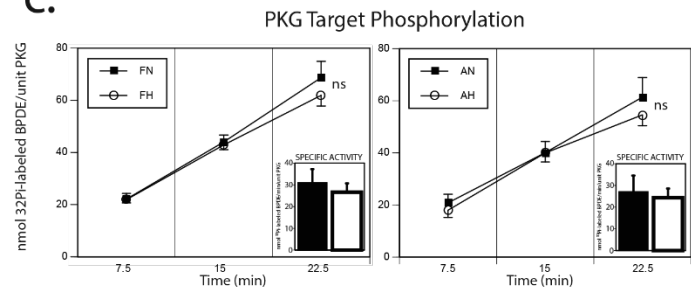
***Figure 3a. PKG isoforms:*** The abundance of total PKG was determined by Western blot analysis using an antibody against a PKG-I epitope common to both the α and β isoforms. All abundances were calculated relative to known amounts of a standard pool prepared from normoxic adult arteries. ***Figure 3b.*** *Relative abundance of PKG-Beta was significantly greater in fetal than adult arteries but was not affected by hypoxia. ***Figure 3c. PKG activity:*** Whole artery PKG activity in middle cerebral arteries (line graphs, pmol-Pi/unit PKG) was not significantly affected by hypoxia in either fetal (left panel) or adult (right panel) homogenates. When whole artery PKG activity was normalized relative to PKG abundance (panel insets), estimates of specific activity (pmol Pi/min/unit PKG) did not vary significantly with hypoxia or between fetal and adult artery homogenates. These data indicate that the ability of hypoxia to ablate the effects of PKG on 5-HT contractions was not due to changes in PKG abundance or kinase activity. Error bars indicate means ± SE for n ≥ 6.
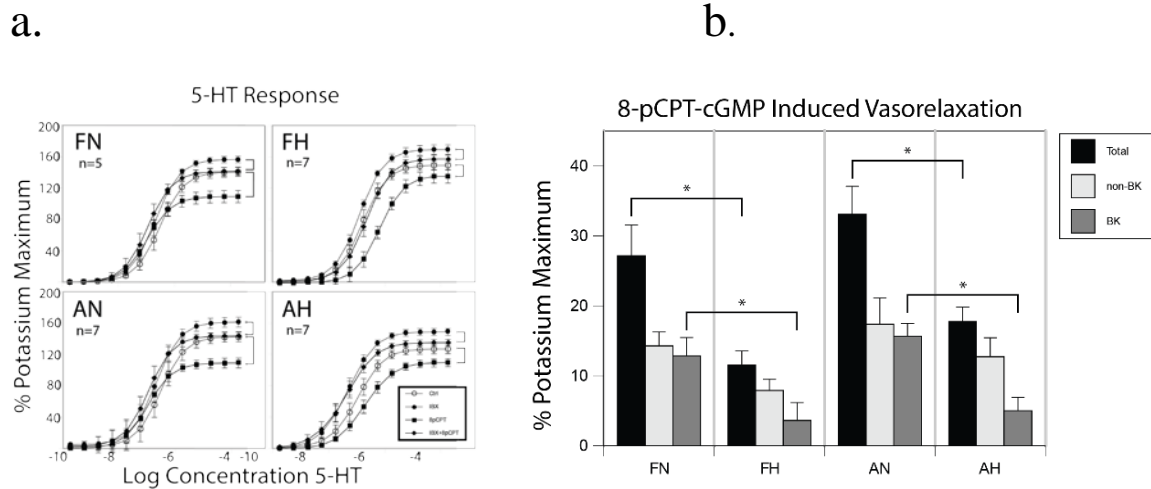
***Figure 4a.*** Effects of iberiotoxin, 8-pCPT-cGMP, age, and hypoxia on 5-HT concentration-response relations. Maximum contractile responses to graded concentrations of 5-HT varied moderately with age and hypoxia in untreated arteries. Maximum contractile responses in iberiotoxin-treated arteries were significantly greater in adult *normoxic* arteries compared with adult *hypoxic* arteries. Pretreatment with 30 µM 8-pCPT-cGMP *significantly attenuated 5-HT efficacy in a concentration-dependent manner in normoxic fetal and adult arteries. In arteries pretreated with 30 μM 8-pCPT-cGMP, 5-HT efficacy was greater in hypoxic compared with normoxic arteries for fetal but not adult arteries. ***Figure 4b.*** Vasorelaxation attributable to the BK channel vs. non-BK channel was determined using an algebraic model. Non-BK channel influence was of greater magnitude in all subgroups (FN, FH, AN, AH); whereas BK channel effects were more sensitive to LTH, particularly in fetal arteries. Error bars indicate mean ± SE for n ≥ 6 for all groups. *Significance ($P < 0.05$)
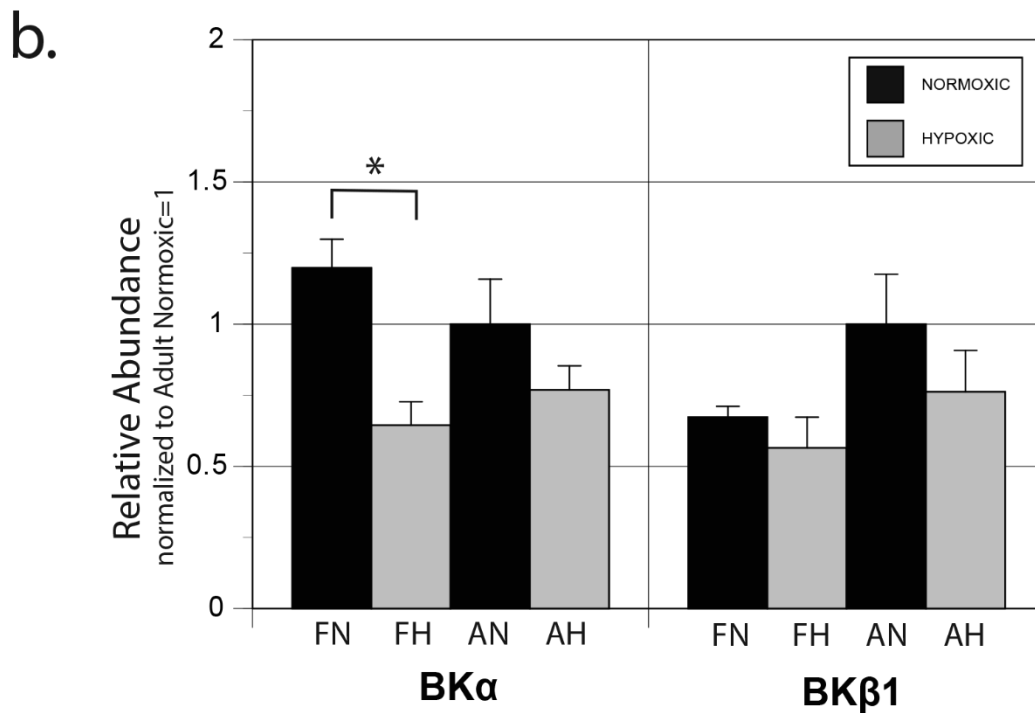
91
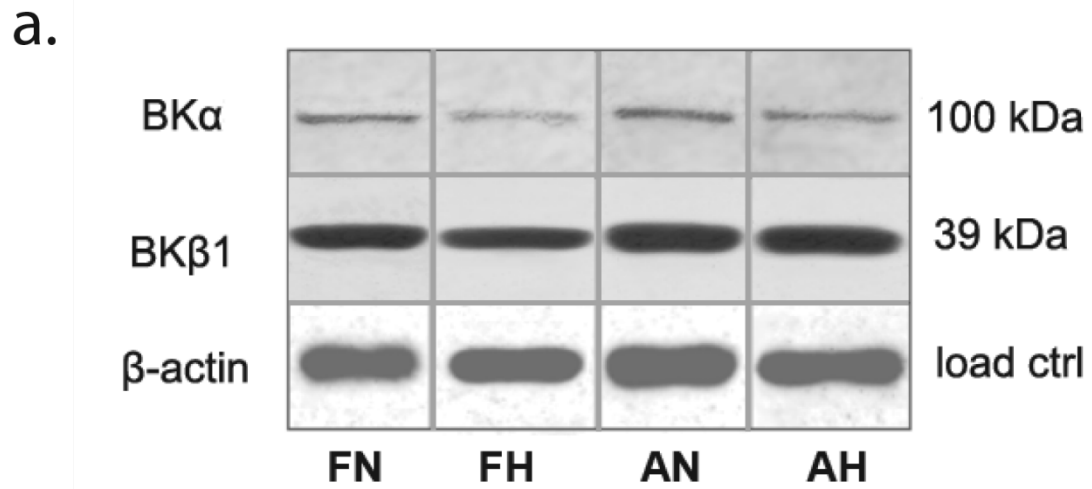
**Figure 5a.** The abundance of total BK-alpha and beta1 isoforms was determined by Western blot analysis using an antibody against a specific epitope in each of the α and β isoforms. **Figure 5b**. All abundances were calculated relative to known amounts of a standard pool prepared from normoxic adult arteries. *Relative abundance of BK-alpha was significantly attenuated 46% by hypoxia in fetal cerebral arteries. Abundance of BK-beta1 was not significantly influenced by chronic hypoxia. ANOVA analysis showed a significant influence of hypoxia on fetal BK-alpha abundance, but no significant interactions of maturity with hypoxia for either BK-alpha or -beta1 protein abundances. Error bars indicate means $\pm$ SE for n $\geq$ 6.

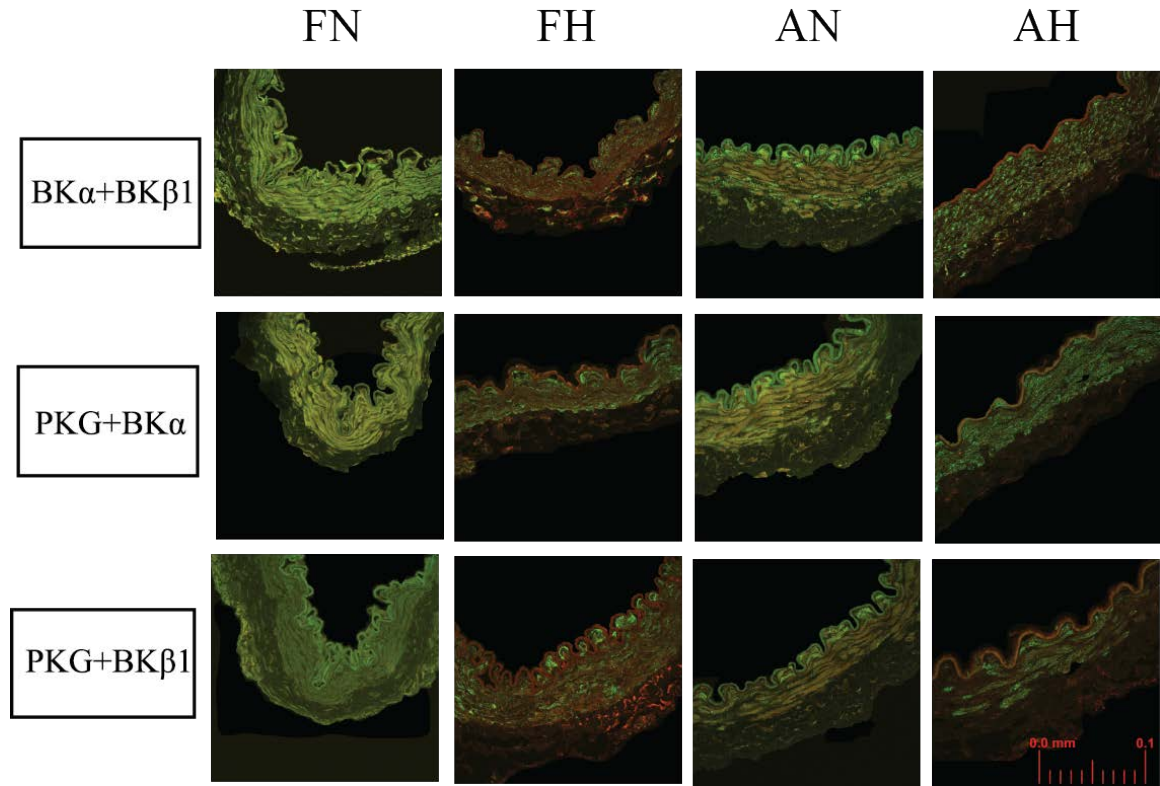| | FN | FH | AN | AH |
| --- | --- | --- | --- | --- |
| BKα+BKβ1 | | | | |
| PKG+BKα | | | | |
| PKG+BKβ1 | | | | |

**Figure 6.** The **first row** in the above graphic represents the distribution of BKα protein (red) and BKβ1 protein (green). The merged images appear as shades of yellow where the two proteins are colocalized within the resolution of the acquired images. Voxel dimensions were ≈146x146x545 nm @488 nm (red); ≈185x185x693 nm @ 633 nm (green). Hypoxic artery segments in both fetal and adult arteries appeared visibly less yellow, and thus represented less colocalization in that protein pair. The **second row** represents PKG (red) and BKα (green). These merged images likewise appeared visually to possess less colocalized protein for both fetal and adult hypoxic arteries. The **third row** represents PKG (red) with BKβ1 (green) protein. Images represent a field of approximately 180x180 µm (scale inset, lower right panel). Histological changes in the hypoxic arteries were also apparent; the medial layer was thinner and less invested in fusiform cell morphology, suggesting a probable shift in smooth muscle phenotype.
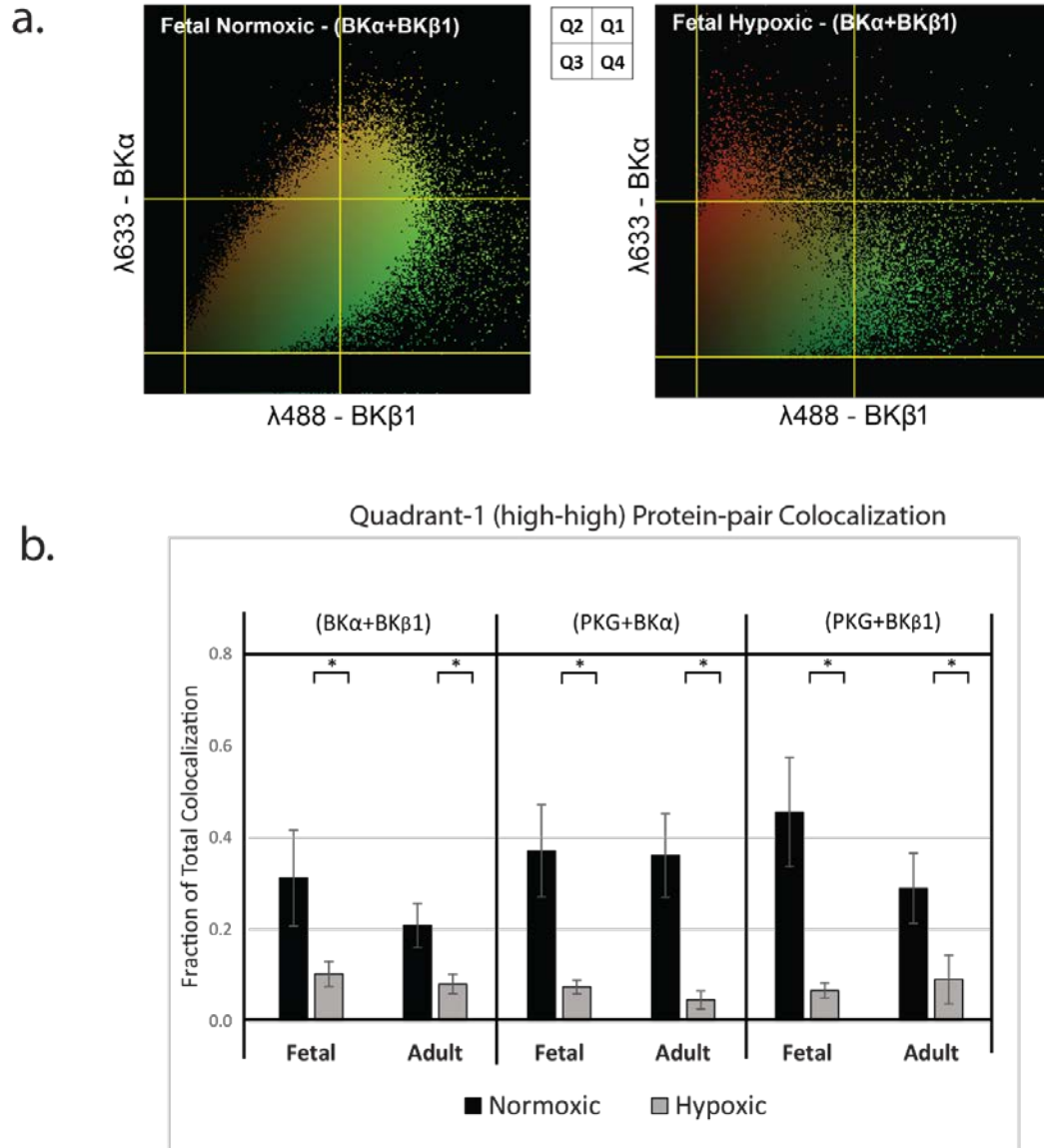
**Figure 7a.** Representative scattergrams for fetal normoxic and hypoxic middle cerebral arteries illustrate a substantial loss of protein colocalization secondary to chronic hypoxia. Likewise, each of the six protein pairs exhibited qualitatively similar scattergrams (not shown). For protein pairs shown (BKα with BKβ1, fetal normoxic and hypoxic), the vertical axis (λ633, red) represents BKα, and the horizontal axis (λ488, green) represents BKβ1 across the full resolution (intensities 0-255) of the analysis software. **Figure 7b.** Quadrant 1 (upper right in each scattergram) colocalization expressed as a fraction of the total shows that chronic hypoxia had a significant effect on all three protein pairs (BKα with BKβ1, PKG with BKα, and PKG with BKβ1) in both fetal and adult arteries. Maturity (fetal vs. adult) displayed a modest but not significant trend in colocalization of BKα with BKβ, and PKG with BKβ1 protein pairs in normoxic cerebral arteries (black bars). Maturity had no significant effect on colocalization of these protein pairs among hypoxic arteries (gray bars). *Significance ($P < 0.05$).
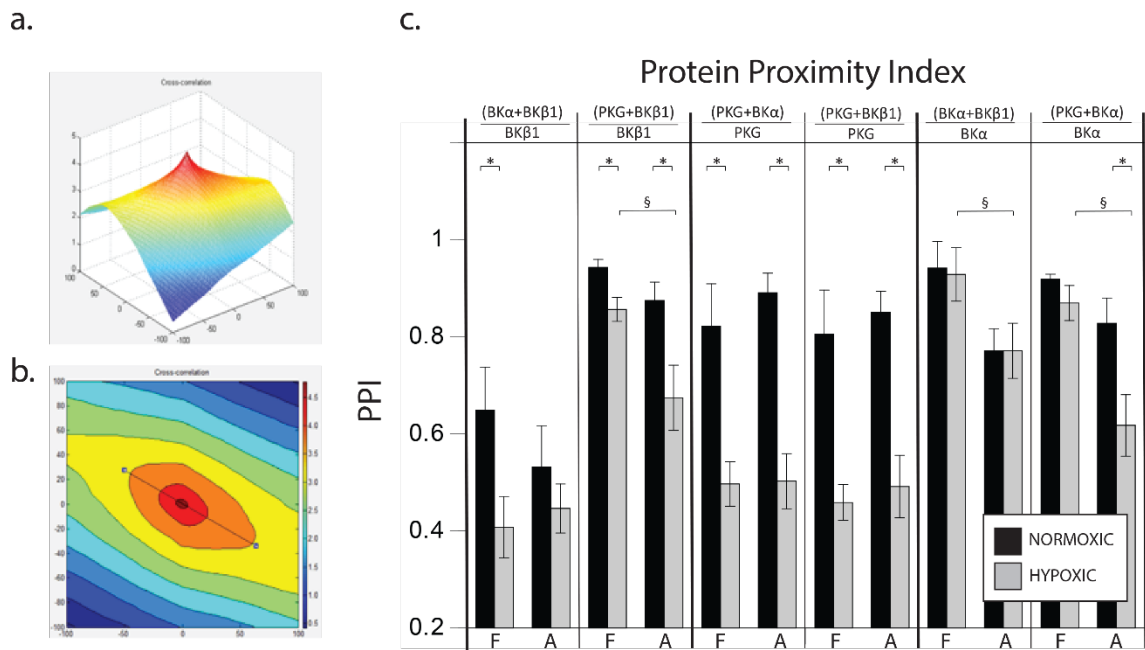
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**Figure 8a**: A three-dimensional mesh plot depicts the cross-correlation surface from an adult normoxic artery image stained for BKα and BKβ1. The horizontal scales represent a measure of Gaussian shift ±100 units from the peak intensity; the vertical scale represents signal intensity. The slow-decay portion of this surface (blues and yellows) represents a pseudo-correlation subset and is filtered from further analysis. The rapid-decay surface (oranges and reds) represents presumed protein-protein interactions (38). This cross-correlation surface, plus the auto-correlation surface for each individual protein of interest (not shown) provides a basis for computing the protein proximity index (PPI). **Figure 8b, Lower left image**: A contour plot from the same data as above, showing the "slice" (black straight line) that defines a cross-section for purposes of equation-fitting of the grid data. The cross-section always bisects the peak of each mesh plot and includes the rapid-decay surface. **Figure 8c, Upper right image**: This PPI histogram shows that long term hypoxia (LTH) significantly (*) depressed colocalization of BKα with BKβ1 as a ratio of total BKβ1 protein [(BKα+BKβ1)/BKβ1] in both fetal (F) and adult (A) arteries (first panel). LTH also depressed the [(PKG+ BKα)/PKG] and [(PKG+ BKβ1)/PKG] PPI indices in both fetal and adult arteries (panels 3 & 4). LTH had less influence on the PPI index of BKα colocalized with BKβ1 expressed as a ratio to total BKα [(BKα+BKβ1)/BKα] (panel 5). Maturity showed a small but significant (§) influence on the [(BKα+BKβ1)/BKβ1] PPI index as well as the [(PKG+BKα)/BKα] and [(PKG+BKβ1)/BKβ1] indexes among hypoxic but not normoxic arteries (panels 2, 5, 6).

## Acknowledgments

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# CHAPTER FOUR

## DISCUSSION

### Future Directions

#### *Investigation of Spontaneous Iberiotoxin-induced Contractions*

It is the nature of the scientific process to formulate hypotheses, and then to design experiments to validate or refute a particular hypothesis. Sometimes in the course of this process there is an unexpected finding that represents alternatively an experimental failure, or a surprising new discovery. Such is the case with early results in this investigation. Based on earlier work with carotid arteries, it was reasonably expected that functional studies in middle cerebral arteries would behave similarly, both with regard to response to the vasocontractant (5-HT) and vasorelaxant (8pCPT-cGMP), as well as changes secondary to chronic hypoxia, albeit of potentially different amplitudes and reactivity given the differences in size and anatomical proximity to the cerebral tissues they service. The following represents heretofore unpublished incidental findings obtained during the course of the main study that deserve further investigation.

In the late summer of 2013 as hypoxic tissues became available from the high-altitude sheep herd, functional contractility studies commenced with careful attention to experimental details in order to minimize variability and to produce accurate results. In order to measure the influence of PKG on the BK channel under conditions of chronic hypoxia, a subset of arteries was pre-treated with iberiotoxin (100nM) to inhibit BK potassium currents, as described in the original carotid article (5). It was expected that contractile forces would *increase* with the iberiotoxin-induced loss of hyperpolarization, when measured as a difference in force with reference to a resting baseline force and

normalized to a percentage of maximum potassium contractile force. Unexpectedly, the opposite occurred. Force differences in iberiotoxin-treated arteries compared with baseline values showed a *decrease* in contractile force, suggesting that iberiotoxin was a powerful vasorelaxant. This could not be true; there was no theoretical framework that could account for such an experimental result. The planned series of experiments was suspended; hypoxic tissues were subsequently invested in organ culture for imaging studies, and a limited budget of hypoxic cerebral arteries were invested in learning what went wrong with the above functional contractility results. The real cost of this was having to wait another full year for the next "hypoxic season" to repeat the set of experiments.

An investigation ensued to learn what went wrong with the iberiotoxin-treated artery functional studies. The protocol at the time was to turn the data recorder on and off at precise intervals when specific reagents were added to the artery bath, or during concentration-response intervals. As a first step, the protocol was revised to leave the recorder on for the full duration of a given experiment, and a variety of experimental conditions were employed to learn what might account for the previous unexpected findings. To our surprise, a subset of hypoxic cerebral arteries exhibited a strong spontaneous contraction, which consistently returned to baseline after several minutes (Figure 1). The start point of these contractions was quite variable, and not all arteries exhibited this phenomenon. The failure or inability to anticipate this "anomaly" previously resulted in "miscalculating" a contractile force as a difference from an observed "baseline" that was not actually representative.

This represents a newly observed phenomenon not previously reported in the literature, and deserves further investigation. Subsequent functional studies by this lab in the "hypoxic season" of late summer 2014 used a modified protocol that fully accounts for unwanted spontaneous contractions; the data is now believed to be correct and accurate (Chapter Three of this dissertation).

Several observations regarding this phenomenon are worth noting: 1) We have observed this only in hypoxic, but not normoxic cerebral arteries, 2) this was not observed in carotid arteries, 3) not all iberiotoxin-treated arteries display this behavior, 4) the onset of a spontaneous contraction is highly variable as measured from the moment of iberiotoxin treatment, 5) this has occurred in both fetal and adult arteries, and 6) each spontaneous contraction returns consistently to, or very near, the original baseline value. It remains to be investigated as to the physiology of the cause and the spontaneous reversal of these contractions. It might be speculated that positive feedback from calcium-dependent calcium release from the SR results in a runaway calcium wave unchecked by BK influence, which might then lead to a depolarization that opens voltage-gated calcium channels. Given that fully iberiotoxin-ligated BK channels remain ~50% conductive (2), these might likely be involved with ultimate repolarization/hyperpolarization of the vascular smooth muscle and return to normal vascular tone. Understanding the physiology of this phenomenon would be a candidate for further investigation.

Given that the above experimental conditions involve potentially lethal doses of iberiotoxin, this may seem clinically irrelevant. But to the contrary, iberiotoxin-ligated BK channels may represent a useful model of BK-deficient VSMC that, by previous

exposure to chronic hypoxia or other pathologies, experience spontaneous strong

contractions, as in the cerebral vasospasm that is known to accompany the migraine

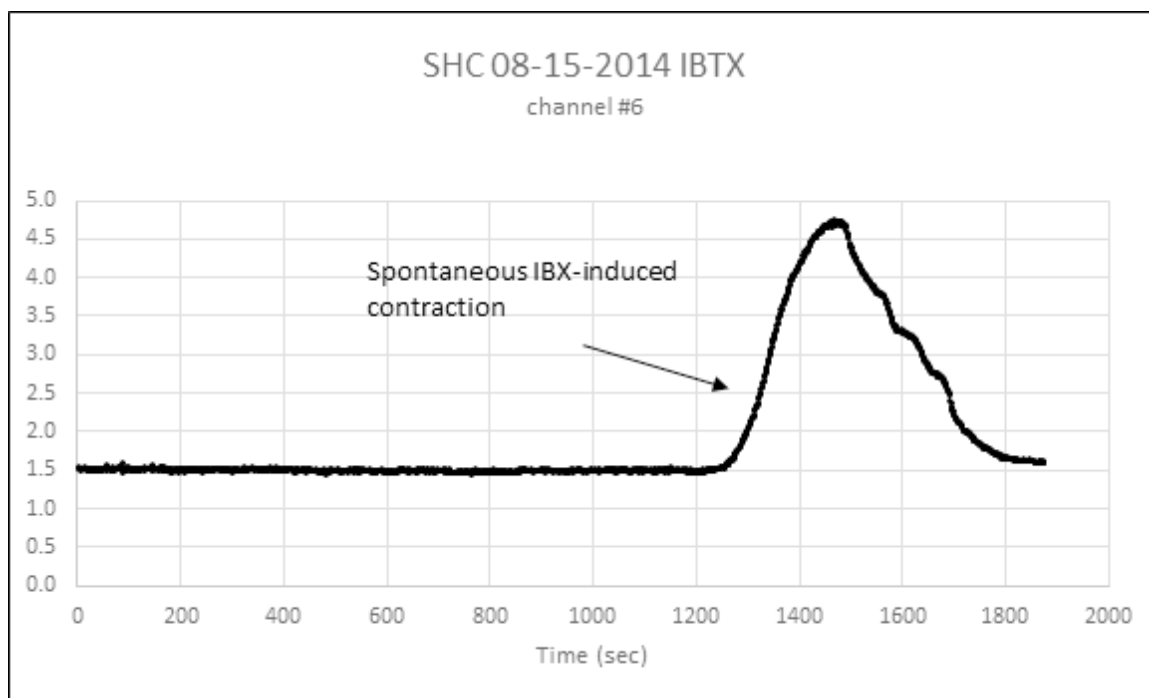phenomenon, and other clinical entities.
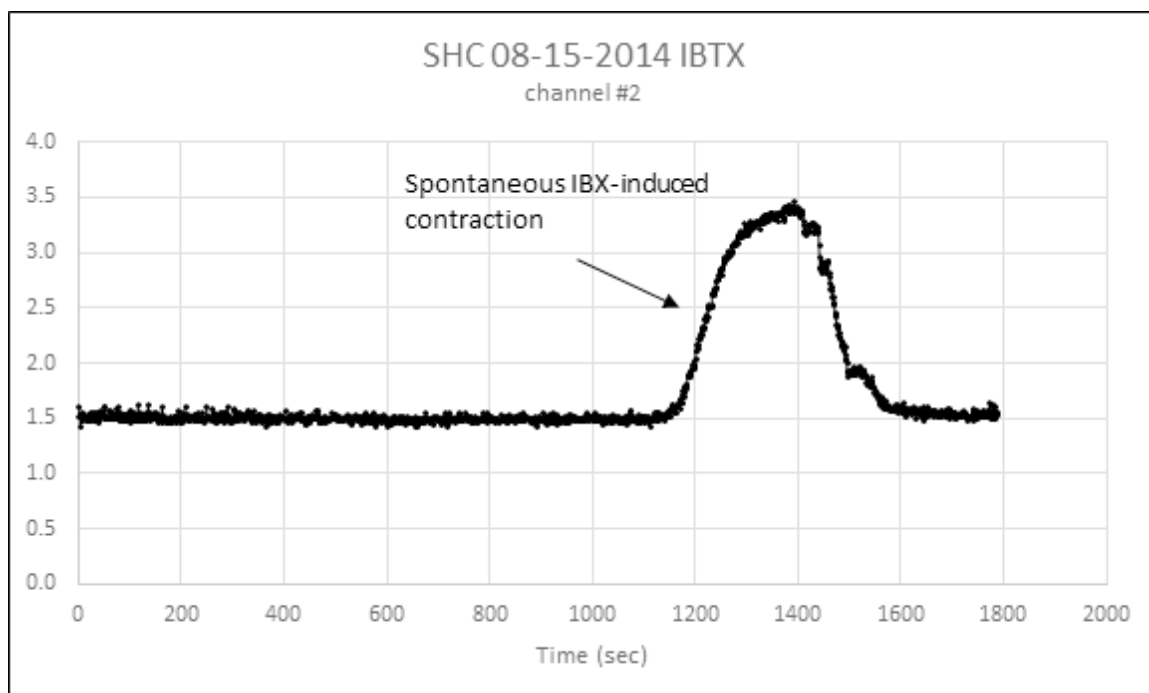
**Figure 1. Iberiotoxin-induced spontaneous contractions**.  Hypoxic adult ovine middle cerebral arteries exhibit spontaneous contractions in the absence of 5-HT.   The horizontal axis represents time (in seconds) from initial application of iberiotoxin. Start times post iberiotoxin treatment is widely variable, but the duration is typically on the order of about 400 seconds, as in the above examples. Contractile force typically returns completely to baseline tension.

### *Examination of BK Channel Protein Phospho Status*

The BK alpha protein has over 200 serine/threonine residues that are potential

targets for AGC kinases (6). Several of these have been studied in great detail with

regards to regulation of channel activity as well as a seeming interaction of several AGC

kinases competing for closely associated serine residues (7). An interesting

"phosphorylation logic" plays out on the BK-alpha protein, whereby action by PKA on

Ser922, or action by PKG on Ser1134 increases calcium sensitivity. These two events are

mutually exclusive. It has been shown that action by PKC on the tandem Ser1151 and

Ser1154 will enable PKG to phosphorylate Ser1134 and disable PKA action on Ser922.

Conversely, inhibition of PKC switches the influence of BK Channel activation from

PKG to PKA (7). Briefly, the effect of prior action by PKC tips the balance of influence

in favor of PKG at the expense of PKA. This has not been investigated in the context of

chronic hypoxia. It would be of interest to examine the phospho status of the BK alpha

protein Ser922, Ser1134, Ser1151 and Ser1154 in hypoxic and normoxic tissues. This

could be accomplished using specific antibodies, or possibly mass spectrometry.

Another line of investigation of this same issue would be to specifically

manipulate the phosphorylation state of the BK-alpha protein by activation of

phosphatases to dephosphorylate the above serine residues, then to selectively activate

PKC followed by PKG activation, and measure the effect of this on colocalization of

PKG/BK-alpha/BK-beta proteins. A separate functional outcome would be the effect of

this on the ability of PKG to induce vasorelaxation on these manipulated arteries. The

experiment would then be repeated in the reverse order: activation of PKG first, followed

by PKC activation, then compare results.  This could potentially replicate *in vitro* the influence of chronic hypoxia, which would be of high theoretical interest.

### *High-Resolution Studies of Contractile Protein Colocalization*

A limitation of the use of confocal microscopy in this investigation was the voxel dimension, as mentioned previously. This is an inherent limitation based on the resolving power of a given wavelength of light. This localizes protein within a linear separation range of 0-583nm. A logical next step would be to repeat the colocalization experiments using a proximity ligation assay (PLA) which has a resolution on the order of 40 nm, sufficient to infer direct protein-protein contact and a probable functional interaction. This could be accomplished using the same tissue blocks that were used for the above confocal microscopy analysis.  Super-resolution microscopy offers a comparable increase in resolving power, although the exact resolution depends on the wavelength and other experimental conditions.

### *Investigation of BK Channel Alternate Splicing in Chronic Hypoxia*

Multiple splice sites exist on the BK-alpha (KCNMA1) gene, which yields no less than twelve, and possibly upwards of twenty or more splice variants (3). The role of hypoxia in controlling splice variants and regulating calcium sensitivity remains to be explored, although it is understood to be tissue-specific and responsive to environmental stress. The refractory nature of the BK channel with regard to PKG-induced vasorelaxation in conditions of chronic hypoxia could well have an underlying splice-variant mechanism; the existence or absence of a PKG serine residue target would

obviate any attempt to activate PKG and expect an observable result. Mass spectrometry determination of BK-alpha protein sequence would be useful to address this issue.

### *Investigation of BK Channel Protein Trafficking by RAB11a*

The BK-beta1 proteins are typically more abundant than the BK-alpha counterparts within vascular smooth muscle. The two proteins are independently regulated. However, the excess abundance of BK-beta1 does not imply that the association of BK-beta1 with BK-alpha is saturated. Rather it is highly regulated. BK-beta1 is rapidly trafficked by RAB11a when it (RAB11a) is activated by PKG (Leo, 2014). This raises an interesting question of whether PKG is ineffective in activating RAB11a under conditions of chronic hypoxia. Failure of RAB11a to efficiently transport intracellular BK-beta1 to the plasmalemma could potentially explain the observed alterations in protein organization that were reported in the above colocalization studies.

And experimental approach might first examine the phospho status of RAB11a in response to PKG activation, and in conditions of hypoxia and normoxia. Additionally, colocalization of BK-beta1 with RAB11a, or PKG with RAB11a could be examined under the same conditions. Of additional interest would be maturation (adult vs. fetal) would influence this dynamic.

### *Potential Therapeutic Rescue of Hypoxia-Damaged Vascular Tissue*

The influence of PKG in promoting and maintaining the contractile phenotype is well-established, and is in part mediated by direct and indirect action on the CREB pathway (4). Loss of contractile phenotype within VSM is accompanied by a loss of

vasorelaxation potential and PKG efficacy. Therapeutic strategies that rely on the NO pathway may likewise fail when the main effector of the pathway, PKG, is itself ineffective, as is the case with hypoxia-damaged arteries. Yet therapies, or even experimental techniques to reverse the loss of PKG efficacy secondary to LTH don't yet exist. It is tempting to speculate that CREB activators might hold the potential to restore the expression of contractile proteins, and thereby the contractile phenotype and PKG efficacy. If that were possible, the protocols used in this investigation would serve as a convenient assay to screen and evaluate candidate therapies.  In particular, the functional contractility methods, and the protein colocalization assay would represent a cost-effective screening tool. Recent reports describe the use of the experimental drug J147, a CREB activator, that shows promise in restoring structure and function among neuronal and related tissues (1). Given the pathways involved, there is at least a theoretical potential for this agent, or another CREB activator, to influence the VSM phenotype, although the effects of this on VSM has not been described.

## Conclusions

It is the nature of scientific investigation that by answering a specific question, more questions appear, seemingly exponentially. This is no exception. This study has confirmed earlier observations regarding the influence of hypoxia on PKG efficacy, and has offered measures of protein abundance and kinase activity that suggest other explanations are needed. Of interest, we have shown that protein organization is profoundly influenced by chronic hypoxia, and that this correlates strongly with the functional endpoints that were measured. Questions that remain unanswered are the

factors that contribute to the observed changes in protein organization. These include

questions about the channel protein phospho-status, trafficking of the BK channel

proteins, alternate splicing, and specific modes of interaction of these with chronic

hypoxia.  That suggests a much larger investigation. By addressing these issues, this

carries the potential for devising new therapeutic strategies that will ultimately reduce the

health risks associated with chronic hypoxia and improve the quality of human life.

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