CHRONIC HYPOXIA MODULATES ENDOTHELIUM-DEPENDENT VASORELAXATION THROUGH MULTIPLE INDEPENDENT MECHANISMS IN OVINE CRANIAL ARTERIES

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1. INTRODUCTION

Of the many external physiological perturbations that may alter blood O_2 transport, chronic hypoxia is probably the most widely studied. Chronic hypoxia is a challenge not only for mountain climbers and pilots, but also for patients with respiratory disease and fetuses compromised by placental insufficiency. Motivated by this clinical relevance, numerous studies have detailed the effects of hypoxic adaptation. Because physiological stresses that influence O_2 delivery stimulate homeostatic responses not only in blood composition, but also in the blood vessels that serve at the interface between oxygen supply and demand, many studies have examined hypoxic modulation of vascular composition and function (Fung, 2003; Fradette and Du Souich, 2004; Moore et al., 2004; Peers and Kemp, 2004; Raguso et al., 2004; Reeves and Leon-Velarde, 2004). For example, high altitude acclimatization has been shown to alter vascular protein content, contractility, receptor profile, and perivascular nerve function (Longo et al., 1993; Ueno et al., 1997; Buchholz et al., 1999). Aside from the intense attention focused on the effects of hypoxia on vascular contractility, however, comparatively little scrutiny has been directed toward the effects of chronic hypoxia on mechanisms of vasorelaxation. This is somewhat surprising, given that vasodilatation is a key initial response to acute hypoxia in most vascular beds (Pearce, 1995; Marshall, 2000). In particular, the effects of chronic hypoxia on endothelial vasodilator function remain largely unexplored, even though the endothelium may significantly augment vasodilator responses to acute hypoxia (Pearce et al., 1989). The present study was designed to address this deficit.

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2. MECHANISMS OF ENDOTHELIUM-DEPENDENT VASODILATATION

To examine the effects of chronic hypoxia on endothelial vasodilator function, we used common carotid arteries taken from young non-pregnant adult sheep that had been maintained for 110 days at an altitude of 3820 m. The arteries were mounted in tissue baths for measurements of contractile responses, as previously described in detail (Pearce et al., 1989; Longo et al., 1993). To enable the study of relaxation responses, the arteries were first contracted with 1 μM serotonin, which is approximately the EC_{50} concentration in this preparation (Teng et al., 1998). To stimulate endothelium-mediated vasorelaxation, we treated the arteries with 1 μM A23187. This calcium ionophore facilitates calcium entry into endothelial cells in a receptor-independent manner, and is thus highly useful for maximally stimulating endothelial NO release (Taniguchi et al., 1999). To verify that all A23187-induced relaxation was due to activation of the enzyme eNOS (endothelial nitric oxide synthase), we also verified that treatment with 100 μM L-Nitro-Arginine Methyl Ester (L-NAME) could completely block all responses to A23187 (see **Figure 1**).

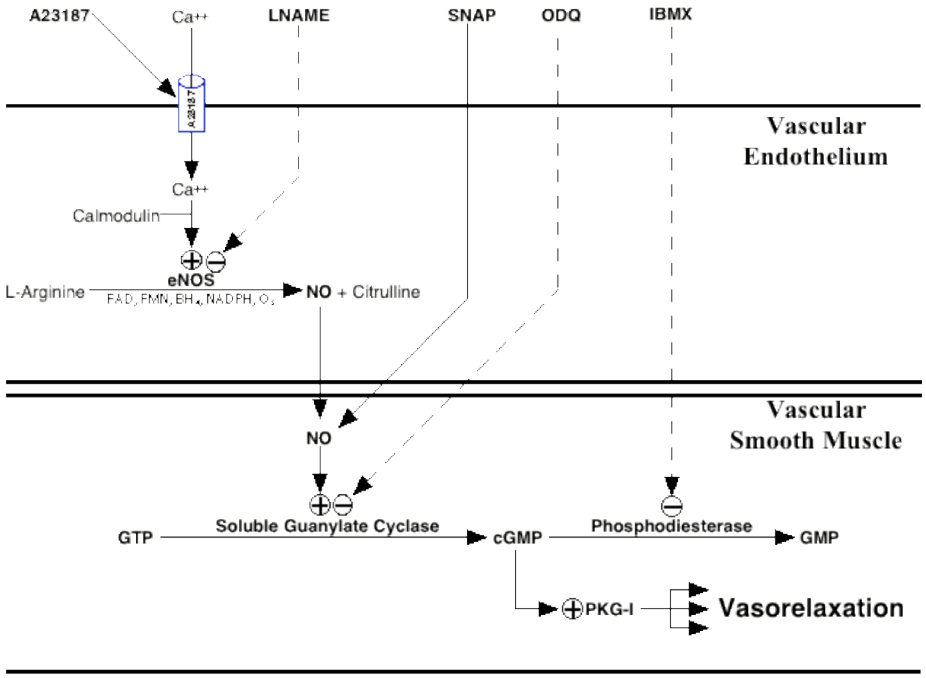

Figure 1. Endothelium-dependent vasodilatation is initiated by a rise in endothelial calcium concentration, which in turn stimulates the enzyme eNOS to synthesize and release NO. The NO is synthesized from L-Arginine and requires the cofactors FAD, FMN, BH4, NAPDPH, and O_2. This NO then diffuses into adjacent smooth muscle where it binds to the heme moiety on soluble guanylate cyclase and activates the synthesis of cGMP from GTP. This cGMP then combines with protein kinase G (PKG) to promote vasorelaxation through multiple mechanisms until is it hydrolyzed by phosphodiesterase. Please see text for additional details.

In response to A23187, carotid arteries from normoxic animals relaxed 65±5% whereas responses from hypoxic animals averaged 71±5%; this difference was not significant. In contrast, when exogenous NO was administered via the donor S-nitroso-N-acetyl-penacilamine (SNAP, see **Figure 1**), magnitudes of relaxation averaged 70±4 and 87±3% in normoxic and hypoxic arteries, respectively. This significant difference revealed that hypoxia enhanced reactivity to NO. This latter finding, together with the parallel finding that the magnitude of endothelium-dependent relaxation was unchanged by hypoxia, and the finding that NO is the main endothelium-dependent vasodilator in this preparation (Williams et al., 2004), suggests that hypoxia must correspondingly depress endothelial synthesis and release of NO.

3. CHRONIC HYPOXIA DEPRESSES NO RELEASE AND eNOS SPECIFIC ACTIVITY

To directly test the hypothesis that chronic hypoxia depresses endothelial NO release, we perfused 4-cm lengths of carotid arteries at 4 ml/min with a 20 mM Hepes buffer at a physiological transmural pressure (60 mm Hg). The perfusate collected was analyzed for NO content using a flurometric assay based on the nitrate reductase method (Kleinhenz et al., 2003). In response to 1 μM A23187 added to the perfusate, NO release was significantly greater in normoxic than in hypoxic arteries (**Figure 2**). To further understand the basis for this effect, we measured the abundance of eNOS in the perfused arteries using standard Western blotting, as previously described (Williams et al., 2004). Values of eNOS abundance were then divided into the corresponding rates of NO release to estimate in situ rates of maximal eNOS activity. Chronic hypoxia had no significant effect on eNOS abundance, but significantly depressed eNOS specific activity (**Figure 2**), suggesting that chronic hypoxia may alter one or more of the many mechanisms now recognized to govern posttranslational modification of eNOS (Boo & Jo, 2003; Fleming & Busse, 2003; Minshall et al., 2003).

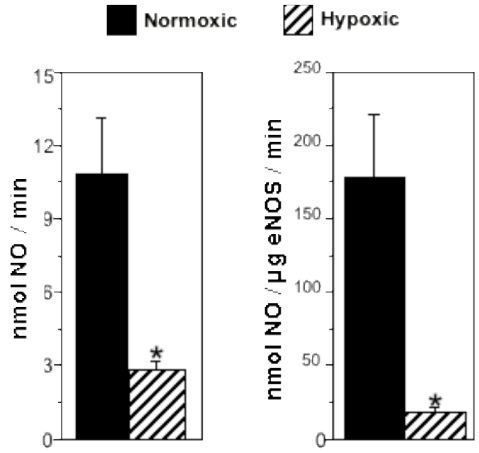

Figure 2. In ovine carotid arteries, chronic hypoxia significantly depressed the maximal rates of NO release induced by A23187 (left panel). Hypoxic depression of NO release was attributable primarily to reductions in the specific activity of the eNOS enzyme (right panel), and did not involve significant changes in eNOS abundance. Vertical error bars indicate standard errors for carotid arteries from 6-8 sheep in each group. Asterisks indicate significant effects of hypoxia at the $P<0.05$ level.

4. CHRONIC HYPOXIA DEPRESSES SOLUBLE GUANYLATE CYCLASE ABUNDANCE AND V_{MAX}

Our finding that NO-induced relaxation was enhanced in arteries from chronically hypoxic animals suggests that chronic hypoxic acts within vascular smooth muscle cells to upregulate mechanisms mediating cGMP-dependent vasodilatation (**Figure 1**). To test this hypothesis, we repeated our measurements of NO-induced relaxation in the presence of 10 μM ODQ, a selective and specific inhibitor of soluble guanylate cyclase (Garthwaite et al., 1995). Treatment with ODQ completely eliminated all relaxation responses to exogenous NO, thus verifying that soluble guanylate cyclase (sGC) was the predominant "NO receptor" in this tissue. Using a broken cell preparation as previously described (White et al., 2000), we next determined substrate-velocity relations for sGC. Although chronic hypoxia had no significant effect on K_m values, it significantly depressed maximal rates of cGMP synthesis (**Figure 3,** left panel). To further understand the basis for this effect, we also measured the abundance of sGC via Western blot as previously described (White et al., 2000). Values of soluble guanylate cyclase abundance were then divided into the corresponding rates of cGMP synthesis to estimate in situ rates of maximal soluble guanylate cyclase activity. Chronic hypoxia significantly depressed abundance in ovine carotid arteries (**Figure 3**, center panel), suggesting that chronic hypoxia must depress rates of transcription of the genes for one or more sGC subunits (Russwurm and Koesling, 2002), or must depress either mRNA stability or efficiency of translation. These are clearly testable hypothesis that offer promise in furthering understanding of how chronic hypoxia modulates mechanisms of vasorelaxation. In contrast, chronic hypoxia had no significant effect on in situ estimates of sGC specific activity (Figure 3, right panel). This absence of physiological regulation of sGC specific activity is consistent with the view that post-translational regulation of sGC activity is rare and has not been elucidated despite considerable investigative effort (Koesling and Friebe, 1999; Andreopoulos and Papapetropoulos, 2000).

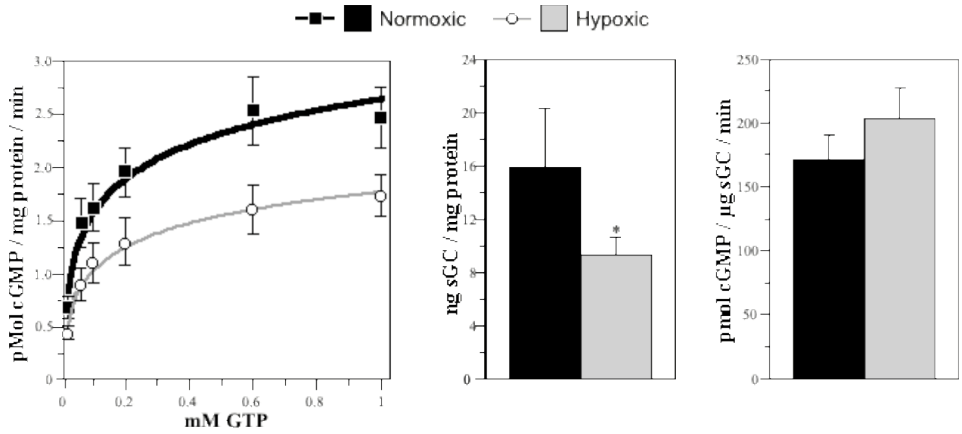

Figure 3. Substrate-velocity measurements for soluble guanylate cyclase (left panel) revealed that chronic hypoxia depressed the Vmax without significantly affecting Km values. Chronic hypoxia also significantly depressed the abundance of soluble guanylate cyclase, as measured via quantitative Westerns and expressed relative to total soluble protein. In situ estimates of soluble guanylate cyclase specific activity (right panel) revealed no significant effect of chronic hypoxia. Vertical error bars indicate standard errors for carotid arteries from 6-8 sheep in each group. The asterisk indicates a significant effect of hypoxia at the $P<0.05$ level.

5. CHRONIC HYPOXIA DEPRESSES PHOSPHODIESTERASE ACTIVITY

Given that relaxation responses to exogenous NO were enhanced by chronic hypoxia, we expected that chronic hypoxia would upregulate sGC activity and rates of cGMP synthesis. Because this is the opposite of what we found, it is clear that hypoxia upregulates some other component of NO-dependent vasorelaxation, downstream from cGMP synthesis. One possible candidate would be rates of cGMP degradation by phosphodiesterase (**Figure 1**). A variety of phosphodiesterase (PDE) subtypes are abundant in vascular smooth muscle (Matsumoto et al., 2003; Rybalkin et al., 2003) and are highly subject to physiological regulation (Francis et al., 2001). To examine the effects of chronic hypoxia on PDE activity, we performed timed measurements of NO-induced cGMP accumulation in the presence and absence of the PDE inhibitor IBMX, as previously described (White and Pearce, 1996). In normoxic arteries, rates of phosphodiesterase activity (in pmol cGMP / min/ mg protein) averaged 12.3±4.0 (N=8), but in hypoxic arteries averaged only 3.4±0.5 (N=7); chronic hypoxia significantly depressed PDE activity against cGMP in ovine carotid arteries. Which isoforms were involved in this effect, and the relative importance of decreased expression and decreased enzyme activity remain unexplored but promising topics for future investigation.

6. SUMMARY

Acclimatization to chronic hypoxia involves numerous compensatory changes in many tissues, including blood vessels. The present data demonstrate that in addition to well-documented changes in contractility, chronic hypoxia also produces important changes in the mechanisms mediating endothelium-dependent vasodilatation. At the level of the endothelium, hypoxia attenuates endothelial release of NO and this appears to be mediated through reductions in eNOS specific activity; chronic hypoxia has little effect on eNOS abundance. In contrast, chronic hypoxia depresses the abundance of sGC, which functions as the downstream vascular receptor for NO released from the endothelium. The decreased abundance of sGC produced by chronic hypoxia occurs without changes in sGC specific activity and results in decreased rates of NO-induced cGMP synthesis. Nonetheless, the vasodilator efficacy of NO is enhanced in hypoxic arteries, which suggests that mechanisms downstream from sGC are upregulated by hypoxia. Consistent with this view, chronic hypoxia significantly depresses PDE activity, which serves to prolong cGMP half-life and enhance its vasodilator effects. It remains possible that chronic hypoxia may also enhance PKG activity and/or the abundance of its substrates; this possibility remains a promising topic for future investigation. Overall, it is important to recognize that the mechanisms of adaptation to chronic hypoxia identified in the present study may be somewhat unique to adult carotid arteries. Adaptive responses to chronic hypoxia can vary considerably between small and large arteries, and also between immature and adult arteries (Longo et al., 1993). Still, the present data clearly demonstrate that both the endothelium and vascular smooth muscle of major arteries are profoundly influenced by chronic hypoxia, and thereby participate fully in whole-body adaptation to reduced oxygen availability.

7. REFERENCES

Andreopoulos, S. and Papapetropoulos, A., 2000, Molecular aspects of soluble guanylyl cyclase regulation, *Gen Pharmacol.* **34**:147-57.

Boo, Y. C. and Jo, H., 2003, Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases, *Am J Physiol Cell Physiol.* **285**:C499-508.

Buchholz, J.,Edwards-Teunissen, K. and Duckles, S. P., 1999, Impact of development and chronic hypoxia on NE release from adrenergic nerves in sheep arteries, *Am J Physiol.* **276**:R799-808.

Fleming, I. and Busse, R., 2003, Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase, *Am J Physiol Regul Integr Comp Physiol.* **284**:R1-12.

Fradette, C. and Du Souich, P., 2004, Effect of hypoxia on cytochrome P450 activity and expression, *Curr Drug Metab.* **5**:257-71.

Francis, S. H.,Turko, I. V. and Corbin, J. D., 2001, Cyclic nucleotide phosphodiesterases: relating structure and function, *Prog Nucleic Acid Res Mol Biol.* **65**:1-52.

Fung, M. L., 2003, Hypoxia-inducible factor-1: a molecular hint of physiological changes in the carotid body during long-term hypoxemia?, *Curr Drug Targets Cardiovasc Haematol Disord.* **3**:254-9.

Garthwaite, J.,Southam, E.,Boulton, C. L.,Nielsen, E. B.,Schmidt, K. and Mayer, B., 1995, Potent and selective inhibition of nitric oxide-sensitive guanylyl cyclase by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, *Mol Pharmacol.* **48**:184-8.

Kleinhenz, D. J.,Fan, X.,Rubin, J. and Hart, C. M., 2003, Detection of endothelial nitric oxide release with the 2,3-diaminonapthalene assay, *Free Radic Biol Med.* **34**:856-61.

Koesling, D. and Friebe, A., 1999, Soluble guanylyl cyclase: structure and regulation, *Rev Physiol Biochem Pharmacol.* **135**:41-65.

Longo, L. D.,Hull, A. D.,Long, D. M. and Pearce, W. J., 1993, Cerebrovascular adaptations to high-altitude hypoxemia in fetal and adult sheep, *Am J Physiol.* **264**:R65-72.

Marshall, J. M., 2000, Adenosine and muscle vasodilatation in acute systemic hypoxia, *Acta Physiol Scand.* **168**:561-73.

Matsumoto, T.,Kobayashi, T. and Kamata, K., 2003, Phosphodiesterases in the vascular system, *J Smooth Muscle Res.* **39**:67-86.

Minshall, R. D.,Sessa, W. C.,Stan, R. V.,Anderson, R. G. and Malik, A. B., 2003, Caveolin regulation of endothelial function, *Am J Physiol Lung Cell Mol Physiol.* **285**:L1179-83.

Moore, L. G.,Shriver, M.,Bemis, L.,Hickler, B.,Wilson, M.,Brutsaert, T.,Parra, E. and Vargas, E., 2004, Maternal adaptation to high-altitude pregnancy: an experiment of nature--a review, *Placenta.* **25 Suppl A**:S60-71.

Pearce, W. J.,Ashwal, S. and Cuevas, J., 1989, Direct effects of graded hypoxia on intact and denuded rabbit cranial arteries, *Am J Physiol.* **257**:H824-33.

Pearce, W. J., 1995, Mechanisms of hypoxic cerebral vasodilatation, *Pharmacol Ther.* **65**:75-91.

Peers, C. and Kemp, P. J., 2004, Ion channel regulation by chronic hypoxia in models of acute oxygen sensing, *Cell Calcium.* **36**:341-8.

Raguso, C. A.,Guinot, S. L.,Janssens, J. P.,Kayser, B. and Pichard, C., 2004, Chronic hypoxia: common traits between chronic obstructive pulmonary disease and altitude, *Curr Opin Clin Nutr Metab Care.* **7**:411-7.

Reeves, J. T. and Leon-Velarde, F., 2004, Chronic mountain sickness: recent studies of the relationship between hemoglobin concentration and oxygen transport, *High Alt Med Biol.* **5**:147-55.

Russwurm, M. and Koesling, D., 2002, Isoforms of NO-sensitive guanylyl cyclase, *Mol Cell Biochem.* **230**:159-64.

Rybalkin, S. D.,Yan, C.,Bornfeldt, K. E. and Beavo, J. A., 2003, Cyclic GMP phosphodiesterases and regulation of smooth muscle function, *Circ Res.* **93**:280-91.

Taniguchi, H.,Tanaka, Y.,Hirano, H.,Tanaka, H. and Shigenobu, K., 1999, Evidence for a contribution of store-operated Ca2+ channels to NO-mediated endothelium-dependent relaxation of guinea-pig aorta in response to a Ca2+ ionophore, A23187, *Naunyn Schmiedebergs Arch Pharmacol.* **360**:69-79.

Teng, G. Q.,Williams, J.,Zhang, L.,Purdy, R. and Pearce, W. J., 1998, Effects of maturation, artery size, and chronic hypoxia on 5-HT receptor type in ovine cranial arteries, *Am J Physiol.* **275**:R742-53.

Ueno, N.,Zhao, Y.,Zhang, L. and Longo, L. D., 1997, High altitude-induced changes in alpha1-adrenergic receptors and Ins(1,4,5)P3 responses in cerebral arteries, *Am J Physiol.* **272**:R669-74.

White, C. R. and Pearce, W. J., 1996, Effects of maturation on cyclic GMP metabolism in ovine carotid arteries, *Pediatr Res.* **39**:25-31.

White, C. R.,Hao, X. and Pearce, W. J., 2000, Maturational differences in soluble guanylate cyclase activity in ovine carotid and cerebral arteries, *Pediatr Res.* **47**:369-75.

Williams, J. M.,Hull, A. D. and Pearce, W. J., 2004, Maturational Modulation of Endothelium-Dependent Vasodilatation in Ovine Cerebral Arteries, *Am J Physiol Regul Integr Comp Physiol.*