Chronic hypoxic decreases in soluble guanylate cyclase protein and enzyme activity are age dependent in fetal and adult ovine carotid arteries

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Chronic hypoxic decreases in soluble guanylate cyclase protein and enzyme activity are age dependent in fetal and adult ovine carotid arteries. J Appl Physiol 100: 1857–1866, 2006. First published February 9, 2006; doi:10.1152/japplphysiol.00662.2005.—The present study tests the hypothesis that chronic hypoxia enhances reactivity to nitric oxide (NO) through age-dependent increases in soluble guanylate cyclase (sGC) and protein kinase G (PKG) activity. In term fetal and adult ovine carotids, chronic hypoxia had no significant effect on mRNA levels for the β1-subunit of sGC, but depressed sGC abundance by 16% in fetal and 50% in adult arteries, through possible depression of rates of mRNA translation (15% in fetal and 50% in adult) and/or increased protein turnover. Chronic hypoxia also depressed the catalytic activity of sGC, but only in fetal arteries (63%). Total sGC activity was reduced by chronic hypoxia in both fetal (69%) and adult (37%) carotid homogenates, but this effect was not observed in intact arteries when sGC activity was measured by timed accumulation of cGMP. In intact arteries treated with 300 μM 3-isobutyl-1-methylxanthine (IBMX), chronic hypoxia dramatically enhanced sGC activity in fetal (186%) but not adult (89%) arteries. This latter observation suggests that homogenization either removed an sGC activator, released an sGC inhibitor, or altered the phosphorylation state of the enzyme, resulting in reduced activity. In the absence of IBMX, chronic hypoxia had no significant effect on rates of cGMP accumulation. Chronic hypoxia also depressed the ability of the cGMP analog, 8-(p-chlorophenylthio)-cGMP, to promote vasorelaxation in both fetal (8%) and adult (12%) arteries. Together, these results emphasize the fact that intact and homogenized sGC activity studies of sGC activity do not always yield equivalent results. The results further suggest that enhancement of reactivity to NO by chronic hypoxia must occur upstream of PKG and can only be possible if changes in cGMP occurred in functional compartments that afforded either temporal or chemical protection to the actions of phosphodiesterase. The range and age dependence of hypoxic effects observed also suggest that some responses to hypoxia must be compensatory and homeostatic, with reactivity to NO as the primary regulated variable.

Cyclic guanosine 3’,5’-cyclic monophosphate; protein kinase G; vascular maturation

Unlike acute hypoxia, which rapidly enhances regional blood flow, chronic hypoxia produces more gradual structural and functional changes that culminate in near normal blood flow and tissue oxygenation (20). Such changes have been observed in individuals residing at altitude as well as those with pathophysiological conditions, such as chronic obstructive pulmonary disease (32). The influences of chronic hypoxia can also be quite dramatic in the growing fetus, particularly when secondary to placental insufficiency or maternal smoking. Chronic fetal hypoxia increases the chance for fetal growth restrictions, a major cause of perinatal morbidity and death (7, 9). In addition, chronic hypoxia causes a broad variety of changes in fetal vascular reactivity that is particularly pronounced in the cerebral circulation (24).

Within the vasculature in general, chronic hypoxia produces a broad spectrum of structural and functional changes that are typically organ specific. For example, chronic hypoxia induces arterial remodeling in the pulmonary vasculature that can lead to the development of pulmonary hypertension (13). The extent of hypoxic pulmonary remodeling also appears to be sensitive to the influence of nitric oxide (NO) and presumably also cGMP (33). In turn, chronic hypoxia has been reported to elevate pulmonary soluble guanylate cyclase (sGC) abundance and activity in some studies (21, 22), but decrease it in others (6, 11). Chronic hypoxia also produces calcium desensitization in pulmonary arteries through effects mediated by depressed RhoA/Rho kinase activity (15). Within the brain, chronic hypoxia alters oxygen exchange by inducing angiogenesis and decreasing intraluminal distances between capillaries and tissue (20). In ovine carotids, chronic hypoxia dramatically alters composition, pharmacomechanical coupling, and contractility (41). In the blood, chronic hypoxia increases erythropoiesis, which, in turn, leads to increased blood viscosity and potentially elevated shear stress with enhanced endothelial NO synthase activation (37). Interestingly, repeated exposure to hypoxia can impart protection against cerebral and cardiac injury caused by acute hypoxia (19, 39), which suggests that some vascular responses to chronic hypoxia may be advantageous and others pathological. The protective vascular mechanisms involved in hypoxic adaptation, however, remain largely unidentified.

To explore how chronic hypoxia could upregulate potentially protective mechanisms governing vascular contractility, our previous studies have focused on hypoxic modulation of vasorelaxant pathways. In ovine carotids, chronic hypoxia depressed endothelial NO release, but enhanced vasorelaxation to NO (31, 45). This enhanced responsiveness to NO appears to be attributable to altered sGC activity, because 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one, a specific inhibitor of sGC, completely ablated all responses to NO. Given these results, the present study was designed to test the hypothesis that chronic hypoxia modulates sGC activity. Because the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
magnitude of the effects of chronic hypoxia varied significantly between fetal and adult arteries, these studies also explore the corollary that the effects of chronic hypoxia on the cGMP-relaxation pathway are dependent on postnatal age. The model chosen for these studies was the ovine carotid, taken from near-term fetal and adult sheep maintained near sea level or at altitude (3,820 m) for ≈110 days. As we have shown previously, the carotid arteries play an important role in responses to hypoxia, particularly in the fetus, where the regulatory capacity of the small cerebral arteries is functionally immature (8, 29). The relatively large mass of the carotids also enables multiple parallel assays from a single animal, which is not possible using smaller, more peripheral arteries. Using carotid arteries, we examined the activity of sGC in whole arteries and homogenates, the abundance of sGC protein and mRNA, and the dose-response characteristics of the cell-permeant but nonhydrolyzable cGMP analog, 8-(p-chlorophenylthio)-cGMP (8-pCPT-cGMP). Together, these studies offer a unique view of the age-dependent mechanisms through which chronic hypoxia modulates cGMP-dependent relaxation of ovine carotids.

MATERIALS AND METHODS

All procedures used in these studies were approved by the Animal Research Committee of Loma Linda University and adhered to the policies and practices outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols utilized segments of common carotid arteries from fetal (139–142 days gestation) and young nonpregnant adult sheep (18–24 mo old). Chronically hypoxic animals were maintained for ≈110 days at the Barcroft Laboratory, White Mountain Research Station (Bishop, CA, altitude 3,820 m). As previously described in detail (16), this acclimatization to hypoxia reduced adult arterial oxygen tensions from ≈102 to ≈64 Torr, reduced fetal arterial oxygen tensions from ≈23 to ≈19 Torr, had no effect on body weight in either age group, and increased blood hemoglobin contents from normoxic values of ≈8.7 to ≈10.5 g/dl in the hypoxic adult, and from normoxic values of ≈9.6 to ≈11.6 g/dl in the hypoxic fetus. Just before experiments, the 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 killed by removal of the heart and rapid exsanguination. Adult animals were killed by intravenous administration of 100 mg/kg pentobarbital. All procedures related to animal surgery and procurement of tissues have previously been described in detail (16). All artery segments were cleaned of extraneous connective and adipose tissue. The endothelium was removed from all artery segments by passing a roughened needle through the lumen. Verification of denudation was determined by lack of vasoconstriction to 1 μM bradykinin. Segments relaxing >10% following contraction with 1 μM 5-HT were excluded.

sGC enzyme activity in artery homogenates. For each enzyme preparation, a frozen artery segment from a single animal (~0.150 g wet wt) was homogenized in 2 ml of ice-cold 50 mM Tris·HCl buffer (pH 7.5), containing 4 mM MgCl₂ and 2 mM D,L-DTT. The homogenization buffer also contained a mixture of protease inhibitors (76.8 nM aprotinin, 83 mM benzamidine, 1 mM iodoacetamide, 1.1 μM leupeptin, 7 μM pepstatin A, and 0.23 mM phenylmethanesulfonyl fluoride). Homogenization was carried out at 4°C using a motor-driven ground glass pestle and mortar. To separate particulate guanylate cyclase from sGC, homogenates were centrifuged for 60 min at 110,000 g, and then an aliquot of supernate was removed for protein determination. The remaining supernate was diluted to the desired protein concentration of 3 mg/ml Tris-soluble protein using homogenization buffer. Fresh homogenate was prepared for each activity measurement.

The activity of sGC was determined by measuring the formation of cGMP from GTP in 200-μl aliquots of the diluted artery homogenate. To remove endogenous cGMP and GTP from the preparation, sample tubes were warmed in a 38°C water bath for a minimum 30-min “clearing period.” As shown previously, this method effectively reduced endogenous levels of cyclic nucleotides and nucleotide triphosphates to negligible levels (44). Following the clearing period, a solution containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.1 mM zaprinast was added to each tube and allowed to warm for an additional 30 min. This addition of phosphodiesterase (PDE) inhibitors prevented the hydrolysis of cGMP during the measurements of sGC velocity, as previously shown (44). Reactions were initiated by the addition of GTP (0.02–1.0 mM GTP final concentration) plus preformed nitrosylated-heme (2 μm final concentration in reaction tube) (43). This use of exogenously nitrosylated heme improved the reproducibility of sGC activation and also minimized any potential influence from trace amounts of NO or carbon monoxide (CO) potentially synthesized de novo (43). The final reaction volume was 350 μl. The samples were incubated for 5 or 15 min, after which 2 ml of ice cold 6% trichloroacetic acid were added to each tube to stop the reaction. The samples were then centrifuged and were extracted with ether, lyophilized, and assayed for cGMP by radioimmunoassay.

Western immunoblotting for sGC protein. Given that total tissue enzyme activity is influenced by enzyme abundance, samples obtained from the sGC enzyme assay were assayed for sGC protein abundance. Owing to the fact that hypoxia influences the abundance and expression of virtually all vascular proteins, loading was not normalized relative to any reference protein, and instead the mass of protein loaded on each lane was carefully controlled. All samples (20 μg per lane) were separated on 10% polyacrylamide SDS gels. The separated proteins were transferred to nitrocellulose using a current of 15 mA for 2.5 h in Towbin’s buffer (25 mM Tris, 192 mM glycine, 20% methanol). Following transfer, the membranes were blocked with 2% milk, 2% BSA, and 0.1% Tween-20 in Tris-buffered saline overnight at 4°C, then probed with a sGC polyclonal antibody (Cayman Chemical, Ann Arbor, MI) at 1:1,500 dilution in blocking buffer for 2 h. The membranes were then washed twice in blocking buffer for 15 min each and probed with a horseradish peroxidase-conjugated goat-anti-rabbit antibody diluted 1:15,000. After 1 h of incubation with the secondary antibody, the membranes were washed as described for the first antibody, followed by 5-min wash in Tris-buffered saline, and then the blots were visualized by using West Femto chemiluminescent substrate (Pierce, Rockford, IL) in an ChemiImager 4400 (Alpha Innotech, San Leandro, CA). Sample abundances were normalized against a pure recombinant sGC enzyme (Alexis Chemical, San Diego, CA). Because it is possible that there may have been very slight differences in the affinity of the antibody we used against ovine and recombinant human sGC, our estimates of the absolute abundance of ovine sGC may not be exact but are probably very close, given the highly conserved structure of the sGC enzyme (35).

Real-time quantitative PCR of sGC β₁- and α-actin mRNA. To assess whether the abundance of sGC protein is transcriptionally and/or transcriptionally regulated, the expression levels of sGC β₁-subunit mRNA were measured. The β₁-subunit was followed because it is a common component of both major vascular isoforms of sGC (35) and thus is insensitive to possible isoform shifts. Total RNA was extracted from common carotid arteries stored in RNA Later (Qiagen, Valencia, CA) using the RNeasy Kit (Qiagen). This extraction method was based on the selective binding properties of silica gel-based membranes with RNA molecules longer than 200 bases. Briefly, arteries were disrupted in liquid nitrogen using a steel mortar and pestle and then homogenized in the presence of guanidine isothiocyanate. The sample was then added to an RNeasy mini column in the presence of ethanol, where the total RNA binds to the membrane and contaminants are efficiently washed away. RNA was then eluted in water and...
Further purified from contaminating genomic DNA using the DNase free kit (Ambion, Austin, TX). The quantity and purity of RNA were determined spectrophotometrically. This extraction method consistently yielded high purity levels with 280/260 ratios greater than 1.8.

Levels of sGC \( \beta_1 \)-subunit mRNA expression were determined using real-time quantitative PCR. The RT-PCR reactions were performed in single step using Quantitect SYBRgreen RT-PCR kit (Qiagen). In brief, the reactions were performed in a final volume of 50 \( \mu \)L containing 200 ng of RNA. The reverse transcription reaction was performed at 50°C for 30 min. After this step, reverse transcriptase was inactivated by heating to 95°C for 15 min at the same time. Hotstar Taq DNA polymerase was activated. Custom primers were designed using published sequences for ovine sGC \( \beta_1 \) (GENE Bank Accession no. AF486295) with the help of the Primer3 software package (http://frodo.wi.mit.edu/primer3/primer3_code.html). The sGC \( \beta_1 \)-subunit primers used were as follows: forward, base position 44, 5'-GGGCCATGAAGATTGTCAAC-3'; and reverse, base position 148, 5'-CCCCAACAGTCTCCACCTTA-3', yielding a 105-bp fragment. \( \alpha \)-Actin was also assayed in the same samples using the following primers: forward, 5'-GTCATCTCCCTGCTCTGT-3'; and reverse, 5'-GCTTTGGATAGGCATGACT-3', yielding a 96-bp fragment. The cDNA fragments were amplified using the iCycler (Bio-Rad, Hercules, CA) system with SYBRgreen PCR reagents. Reactions were performed for 35 cycles (denaturation for 15 s at 94°C, annealing for 30 s at 50°C, and extension for 30 s at 72°C). PCR products were directly monitored by measuring the increase in fluorescence caused by binding of SYBRgreen to double-stranded DNA. The threshold cycle (C\(_T\)) number was recorded for each sample and represented the number of cycles required to reach a standardized level of fluorescence. The C\(_T\) values were obtained for all samples in duplicate.

sGC enzyme activity in whole arteries. Because compartmentalization can influence the activity of enzymes in vivo, the enzyme activity of sGC was measured in endothelium-denuded whole artery segments. Arteries were cut into segments 3–5 mm in length and mounted on paired wires between a low-compliance force transducer (Radnoti, Monrovia, CA) and a post attached to a micrometer used to vary resting tension. Segments were equilibrated at least 1 h at 38.5°C (normal ovine core temperature) in a bicarbonate Krebs solution containing (in mM) 122 NaCl, 25.6 NaHCO\(_3\), 5.56 dextrose, 5.17 KCl, 2.49 MgSO\(_4\), 1.60 CaCl\(_2\), 0.114 ascobic acid, and 0.027 disodium EDTA containing 100 \( \mu \)M l-nitro arginine methyl ester and 100 \( \mu \)M l-nitro arginine, continuously bubbled with 95% \( \text{O}_2\)-5% \( \text{CO}_2\). During equilibration, segments were maintained at optimum resting tensions, as previously described in detail (30).

Arteries initially were contracted by exposure to an isotonic potassium Krebs solution containing 122 mM K\(^+\) and 31 mM Na\(^+\). After peak tensions were obtained, segments were washed with normal sodium Krebs solution and allowed to return to baseline levels of tension for 30 min. Artery segments for basal cGMP levels were then frozen in liquid N\(_2\). Artery segments for sGC activity were then contacted with 3 \( \mu \)M serotonin following incubation with 1 \( \mu \)M prazosin to inhibit \( \alpha_1 \)-adrenergic receptor activation and 0.2 \( \mu \)M cocaine to inhibit neuronal reuptake, and in the presence or absence of 300 \( \mu \)M IBMX to inhibit PDE activity. This concentration of IBMX was found to maximally inhibit PDE activity in whole arteries (44). Following attainment of a stable level of initial tone, segments were exposed to 10 \( \mu \)M S-nitroso-N-acetyl-penicillamine (SNAP) and frozen at various time intervals (15, 30, 45 s).

The contents of cGMP in frozen artery segments were determined as previously described (44). Briefly, arteries were homogenized in 6% TCA, and the supernatants were washed with water-saturated diethyl ether and then lyophilized. The content of cGMP in each sample was then measured, as described in MATERIALS AND METHODS for sGC enzyme activity in artery homogenates. Levels of cGMP were normalized to base-soluble protein obtained from TCA precipitates.

8-pCPT-cGMP dose-response studies. To assess the effects of chronic hypoxia on cGMP-dependent vasorelaxation, dose-response studies with a cGMP analog were measured using the cGMP analog 8-pCPT-cGMP, a membrane-permeable and nonmetabolizable molecule (4). Artery segments were prepared as previously described. Following contraction by potassium Krebs solution, segments were contracted with 3 \( \mu \)M 5-HT. Following attainment of a stable level of initial tone, cumulative doses of 8-pCPT-cGMP were added to the baths to produce concentrations ranging from 1 \( \mu \)M to 10 mM.

Data analysis and statistics. \( K_m \) and \( V_{max} \) values were calculated by fitting the substrate-product data to the Michaelis-Menten equation. Rates of cGMP production in whole arteries were calculated from the slope values obtained by a best-fit linear curve. So that all values normalized relative to protein could be compared, parallel determinations of Tris-soluble and base-soluble protein were carried out in each artery type and age. The ratios of Tris- to base-soluble proteins were then used to convert all protein denominators into units of base-soluble protein. Maximum relaxation responses were calculated as percentage relaxations of the maximum initial contractile tension. For determination of agonist sensitivity (\( E_{D50} \)) and \( pD_2 \) (−log \( ED_{50} \)), the relaxation data were normalized relative to maximum percentage relaxation and then fitted to the logistic equation using nonlinear regression, as previously described (45). To estimate mRNA translation efficiency, protein abundance was multiplied by the \( C_T \) obtained in corresponding samples. All data were each independently analyzed using a two-way analysis of variance with oxygen level as one factor (normoxic vs. hypoxic) and maturation as the other (fetal vs. adult). Homoscedasticity was verified for all ANOVA comparisons using Bartlett’s test. The individual effects of hypoxia and maturation were determined by post hoc Duncan’s tests.

RESULTS

In total, 492 segments were obtained from 36 normoxic and 34 hypoxic adults and 29 normoxic and 28 hypoxic fetal sheep. When the same protocol was run using multiple segments of the same artery type from the same animal, the results were averaged by animal. Throughout the text, the given values of \( N \) refer to the number of animals studied and not the number of arterial segments.

Homogenate sGC enzyme activity. Activation of sGC with NO-heme in the presence of various GTP concentrations yielded cGMP formation that followed Michaelis-Menten kinetics (Fig. 1). Homogenate \( V_{max} \) values for sGC averaged 0.93 ± 0.1 and 0.59 ± 0.08 pmol cGMP·mg protein\(^{-1} \cdot \text{min}^{-1} \) in normoxic and hypoxic arteries, respectively, and these values were significantly greater than corresponding fetal values (0.43 ± 0.03 and 0.13 ± 0.02 pmol cGMP·mg protein\(^{-1} \cdot \text{min}^{-1} \), respectively). Chronic hypoxia also significantly depressed \( V_{max} \) values in both adult and fetal carotids. Regarding normoxic and hypoxic homogenate \( K_m \) values for sGC, these averaged 0.077 ± 0.01 and 0.071 ± 0.01 mM GTP, respectively, in the adult, and these values were significantly greater than those observed in normoxic (0.034 ± 0.004 mM GTP) and hypoxic (0.043 ± 0.008 mM GTP) fetal homogenates.

Abundance and specific activity of sGC in ovine common carotid arteries. Estimates of sGC protein averaged 5.4 ± 1.5 and 2.7 ± 0.4 ng/mg protein in normoxic and hypoxic adult homogenates, respectively, and these values were significantly less than those observed in normoxic (7.1 ± 0.9 ng/mg protein) and hypoxic (6.0 ± 0.5 ng/mg protein) fetal homogenates (Fig. 2A). Chronic hypoxia significantly decreased sGC abundance by 50 and 16% in adult and fetal homogenates, respectively.

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To assess how chronic hypoxia influenced the specific activity of sGC, the $V_{\text{max}}$ values obtained from the homogenate assays were normalized relative to estimated sGC abundance to calculate enzyme-specific activity (Fig. 2B) in units of picomoles cGMP per micrograms sGC per minute. Values of sGC-specific activity averaged 171.4 ± 19.1 and 219.9 ± 28.3 pmol cGMP·μg sGC⁻¹·min⁻¹ in normoxic and hypoxic adult homogenates, respectively. These adult values were significantly greater than observed in either normoxic (60.2 ± 4.2 pmol cGMP·μg sGC⁻¹·min⁻¹) or hypoxic (21.9 ± 3.8 pmol cGMP·μg sGC⁻¹·min⁻¹) fetal artery homogenates. Hypoxia had no significant effect on sGC-specific activity in the adult homogenates, but significantly depressed sGC-specific activity in the fetal homogenates.

Expression levels of sGC $\beta_1$-subunit and $\alpha$-actin mRNA. $C_T$ times obtained from real-time quantitative PCR for sGC $\beta_1$-subunit mRNA averaged 18.5 ± 0.1 and 18.8 ± 0.4 (adult) and 19.0 ± 0.2 and 19.2 ± 0.1 (fetal) in normoxic and hypoxic arteries, respectively (Fig. 3A). Given that $C_T$ numbers are inversely proportional to mRNA abundance, these results indicate that hypoxia had no significant effect on sGC $\beta_1$-subunit mRNA abundance, and maturation enhanced mRNA abundance but only modestly (41% for normoxic and 32% for hypoxic arteries).

In parallel with the assays for sGC $\beta_1$-subunit mRNA, the abundances of $\alpha$-actin mRNA were also determined. Previous studies have suggested that $\alpha$-actin protein levels in ovine cranial arteries remain stable throughout development (48), and thus $\alpha$-actin mRNA levels might also change negligibly during this period, which would make $\alpha$-actin mRNA a useful internal reference for vascular mRNA measurements. $C_T$ times for $\alpha$-actin mRNA averaged 28.2 ± 0.3 and 29.2 ± 0.2 (adult) and 27.7 ± 0.2 and 28.7 ± 0.4 (fetal) in normoxic and hypoxic arteries, respectively (Fig. 3B). These values revealed that chronic hypoxia modestly but significantly depressed actin mRNA abundance by $\approx$50% in both adult and fetal arteries. Age alone had no significant effect on $\alpha$-actin mRNA abundance. Interestingly, the abundance of sGC $\beta_1$ mRNA appeared to be more than 400-fold greater than the abundance of $\alpha$-actin mRNA in both fetal and adult arteries.
The translation of mRNA into protein is highly regulated and potentially subject to environmental perturbation. To obtain an index of the translational efficiency for sGC mRNA, we calculated the product of absolute protein abundance (Fig. 2A) and CT number (Fig. 3A). This calculation yielded an index whose value was directly proportional to apparent translational efficiency. The values averaged 100.4 ± 28.6 and 50.2 ± 7.1 (adult) and 135.4 ± 16.2 and 115.5 ± 10.1 (fetal) in normoxic and hypoxic arteries, respectively (Fig. 3C). These results revealed that maturation significantly decreased translational efficiency for sGC β1 mRNA, and this effect was individually significant in hypoxic arteries. In addition, hypoxia also significantly depressed apparent translational efficiency, and this effect was individually significant in the adult arteries.

**sGC enzyme activity in whole arteries.** Owing to the potential influence of compartmentalization on enzyme activity, the influence of chronic hypoxia on sGC activity was examined in situ through the use of the PDE inhibitor IBMX and exogenously added NO. Addition of 10 μM SNAP to treated artery segments resulted in linear increases in cGMP content for up to 45 s in all groups (Fig. 4). Rates of cGMP synthesis, as indicated by the slopes of the relations between time and cGMP content, averaged 8.3 ± 1.4 and 15.8 ± 4.8 (adult) and 29.5 ± 5.6 and 84.5 ± 16.4 (fetal) pmol cGMP·mg protein⁻¹·min⁻¹ in normoxic and hypoxic arteries, respec-

Fig. 3. Effects of chronic hypoxia on sGC β1-subunit and α-actin mRNA expression. Shown here are the results of real-time PCR expressed as threshold cycle (C_t) values for sGC β1-subunit mRNA (A) and α-actin mRNA (B) from hypoxic and normoxic adult and fetal common carotid arteries. C: ratio of sGC protein to corresponding mRNA levels as an estimate of translational efficiency. Values are means ± SE for 6 animals in each case. Maturation significantly enhanced sGC mRNA expression (ANOVA), as reflected by lower C_t values in adult than in fetal arteries. This effect was individually significant by post hoc analysis in normoxic animals. Hypoxia significantly depressed α-actin expression, and this effect was individually significant in both age groups. The ratio of sGC protein to mRNA revealed that both chronic hypoxia and maturation significantly decreased transcriptional efficiency (ANOVA). The maturation effect was individually significant in hypoxic animals. *Significant hypoxic effects. Significant age-related effects for corresponding treatments. Significance implies P < 0.05 for all comparisons.

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Fig. 4. Effects of chronic hypoxia on sGC enzyme activity in whole artery segments. Rates of cGMP synthesis varied significantly among whole artery segments from normoxic and hypoxic adult (A) and fetal (B) common carotid arteries. Artery segments were precontracted with 3 μM 5-HT and frozen at various times following exposure to 10 μM S-nitroso-N-acetyl-penicillamine in the presence of 300 μM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity, then assayed for cGMP content. Insets: rates of cGMP synthesis were taken as the slope values of the relations between cGMP contents and time and were expressed as pmol cGMP·mg protein⁻¹·min⁻¹. Values are means ± SE for 8–13 animals in each case. Chronic hypoxia significantly increased the rates of cGMP synthesis (ANOVA), and this difference was individually significant within fetal arteries. Maturation significantly decreased rates of cGMP synthesis, and this difference was individually significant in both normoxic and hypoxic animals. *Significant hypoxic effects. Significant age-related effects. Significance implies P < 0.05 for all comparisons.
Interestingly, these in situ values were 3- to 10-fold greater than observed in homogenate enzyme activity measurements. In addition, the values were significantly (ANOVA) enhanced in hypoxic, compared with normoxic, arteries in both age groups (Fig. 4, inset). In contrast, maturation significantly decreased rates of cGMP synthesis in both normoxic and hypoxic animals.

**Net accumulation of cGMP in whole arteries.** In terms of vasorelaxation, the most important variable is overall cGMP concentration, which reflects the balance between its parallel rates of synthesis and degradation. To explore how chronic hypoxia influenced net cGMP accumulation, cGMP contents were determined in artery segments at 0, 15, 30, and 45 s after the addition of 10 μM SNAP. The rates of cGMP accumulation were linear between 15 and 45 s in all arteries (Fig. 5). Rates of cGMP accumulation were not influenced by either chronic hypoxia or maturation and averaged 4.1 ± 0.9 and 7.9 ± 3.2 (adult) and 4.5 ± 1.1 and 2.5 ± 1.1 (fetal) pmol cGMP·mg protein⁻¹·min⁻¹ in normoxic and hypoxic arteries, respectively. Although rates of cGMP accumulation were not altered, basal cGMP content was significantly depressed by maturation in both normoxic and hypoxic arteries. Values of basal cGMP content expressed as pmol cGMP/mg protein averaged 0.12 ± 0.02 and 0.11 ± 0.01 (adult) and 0.82 ± 0.35 and 0.54 ± 0.28 (fetal) in normoxic and hypoxic arteries, respectively.

**8-pCPT-cGMP dose-response relations.** Aside from potential effects on cGMP synthesis, it is quite possible that chronic hypoxia could alter the ability of cGMP to promote vasorelaxation. To explore this possibility, relaxation responses to 8-pCPT-cGMP, a membrane-permeant and nonmetabolizable cGMP analog, were examined. In dose-response experiments, 8-pCPT-cGMP elicited dose-dependent relaxations in all arteries studied (Fig. 6). Maximal efficacy (defined as % maximal relaxation) values for 8-pCPT-cGMP averaged 89.3 ± 4.6 and 78.9 ± 3.8% (adult) and 99.2 ± 0.8 and 91.2 ± 2.3% (fetal) in normoxic and hypoxic arteries, respectively. Both chronic hypoxia and maturation significantly decreased the maximal...
efficacy for 8-pCPT-cGMP (ANOVA). Sensitivity to 8-pCPT-
cGMP, as reflected by its pD2 values, averaged 3.8 ± 0.2 and
3.3 ± 0.2 (adult) and 4.0 ± 0.1 and 3.9 ± 0.1 (fetal) in
normoxic and hypoxic arteries, respectively. Thus sensitivity to
8-pCPT-cGMP was enhanced in fetal arteries, and this effect
was individually significant by post hoc analysis in both
normoxic and hypoxic arteries (Fig. 6, inset).

DISCUSSION

In relation to the vascular effects of chronic hypoxia, very
few studies have yet examined the involvement of sGC and the
cGMP-relaxation pathway, and, among those studies published
in this area, most have focused on the adult pulmonary circu-
lation. For example, the extent of hypoxia-induced pulmonary
vascular remodeling can be attenuated by NO (33), which
suggests the possible involvement of sGC and cGMP in this
process. Studies of the effects of chronic hypoxia on the
pulmonary vasculature, however, have yielded inconsistent
findings, with some authors reporting decreases in whole tissue
sGC activity (6, 11), whereas others have reported hypoxic increases in sGC abundance and activity (21, 22). Aside from
these studies of pulmonary vessels, virtually no studies have
yet examined the effects of chronic hypoxia on sGC in cranial
arteries, or in fetal arteries of any kind. Our previous results do
demonstrate, however, that NO-induced vasorelaxation is po-
tentiated by chronic hypoxia (45), suggesting that some step
coupling activation of sGC to relaxation is modified by hy-
ppoxic acclimatization. Given these findings, the present study
was designed to test the hypothesis that chronic hypoxia enhances reactivity to NO through age-dependent increases in
sGC and protein kinase G activity.

In contradiction to our main hypothesis, the present results
revealed that chronic hypoxia decreased the velocity of sGC in
artery homogenates (Fig. 1). This depression was observed in
both adult and fetal artery homogenates and was not accom-
panied by any significant change in substrate affinity. These
findings thus raised questions about the mechanisms through
which chronic hypoxia could attenuate the velocity of sGC in
artery homogenates. Classically, differences in enzyme veloc-
ity are attributable to changes in three main variables: 1) en-
zyme concentration, 2) enzyme-specific activity, or 3) the
fractional extent of activation of the enzyme.

As shown in Fig. 2A, chronic hypoxia depressed the esti-
mated abundance of sGC in both fetal and adult arteries. This
difference was probably not attributable to apparent changes in
transcription, because chronic hypoxia had no significant effect
on the abundance of mRNA for the sGC β1-subunit (Fig. 3A).
In contrast, chronic hypoxia did significantly depress mRNA
abundance for α-actin (Fig. 3B), which suggests that chronic
hypoxia does exert effects at the translational level for at
least some vascular proteins, but not for the sGC β1-subunit
gene (23). Instead, chronic hypoxia appeared to depress the
rate of translation of the β1-subunit mRNA, as indicated by the
ratio of β-subunit protein to mRNA (Fig. 3C). Other recent
studies have also suggested that chronic hypoxia may influence
protein expression by influencing mRNA stability and the
initiation of mRNA translation (23, 46). However, it remains
possible that chronic hypoxia accelerated the rate of protein
turnover for sGC, which would also reduce the ratio of protein
to mRNA for the sGC β1-subunit.

Whereas hypoxic decreases in sGC abundance could help
explain the corresponding decreases in sGC enzyme velocity
observed in artery homogenates (Fig. 1), changes in enzyme-
specific activity might also have been involved. Consistent
with this possibility, the rate of cGMP production, when
normalized relative to the mass of sGC present, was signifi-
cantly depressed by chronic hypoxia in fetal artery homoge-

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sGC activity may differ considerably, as previously reported (3). To explore this idea, sGC activity was measured via timed measurements of cGMP accumulation in intact arteries. These measurements yielded estimates of sGC activity that were dramatically greater than observed in homogenates, even when both estimates were normalized relative to base-soluble protein (Fig. 4). Because homogenization buffers included effective concentrations of multiple protease inhibitors and were maintained at 4°C during homogenization, it is unlikely that subunit dissociation, digestion, or protein oxidation limited homogenate activity. Intact artery cGMP levels could conceivably have been augmented by particulate guanylate cyclase activity, although this is highly unlikely, given that particulate guanylate cyclase is unresponsive to NO (40), which was used to activate sGC in the intact artery measurements. Most importantly, the absolute activities measured for homogenate sGC were quite similar to those reported for other broken cell preparations (47), suggesting that additional factors were involved in intact arteries. The homogenate-intact differences are also not attributable to variable involvement of PDE activities, because these were optimally inhibited in both preparations using IBMX. Although ATP has recently been suggested to inhibit sGC activity in situ (34), it is also doubtful that this mechanism can explain our results: because no ATP was added to the homogenate preparations, endogenous ATP was hydrolyzed prior to commencing the assay, and thus the potentially inhibitory ATP concentrations should have been greater in the intact arteries than in the homogenates. Together, these considerations suggest that homogenization either released a compartmentalized inhibitor (e.g., CCTγ, biliverdin IX, t-ascorbic acid, etc.), removed a soluble activator or stabilizer of sGC (e.g., HSP70, HSP90), and/or activated phosphatase or kinase (e.g., PKG, PKA, PKC, etc.) activities that altered sGC activity (2, 10, 25, 27, 34, 42). This interpretation is consistent with other published studies of sGC activity, in which activity estimates in intact tissue are dramatically different than those obtained in broken cell preparations (3). Indeed, this feature of the results constitutes a major finding of the present study.

To more closely examine the possibility that hypoxic changes in PDE activity may contribute to hypoxic enhancement of reactivity to NO (45), we repeated the intact artery measurements of cGMP accumulation in the absence of PDE inhibition. These measurements revealed that, in the presence of PDE activity, neither chronic hypoxia nor postnatal age significantly affect rates of cGMP accumulation (Fig. 5). Indirectly, this pattern of results suggests that hypoxia dramatically upregulated PDE activity and more so in fetal than adult arteries. Perhaps more importantly, these results suggest that chronic hypoxia produces multiple important changes in carotid cGMP metabolism, but that these are closely regulated and compensated for in terms of overall vascular reactivity. These results do not preclude important vasorelaxant consequences of chronic hypoxia, but strongly suggest that, at least in terms of cGMP, they must be highly compartmentalized if they are to significantly alter patterns of vasorelaxation.

In light of our original hypothesis that chronic hypoxia may enhance NO-induced relaxation through changes in either sGC activity or PKG, we examined the concentration-dependent relaxant effects of the cell-permeant nonhydrolyzable cGMP analog, 8-pCPT-cGMP. In contradiction with our original hypothesis, chronic hypoxia depressed both sensitivity to and the efficacy of 8-pCPT-cGMP in intact fetal and adult carotids precontracted with 5-HT (Fig. 6). That chronic hypoxia can influence vascular PKG activity has been previously reported, although only in pulmonary arteries (14). Given this key finding, the ability of chronic hypoxia to enhance the relaxant effects of NO in ovine carotids cannot be explained by changes in the relaxant efficacy of cGMP mediated through PKG. Thus hypoxic potentiation of relaxation to NO must occur upstream of PKG. Although cGMP could potentially influence relaxation through PKG-independent mechanisms, such as activation of PKA or cGMP-sensitive PDE (1, 36), these mechanisms are probably negligible in ovine carotids, because selective PKG inhibitors effectively ablate relaxation responses to cGMP in this preparation (28). Another possibility is that chronic hypoxia facilitates NO-induced relaxation through cGMP-independent mechanisms, but this can be ruled out because 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, a selective inhibitor of guanylate cyclase, also eliminates responsiveness to NO in ovine carotids (45). Together, these considerations reinforce the view that NO-induced changes in cGMP must be highly compartmentalized and are probably also quite heterogeneous in their proximity to hydrolysis by PDE. This would enable the hypoxic enhancement of sGC activity to translate into improved responsiveness to NO, despite modestly depressed PKG reactivity to cGMP. Consistent with this view, rates of cGMP synthesis in intact arteries were quite variable in the absence of PDE inhibition. How and where the key cGMP compartments may reside within ovine carotids is uncertain, but it is, nonetheless, clear that the coupling between cGMP synthesis and degradation is somehow influenced by chronic hypoxia in an age-dependent manner.

Overall, the present results support the hypothesis that chronic hypoxia increases NO-induced vasorelaxation through increases in sGC activity that were apparent only in intact arteries and much more so in fetal than adult arteries. These increases, however, cannot be explained by changes in sGC abundance, because hypoxia decreased abundance in both fetal and adult arteries; even though sGC mRNA was not affected by hypoxia, hypoxia appeared to either inhibit mRNA translation or enhance sGC turnover. Hypoxic enhancement of NO-induced relaxation could not be explained by an increased ability of PKG to affect relaxation, because hypoxia depressed relaxant responses to 8-pCPT-cGMP, a nonmetabolizable analog of cGMP. A key feature of these findings is that hypoxic enhancement of sGC activity was not evident in homogenized arteries, suggesting that homogenization either removed an sGC activator, released an sGC inhibitor, or altered the phosphorylation state of the enzyme, resulting in reduced activity. In intact arteries treated with PDE inhibitors, chronic hypoxia dramatically enhanced sGC activity in fetal but not adult arteries, reinforcing the idea that hypoxia may have enhanced the availability of an activator that was more abundant in fetal arteries, and was upregulated by hypoxia. In the absence of PDE inhibitors, chronic hypoxia had little effect on rates of cGMP accumulation, suggesting that hypoxia significantly upregulated PDE activity and more so in fetal than adult arteries. Indeed, the data suggest that one of the main effects of chronic hypoxia is modulation of PDE activity; further exploration of this possibility is well warranted. The range and age dependence of hypoxic effects observed also suggest that some responses to hypoxia must be compensatory and homeostatic.

