Chronic hypoxia alters the function of NOS nerves in cerebral arteries of near-term fetal and adult sheep

EMMANUEL M. MBAKU,1 LUBO ZHANG,1 WILLIAM J. PEARCE,1 SUE P. DUCKLES,2 AND JOHN BUCHHOLZ1

1Department of Physiology and Pharmacology, School of Medicine, Loma Linda University, Loma Linda 92350; and 2Department of Pharmacology, College of Medicine, University of California, Irvine, California 92697

Submitted 23 August 2002; accepted in final form 23 October 2002

Abstract

Chronic hypoxia alters the function of NOS nerves in cerebral arteries of near-term fetal and adult sheep. J Appl Physiol 94: 724–732, 2003. First published October 25, 2002; 10.1152/japplphysiol.00771.2002.—In addition to adrenergic innervation, cerebral arteries also contain neuronal nitric oxide synthase (nNOS)-expressing nerves that augment adrenergic nerve function. We examined the impact of development and chronic high-altitude hypoxia (3,820 m) on nNOS nerve function in near-term fetal and adult sheep middle cerebral arteries (MCA). Electrical stimulation-evoked release of norepinephrine (NE) was measured with HPLC and electrochemical detection, whereas nitric oxide (NO) release was measured by chemiluminescence. An inhibitor of NO synthase, N^\text{-} \text{nitro-}L\text{-arginine methyl ester (L-NAME)}, significantly inhibited stimulation-evoked NE release in MCA from normoxic fetal and adult sheep with no effect in MCA from hypoxic animals. Addition of the NO donor S-nitroso-N-acetyl-DL-penicillamine fully reversed the effect of L-NAME in MCA from normoxic animals with no effect in MCA from hypoxic animals. Electrical stimulation caused a significant increase in NO release in MCA from normoxic animals, an effect that was blocked by the neurotoxin tetrodotoxin, whereas there was no increase in NO release in MCA from hypoxic animals. Relative abundance of nNOS as measured by Western blot analysis was similar in normoxic fetal and hypoxic animals. Relative abundance of nNOS as measured by Western blot analysis was similar in normoxic fetal and adult MCA. However, after hypoxic acclimatization, nNOS levels dramatically declined in both fetal and adult MCA. These data suggest that the function of nNOS nerves declines during chronic high-altitude hypoxia, a functional change that may be related to a decline in nNOS protein levels.

Although the cerebral vasculature is well innervated by sympathetic nerves, their contribution to the control of cerebral blood flow under normal physiological conditions is unclear (1, 4, 5). Variations in blood pressure elicit autoregulatory adjustments of arterial diameter, maintaining constant cerebral blood flow (5). When arterial pressure is elevated (150–180 mmHg), reflex sympathetic nerve activation blunts increases in cerebral blood flow and reduces the risk of blood-brain barrier disruption (6, 15). Thus sympathetic nerves have been suggested to protect an organism from various stresses such as severe hypertension or hypercapnia (5, 11). The development of elevated arterial pressure in fetal sheep at high altitude has been noted (21). However, cerebral blood flow did not increase in the face of arterial hypertension. It is possible that adrenergic innervation may play an important role in protecting the fetal cerebral circulation at high altitude (21).

In addition to sympathetic nerves, cerebral blood vessels are innervated by nerves that contain neuronal nitric oxide synthase (nNOS) and release nitric oxide (NO) (18, 33). In dog middle cerebral artery (MCA) strips denuded of endothelium, relaxation induced by transmural nerve stimulation (TNS) or nicotine is blocked either by NOS antagonists or by the neuronal poison TTX (32, 33). Thus, in cerebral arteries, NO is liberated during nerve stimulation. In perfused sheep MCAs, TNS increases the overflow of both norepinephrine (NE) and NO. In contrast, in the facial artery TNS increases overflow of NE but not NO (24). These studies suggest that NE and NO are simultaneously released with nerve activation in the cerebrovasculature.

Immunohistochemical techniques have identified structural apposition between adrenergic and nNOS neurons in both the porcine cerebrovasculature and rat superior cervical ganglion (10, 19, 29, 35, 39). Furthermore, nicotine relaxes constricted basilar artery strips, an effect abolished by adrenergic denervation (39). Blockade of β_2-adrenoceptors antagonizes nicotine-induced relaxation in the same artery model (19). Overall, these data suggest that the release of NE subsequently activates adjacent nNOS nerves, and adrenergic nerves are necessary for the normal function of nNOS nerves in cerebral arteries.

The release of NO from nNOS nerves has been shown to augment stimulation-evoked NE release. In
rat cerebral cortex, N-methyl-D-aspartate or K+-evoked NE release from hippocampal slices is blocked by NOS antagonists (23, 25). Consistent with these studies, NOS antagonists have been shown to inhibit NE release in sheep MCAs (4, 24).

Little is known about the physiological role of the interaction between nNOS-containing and adrenergic nerves. As mentioned above, adrenergic innervation of cerebral vessels is thought to be particularly important during cardiovascular stresses such as hypertension. Our laboratory has had a long-term interest in the impact of chronic high-altitude hypoxia on the function of the cerebral circulation of both the fetus and adult (3, 4, 20, 21). Therefore, we used this model of chronic high-altitude hypoxemia in the pregnant ewe to investigate the functional relation between endogenous nNOS and adrenergic nerves in cerebral arteries. We compared stimulation-evoked NE and NO release in the MCAs from normoxic and hypoxic near-term fetal and adult sheep. The facial artery was used for comparison because it lacks nNOS-containing nerves (4, 20, 21). Several time controls were used to ensure that equivalent current and voltage were being delivered to each set of electrodes in each bath.

Materials and Methods

Forty pregnant and forty nonpregnant ewes of mixed breed were obtained from one supplier (Nebeker Ranch, Lancaster, CA). These animals were randomly separated into two groups: control normoxic (20 pregnant and 20 nonpregnant) and hypoxic ewes (20 pregnant and 20 nonpregnant). Animals in the hypoxic group were transferred to the White Mountain Research Station (Bishop, CA; altitude of 3,280 m) and separated on an ESA MD-TM eluting mobile phase contained 75 mM NaHCO3, 1.2 MgSO4, 0.3 ascorbic acid, and 11.5 glucose. Animals in the hypoxic group remained at Nebeker ranch (718 m). All normoxic ewes, nonocclusive tracheal catheters were surgically implanted (14) to allow the administration of N2 gas. This was done to ensure that equivalent current and voltage were being delivered to each set of electrodes in each bath.

Tissue preparation. Three- to four-centimeter lengths of the MCAs and the comparison tissue, the facial artery, were cannulated at both ends and mounted on a low-volume perfusion system as previously described (2). The MCA segment used was the main branch from the circle of Willis. The diameters of the MCAs and facial arteries ranged from 0.8 to 1.0 mm and from 1.0 to 1.4 mm, respectively. Arteries were perfused at a rate of 1 ml/min with aerated (95% O2-5% CO2) Krebs at a perfusion pressure of ~60–70 mmHg in either artery type. The entire assembly was immersed in a circulating water bath kept at 37°C. In all experiments, a Grass model S-48 stimulator (Grass Instruments, Quincy, MA) delivered electrical field stimulation to perivascular nerves through a pair of platinum electrodes. The stimulation parameters were 8 Hz, 60 V (supermaximal voltage), 1-ms duration, and 480 pulses (1-min stimulation). We measured voltage and current delivery to the platinum electrodes in each tissue bath before and during each experiment with an ohmmeter-ammeter (Wavetek, San Diego, CA). This was done to ensure that equivalent current and voltage were being delivered to each set of electrodes in each bath.

Western blot analysis. Two hypotheses were addressed: 1) ability of NOS nerves to modulate adrenergic transmitter release will be altered by high-altitude acclimatization in an age-dependent manner, and 2) high-altitude acclimatization will alter nNOS abundance in cerebral arteries.

Effect of N⁰-nitro-L-arginine methyl ester and S-nitroso-N-acetyl-DL-penicillamine on stimulation-evoked NE release. This protocol was designed to test the effects of NOS inhibition with l-NAME on NE release. Perivascular nerves in the control normoxic and hypoxic arteries were activated three consecutive times (S1–S3) for 1 min with a 45-min rest between stimulations. These time controls were used to establish the consistency of NE release over the experimental time course. In treatment tissues, perivascular nerves were activated once for 1 min (S1) and then equilibrated for 45 min in buffer containing 10 μM N⁰-nitro-L-arginine methyl ester (l-NAME). For 1 min (S2) followed by another 45-min equilibration period where tissues were exposed to buffer containing 10 μM l-NAME and the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP), and again the nerves were activated for 1 min (S3). Control and treatment tissues were exposed throughout the experiment to deoxycorticosterone and cocaine (10⁻⁵ M) to block extraneuronal and neuronal uptake of NE, respectively. A 5-ml sample was collected before each nerve activation to enable monitoring of basal NE and NO release.

Measurement of NE. In each perfusate sample, NE was extracted with alumina at pH 8.6. The internal standard 3,4-dihydroxybenzylamine (DHBA; 400 pg) was then added by using a protocol described previously (2). A 100-μl sample of the extracted amines was injected into an ESA Coulomb II high-performance liquid chromatograph (ESA, Bedford, MA) and separated on an ESA reverse-phase C₁₈ column. The ESA MD-TM eluting mobile phase contained 75 mM
Na$_2$H$_2$PO$_4$, 0.5 M sodium dodecyl sulfate, 0.025 mM EDTA, 20% acetonitrile, and 5% methanol. The amount of NE in the injected samples was calculated with the following equation:

$$\text{NE (pg)} = \frac{\text{NE peak Ht sample}}{\text{NE peak Ht standard}} \times 100 \text{ pg DHBA} \times \frac{\text{DHBA peak Ht standard}}{\text{DHBA peak Ht sample}}$$

where Ht is height. Recovery averaged 92%. Tissue NE content was quantified by homogenizing arteries in 3 ml of 0.1 N perchloric acid, followed by centrifugation. A 300-μl aliquot of the supernatant was taken, and NE was extracted in a similar manner as the perfusate. NE content was used to calculate fractional NE release per pulse.

**Fractional NE release**

$$\text{Fractional NE release} = \frac{\text{total NE release (pg)} \times \text{number of stimulation pulses}}{\text{NE tissue content (pg)}}$$

**Measurement of NO.** NO was measured with the Seive 280B NO analyzer. Before the measurement of NO in the samples, a series of standard NaNO$_3$ solutions were run from which a standard curve was obtained. Frozen samples of perfusate were thawed, and NO was measured as previously described (36). In brief, NO was measured by injection of a 100-μl aliquot of the perfusate into a Sievers 280B NO analyzer (Sievers Instruments, Boulder, CO). In this instrument, stable nitrates and nitrites are reduced to NO. NO is then measured by reaction with ozone, which yields photons detected by a photomultiplier tube. Calibration curves were run during each analysis, and individual standard curves (0–200 pmol) were used to convert NO signals from samples into picomoles of NOx.

**Isolation of soluble protein in cerebral blood vessels.** Cerebral artery homogenates were prepared as previously described (26). Briefly, cerebral arteries were dissected from two to three brains and were weighed, pooled and homogenized in 1 ml of ice-cold N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (50 mM, pH 7.0). Facial arteries were also pooled and prepared in the same manner as cerebral arteries. Homogenates were centrifuged for 30 min at 10,000 g. The supernatant was decanted and centrifuged for 75 min at 180,000 g. Total tissue soluble-protein content was calculated from spectrophotometric measurements of soluble protein in a 10-μl aliquot of the 180,000-g supernatant by using a bicinechonic acid protein assay kit and bovine serum albumin standard obtained from Pierce (Rockford) (30).

**Western blot analysis.** Western immunoblotting techniques were used to detect and quantify the relative levels of nNOS in cerebral artery homogenates from fetal and adult normoxic and hypoxic sheep (34). Soluble protein samples and recombinant rat nNOS (brain cell Nos) standards (Biomol, Plymouth Meeting, PA) were prepared in Laemmli buffer (Bio-Rad, Hercules, CA) containing 5% 2-mercaptoethanol. Samples and standards were heated to 100°C for 5 min, separated by 7.5% Tris·HCl sodium dodecyl-sulfate polyacrylamide gels (150 V, 90 min), and transferred onto polyvinylidene difluoride membranes with a semidy transfer cell (15 V, 55 min, Bio-Rad). Completion of electrophoretic transfer of samples and nNOS standards was verified by staining the gel with Coomassie blue. After blotting was complete, gels consistently stained negative for protein bands, demonstrating complete protein transfer. Membranes were blocked for 12 h (14 h maximum) at 4°C in blocking buffer containing 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, 0.1% Tween 20, and 6% nonfat dry milk. The blocking buffer was replaced with fresh blocking buffer containing rabbit anti-rat polyclonal nNOS IgG antibody (Biomol) diluted 1:1,000 and then incubated for 1 h. Membranes were washed three times (20 min each) with buffer containing 10 mM Tris·HCl, 2 mM NaCl, and 0.1% Tween 20 and incubated for 1 h in washing buffer containing 6% nonfat dry milk and secondary alkaline phosphatase-conjugated antibody (1:1,000 dilution, Bio-Rad). Membranes were again washed three times, 20 min each, and protein bands were detected by enhanced chemiluminescence and exposed to film (Amersham Pharmacia Biotech, Newark, NJ). On each gel, a molecular weight ladder (Bio-Rad) was used to confirm the relative molecular weight of nNOS in samples and nNOS recombinant standards. Sample and recombinant nNOS standards consistently yielded a single band migrating between the molecular weight ladder standards of myosin (199) and β-galactosidase (128). The approximate molecular mass of 155 kDa in this single-sample band corresponded to the molecular weight of the rat nNOS recombinant standard (34).

**Quantification of the relative abundance of nNOS.** Densitometric analysis of autoradiographic films was used to determine relative abundance of nNOS in cerebral artery homogenates. Plots of optical density vs. nanograms of recombinant nNOS for each Western blot were used as a standard curve to quantify the relative amount of nNOS in each sample. Standard curves were linear from 4 to 30 ng of recombinant nNOS standard with a lower limit of detection of 2.5 ng. A linear equation ($r = 0.996$) was used to convert the optical density of nNOS bands in the samples to nanograms of nNOS. Relative abundance was determined simply by the ratio of nanograms nNOS detected in sample to micrograms of total soluble protein.

**Statistical analysis.** Two-way ANOVA and Fisher’s protected least significant difference test were used to analyze the impact of development and chronic hypoxia on NE content, abundance of nNOS, and stimulation-evoked NE and NO release. All data were analyzed for heterogeneity of variance by Cochran’s test. If significant heterogeneity was detected, then the data were transformed to log values and the tests repeated. The effects of L-NAME and SNAP treatment on stimulation-evoked NE release relative to controls within the groups were determined by paired Student’s t-test. Comparison of stimulation-evoked NE and NO release to basal overflow and the effect of TTX were also determined by paired t-test. In this study, a value of $P < 0.05$ was considered to be statistically significant.

**RESULTS**

**Stimulation-evoked NE and NO release in normoxic MCAs.** Activation of perivascular nerves in fetal and adult normoxic MCAs significantly increased fractional NE release (Fig. 1, A and B). In the same tissues, activation of perivascular nerves significantly increased NO overflow compared with basal release (Fig. 1, C and D). The neurotoxin TTX completely blocked stimulation-evoked NE and NO release, confirming a neuronal source for both NE and NO. Activation of perivascular nerves in fetal and adult normoxic facial arteries also significantly increased fractional NE release, and this increase was blocked with TTX (data not shown). However, in contrast to results with MCAs, in the facial artery there was no increase in NO overflow during nerve stimulation, as we have previ-
ously shown (24). TTX also had no effect on no overflow in the facial artery.

**Effects of development and chronic hypoxia on stimulation-evoked NE release.** Effects of the NOS antagonist L-NAME and the NO donor SNAP on stimulation-evoked fractional NE release from normoxic fetal and adult MCAs are shown in Fig. 2. To validate the effects of L-NAME and SNAP, time controls were used to ensure that stimulation-evoked NE release from adrenergic nerves was consistent over the experimental time course. Stimulation-evoked NE release in these time controls was consistent for both normoxic and hypoxic fetal and adult MCAs from hypoxic fetal and adult arteries (Table 1). In the presence of L-NAME, stimulation-evoked NE release significantly declined in normoxic fetal and adult MCAs. This effect was completely reversed by application of the NO donor SNAP (Fig. 2, A and B). In contrast, in MCAs from hypoxic fetal or adult animals, L-NAME and SNAP no longer had any effect on stimulation-evoked NE release (Fig. 2, C and D). In the facial artery, L-NAME had no significant effect on stimulation-evoked NE release from either normoxic or hypoxic fetal and adult animals (Table 2).

**Effect of development and chronic hypoxia on nNOS levels.** As shown in Fig. 3, A and C, soluble nNOS was detected in supernatants isolated from normoxic fetal and adult cerebral artery homogenates. There was no detectable nNOS in the pellet fraction, confirming that the homogenization method we employed efficiently extracted all nNOS into the soluble aqueous phase (Fig. 3, A–D). Interestingly, nNOS levels were significantly lower in supernatants from hypoxic than from normoxic homogenates, regardless of age (Fig. 3, B and D). In contrast to the MCAs, there was no detectable nNOS in supernatant or pellet fractions from normoxic or hypoxic fetal and adult facial arteries (data not shown).

By using the standard curve generated from nanograms of recombinant nNOS vs. optical density (Fig. 3, E and F), we quantified the relative abundance of nNOS per microgram of soluble protein in cerebral artery supernatants (Fig. 4A). The relative abundance of nNOS was similar in both normoxic fetal and adult cerebral arteries. However, the relative abundance of nNOS significantly declined in hypoxic compared with normoxic cerebral arteries but did not vary significantly with age in the hypoxic group (Fig. 4A). Stimulation-evoked NO release normalized to total soluble protein was not significantly different in normoxic fetal compared with adult MCAs (Fig. 4B). Consistent with the decline in relative nNOS abundance in cerebral arteries from hypoxic fetus and adult, stimulation-evoked release of NO dramatically declined in MCAs from hypoxic fetal and adult sheep compared with normoxic (Fig. 4B).

**NE content and basal NE release.** Table 3 summarizes NE content in MCAs from fetal and adult normoxic and hypoxic sheep. NE content in the MCAs of normoxic and hypoxic fetal sheep was significantly lower compared with the MCAs from both the normoxic and hypoxic adult. NE content was depressed in MCAs from both fetal and adult sheep acclimatized to chronic hypoxia, although this effect reached statistical significance only in adult animals.

In Table 4, the impact of development and drug treatment on basal NE overflow in the MCAs is summarized. Exposure to L-NAME or SNAP did not significantly affect basal NE overflow in arteries from either normoxic or hypoxic animals. Basal NE overflow from the MCAs was significantly greater in normoxic and hypoxic fetal arteries compared with adult. Chronic hypoxia did not significantly alter basal NE overflow in MCAs from fetal or adult animals.
DISCUSSION

The most interesting result of this study is that, in cerebral arteries from adult and fetal sheep, chronic hypoxia causes a loss of stimulation-evoked NO release and a concomitant decline in nNOS levels. To our knowledge, this is the first time that a substantial loss of function of NOS-containing nerves in the cerebral circulation has been demonstrated after high-altitude hypoxia. Despite the hypoxic-induced loss of nNOS nerve function that normally augments NE release,

Table 1. Time controls establishing consistency of stimulation-evoked fractional NE release in MCA from normoxic and hypoxic fetal and adult sheep

<table>
<thead>
<tr>
<th></th>
<th>Fractional NE Release, pg/pg content/pulse × 10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td>Fetus</td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>108 ± 32</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>112 ± 16</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>32 ± 4.3</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>48 ± 6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 11–13 arteries from 11–13 animals. All arteries were treated with deoxycorticosterone and cocaine throughout the experiment. NE, norepinephrine; MCA, middle cerebral arteries; S1, S2, and S3, first, second, and third stimulation, respectively.

Table 2. Lack of effect of l-NAME and SNAP on stimulation-evoked fractional NE release in facial arteries from normoxic and hypoxic fetal and adult sheep

<table>
<thead>
<tr>
<th></th>
<th>Fractional NE Release, pg/pg content/pulse × 10⁻⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S1</td>
</tr>
<tr>
<td>Fetus</td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>65 ± 16</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>90 ± 15</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>21.2 ± 5.2</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>18.2 ± 2.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–13 arteries from 6–13 animals. All arteries were treated with deoxycorticosterone and cocaine throughout the experiment. l-NAME, N⁶-nitro-l-arginine methyl ester; SNAP, S-nitroso-N-acetyl-dl-penicillamine.
stimulation-evoked NE release was not significantly different in arteries from normoxic and hypoxic animals. These results underscore the adaptive potential of the cerebral circulation during moderate, chronic high-altitude hypoxia. Our laboratory’s previous studies have shown that during high-altitude hypoxia, blood pressure rises both in the fetus and adult (21). The present results further suggest that hypoxia induced attenuation of the function of nNOS nerves contributes to the pressor effects of altitude acclimatization.

Model of chronic hypoxia in adult and fetus. Adaptation to physiological stresses such as chronic hypoxia involves the sympathetic nervous system (5, 28). Stress imposed by chronic hypoxia can be particularly intense in fetal mammals confined to a lowered O2 tension environment. To compensate for this O2 deficit, normoxic mammalian fetuses exhibit high cardiac output and O2 delivery (22). In this study, pregnant sheep were maintained at 3,820 m, resulting in moderate maternal and fetal hypoxia, well in the range of successful adaptation in sheep. Whereas the compensatory cardiovascular, pulmonary, and hematological consequences of high-altitude hypoxemia are well defined (17), there appears to be a “threshold” of O2 deprivation, which, if exceeded, leads to pathological consequences (21). During ovine gestation at 3,820 m, adult and fetal arterial PO2 values decline, whereas arterial pH remains unchanged, and hematocrit increases to maintain O2 delivery (16). Hypoxic fetal weights increase during gestation, and near-term fetal weights are comparable to control fetuses maintained at 718 m. Fetal morbidity, mortality, and abortion do not increase in this model. Animals in the present study were exposed to the same degree of hypoxic stress as in our laboratory’s previous studies, with arterial PO2 values similar to previously published values in both age groups (3, 4, 20, 21). Overall, the high-altitude model we employed in these studies represents moderate chronic hypoxia, enabling adaptive responses to be studied.

**Function of adrenergic and nNOS nerves.** Activation of perivascular nerves in MCAs from normoxic fetal and adult sheep caused a significant increase in both NE and NO over flow compared with the respective basal overflows. Stimulation-evoked NE and NO release were antagonized by the neurotoxin TTX, confirming a neuronal source of both substances. Furthermore, in MCAs from normoxic fetal and adult sheep, stimulation-evoked NE release was antagonized by the NOS inhibitor L-NAME, an effect reversed by the NO donor SNAP. These data confirm that cerebrovascular arteries are unique, containing both adrenergic and nNOS neurons, and that NE release is facilitated by NO. The conclusions derived from the MCAs are strengthened by studies of our comparison model, the facial artery. Electrical stimulation has been previously shown to increase NE release in the facial artery of adult animals, but there is no increase in NO overflow and no effect of L-NAME on NE release (4, 24). In the present study, we confirm and extend these results showing a similar lack of NOS function in facial arteries from the normoxic fetal sheep. Overall, the present data are in agreement with our previous stud-
ies suggesting that MCAs contain both adrenergic and nNOS nerves (4, 24). In addition, these data are consistent with other studies suggesting that cerebral vessels contain nNOS neurons (19, 29, 33, 39).

Given the close proximity of adrenergic and nNOS nerves, functional interactions between these nerves have been a topic of interest (18, 28). In rat atria

**Table 3. Effect of development and chronic hypoxia on NE content in MCA**

<table>
<thead>
<tr>
<th></th>
<th>NE Content, ng/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetus</td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>26 ± 6†</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>50 ± 8*</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
</tr>
<tr>
<td>Normoxic</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Hypoxic</td>
<td>50 ± 8*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 11–13 arteries from 11–13 animals. *Significantly different from hypoxia, P < 0.01. †Significantly different from adult, P < 0.01.

mesenteric arteries and cerebral cortex and in sheep MCAs, stimulation-evoked NE release is inhibited by NOS antagonists (4, 13, 24, 25, 37, 38). These studies suggest that NO from nNOS nerves serves to augment adrenergic nerve activity. Indeed, our present study shows that, in both normoxic fetal and adult MCAs, L-NAME significantly inhibits stimulation-evoked NE release. These results demonstrate the facilitatory effect of NO on adrenergic nerve activity in the cerebral vasculature.

The mechanism underlying this facilitatory effect of NO is unclear, but studies have begun to illuminate this process. In superior cervical ganglion cells, NO donors such as SNAP increase calcium current amplitude, whereas in PC12 cells NO donors have been shown to increase the sensitivity of intracellular calcium release mechanisms to calcium (7, 9). These effects are mimicked by the cGMP analog 8-bromo-cGMP, suggesting that NO signaling through the cGMP pathway may elevate stimulation-evoked increases in intracellular calcium. This provides at least one explanation for the ability of NO to augment stimulation-evoked NE release.

**Hypoxia and nNOS in cerebral arteries.** In complete contrast to MCAs from normoxic sheep, stimulation-evoked NO release as well as the effect of L-NAME were selectively abolished in MCAs from hypoxic fetal and adult sheep. These data are consistent with our laboratory’s previous study demonstrating that the effect of L-NAME to depress stimulation-evoked NE release in adult and fetal MCAs is abolished with chronic hypoxia (4). Despite a decline in function of facilitatory nNOS nerves after hypoxic acclimatization, stimulation-evoked NE release was not significantly altered in fetal or adult MCAs (Fig. 2). NE content in both fetal and adult MCAs declined during chronic hypoxia (Table 3); despite this decline, NE release was maintained. These data suggest that adrenergic nerves in fetal and adult cerebral arteries may compensate for hypoxic declines in function of nNOS nerves and NE content to maintain their overall function. Further studies are necessary to define the mechanisms underlying sustained adrenergic nerve function during chronic hypoxia.
To further our understanding of the impact of chronic hypoxia at high altitude, we measured the relative abundance of nNOS in cerebral arteries in fetal and adult sheep and expressed stimulation-evoked NO overflow as a fraction of total soluble protein. We found that the abundance of nNOS in cerebral arteries dramatically declined after high-altitude chronic hypoxia in both the fetus and adult. Consistent with these data, stimulation-evoked NO release as a fraction of soluble protein also declined, and there was no significant difference between hypoxic fetus and adult. These data suggest that at least one mechanism for the decline in NO release and the corresponding loss of the effect of l-NAME on stimulation-evoked NE release is the marked decline in the levels of perivascular nNOS. To our knowledge, this is the first study to report such results.

In normoxic animals, the relative abundance of nNOS was similar in fetal and adult cerebral arteries. Second, stimulation-evoked NO release as a fraction of total soluble protein was also similar in fetal and adult cerebral arteries. Thus, although total stimulation-evoked NO overflow in fetal was lower than adult MCAs, when corrected for total soluble protein content stimulation-evoked NO release was similar in the fetus and adult. These data contrast a previous study demonstrating that maximal nNOS activity is greater in the brain of adult rats compared with 14- to 18-day-old pups (26). This study, carried out in the presence of saturating levels of cofactors, suggested that a change in the function of NO with development could be due in part to changes in cerebral nNOS levels during prenatal and early postnatal life. Consistent with this idea, it has been shown that hormones known to change during pregnancy and development affect nNOS levels in the rat brain (12). However, the study of Pearce et al. (26) focused on brain parenchyma, which is undergoing rapid growth and development, whereas our study focuses on vascular innervation, which might have a very different timetable for developmental patterns of protein expression. Overall, the present study suggests that mechanisms controlling cerebrovascular nNOS protein levels appear to be near or fully developed in the near term fetus.

Effect of development on NE release. There is little information on the impact of development specifically on stimulation-evoked fractional NE release. In this study, both basal and stimulation-evoked fractional NE release from MCAs were significantly greater in the fetus than in the adult. Thus, during development, adrenergic nerve function appears to decline. The data in this study are consistent with our laboratory’s previous studies suggesting that adrenergic nerve activity rises just before birth (3, 4). Moreover, the data are consistent with rising plasma levels of NE in both rats and sheep (8, 31). The rise in sympathetic nerve activity before birth suggests that the fetus must prepare to transition from a highly modulated environment to the external environment where multiple vascular mechanisms become essential for homeostasis (27).

In conclusion, we have shown that nNOS nerves augment stimulation-evoked NE release in cerebral vessels of both adult and fetal sheep. Furthermore, we confirm that both our initial hypotheses are correct: 1) acclimatization to chronic high-altitude hypoxia during gestation markedly attenuates the function of nNOS nerves and 2) high-altitude hypoxia drastically reduces nNOS abundance in cerebral arteries. Despite the loss of nNOS nerve function, adrenergic nerves maintain their activity, supporting successful adaptation to high-altitude hypoxia. Our study demonstrates that at least one mechanism underlying the decline in nNOS nerve function is a significant decline in nNOS levels during chronic hypoxia. Although in our study successful adaptation to high-altitude hypoxia appears to have occurred, the loss of function of nNOS nerves may still have consequences for the distribution of cerebral blood flow during acclimatization at high altitude.

This work was supported in part by National Institute of Child Health and Human Development Grant P01-HD-31226, American Heart Association National Affiliate Established Investigator Award 0040621N, and National Heart, Lung, and Blood Institute Grant RO1-HL-50775.

REFERENCES

FUNCTION OF NOS NEURONS IN SHEEP CEREBRAL ARTERIES


