Pre- and postjunctional \(\alpha_2\)-adrenergic receptors in fetal and adult ovine cerebral arteries

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Bishai, John M., Luit Penninga, Roel Nijland, Rogier Meulenaar, Ciprian P. Gheorghe, Yu Zhao, John N. Buchholz, Lubo Zhang, and Lawrence D. Longo. Pre- and postjunctional \(\alpha_2\)-adrenergic receptors in fetal and adult ovine cerebral arteries. Am J Physiol Regulatory Integrative Comp Physiol 282: R1654–R1662, 2002; 10.1152/ajpregu.00475.2001.—In ovine cerebral arteries, adrenergic-mediated vasoconstrictor responses differ significantly with developmental age. We tested the hypothesis that, in part, these differences are a consequence of altered \(\alpha_2\)-adrenergic receptor (\(\alpha_2\)-AR) density and/or affinity. In fetal (\(\sim\)140 days) and adult, we measured \(\alpha_2\)-AR density and affinity with the antagonist \(\text{[\text{H]}idazoxan in main branch cerebral arteries and other vessels. We also quantified contractile responses in middle cerebral artery (MCA) to norepinephrine (NE) or phenylephrine in the presence of the \(\alpha_2\)-AR antagonists yohimbine and idazoxan and contractile responses to the \(\alpha_2\)-AR agonists clonidine and UK-14304. In fetal and adult cerebral arteries homogenates, \(\alpha_2\)-AR density was \(201 \pm 18\) and \(52 \pm 6\) fmol/mg protein, respectively (\(P < 0.01\); however, antagonist affinity values did not differ. In fetal, but not adult, MCA, \(10^{-7}\) M yohimbine significantly decreased the \(pD_2\) for NE-induced tension in the presence of \(3 \times 10^{-5}\) M cocaine, \(10^{-5}\) M deoxycorticosterone, and \(10^{-6}\) M tetrodotoxin. In fetal, but not adult, MCA, UK-14304 induced a significant decrease in \(pD_2\) for the phenylephrine dose-response relation. In addition, stimulation-evoked fractional NE release was significantly greater in fetal than in adult cerebral arteries. In the presence of \(10^{-6}\) M idazoxan to block \(\alpha_2\)-AR-mediated inhibition of prejunctional NE release, the fractional NE release was significantly increased in both age groups. We conclude that in fetal and adult ovine cerebral arteries, \(\alpha_2\)-AR appear to be chiefly prejunctional. Nonetheless, the fetal cerebral arteries appear to have a significant component of postjunctional \(\alpha_2\)-AR.

cerebrovascular circulation; vascular smooth muscle; norepinephrine; clonidine; UK-14304; yohimbine; idazoxan; tetrodotoxin; fetus

THE CEREBRAL VASCULATURE is rich in adrenergic innervation, which arises predominantly from the superior cervical ganglia (5, 9, 25). Under normal conditions, these nerves may not play an important role, inasmuch as myogenic autoregulatory mechanisms maintain cerebrovascular tone (9). Nonetheless, cerebrovascular adrenergic nerves appear to help maintain cerebral vascular tone under conditions of hypoxia and hypertension (9, 45). In addition, considerable evidence supports the importance of the \(\alpha\)-adrenergic system in cerebrovascular reactivity (3, 17, 21–23). Cerebral blood vessels are reported to contract in response to norepinephrine (NE) via stimulation of postjunctional \(\alpha_1\)- and/or \(\alpha_2\)-adrenergic receptors (AR) (3, 32). \(\alpha_2\)-AR, which also may be prejunctional, have been shown to play an important role in cerebrovascular reactivity in several species (3, 4, 10, 11, 27, 33, 35, 36, 40, 42). However, no unified role for cerebrovascular \(\alpha_2\)-AR has been shown to exist throughout all species (18). Activation of prejunctional \(\alpha_2\)-AR inhibits NE release from sympathetic nerves by negative feedback (14, 34) and, thus, may play a role in inhibiting NE release under conditions of hypoxia and other stress (20).

Cerebral artery adrenergic reactivity varies as a function of vessel size (17, 22) and age (17, 21, 23, 31). For instance, in the human saphenous vein, \(\alpha_2\)-AR activity decreases with age (15); however, in Fischer 344 rat tail artery, there appears to be no effect of age on postjunctional \(\alpha_2\)-AR (41). Nonetheless, the functional role of \(\alpha_2\)-AR in the cerebral arteries of sheep, a species subject to considerable experimental study, and how this role might vary with developmental age are unknown. Thus the present study was designed to test the hypothesis that, in ovine cerebral arteries, \(\alpha_2\)-AR density and/or affinity, \(\alpha_2\)-AR-mediated contractile responses, and \(\alpha_2\)-AR-mediated NE release vary as a function of age from fetus to adult.

METHODS

Experimental animals and tissues. We obtained cerebral arteries from 32 near-term fetuses (\(\sim\)140 days gestation) and 30 nonpregnant adult sheep (\(\leq 2\) yr) from Nebeker Ranch (Lancaster, CA). When twin fetuses were obtained, we used only one fetus of the pair. For comparative purposes, we also obtained vessels from 18 newborn lambs (2–5 days old). All 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.
experiments were performed in accordance with approved institutional animal care guidelines. We anesthetized and killed the ewes and newborn lambs with pentobarbital sodium (100 mg/kg iv); then we obtained isolated cerebral artery segments. We previously showed that death by this method has no significant effect on vessel reactivity compared with death by other anesthetic agents (21, 31). All contractility studies were performed in isolated main branch middle cerebral arteries (MCA; 450 ± 25 and 350 ± 25 μm diameter in adult and fetus, respectively) cleaned of adipose and connective tissue.

α₂-AR binding. For measurement of α₂-AR density and affinity, we obtained main branch, anterior, middle, and posterior cerebral arteries. In addition, we prepared cerebral cortex microvessel and capillary fractions from fetal and adult brain parenchyma, as we described previously (22). Vessels used for receptor analysis were rapidly removed, cleaned, and frozen in liquid nitrogen and kept at −80°C until assay. Cerebral artery samples from four to six brains were pooled separately to obtain enough membrane protein for a single assay (22). All vessels were homogenized (Polytron 10/35, Brinkmann) in 10 volumes (wt/vol) of iced 50 mM Tris-HCl, pH 7.4 (with the tube immersed in a beaker with ice and care taken to avoid foaming), and centrifuged at 300 g for 5 min at 4°C (IEC-DPR-6000) to remove cellular debris and large vessel fragments. The supernatant was then centrifuged at 48,000 g for 60 min at 4°C. After the supernatant was discarded, twice the volume of iced buffer, pH 7.4, was added to obtain a protein concentration of 1 mg/ml (6). Unless otherwise noted, all chemical compounds were purchased from Sigma Chemical (St. Louis, MO).

For α₂-AR saturation binding, we used the selective α₂-AR antagonist [3H]idazoxan (New England Nuclear, Boston, MA). Nonselective binding was determined with phenotamine (10−8 M final concentration). Each assay tube contained 0.25 ml of [3H]idazoxan (0.02–4 nM), 10−5 M phenotamine, and 2.0 ml of membrane protein (1.0 mg/ml final concentration). The membrane suspension was added in a timed sequence to start the binding. The tubes were mixed and incubated for 2 h at 30°C; then cold Tris (2.0 ml) was washed over Whatman GF/B filters in a cell harvester (Brandel, Gaithersburg, MD) according to standard protocol. The filters were counted in 3.8 ml of Hydrofluor (National Diagnostics, Atlanta, GA).

Saturation curves were analyzed by use of nonlinear least-squares regression to fit binding data to a rectangular hyperbola. This fitting generated both the maximal radioligand binding or receptor density (Bmax) and the dissociation constant (KD) values (Inplot, version 3.0, Graphpad Software, San Diego, CA).

Measurement of isometric tension. As noted above, we isolated and removed, without stretching, MCA from near-term fetal and nonpregnant adult sheep. From each animal, four artery segments (1 for each of 4 vessel baths) were obtained and used as previously described (21, 23). To avoid the complication of endothelium-mediated effects, we removed the endothelium by carefully inserting a small wire three times. To confirm endothelium removal, we contracted the vessel with 10−5 M 5-hydroxytryptamine and, at the plateau, added 10−6 M ADP. Vessels that relaxed >20% after this treatment were excluded from further study (21). Segments (5 mm) of each vessel were cannulated with tungsten mounting wires and suspended between a force transducer (Kulite BG-10) and a micrometer-driven post used to control resting tension. The vessels were suspended in an oxygenated standard Krebs solution containing (in mM) 122 NaCl, 25.6 NaHCO3, 5.56 dextrose, 5.17 KCl, 2.49 MgSO4, 1.60 CaCl2, 0.114 ascorbic acid, and 0.027 disodium EDTA. In addition to control experiments under these conditions, in about two-thirds of the experiments, we added 10−5 M cocaine and 10−6 M deoxycorticosterone to block neural and extraneuronal uptake of NE, respectively, 10−6 M propranolol to inhibit β-AR, and 3.6 × 10−4 M iproniazid to block monoamine oxidase metabolism of NE. Iproniazid was added for 40 min, and tissues were washed four times over 30 min with fresh Krebs buffer. Cocaine, deoxycorticosterone, and propranolol were added 15 min before addition of agonist. Also in one-third of these studies, to minimize interference of presynaptic α₁-AR, tissues were equilibrated in Na+ -Krebs buffer containing 10−6 M tetrodotoxin. The bath chambers were bubbled with 95% O2-5% CO2 at 38°C. We allowed 30 min for equilibration at optimum resting tension. On the basis of our previous studies, the optimum resting tensions were 0.4 and 0.6 g for fetal and adult MCA, respectively (31). With these general techniques, we performed the following studies.

NE dose-response relations and effects of antagonists. To establish control NE dose-response curves of fetal and adult cerebral arteries and to confirm our previous results, four vessel segments from each age group were mounted and incubated for 20 min in Krebs buffer. Then we contracted the endothelium-denuded arteries by exposure to isotonic K+-Krebs solution containing 120 mM KCl and 31 mM NaCl (i.e., to depolarize the vascular smooth muscle). After peak tension was obtained, we washed the vessels with normal Na+ -Krebs solution and allowed them to return to baseline tension for 20 min. We repeated the K+ depolarization three times, allowing 15 min after each K+ washout. We then induced contraction with 10−6 M NE. After washout and reequilibration for 40 min, we added cumulative doses of NE in half-log increments (10−8–10−3 M). We also performed time-control studies on these vessels by repeating the dose responses at least three times over a 2- to 3-h period. These experiments confirmed the NE pD2 (negative logarithm of the EC50, or half-maximal contraction for NE, and an index of tissue “sensitivity” or “potency”), the maximum response (NEmax), and the reproducibility of the response in these vessels (n = 5 each).

In addition, we examined NE dose response in the presence of the α1-AR antagonist prazosin (10−8 M) or the α₂-AR antagonist yohimbine (10−7 M), each added 15 min before NE. For instance, in bath 1, to obtain control values, we performed two to three NE dose-response curves (10−8–10−3 M). In bath 2, after the control NE dose response, we repeated it in the presence of 10−7 M prazosin. In bath 3, after the control NE dose response, we repeated it in the presence of 10−7 M yohimbine (n = 5 each). In bath 4, we repeated the protocol for bath 2 or 3. Again, in a companion series of studies, after the control NE dose response, we repeated the NE dose response in the presence of cocaine, deoxycorticosterone, and tetrodotoxin (referred to as the “cocktail”). To establish that the α₂-AR were indeed blocked, we repeated the NE dose-response curves in vessels pretreated with the α₂-AR antagonist idazoxan (3 × 10−7 M) for 15 min.

α₂-AR agonist dose-response study. In fetal and adult cerebral arteries, we quantified the dose-response curves for the relatively selective α₂-AR agonists clonidine and 5-bromo-6-[2-imidazolin-2-yl-amino]-quinoxaline (UK-14304, brimonidine) at 10−8–10−3 M in half-log increments (n = 4 each). To minimize interference of presynaptic α₂-AR in these studies, tissues were equilibrated in Na+ -Krebs buffer containing the cocaine-deoxycorticosterone-tetrodotoxin cocktail. These experiments were designed to define the agonist pD2, the NEmax, and the reproducibility of the response. Again, we determined the maximum contraction produced by KCl, which was used to normal-
ize the α2-AR agonist-induced response, e.g., as percent K+ maximum (K+max). We also examined the effect of α2-AR blockade on MCA before adding clonidine or UK-14304. We administered the α2-AR antagonist yohimbine (10^-7 M); then after 15 min we examined the clonidine (10^-8–10^-3 M) dose-response relation (again, in the presence of the cocktail). In another series of arteries, we examined the clonidine dose response in the presence of the α1-AR antagonist prazosin (10^-8 M) added 15 min before clonidine. We compared these responses with the several agonists and antagonists, clonidine and yohimbine, NE, and prazosin (n = 5 ea).

**Effect of α2-AR agonist on α1-AR agonist dose response.** In a control phenylephrine dose-response curve (10^-8–10^-3 M), the vessel was then rinsed five times, and fresh buffer was added. Then we added the α2-AR agonist clonidine or UK-14304 (10^-8 or 3 × 10^-7 M, respectively); both of these doses were "subthreshold," in that they did not cause contraction by themselves and, after 15 min, repeated the phenylephrine dose response (10^-8–10^-3 M in half-log increments).

**Fractional NE release.** Segments of MCA (3–4 cm) were cannulated at both ends with polyethylene tubing and mounted in a low-volume perfusion system as described by Buchholz and Duckles (8). The MCA segment was the main branch from the circle of Willis. MCA were 0.8–1.1 mm in diameter in fetus and adult and were perfused with aerated (95% O2-5% CO2) Krebs solution at a rate of 1.0 ml/min, creating a perfusion pressure of 55–65 mmHg. The perfusion assembly was immersed in a circulating water bath and kept at 37°C.

Electrical field stimulation was delivered to perivascular nerves through a pair of platinum electrodes linked to a stimulator (model S-48, Grass Instruments, Quincy, MA). The stimulation parameters were 8 Hz, 60 V, 1-ms duration, and 480 pulses (1-min stimulation). In each experiment, one MCA served as a time control. Mbaku and co-workers (24) recently showed that nitric oxide released from nitric oxide synthase (NOS)-containing nerves augments stimulation-evoked NE release. Therefore, to remove the influence of NOS-containing nerves, tissues were continuously exposed to an inhibitor of NOS, N^ω-nitro-L-arginine methyl ester (t-NAME, 10^-5 M).

Consistency of stimulation-evoked NE release over the duration of the experiment was established by activation of perivascular nerves in the control MCA two consecutive times for 1 min, with a ≥30-min equilibration separating each stimulation. Control tissues were activated for 1 min in the absence of NE reuptake blockade, and, after a 30-min equilibration, tissues were activated for 1 min in the presence of cocaine and deoxycorticosterone, together with the α2-AR antagonist idazoxan (10^-7 M). Perfusates were collected at the start of each stimulation period until 5 ml were collected. Basal NE release was monitored by collecting 5 ml of perfusate before each stimulation. Perfusates were extracted with alumina and quantified with dihydroxybenzylamine as an internal standard (300 pg), as previously described (8). A 100-μl sample of extracted amines was then injected into a high-pressure liquid chromatograph (Coulochem II, ESA, Bedford, MA) and separated on a reverse-phase C18 column (ESA) with MD-TM aqueous mobile phase (ESA). The mobile phase contained (in mM) 75 Na2HPO4, 500 sodium dodecyl sulfate, 0.025 EDTA, 20% acetonitrile, and 5% methanol. The amount of NE in the injected and collected samples was calculated as follows: stimulation-evoked fractional NE release = picograms of NE released + picograms of NE tissue content × number of stimulation pulses / ng of tissue. Recovery varied from 85 to 98%.

**Statistical analysis.** Values are means ± SE. Except for the pooled tissues used for the receptor density and affinity assays, in all cases, n refers to the number of vessel segments (which corresponds to the number of animals) studied. Because of the nature of these studies, several tests were used to test for significant differences. For testing differences between two groups, we used a simple unpaired Student’s t-test. For multiple comparisons, one-way analysis of variance (ANOVA; age), coupled with Duncan’s multiple range test, was used. Where appropriate, we used ANOVA with repeated measures. The effect of the α2-AR blocker idazoxan on NE release was analyzed by paired t-test. The impact of development on NE release between the treatment groups was analyzed by two-way ANOVA and Fisher’s protected least significant difference test. P < 0.05 was considered significant.

**RESULTS**

**α2-AR binding.** To examine the extent to which main branch and other cerebral arteries possess α2-AR, we quantified α2-AR density. Figure 1A shows representative α2-AR binding curves for fetal sheep main branch arteries.

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**Fig. 1.** A: saturation binding of [3H]idazoxan to near-term fetal main branch cerebral arteries (combined anterior, middle, and posterior). Aliquots of plasma membranes were incubated with various concentrations of [3H]idazoxan. •, Total binding; ●, specific binding; ▼, nonspecific binding. Nonspecific binding was determined with phen tolamine (see METHODS). B: left, α2-adrenergic receptor (α2-AR) density (Bmax) as determined with [3H]idazoxan in main branch cerebral arteries of fetal and adult sheep. Values are means ± SE. Receptor density was significantly greater for fetal than for adult vessels (P < 0.01). Right, α2-AR affinity (Kd) values for fetal and adult cerebral arteries.
cerebral arteries. We observed saturable binding for fetus and adult. Nonspecific binding was linear in all preparations and accounted for 12 and 22% of total binding in fetal and adult cerebral vessels, respectively, at the [3H]idazoxan concentrations that equaled the mean $K_D$ (0.55 ± 0.10 nM). Scatchard plots were linear in all studies, with Pearson correlation coefficients of 0.930–0.996. Similarly, corresponding Hill coefficients ranged from 0.987 to 0.997 in each experiment. This analysis indicated the presence of a single class of $\alpha_2$-AR binding sites in each of the two age groups.

Table 1. $B_{\text{max}}$ and $K_D$ in ovine cerebral artery $\alpha_2$-AR

<table>
<thead>
<tr>
<th>Common carotid</th>
<th>Fetus</th>
<th>Newborn</th>
<th>Adult</th>
</tr>
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<tbody>
<tr>
<td>$B_{\text{max}}$, fmol/mg</td>
<td>13 ± 2*</td>
<td>11 ± 1*</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>$K_D$, nM</td>
<td>0.65 ± 0.10(4)</td>
<td>0.64 ± 0.10(4)</td>
<td>0.69 ± 0.10(4)</td>
</tr>
<tr>
<td>Circle of Willis</td>
<td></td>
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<tr>
<td>$B_{\text{max}}$, fmol/mg</td>
<td>115 ± 10*</td>
<td>36 ± 4</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>$K_D$, nM</td>
<td>0.45 ± 0.10(4)</td>
<td>0.45 ± 0.10(4)</td>
<td>0.42 ± 0.10(4)</td>
</tr>
<tr>
<td>Anterior, middle, and posterior cerebral vessels</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>$B_{\text{max}}$, fmol/mg</td>
<td>201 ± 18*</td>
<td>71 ± 9</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>$K_D$, nM</td>
<td>0.54 ± 0.10(7)</td>
<td>0.71 ± 0.10(4)</td>
<td>0.47 ± 0.10(8)</td>
</tr>
<tr>
<td>Cerebral microvessels</td>
<td></td>
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</tr>
<tr>
<td>$B_{\text{max}}$, fmol/mg</td>
<td>135 ± 15*</td>
<td>†</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>$K_D$, nM</td>
<td>0.71 ± 0.10(3)</td>
<td>†</td>
<td>0.63 ± 0.10(3)</td>
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</table>

Values are means ± SE of number of individual assays in parentheses, with vessels from 4–6 brains per assay. $B_{\text{max}}$, maximal radioligand binding or receptor density; $K_D$, dissociation constant; $\alpha_2$-AR, $\alpha_2$-adrenergic receptor. †Newborn cerebral microvessels were not assayed. *P < 0.01 vs. adult.

Figure 1B shows the $\alpha_2$-AR density values ($B_{\text{max}}$), as measured with saturation binding of [3H]idazoxan for main branch cerebral arteries of near-term fetus and adult: 201 ± 18 and 52 ± 6 fmol/mg protein, respectively ($P < 0.01$; Table 1). For comparison, Table 1 gives the corresponding $B_{\text{max}}$ value for newborn cerebral arteries. For the fetus and adult, [3H]idazoxan affinity ($K_D$) was 0.54 ± 0.10 and 0.47 ± 0.10 nM, respectively (not significantly different). In cerebral microvessels of fetus and adult, $B_{\text{max}}$ values were not greatly different from those in the main branch cerebral arteries (Table 1). In contrast, in fetal, newborn, and adult common carotid arteries, the $\alpha_2$-AR densities were 13 ± 2, 11 ± 1, and 6 ± 1 fmol/mg, respectively (Table 1).

**NE-induced contractile responses.** To examine the effects of $\alpha_2$- or $\alpha_1$-AR blockade on NE-induced tension, we examined NE (10$^{-8}$–10$^{-3}$ M) dose-response relations in the presence of 10$^{-7}$ M yohimbine (an $\alpha_2$-AR antagonist) or 10$^{-8}$ M prazosin (an $\alpha_1$-AR antagonist). Figure 2A shows the percent NE-induced tension (as percentage of NEmax) of adult MCA in response to increasing NE concentrations under control conditions and in the presence of yohimbine or prazosin. Neither the percent maximum values at 10$^{-4}$ M NE nor the pD2 values, under control conditions or in the presence of yohimbine, were significantly different (Table 2). Prazosin significantly decreased pD2 (Fig. 2A, Table 2). To examine the effects of pretreatment with 3 × 10$^{-7}$ M idazoxan on NE-induced contraction, we studied the contractile response in absolute terms and as a percentage of NEmax. For the adult, the NE responses in...
the presence of idazoxan were not significantly different from those for yohimbine (Table 2).

To examine the role of blockade of neuronal and extraneuronal NE uptake and to minimize sympathetic nerve-associated presynaptic α2-AR, we also quantified NE-induced responses in the presence of 3 × 10⁻⁵ M cocaine, 10⁻⁵ M deoxycorticosterone, and 10⁻⁶ M tetrodotoxin. Figure 2B shows the NE concentration-response relations for adult MCA in the presence of cocaine, deoxycorticosterone, and tetrodotoxin. Under these conditions, the percent maximum tension values at 10⁻⁴ M NE for NE alone or in the presence of yohimbine or prazosin were markedly attenuated, as were the corresponding pD₂ values (Table 2). When calculated as percentage of NE max, however, the value for yohimbine was only slightly decreased. Under all conditions, contractile responses in the presence of tetrodotoxin (but not cocaine and deoxycorticosterone alone) were 33–53% of control (Table 2).

Figure 2C shows the NE-induced contractile responses of fetal MCA under control conditions and in the presence of 10⁻⁷ M yohimbine and 10⁻⁸ M prazosin. The maximum values at 10⁻⁴ M NE were similar with or without yohimbine. The corresponding pD₂ values also did not differ, although that in the presence of prazosin was much less (Table 2). In fetal MCA treated with 3 × 10⁻⁷ M idazoxan followed by 10⁻⁸–10⁻³ M NE, the responses did not differ significantly from those for yohimbine (Table 2).

Figure 2D shows the NE concentration-response relations for fetal MCA in the presence of yohimbine and prazosin, as well as in the presence of the cocaine-deoxycorticosterone-tetrodotoxin cocktail. Under these conditions, the maximum tensions at 10⁻⁴ M NE were significantly decreased. Again, and as with the adult, under all conditions in the presence of TTX (but not cocaine and deoxycorticosterone alone), the contractile responses were 29–55% of control. The corresponding pD₂ values in the presence of NE alone and in the presence of NE and yohimbine were 5.3 ± 0.1 and 4.7 ± 0.1, respectively (Table 2), with the value in the presence of 10⁻⁷ M yohimbine significantly different from the NE control (P < 0.05).

Lack of clonidine-induced responses. To examine the effect of the α2-AR agonist clonidine (10⁻⁸–10⁻³ M) on MCA tension under control conditions, we measured the dose-response relations. Under control conditions and in the presence of the cocktail, clonidine failed to produce a significant increase in tension in adult or fetal MCA, except at the highest concentration (data not shown). To verify this lack of response to α2-AR agonists, we repeated these studies using UK-14304. As in the case of clonidine, neither adult nor fetal MCA responded to 10⁻³–10⁻⁴ M UK-14304 under control conditions or in the presence of the cocktail (data not shown).

Effect of α2-AR agonist on adrenergic-induced response. To examine the effect of α₂-AR agonist on α₁-AR agonist-induced contraction, we incubated the vessels in 3 × 10⁻⁷ M UK-14304 and quantified the phenylephrine-induced tension. Figure 3A shows the phenylephrine concentration-response curves for adult MCA as a control and in the presence of 3 × 10⁻⁷ M UK-14304. Under these conditions, the phenylephrine-induced tensions and pD₂ values were not significantly different (Table 3).

Figure 3B shows the phenylephrine dose-response curves for fetal MCA under control conditions and in the presence of 3 × 10⁻⁷ M UK-14304. At 10⁻⁴ M phenylephrine, the maximum tension values were somewhat less, and corresponding pD₂ values differed significantly (5.3 ± 0.1 and 4.9 ± 0.1, respectively, P < 0.05; Table 3).

Although not shown, similar results were observed after pretreatment of the vessels with 10⁻⁶ M clonidine followed by 10⁻⁸–10⁻³ M phenylephrine. For the adult MCA under these conditions, the maximum tension values at 10⁻⁴ M phenylephrine were similar, as were the pD₂ values (Table 3). In contrast to 10⁻⁶ M clonidine pretreatment of fetal MCA, the maximum tensions at 10⁻⁴ M phenylephrine and pD₂ values were significantly less than control (Table 3).

Fractional NE release. To assess the effect of α₂-AR inhibition on NE release, we measured stimulation-evoked fractional NE release in the presence of cocaine and deoxycorticosterone to block neuronal and extra-

Table 2. Peak responses of vascular tension in MCA to NE with and without α-AR₂ antagonists

<table>
<thead>
<tr>
<th></th>
<th>Adult</th>
<th>Fetus</th>
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<tr>
<td></td>
<td>Control</td>
<td>Cocktail</td>
</tr>
<tr>
<td><strong>K</strong>&lt;sub&gt;max&lt;/sub&gt;, g</td>
<td>1.9 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>NE (10⁻⁴ M), g</td>
<td>1.5 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td><strong>pD₂</strong></td>
<td>6.0 ± 0.1</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Yohimbine + 10⁻⁴ M NE, g</td>
<td>1.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td><strong>pD₂</strong></td>
<td>5.7 ± 0.1</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Idazoxan + 10⁻⁴ M NE, g</td>
<td>1.1 ± 0.1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td><strong>pD₂</strong></td>
<td>5.7 ± 0.1</td>
<td>5.4 ± 0.1</td>
</tr>
<tr>
<td>Prazosin + 10⁻⁴ M NE, g</td>
<td>0.8 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td><strong>pD₂</strong></td>
<td>4.3 ± 0.2*</td>
<td>5.1 ± 0.1*</td>
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</table>

Values are means ± SE, expressed as absolute values. MCA, middle cerebral arteries; NE, norepinephrine; pD₂, negative logarithm of EC₅₀, or half-maximal contraction for NE, and an index of tissue "sensitivity" or "potency"; control, isotonic Krebs buffer (5.17 mM K⁺; see METHODS for details). K<sup>max</sup>; values obtained in Krebs solution containing 120 mM K⁺; cocktail, Krebs buffer with 3 × 10⁻⁵ M cocaine, 10⁻⁵ M deoxycorticosterone, 10⁻⁶ M propranolol, 3.6 × 10⁻⁶ M iproniazid, and 10⁻⁶ M tetrodotoxin. In each instance, values were significantly different (P < 0.01) from control. *P < 0.01 vs. NE alone.
**DISCUSSION**

The presence of postjunctinal $\alpha_2$-AR has been convincingly demonstrated with radioligand binding studies in several vessel types (1, 18, 47). Furthermore, in the rat, $\alpha_2$-AR agonists have been shown to increase vascular resistance in vivo (38). Despite these in vivo studies, in vitro functional studies have not always been as clear-cut. For example, in the normotensive Wistar-Kyoto rat, contractile responses to adrenergic nerve stimulation were not sensitive to the $\alpha_2$-AR antagonist idazoxan; however, in the spontaneously hypertensive rat strain, idazoxan depressed the contractile responses (28). In cerebral vessels in the dog, postjunctional $\alpha_2$-AR may play only a minor role in smooth muscle contractility (27). These studies suggest that species, strain, and/or vascular model may be important in the expression and function of postjunctional $\alpha_2$-AR. Furthermore, the species and vascular models within a given species that exhibit functional $\alpha_2$-AR have not been completely documented. Given that there are no previous studies on the function of postjunctional $\alpha_2$-AR in the sheep cerebrovasculature,

neuronal NE uptake, respectively. We then repeated these measurements with the addition of $10^{-6}$ M idazoxan (see METHODS). In the presence of cocaine, deoxycorticosterone, and L-NAME, fractional NE release (pg·pg NE$^{-1}$·pulse$^{-1}$ $\times 10^{-6}$) in fetal and adult cerebral arteries was 51.5 ± 18.9 and 10.8 ± 3.6, respectively ($P < 0.01$). The percent increase of fractional NE release in the presence of idazoxan was similar in fetal and adult MCA: 79 and 105%, respectively. In addition, stimulation-evoked fractional NE release was greater in fetal than in adult MCA under both treatment conditions ($P < 0.01$).

Table 3. Peak responses of vascular tension in MCA to phenylephrine with and without $\alpha_2$-AR agonists

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Adult</th>
<th>Fetus</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{max}$ g</td>
<td>15</td>
<td>1.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>PHE (10$^{-4}$ M), g</td>
<td>5</td>
<td>1.4 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>pD2</td>
<td>5</td>
<td>4.9 ± 0.1</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>Clonidine + 10$^{-4}$ M PHE, pD2</td>
<td>5</td>
<td>1.1 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>UK-14,304 + 10$^{-4}$ M PHE, g</td>
<td>5</td>
<td>4.7 ± 0.1</td>
<td>4.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as absolute values. PHE, phenylephrine; see Table 2 footnote for definition of other abbreviations. *P < 0.01 vs. PHE alone.

**Fig. 4.** Stimulation-evoked fractional NE release in fetal and adult MCA. Lighter hatched and stippled bars, fractional NE release from fetal and adult cerebral arteries in the presence of $10^{-5}$ M cocaine, $10^{-6}$ M deoxycorticosterone, and $10^{-8}$ M N$^\text{n}$-nitro-L-arginine methyl ester. Difference in NE release between fetus and adult was significant ($P < 0.01$). Darker hatched and stippled bars, NE release from fetal and adult MCA in the presence of $10^{-5}$ M cocaine, $10^{-6}$ M deoxycorticosterone, $10^{-5}$ M N$^\text{n}$-nitro-L-arginine methyl ester, and $10^{-6}$ M idazoxan. Difference between fetus and adult was significant ($P < 0.01$), as was difference in NE release in the presence of idazoxan compared with its absence ($P < 0.05$) for fetus and adult.
we questioned whether postjunctional $\alpha_2$-AR are involved in mediating in vitro contractile responses.

The present studies offer several unique observations. First, $\alpha_2$-AR density values were significantly greater in fetal than in adult cerebral arteries (Fig. 1B, Table 1). Also, $\alpha_2$-AR density values were significantly greater in smaller arteries (combined anterior, middle, and posterior cerebral) than in a larger extracranial artery (common carotid). Second, stimulation-evoked NE release from fetal cerebral arteries was significantly greater than that from adult vessels (Fig. 4). Nonetheless, when prejunctional $\alpha_2$-AR were inhibited by idazoxan, thereby blocking NE negative feedback on further NE release, the proportional increase in fractional NE release was similar in vessels of both age groups. Third, adult MCA showed no significant change in adrenergic-induced tension or pD$_2$ values in the presence of an $\alpha_2$-AR antagonist (Fig. 2A). Furthermore, with tetrodotoxin blockade of the adrenergic nerves (and prejunctional $\alpha_2$-AR) and inhibition of NE uptake, the lack of yohimbine inhibition of the NE-induced contraction (Fig. 2B) further suggests the absence of functional postjunctional $\alpha_2$-AR in adult vessels. In the fetal cerebral arteries, in contrast, in the presence of $10^{-7}$ M yohimbine, cocaine, deoxycorticosterone, and tetrodotoxin, the NE dose response was shifted 0.5 log unit to the right (Fig. 2D). Fourth, the $\alpha_2$-AR agonists clonidine and UK-14304 did not elicit a contractile response in adult or fetal MCA, except at a relatively high dose. However, in the presence of $3 \times 10^{-7}$ M UK-14304, the fetal, but not adult, phenylephrine dose response was significantly shifted to the right (Fig. 3B). These studies suggest that the majority of functional $\alpha_2$-AR in ovine cerebral arteries are prejunctional; however, in the fetal vessels, some appear to be postjunctional.

Cerebral artery $\alpha_2$-AR. During the past decade, considerable progress has been made in understanding the role of $\alpha_2$-AR in the vasculature. The role of the adrenergic system in regulation of cerebral blood flow (CBF) remains controversial because of differences between the anatomic demonstration of receptor location and the relative lack of change of CBF after adrenergic stimulation. $\alpha_2$-AR are widely distributed in large and small cerebral vessels of many species (3, 35). Edvenson and colleagues (16) proposed tonic $\alpha_2$-AR-mediated cerebral artery vasoconstriction in the cat. The relatively selective $\alpha_2$-AR agonist dexmedetomidine has been shown to decrease CBF $\sim$30% in isoflurane-anesthetized dogs (27).

A key factor in arterial contractility to NE and/or other agonists is agonist/receptor activation, as well as the intrinsic efficacy of the signal transduction process, which couples receptor activation to vascular smooth muscle contraction. Cerebral blood vessels are sensitive to NE via stimulation of $\alpha_1$- and/or $\alpha_2$-AR (3, 40, 44). $\alpha_2$-AR exist in at least three subtypes, which have been defined pharmacologically and by molecular cloning, and each has been shown to inhibit adenylate cyclase activation and, thus, reduce intracellular cAMP activity (12, 18). $\alpha_2$-AR appear to play a key role in the regulation of cerebrovascular tone in many species, including the cat (29, 35, 36), dog (27, 33, 40), pig (3, 10, 11), cow (42), rat (4), monkey (44), and human (43, 44). In cat MCA, $\alpha_2$-AR may be the chief mediator of NE-induced contraction (29). Prejunctional $\alpha_2$-AR develop rapidly in the fetal rat brain, paralleling sympathetic innervation (48), and have been reported in virtually every vascular tissue examined (18).

Some have argued that $\alpha_2$-AR play a greater role in vascular contraction in vivo than in vitro (2, 38, 39). In part, this may be because it has been difficult to demonstrate the functional role of $\alpha_2$-AR in vitro (41). Part of the difficulty lies in the fact that $\alpha_2$-AR agonists do not directly produce vasoconstriction but provide an ancillary drive to the vasoconstrictor stimulus produced by $\alpha_1$-NE agonists (18, 49, 50). In vitro the proximal tail artery of rats has postsynaptic $\alpha_2$-AR (28), as do cerebral arteries (29, 33, 36) from several species. Rat tail artery displayed contractile responses to NE and phenylephrine that were potentiated by $\alpha_2$-AR agonists, a potentiation blocked by $\alpha_2$-AR antagonists (49, 50). In the tail artery of Fischer 344 rats, the $\alpha_2$-AR antagonists idazoxan and rauwolscine shifted the NE concentration-response curves to the right, while the $\alpha_2$-AR agonists UK-14304 and BHT-920 shifted the $\alpha_1$-AR agonist methoxamine dose-response curves to the left (41). Other studies have suggested that the effects of postjunctional $\alpha_2$-AR may be muted and are only observed when another type of agonist is present (18, 26).

Also, as suggested by Nielsen et al. (30), in vitro studies of $\alpha_2$-AR-mediated contractions have not always been clear because of species differences. They examined NE effects in similar-sized mesenteric arteries (200 $\mu$m diameter) from several species and demonstrated $\alpha_2$-AR effects in porcine and human vessels but not in rabbit or rat. Finally, $\alpha_2$-AR-mediated vascular contraction is complicated by release of vasodilator prostaglandins from the endothelium, in vivo and in vitro, during adrenergic stimulation (13). In part for this reason, endothelium-denuded vessels were used in the present studies.

Cerebral vessel $\alpha_2$-AR density and development. Several studies have examined vascular $\alpha_2$-AR density and affinity; however, we are not aware of such quantification in adult or fetal cerebral arteries. The present studies examined the extent to which age-related differences in cerebral artery contraction may be a function of changes in $\alpha_2$-AR density and/or affinity. Our values of $\alpha_2$-AR density, as determined with $[^{3}H]$Idazoxan, are similar in magnitude to those reported for the rat tail artery (47) and human and monkey posterior communicating artery (44). Those studies used $\alpha_2$-AR antagonists other than idazoxan; thus the antagonist affinity values differ. The present studies demonstrate that $\alpha_2$-AR density varies dramatically as a function of developmental age (Fig. 1B) and vessel size (Table 1), although the biological meaning of these differences remains unclear. The higher $\alpha_2$-AR density values in the small vessels suggest more sympathetic nerve innervation than in the larger arteries; regula-
tion of the cerebral microcirculation is important for neuronal integrity and its role in permeability of the blood-brain barrier. In several species, these vessels receive considerable adrenergic innervation. As demonstrated in this study, α2-AR density values in fetal and adult microvessels were similar to those in main branch cerebral arteries. The high α2-AR density values in fetal main branch cerebral arteries compared with the adult also demonstrate the independent regulation of this receptor as a function of developmental age. The values of α2-AR density in the fetal and adult main branch cerebral vessels bore no relation to their NE-induced maximal tension (expressed as grams or %K\textsubscript{max}), suggesting that the majority of functional α2-AR in ovine cerebral arteries may be prejunctional. Alternatively, they may be postjunctional and act to potentiate the α1-AR response or be uncoupled. Also, as with any tissue, the cerebral arteries do not consist of a single cell type but, rather, of neurons and other cells in addition to vascular smooth muscle, and the distribution of these cell types may vary with developmental age.

α-AR agonists, antagonists, and adrenergic-induced contraction. As we reported previously, α1-AR and its second messenger inositol 1,4,5-trisphosphate play a key role in NE-induced contraction in main branch cerebral and common carotid arteries of the adult sheep (22) and the developing fetus (23). As anticipated in the present study, the NE-induced increase in tension was markedly attenuated by the α1-AR antagonist prazosin in adult and fetal sheep MCA (Fig. 2). In the adult MCA, neither the α2-AR antagonists yohimbine and idazoxan nor the α2-AR agonist UK-14304 showed a significant effect on vascular tension. In the fetal arteries, in contrast, agonist and antagonist significantly affected the dose-response curves (Figs. 2D and 3B). As shown in Fig. 2D, one might expect the α2-AR antagonist yohimbine to decrease the sensitivity to adrenergic-induced contraction. However, it is not entirely unexpected that an agonist such as UK-14304 would have a similar effect on postjunctional α2-AR (Fig. 3B), because UK-14304 and clonidine are partial agonists and may have low efficacy on postjunctional α2-AR in fetal MCA. Therefore, they function as competitive blockers to NE-induced contraction, although this was not observed in the present study.

Fractional NE release. An important finding of the present study was that stimulation-evoked NE release was severalfold greater in the fetal than in the adult MCA (Fig. 4). Additionally, the α2-AR antagonist idazoxan, by inhibiting NE-mediated negative feedback on further NE release, essentially doubled fractional NE release in fetal and adult cerebral arteries. This suggests a similar function of α2-AR in fetal and adult cerebral arteries.

Perspective

The idea that NE or other neurotransmitter release is regulated at individual neuron terminals at their junction with vascular smooth muscle cells and that α2-AR and other receptors function by negative-feedback control is widely held (37). Nonetheless, many questions remain regarding this regulation (19) and, particularly, the role of α2-AR in the regulation of the cerebral circulation (26, 27). The present studies suggest that in adult ovine cerebral arteries the majority of the α2-AR are pre- rather than postjunctional. Alternatively, those that are postjunctional may act to potentiate the α1-AR response or be uncoupled. In contrast, although in the fetus a large number of α2-AR are probably prejunctional, a significant number also appear to be postjunctional, as noted by the evidence cited above. In addition, α2-AR density values were significantly greater in fetal than in adult arteries and also were greater in small than in large arteries. Finally, idazoxan inhibition of postjunctional α2-AR resulted in a similar percent increase in fractional NE release. Overall, α2-AR appear to play a role in mediating adrenergic contractions in the cerebral arteries of the fetus, but their role in the adult is less clear. In fact, the physiological role of α2-AR continues to be debated (19, 37). Although α2-AR may play a role in regulating cerebral artery tone for the ovine adult and fetus in vivo, especially under conditions of hypoxic or other stress, evaluation of this possibility must await studies in the intact preparation.

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