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Maturational modulation of endothelium-dependent vasodilatation in ovine cerebral arteries

James M. Williams, Andrew D. Hull, and William J. Pearce

Departments of Physiology, Pharmacology, and Biochemistry, Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda, California

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Williams, James M., Andrew D. Hull, and William J. Pearce. Maturational modulation of endothelium-dependent vasodilatation in ovine cerebral arteries. Am J Physiol Regul Integr Comp Physiol 288: R149–R157, 2005. First published September 9, 2004; doi:10.1152/ajpregu.00427.2004.—To address the hypothesis that maturation enhances endothelial vasodilator function in cerebral arteries, relaxant responses to ADP and A-23187 were determined in ovine carotid and cerebral arteries harvested from 25 newborn lambs (3–7 days) and 23 adult sheep. Maturation significantly increased pD2 values for A-23187 (newborn range: 4.9 ± 0.3 to 5.4 ± 0.3; adult range: 6.0 ± 0.2 to 7.1 ± 0.2) and the maximal vasodilator response to A-23187 by 10–18%. In contrast, maturation decreased maximum responses to ADP by 5–25% with no change in pD2. The magnitudes of endothelium-dependent relaxation were not affected by 10 μM indomethacin but were virtually abolished by 100 μM Nω-nitro-L-arginine methyl ester/L-nitro arginine, indicating that nitric oxide (NO) is the primary endothelium-dependent vasodilator in these arteries. Maturation also modestly decreased endothelial NO synthase (eNOS) abundance in both carotid (32%) and cerebral (26%) arteries. Together, these findings reinforce the view that receptor coupling to endothelial activation is tightly regulated and may offset underlying changes in maximal endothelial vasodilator capacity. This capacity, in turn, appears to increase with postnatal age despite major growth and expansion of endothelial cell size and vascular wall volume. In ovine cerebral arteries, endothelial vasodilator capacity appears completely dependent on eNOS activity but not on cyclooxygenase activity. In turn, eNOS activity appears to be postnatally regulated by mechanisms independent of changes in eNOS abundance alone.

A-23187; ADP; indomethacin; Nω-nitro-L-arginine methyl ester; l-nitro arginine; newborn; ontogeny; vascular morphometry

BRAIN DAMAGE FROM CEREBROVASCULAR disturbances contributes to many neonatal disorders that evolve into permanent disability (23). The vulnerability to this damage, in turn, is often strongly associated with the organization of the maturing cerebrovascular network that homeostatically couples brain metabolic demand to vascular supply during adaptation to extrauterine life and, more importantly, during cerebrovascular insults such as asphyxia (20, 33). This coupling is complex and unique from that in the adult not only because cerebral blood flow is lower in neonates than in adults (19, 29), but also because the influences of blood gases, arterial pressure, the adrenergic innervation, and many other mediators are quite different in neonates and adults (19, 36, 37, 49). In addition, the larger cerebral arteries that contribute significantly to cerebrovascular regulation in adults (12) may play an even more important regulatory role in the immature cerebral circulation (9). Superimposed on these basic age-related changes in cerebrovascular reactivity are potential changes in endothelial function that is a critical component of the responses to many physiological agonists (14). Unlike age-related changes in reactivity to contractile agonists such as norepinephrine and vasodilators such as adenosine, the effects of maturation on endothelial function in the cerebral circulation have received relatively little attention.

To date, most studies of cerebral endothelial maturation have focused on blood-brain barrier properties. These studies showed that endothelial tight junctions form early in fetal life but that blood-brain barrier characteristics change throughout gestation, are not fully mature at birth, and change rapidly during early postnatal life (11). Nevertheless, the endothelium is capable of modulating vascular tone in fetal and newborn arteries. To date, endothelium-dependent vasodilatation has been demonstrated in immature coronary (31), renal (45), pulmonary (22), and cerebral (28, 53) arteries. However, these studies generally have not included parallel studies in corresponding adult arteries and have varied considerably in their suggestions of which endothelial vasoreactive factors are released.

In light of multiple suggestions that the magnitude of endothelium-dependent relaxation increases with postnatal age (7, 28, 45) and is highly heterogeneous among different vascular beds (50), the present studies were carried out to test the hypothesis that postnatal age and artery type significantly influence endothelium-dependent vasorelaxation in ovine carotid and cerebral arteries. Because a variety of evidence suggests that responses to physiologically relevant endothelium-dependent vasodilators can differ markedly from responses to pharmacological activators of the vascular endothelium (47), we used both receptor-dependent and receptor-independent agents to elicit endothelium-dependent vasodilatation. Given that the size and organization of vascular endothelial and smooth muscle cells change dramatically during postnatal maturation (21, 25), we also used vessel morphometry to determine if endothelial vasodilator capacity varies as a function of the smooth muscle wall volume served by the endothelial layer. Finally, because the apparent roles of prostanooids and nitric oxide (NO) in neonatal endothelium-

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dependent vasodilatation have been highly inconsistent in other preparations (7, 34, 44), we used inhibitors of both cyclooxygenase and NO synthase, as well as Western blot analysis, to identify the relative contributions of these factors to endothelium-dependent vasodilatation in ovine cerebral arteries.

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 used segments of common carotid (COM), basilar (BAS), posterior communicating (PCA), and middle cerebral (MCA) arteries from newborn lambs (3-7 days old) and young nonpregnant adult sheep (18-24 mo old). After the vessels were cleaned of extraneous connective and adipose tissue, the arteries were cut into segments 3-5 mm in length and mounted on paired wires between a low-compliance force transducer (Kulite BG-10) and a post attached to a micrometer used to vary resting tension. The segments then 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 NaHCO3, 5.56 dextrose, 5.17 KCl, 2.49 MgSO4, 1.60 CaCl2, 0.114 ascobic acid, and 0.027 disodium EDTA, continuously bubbled with 95% O2-5% CO2. During equilibration, segments were maintained at optimum resting tensions as previously described in detail (38).

Dose-response studies. During all dose-response experiments, an on-line computer continuously digitized, recorded, and normalized vessel tensions. First, we briefly contracted the arteries by exposure to an isotonic potassium Krebs solution containing 122 mM K+ and 31 mM Na+. After peak tensions were obtained, we washed the vessels with normal sodium Krebs solution and allowed them to return to baseline levels of tension for 30 min. We then induced a second contraction using a mixture of 10 μM serotonin and 20 μM histamine. In previous studies, this mixture has produced maximal contractions with greater stability than any other method of contraction, particularly in newborn lamb cerebral arteries (38).

After attainment of a stable level of initial tone, the capacity of the endothelium to produce vasorelaxation was tested via responses to ADP (receptor dependent) and A-23187 (receptor-independent). We used ADP instead of another widely used endothelium-dependent relaxant, acetylcholine, because separate experiments revealed that responses of either adult or newborn ovine cerebral arteries to acetylcholine were of low magnitude in this species (<20%, n = 6 for both age groups). Cumulative doses of either ADP or A-23187 were added to the baths to produce concentrations ranging from 10^-9 to 10^-3 M. ADP was dissolved in distilled water and the A-23187 was dissolved in DMSO. The ADP experiments were performed in the presence of 10 μM 8-phenyl-theophylline to block the action of any adenosine derived from ADP by the action of ectonucleotidases. In separate experiments, this concentration of 8-phenyl-theophylline was sufficient to completely block vasodilator responses to 1 μM adenosine. The 8-phenyl-theophylline administered was dissolved in DMSO and the maximal bath concentration of DMSO attained during any experiment was 0.3%, which had no independent effect on vessel tension.

Endothelial and vascular smooth muscle cell staining. To determine the effects of maturation on the vessel wall volume per endothelium cell, unmounted arterial segments without tone were stained en face using silver nitrate. For this treatment, each segment was carefully opened longitudinally with fine scissors, washed with 5% dextrose in saline, and then incubated for 3 min in 0.25% AgNO3. After the silver nitrate exposure, the segments were again washed with 5% dextrose in saline and then incubated in 3% CoBr2 in 1% NH4Br for 10 min. The segments were again washed with 5% dextrose in saline and then fixed in 4% formaldehyde. The fixed segments were mounted in glycerin and examined using light microscopy under medium power. A video camera attached to the microscope provided signals to a Quantex image analyzer, which enabled calibrated measurements of endothelial cell dimensions. From each segment examined, the widths and lengths of at least 10 individual endothelial cells were recorded, and these data were averaged to produce a single average measurement of cell length and width per segment. Multiple segments of the same artery type from the same animal were routinely examined, and these data were averaged across segments to produce a single average measurement of cell length and width for each artery type from each individual animal. For each artery examined, the average endothelial cell cross-sectional area was calculated as π4 times the product of cell width and length, assuming that the cells were all longitudinally ellipsoidal in shape. Endothelial cell cross-sectional areas were also averaged across multiple segments from the same animal, as described for cell length and width. Vascular smooth muscle cell dimensions and cross-sectional areas were measured as described for endothelial cells in adjacent segments of the same arteries used for the endothelial cell measurements, with the exception that the vascular endothelium was first removed by mild mechanical abrasion. All methods used for measurements of cell dimensions have been previously described (10).

From each artery sample used to obtain measurements of cell dimensions, a separate segment was also taken for measurement of wall thickness using methods previously described (38). Briefly, arteries were cleaned of fat and connective tissue after which thin coronal sections were cut from each segment. These ring-shaped coronal sections were then mounted on slides in saline, placed under coverslips, and then placed on a microscope stage. Distances from the outer to the inner surface were then measured using projection microscopy. All measurements were calibrated using a stage micrometer. For each of the vessel segments studied, wall thickness was expressed as the mean of at least 10 measurements.

Measurements of endothelial cell cross-sectional area and wall thickness were combined to enable calculation of the mean wall volume per endothelial cell. For this calculation, we first estimated the number of endothelial cells per unit length L of artery as the lumenal area per length L divided by the cross-sectional area per endothelial cell

\[
\frac{L \cdot \pi \cdot D}{A}
\]

where L is the artery segment length in micrometers, D in the lumenal diameter in micrometers, and A is the average cross-sectional area per endothelial cell in units of square micrometers. Next, we calculated the volume of artery wall per unit length L of artery assuming cylindrical geometry

\[
L \cdot \pi \cdot T \cdot (D + T)
\]

where T is the artery wall thickness in micrometers and all other variables are as previously defined. Finally, we calculated the ratio of wall volume per endothelial cell as the ratio of wall volume in cubic micrometers per unit length L divided by the number of endothelial cells per length L. This expression simplified to

\[
\frac{A \cdot T \cdot (D + T)}{D}
\]

This derivation implies that the ratio of wall volume per endothelial cell will increase directly with increases in either average endothelial cell cross-sectional area (A) or artery wall thickness (T). From another perspective, this ratio represents the smooth muscle volume served by each endothelial cell.

Effects of indomethacin, L-nitro arginine/N\textsuperscript{G}-nitro-L-arginine methyl ester, and endothelium removal on relaxation responses. To assess the possible contribution of either eicosanoids or NO in the endothelium-dependent vasorelaxation responses, separate experiments were per-
formed in newborn and adult artery segments. Artery segments were prepared as described for the dose-response experiments and then contracted with an isotonic potassium Krebs solution containing 122 mM K⁺ and 31 mM Na⁺. After peak tensions were obtained, vessels were washed with normal sodium Krebs solution and allowed to return to baseline levels of tension for 30 min. The artery segments were then contracted with a mixture of 10 μM serotonin and 20 μM histamine. Next, the arteries were relaxed with either 10 μM ADP or 1 μM A-23187. Both agents were used at the concentrations approximately equal their EC₅₀ values. The ADP experiments were performed in the presence of 10 μM 8-phenyl-theophylline to block the action of any adenosine derived from ADP by the action of ectonucleotidases.

To assess the contribution of eicosanoids to endothelium-dependent relaxation in these arteries, responses to ADP and A-23187 were also obtained following incubation for 20 min in 10 μM indomethacin (16). Validation experiments showed this duration of treatment with this concentration of indomethacin sufficient to completely block responses to 1 μM arachidonic acid. To assess the contribution of NO, responses to ADP and A-23187 were also obtained following incubation for 30 min in 100 μM N⁵-nitro-l-arginine methyl ester and 100 μM l-nitro arginine (l-NAME/l-NA). In validation experiments, treatment for this duration with these concentrations was sufficient to optimally inhibit NO production, as measured by nitrate analysis. The combination of 100 μM l-NAME and 100 μM l-NA was more effective at inhibiting NO production than 200 μM of either agent alone, and thus this combination was used in all experiments.

In representative segments of both newborn and adult arteries, the endothelium was removed by gentle mechanical abrasion before mounting the arteries for contractility measurements. Denudation was verified morphologically by the complete absence of endothelial cells following silver staining and microscopic examination. All relaxant responses to A-23187 and ADP were absent (<8%) in denuded arteries.

Western immunoblotting for endothelial NO synthase protein. Segments of newborn and adult ovine common carotid and cerebral arteries were cleaned of extraneous connective and adipose tissue, frozen in liquid nitrogen for 20 min, and then ground under liquid nitrogen. The powder was incubated for 30 min in a lysis buffer (150 mM NaCl, 50 mM Tris, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 5 mg/ml pepstatin, 5 mg/ml aprotinin, and 5 mg/ml benzamidine at pH 7.4). After this incubation, the samples were sonicated briefly and spun at 12,000 g for 30 min at 4°C, after which protein concentrations were determined using the BCA assay (Pierce, Rockford, IL).

Samples (10–15 μg/lane) were separated on 8% polyacrylamide SDS gels. Carotid and cerebral samples were normalized against a kidney homogenate standard; the same standard was used for all gels. The separated proteins were transferred to nitrocellulose using a current of 400 mA for 3 h in Towbin’s buffer (25 mM Tris, 192 mM glycine, 20% methanol). After transfer, the membranes were blocked with 5% nonfat dry milk in Tris-buffered saline (M-TBS) overnight and then probed with an endothelial NO synthase (eNOS) monoclonal antibody (Transduction Labs, San Diego, CA) at 1:750 dilution in M-TBS for 3 h. The membranes were then washed for 30 min and probed with a phosphate-conjugated goat-anti-mouse antibody diluted 1:2,000 with M-TBS. After 1-h incubation with the second antibody, the membranes were washed as described for the first antibody, and then the blots were visualized by film (Amersham, Piscataway, NJ) using exposure times from 30 s to 3 min. The resulting films were analyzed using a Bio-Rad model 7700 image analyzer.

Data analysis and statistics. Maximum relaxation responses were calculated as percentage reductions in the maximum initial contractile tension. Thus a percent relaxation of 100% would return the value of contractile tension to baseline. For determination of pD₂ (−log EC₅₀), the relaxation data were normalized relative to maximum percentage relaxation and then fitted to the logistic equation (43) using nonlinear regression. Percent maximum relaxation, pD₂ values, and endoNO abundance were each independently analyzed using a two-way ANOVA with age as one factor (newborn vs. adult) and artery type as the other (COM, BAS, PCA, and MCA). Homoscedasticity was verified for all ANOVA comparisons using Bartlett’s test. The effects of maturation (newborn vs. adult) within each artery type were determined by post hoc Duncan’s tests. For the ADP/A-23187 ratio and endothelial cell cross-sectional data, standard errors were calculated using a pooled variance within each age-artery combination. Statistical differences between ratios for endothelial cell cross-sectional area were analyzed using a Behrens–Fisher analysis. 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.

RESULTS

In total, 956 segments were obtained from 23 adult and 25 newborn sheep. Contractile tensions produced in response to 122 mM potassium-Krebs averaged 6.53 ± 0.72, 2.36 ± 0.27, 2.58 ± 0.23, and 2.70 ± 0.25 g in newborn COM, BAS, PCA, and MCA segments, respectively (average segment length: 5 mm). Corresponding values in adult arteries averaged 4.01 ± 0.65, 1.95 ± 0.35, 3.14 ± 0.29, and 2.66 ± 0.37 g, respectively (average segment length: 3 mm). None of these values were significantly influenced by endothelium removal (ANOVA). The percent difference in potassium-induced tone produced by denudation averaged 7.9 ± 7.6% across all arteries. The corresponding value for agonist-induced tone averaged 9.5 ± 7.0%.

ADP dose-response relationships. ADP produced consistent dose-dependent relaxations in both newborn and adult arteries (Fig. 1). Because these responses were absent in denuded arteries, we attribute them to activation of endothelial P₂ purinergic receptors. Values of maximum ADP efficacy were generally greater in newborn than adult arteries (ANOVA), and these differences were significant (Duncan’s analysis) in COM and MCA (Fig. 2). Adult values of maximal ADP efficacy ranged between 54.7 ± 5.2% (COM) and 77.4 ± 6.2% (BAS), and newborn values ranged from 47.3 ± 3.7% (COM) to 72.1 ± 6.8% (PCA).

In contrast to ADP efficacy, sensitivity to ADP as reflected by pD₂ values varied little with age. A significant post hoc age-related difference in pD₂ was observed only in COM arteries (adult = 5.4 ± 0.2, newborn = 4.8 ± 0.2). Independent of age, sensitivity to ADP was significantly greater in intracranial cerebral arteries (combined pD₂ range: 5.8 ± 0.1 to 6.1 ± 0.1) than in the extracranial COM arteries (combined pD₂ range: 4.8 ± 0.2 to 5.4 ± 0.2; Fig. 2).

A-23187 dose-response relationships. The calcium ionophore A-23187 elicited dose-dependent relaxation in all arteries studied (Fig. 3). Because these responses were absent in denuded arteries, we attribute them to enhanced calcium entry into endothelial cells. In contrast to ADP, values of maximal efficacy for the receptor-independent, endothelium-dependent vasodilator A-23187 were significantly greater (ANOVA) in adult than in neonatal arteries (Fig. 2). Newborn values of maximum efficacy for A-23187 ranged from 81.1 ± 5.7% (COM) to 89.7 ± 4.2% (BAS) and adult values ranged from 90.7 ± 2.6% (MCA) to 95.7 ± 2.0% (COM). After post hoc analysis (Duncan’s analysis), efficacy varied significantly with
Fig. 1. Effects of maturation on ADP dose-response relationships. Shown here are the ADP concentration-relaxation relationships observed in newborn and adult common carotid (COM), basilar (BAS), posterior communicating (PCA), and middle cerebral (MCA) arteries. Relaxation magnitudes are expressed as percentage reductions in the contractile tone produced by 10 μM serotonin plus 20 μM histamine. The solid lines indicate curves of best fit to the logistic equation, as determined by nonlinear regression. The vertical error bars indicate SE for 10–12 animals in each case. Statistical analysis of these data is summarized in Fig. 2.

Fig. 2. Effects of maturation on dose-response coefficients for A-23187 and ADP. Summarized here are the averages of maximum efficacy (left) and pD₂ (right) obtained by nonlinear regression for responses to both ADP (top) and A-23187 (bottom) in each of the 4 artery types. Factorial ANOVA revealed significant differences due to age across all arteries for both coefficients for both ADP and A-23187. In addition, post hoc Duncan’s analyses revealed significant (*P < 0.05) individual age-related differences within artery type. Vertical error bars indicate SE for 10–12 animals in each case.

Sensitivity to A-23187 was significantly less (ANOVA) in neonatal than adult arteries (Fig. 2). Newborn pD₂ values for A-23187 ranged between 4.9 ± 0.3 (COM) to 5.4 ± 0.3 (BAS) and adult values ranged from 6.0 ± 0.2 (COM) to 7.1 ± 0.2 (PCA). Post hoc analyses revealed significant individual age-related differences in each artery type (Fig. 2).

ADP/A-23187 ratio. To examine possible maturational influences on ADP receptor coupling, values of maximal ADP...
efficacy were expressed relative to corresponding values for maximal A-23187 efficacy (Fig. 4). This ratio thus normalized responses to ADP relative to the maximal endothelium-dependent vasodilator capacity. As indicated by a Behren’s-Fisher analysis, significant age-related differences in the ADP/A-23187 response ratio were observed in all arteries except the PCA. Compared with newborn values, corresponding adult values were 27, 14, 14, and 32% less in COM, BAS, PCA, and MCA, respectively.

Smooth muscle volume per endothelial cell. To evaluate relationships between maturational changes in the endothelial cell cross-sectional area and vessel wall volume, we calculated mean wall volume per endothelial cell (Fig. 5). Maturation increased the ratio of vascular wall volume per endothelial cell

Fig. 3. Effects of maturation on ADP/A-23187 efficacy ratios. Shown here are the ratios of the average efficacies for ADP relative to those for A-23187 in each of the 4 artery types used. These ratios indicate the vasodilator efficacy for ADP normalized relative to the maximum endothelial vasodilator capacity, as defined by responses to A-23187. Vertical error bars indicate SE calculated using a pooled variance; *significant differences as detected by a Behren’s-Fisher analysis. Overall, normalized responses to ADP were significantly greater in newborn than adult arteries.

Fig. 5. Mean vessel wall volume per endothelial cell in ovine cerebral arteries. In newborn and adult COM, BAS, PCA, and MCA arteries, endothelial cell cross-sectional areas and vessel wall thicknesses were used to calculate mean wall volume in cubic micrometers per endothelial cell. This value represents the smooth muscle volume of distribution for endothelial vasoactive molecules released in the serosal direction. All vertical error bars indicate SE for 6 animals. All adult values were significantly greater (*P < 0.05) than corresponding newborn values for each artery type, as determined by a Behren’s-Fisher analysis.
in all artery groups. In the newborn, these volumes ranged from 308 ± 14 × 10^3 μm^3 (COM) to 31 ± 2 × 10^4 μm^3 (MCA), whereas adult values ranged from 770 ± 20 × 10^3 μm^3 (COM) to 160 ± 9 × 10^5 μm^3 (BAS) (Fig. 1). Overall, maturation enhanced mean wall volume per endothelial cell by 150, 231, 156, and 765% in COM, BAS, PCA, and MCA, respectively.

Effects of indomethacin on ADP- and A-23187-induced relaxation. Across all arteries in both age groups, relaxation responses to ADP in endothelium-intact arteries averaged 28.5 ± 2.6% (n = 50) and 26.3 ± 3.1% (n = 51) in the presence and absence of indomethacin, respectively. Similarly, combined relaxation responses to A-23187 averaged 42.5 ± 4.4% (n = 51) and 39.9 ± 3.9% (n = 57) in the presence and absence of indomethacin, respectively. Neither of these differences was statistically significant even though overall statistical power reached 0.981. When the individual effects of indomethacin were subjected to post hoc analysis, again, pretreatment with 10 μM indomethacin did not significantly alter the magnitudes of either ADP- or A-23187-induced vasodilation in any artery or age combination.

Effects of L-NAME/L-NA on ADP- and A-23187-induced relaxation. Pretreatment of artery segments with 100 μM L-NAME with 100 μM L-NA significantly attenuated responses to both ADP and A-23187 in all arteries as determined by factorial ANOVA. In addition, post hoc Duncan’s analyses revealed significant (P < 0.05) individual treatment differences within each artery type. Responses to ADP were attenuated by L-NAME/L-NA in the newborn and adult, respectively, by 91.5 and 91.5% (COM), 100 and 97.8% (BAS), 96.1 and 98.4% (PCA), and 66.8 and 87.2% (MCA). Similarly, responses to A-23187 were attenuated by 65 and 69% (COM), 92.1 and 88.9% (BAS), 94.1 and 94.7% (PCA), and 90.3 and 79.9% (MCA) in newborn and adult, respectively (Fig. 6).

Abundance of eNOS protein in ovine cerebral arteries. Measurement of eNOS protein abundances in ovine cerebral arteries with Western immunoblotting and a specific monoclonal antibody revealed that the relative amounts of eNOS were less in adults than newborns (Fig. 7). Compared with the eNOS abundance in our kidney standard (arbitrary value of 1), eNOS...
levels averaged 2.2 ± 0.3 and 1.5 ± 0.4 (common carotid) and 3.1 ± 0.2 and 2.3 ± 0.2 (cerebral) in newborn and adult artery homogenates, respectively. This corresponds to levels of eNOS abundance that were 32% (common carotid) and 26% (cerebral) less in adult than in newborn arteries.

DISCUSSION

During the past five decades, the vascular endothelium has been the focus of literally thousands of studies, many of which have used isolated or cultured endothelial cells. Owing to the fact that endothelial cell phenotype has long been known to be highly dynamic, can undergo major changes in culture, and is functionally heterogeneous (18, 50), studies focusing specifically on the mechanisms governing the magnitude of endothelium-dependent vasodilatation have largely examined intact artery responses. Among this much smaller subset of publications, relatively few have directly examined endothelium-dependent relaxation in fetal, neonatal, or immature arteries of any kind, and the majority of these have explored pulmonary endothelial function. Only a dozen or so published studies have yet directly examined endothelium-dependent vasodilatation in immature arteries of the cerebral circulation. Thus, despite major scientific interest in the vascular endothelium and its well-established importance in cerebrovascular homeostasis, the mechanisms enabling endothelial regulation of vascular tone remain poorly studied in the immature cerebral circulation. The present study was conducted to help address this deficit.

To maximize physiological relevance, the present experiments explored the vasodilator effects of ADP, one of many endogenous endothelium-dependent vasodilators. ADP is continuously present at low concentrations in plasma, is released in quantity by aggregating platelets, and activates endothelial cells via purinergic receptors on the endothelial cell surface (4, 30). In the ovine carotid and cerebral arteries we examined, ADP produced a potent endothelium-dependent vasodilatation of similar magnitude in newborn and adult arteries (Figs. 1 and 2). Age-dependent differences in responses to ADP were observed only in the common carotid and middle cerebral arteries. In contrast to previous suggestions that the magnitude of endothelium-dependent relaxation in porcine coronary (31), human vertebral (7), guinea pig renal (45), and ovine pulmonary (26) arteries is less in immature than mature animals, neonatal responses to ADP were equal to or greater than adult responses in all ovine cranial arteries examined. Whereas this result reinforces the view that endothelial vasodilator function is highly heterogeneous among different vascular beds (50), it also suggests that endothelial reactivity to ADP is carefully and uniquely regulated throughout postnatal maturation in the ovine cerebrovascular circulation.

Because maturational changes in purinergic receptor type, density, and coupling to intracellular signaling pathways regulating endothelial vasodilator release could all potentially influence responses to ADP, we also examined endothelium-dependent relaxation responses to the ionophore A-23187. This pharmacological tool is a divalent cation ionophore that effectively bypasses the plasmalemmal receptor apparatus, directly increases endothelial cytosolic calcium through multiple mechanisms, and potently stimulates endothelial vasodilator release (8, 51). In response to A-23187, both magnitude of relaxation and potency (pD2) were significantly greater in adult than in newborn arteries, regardless of artery type (Figs. 2 and 3). In addition, the maximum magnitudes of relaxation to A-23187 were significantly greater than those for ADP and approached 100% of induced tone in all artery types; responses to A-23187 appeared to reflect the maximum endothelium-dependent vasodilator capacity of each artery type. Together with the ADP results, these data suggest that important age-dependent regulation of endothelial responsiveness occurs upstream of the mechanisms regulating responses to cytosolic calcium within the endothelial cell.

To estimate the effects of postnatal maturation on the coupling between plasmalemmal purinergic receptors and cytosolic calcium, we normalized the maximum relaxation magnitudes obtained with ADP relative to those obtained with A-23187 for all arteries (Fig. 4). This normalization revealed that the ability of ADP to elicit endothelium-dependent relaxation is increased in neonatal ovine carotid and cerebral arteries. Whereas this upregulation could serve to minimize age-related differences, vasodilator responsiveness to ADP and the mechanisms involved remain uncertain. As stated above, age-related differences in purinergic receptor type could play a role, although changes in receptor subtype should yield major differences in sensitivity to ADP (27) and such shifts were not observed in the cerebral arteries (Fig. 2). A simpler and more probable explanation is that postnatal maturation decreases P2Y receptor density. The sophisticated methodology necessary to directly test this hypothesis has been described (41) but has yet to be applied to age-related changes in endothelial purinergic receptor densities.

Aside from suggesting possible age-related changes in the coupling of ADP to endothelium-dependent vasodilatation, the present data also revealed significant age-related differences in endothelial sensitivity to A-23187. As shown in Fig. 2, A-23187 sensitivity (pD2) was significantly increased in adult compared with neonatal arteries, regardless of artery type. The simplest explanation for this finding is that endothelial sensitivity to calcium was greater in the adult arteries, leading to greater vasodilator release at the same calcium concentration. Consistent with this idea, age-related changes in cerebrovascular calcium sensitivity are well documented (2, 3). However, many other mechanisms remain possible, including age-related differences in the ability of the adjacent smooth muscle to respond to endothelium-dependent vasodilators and/or a change in the type and quantity of endothelial vasodilators released in response to activation. In addition, structure-function relationships between endothelial and vascular smooth muscle cells change dramatically during the burst of rapid vascular growth typical of the immediate postnatal period (21, 25). Such structural changes are also characteristic of postnatal growth of ovine cerebral arteries (10, 40) and could potentially influence the distances endothelial vasodilators must travel to effect vasorelaxation.

To test the hypothesis that postnatal arterial growth influences the magnitude of endothelium-dependent vasodilatation by altering the diffusional distances and volumes of distribution for endothelial vasodilators within the cerebral artery wall, we measured smooth muscle and endothelial cell dimensions and calculated the ratio of artery wall volume per endothelial cell. As shown in Fig. 5, postnatal maturation dramatically increased the artery wall volume served by each individual
endothelial cell. Thus the maximum magnitude of endothelium-dependent vasodilatation increased in tandem with increased intramural distances and volumes of distribution for endothelium-dependent vasodilators. This suggests that age-related increases in the magnitude of endothelium-dependent vasodilatation cannot be simply explained by improvements in endothelial smooth muscle geometry. Instead, the data suggest that postnatal maturation increases either 1) the type, amount, or half-life of endothelial vasodilator molecules released by each endothelial cell or 2) the smooth muscle reactivity to these molecules.

Given that prostacyclin and other products of cyclooxygenase metabolism have long been recognized as endothelium-dependent vasodilators in certain vascular beds (13) and that endothelial release of prostacyclin has been suggested to be age dependent (53), we examined the effects of cyclooxygenase inhibition on the magnitude of endothelium-dependent vasodilatation. Cyclooxygenase was inhibited with 10 μM indomethacin, which in validation studies completely blocked relaxant responses to 10 μM arachidonic acid. This concentration of indomethacin, however, had no significant effects on the magnitude of endothelium-dependent vasodilatation in our preparations, suggesting that prostacyclin is not a primary endothelium-dependent vasodilator in ovine cerebral arteries, regardless of postnatal age. Similar results have previously been reported in studies of guinea pig (1), porcine (42), and mouse (17) arteries, indicating a species-dependent role for cyclooxygenase products as endothelium-dependent vasodilators.

In contrast to indomethacin, 100 μM 1-NAME/L-NAME virtually abolished endothelium-dependent vasodilatation elicited by either ADP or A-23187 in all arteries of both age groups (Fig. 6). These results clearly suggest that NO is the primary endothelium-dependent vasodilator in ovine cerebral arteries. If so, then age-related increases in the magnitude of endothelium-dependent vasodilatation most likely involve either 1) an increase in endothelial NO release or 2) an increase in vascular reactivity to NO. Regarding vascular reactivity to NO, we previously showed that vasodilator responses to NO (39), the ability of NO to stimulate cGMP synthesis (52), and the ability of cGMP to induce vasorelaxation (32) are all increased in immature compared with mature cerebral arteries. Together, these data suggest that enhanced reactivity to NO cannot explain age-related improvements in endothelium-dependent relaxation. Alternatively, the combined data suggest that postnatal increases in the magnitude of endothelium-dependent vasorelaxation are best explained by increased endothelial NO release.

A main determinant of endothelial NO release is the abundance of eNOS present within endothelial cells. However, Western blot measurements demonstrated that the abundance of eNOS relative to the total mass of soluble cellular protein present in the artery wall was actually significantly greater in newborn than adult cerebral arteries (Fig. 7). This result suggests that possible age-related increases in NO release are not simply explained by parallel increases in the relative abundance of eNOS. Other potential mechanisms include age-related increases in eNOS-specific activity, possibly mediated by differences in eNOS isoform (35) or by differences in the type and extent of posttranslational modification of eNOS (6, 15). In addition, age-related differences in eNOS cofactor availability (48), substrate availability (46), or concentration of endogenous NOS inhibitors such as asymmetric dimethylarginine (5) could also significantly influence maximal rates of endothelial NO synthesis and release. Finally, age-related differences in NO half-life and rates of oxidation (24) could also alter rates of NO release. Clearly, exploration of the relative importance of these latter mechanisms as well as direct verification of the effects of postnatal age on endothelial NO release appear warranted.

In overview, the present experiments demonstrate that postnatal maturation significantly alters multiple key features of endothelium-dependent vasodilatation in ovine cerebral arteries. Whereas the coupling of ADP to endothelial relaxation changes relatively little with age, the ability of A-23187 to induce endothelium-dependent relaxation improves significantly with postnatal maturation. These findings reinforce the view that receptor coupling to endothelial activation is tightly regulated and may offset underlying changes in maximal endothelial vasodilator capacity. This capacity, in turn, appears to increase with postnatal age despite major growth and expansion of endothelial cell size and vascular wall volume. In ovine cerebral arteries, endothelial vasodilator capacity appears completely dependent on eNOS activity but not on cyclooxygenase activity. In turn, eNOS activity appears to be postnatally regulated by mechanisms independent of changes in eNOS abundance alone. Further elucidation of how these changes are coordinated, what molecular mechanisms are involved, and how they influence overall cerebrovascular homeostasis remain as promising topics for future investigations of the effects of postnatal age on endothelial vasodilator function.

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REFERENCES


