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Maturation Alters Cyclic Nucleotide and Relaxation Responses to Nitric Oxide Donors in Ovine Cerebral Arteries

Surya M. Nauli Charles R. White Andrew D. Hull William J. Pearce

Division of Perinatal Biology, Department of Physiology and Pharmacology, Loma Linda University School of Medicine, Loma Linda, Calif., USA

Key Words

Cerebrovascular circulation · Cyclic adenosine monophosphate · Cyclic guanosine monophosphate · Sheep

Abstract

To examine the hypothesis that maturation modulates nitric oxide (NO)-induced relaxation in cerebral arteries, we quantified concentration-relaxation relations and the corresponding dynamic responses of guanosine 3′:5′-cyclic monophosphate (cGMP) and adenosine 3′:5′-cyclic monophosphate (cAMP) levels following administration of nitroglycerin and S-nitroso-N-acetyl-penicilamine (SNAP), an NO donor, in posterior communicating and middle cerebral arteries from newborn (3–7 days) and adult sheep. The results offer 5 main observations: (1) the efficacy and potency of NO donors were generally greater in newborn than in adult cerebral arteries; (2) rates of relaxation, and presumably rates of NO release, were faster for equimolar concentrations of SNAP than for nitroglycerin in both newborn and adult arteries; (3) basal concentrations were greater for cAMP than for cGMP, and both were greater in newborn than adult cerebral arteries; (4) in adult cerebral arteries, NO-induced increases in cGMP occurred faster but relaxation developed more slowly than in newborn cerebral arteries, and (5) responses to NO donors involved significant cross-reactivity between cGMP and cAMP, the characteristics of which were age, artery, and agent specific. From these results, we conclude that postnatal changes in reactivity to NO reflect corresponding changes in soluble guanylate cyclase activity and possible decreases in NO half-life. We also conclude that maturation slows the mechanisms mediating NO-induced relaxation, and that this effect is more pronounced in distal than in proximal cerebral arteries. The data also suggest that the rate-limiting step governing rates of response to NO is probably downstream from cGMP synthesis. From the basal cyclic nucleotide levels, we conclude that basal ratios of synthesis to hydrolysis were greater in fetal than adult arteries. Because NO increased both cGMP and cAMP, we speculate that Type III phosphodiesterase has a possible influence upon cerebrovascular responses to NO, and that this influence varies with postnatal age and artery type. Together, these findings emphasize that the cerebrovascular effects of NO are highly age dependent and artery specific, and should be carefully considered when administering NO therapeutically in the neonate.

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William J. Pearce, PhD
Division of Perinatal Biology
Loma Linda University School of Medicine
Loma Linda, CA 92350 (USA)

Introduction

Owing to the many important physiological and pharmacological roles attributed to nitric oxide (NO), NO donors are becoming increasingly popular for clinical treatment of a growing number of cardiovascular pathologies [1, 2]. One of the most important of these applications is treatment of neonatal pulmonary hypertension [3]. Relevant to such applications, however, are numerous indications that NO is generally more potent in immature than in mature individuals [4–7], suggesting that therapeutic use of inhaled NO in neonates may involve cardiovascular complications, particularly in the cerebral circulation [8]. Despite the clinical utility of NO in pediatric patients, the reasons why reactivity to NO is highly age dependent remain poorly understood and largely unstudied.

In contrast to inhaled NO, the effects of NO donors have been studied for many years. The most common of all NO donors is nitroglycerin, which has long been recognized as a source of NO following conversion by endogenous enzymes [9–11]. The expression and activity of the enzymes responsible for the biotransformation of nitroglycerin appear to be under physiological control because repeated administration of nitroglycerin induces tolerance to nitroglycerin, but not to NO from donors such as S-nitroso-N-acetyl-penicilamine (SNAP) that require no intracellular enzymatic activation [9, 10, 12]. Other enzymes, such as protein kinase C and generators of free radicals can also influence the development of nitrate tolerance, as well as the production of, and reactivity to, NO [11, 13]. Because the activity and expression of many of these enzymes change with age, it remains possible that such age-related changes contribute to maturational differences in reactivity to NO and NO donors.

Once within the vascular smooth muscle cell, the main vasoactive effect of NO is to activate the enzyme soluble guanylate cyclase and thereby increase the cytosolic concentration of guanosine 3′:5′-cyclic monophosphate (cGMP) [14]. Increases in cGMP concentration are typically highly dynamic, tissue specific, and depend heavily on the types and amounts of phosphodiesterases present [15]. In turn, cGMP activates cGMP-dependent protein kinase, which can phosphorylate multiple different proteins to induce relaxation [16]. Again, the levels of the proteins that govern these vasorelaxant effects of NO vary with age and thus may help to explain why reactivity to both NO and cGMP change with postnatal age [17, 18].

Whereas the effects of NO donors are commonly attributed mainly to cGMP-dependent effects, it is also possible that changes in adenosine 3′:5′-cyclic monophosphate (cAMP) might be involved. Given the possible presence of phosphodiesterases Type II (cGMP-stimulated) and Type III (cGMP-inhibited) within cerebrovascular smooth muscle, it is possible that changes in cGMP levels may produce attendant changes in cAMP [19, 20]. In addition, cAMP and cGMP cross-activate both cAMP-dependent and cGMP-dependent protein kinase [21], suggesting further possibilities for interaction among the cyclic nucleotide signaling systems. The extents to which such interactions change during postnatal age, however, have not yet been explored in any vascular bed.

The present study addresses three hypotheses focused on the mechanisms whereby reactivity to NO changes with postnatal age. The first hypothesis predicts that rates of biotransformation and liberation of NO from indirect NO donors such as nitroglycerin change with age. To test this hypothesis, we performed and compared parallel studies with nitroglycerin, which requires biotransformation to release NO, with SNAP, which releases NO spontaneously upon hydration. The second hypothesis predicts that the magnitudes of cGMP responses to NO donors will change with age in an artery-specific manner. To test this hypothesis, we carried out parallel experiments in middle cerebral and posterior communicating arteries from newborn and adult sheep. The third hypothesis predicts that interaction between the cGMP and cAMP pathways changes with postnatal age. To assess this hypothesis, we compared the simultaneous time courses and magnitudes of increases in both cAMP and cGMP to NO donors.

Methods

All experimental protocols were approved by the Animal Research Committee of Loma Linda University, Loma Linda, Calif., USA, and followed all guidelines in the NIH Guide for the Use of Laboratory Animals. Posterior communicating and middle cerebral arteries from newborn lambs (age 3–5 days) and young nonpregnant adult female sheep (age 18–24 months) were harvested for these experiments. The dimensions of the arteries used were closely similar to our previously reported values for these vessels; in these arteries, unstressed diameters average 0.7 mm in the fetal posterior communicating, 0.5 mm in the fetal middle cerebral, 0.8 mm in the adult posterior communicating, and 0.6 mm in the adult middle cerebral arteries [22]. Up to 8 ring segments of each artery type were cut from each animal. We used multiple segments from the same animal in the various protocols, but always averaged replicate results into a single value for statistical analysis. Therefore, in our data, 'n' refers to the number of animals, not the number of segments.

The arteries were cleaned of adhering tissues, cut into ring segments 3 mm in length, then denuded of vascular endothelium by

mild mechanical abrasion, as previously described [5, 17, 18]. Briefly, this method involves passing an appropriately sized stainless steel hypodermic needle through the lumen of each arterial segment. Each vascular ring was mounted on paired wires placed between a force transducer (Kulite BG-10) and a post attached to a micrometer controlling resting tension. These preparations were immersed in a Krebs bicarbonate solution containing (in m*M*) 122 NaCl, 25.6 $NaHCO_3$, 5.56 dextrose, 5.17 KCl, 2.49 $MgSO_4$, 1.60 $CaCl_2$, 0.114 ascorbic acid and 0.027 disodium EDTA, continuously bubbled with 95% O_2 plus 5% CO_2 and maintained at 38.5°C (normal ovine core temperature). The arteries were stretched to their optimum diameters, as previously determined [22], which typically yielded resting tensions of approximately 0.5 g. All arteries were equilibrated until resting tensions remained stable for at least 30 min. Endothelial denudation was verified by the absence of a vasodilator response to the endothelium-dependent vasodilator A23187 (calcium ionophore, 1 μM). Following equilibration, the arteries were repeatedly contracted by exposure to an isotonic potassium Krebs solution containing 122 mM K^+ and 31 mM Na^+. After reproducible peak tensions were obtained, the preparations were washed and reequilibrated at baseline tension for another 30 min. Contractile tensions were continuously digitized, normalized, and recorded using an on-line computer as previously described [5].

Three experimental protocols were performed, one of which addressed each of the main hypotheses. First, a dose response relationship for the vasorelaxants, nitroglycerin and SNAP were determined. The second protocol measured the relaxation time course for each agent at the concentration of 10 μM. The 10 μM concentration was chosen as previous studies have determined it to evoke a near-maximal relaxation in this preparation [5]. The third and final protocol examined the relationship between the duration of exposure to SNAP or nitroglycerin and the levels of both cGMP and cAMP.

Protocol 1

The first protocol assessed the concentration-response relations for SNAP and nitroglycerin in all experimental groups. Following initial equilibration, we contracted the arteries by exposure to a mixture of 20 μM histamine with 10 μM serotonin. As shown previously, this combination yields maximal contractions that are quite stable [22]. Once contractile responses had stabilized, cumulative concentrations of either SNAP or nitroglycerin in aqueous solution were added to the tissue baths. Both agents were applied in half-log increments to yield bath concentrations between 10^{-11} and 10^{-4} M. Because SNAP is unstable in solution, it was freshly prepared immediately before use and held on ice. Relaxant responses were calculated as the percentages of the maximum initial tone attained in each vessel type. In addition, pD_2 values (negative log of the EC_{50}) for each concentration-response relation were determined by fitting the normalized concentration-response relations with the logistic equation using computerized nonlinear regression.

Protocol 2

The second protocol quantified rates and magnitudes of relaxation observed in response to application of either SNAP or nitroglycerin. Arteries were contracted with the mixture of serotonin and histamine, as described for protocol 1, after which 10 μM of either SNAP or nitroglycerin was administered to the baths. The resulting changes in tension were monitored for 120 s. All relaxation responses were normalized relative to maximum initial contractile tension. Rates of relaxation were determined by fitting the relaxation time courses to a monoexponential decay model [$y = (A - B)\,e^{-kt} + B$] with a nonzero asymptote using computerized nonlinear regression. In this model y is the percentage of maximum normalized tension remaining at time t, A is the percentage of maximum normalized tension at time zero (approximately 100%), k is the exponential rate constant of relaxation, and B is the percentage of maximum tension at steady state, as previously described [23].

Protocol 3

The experiments of the third protocol examined the temporal relations between cGMP and cAMP as functions of the duration of exposure to either 10 μM SNAP or 10 μM nitroglycerin. Eight segments of each artery type from each animal were studied simultaneously. The arteries were prepared, equilibrated, and contracted as described for protocol 1. When contractile tensions had stabilized, we added either 10 μM SNAP or 10 μM nitroglycerin and flash-froze the segments in liquid nitrogen at varying durations of exposure to the relaxants. The durations used ranged from zero (baseline) to 100 s in 10-second increments. All segments were then subsequently analyzed for cGMP and cAMP content. Thus, from each animal, we obtained a set of artery segments whose treatment varied only by the duration of exposure to the vasorelaxant used.

Cyclic Nucleotide and Protein Determinations

Segments were individually homogenized in 1 ml ice-cold 6% trichloroacetic acid using a motor-driven ground glass pestle and mortar (Lurex, Vineland, N.J., USA). After centrifuging the homogenates for 60 min at 3,000 *g*, we reserved the resultant pellet for protein determination and decanted the supernates for subsequent cyclic nucleotide assay. For protein content determinations, we used an extraction (60 min in 0.1 *N* NaOH at 37°C) designed to exclude connective tissue and structural proteins, as previously described [22]. We quantified protein using the Bradford Coomassie brilliant blue assay. As we have shown previously, this assay produces protein values that are both consistent and uniform in the vessel types studied [22].

For cyclic nucleotide determinations, we extracted the reserved supernates with water-saturated diethyl ether, then lyophilized aliquots of the aqueous phase, reconstituted them in 50 mM acetate buffer, and assayed for cyclic nucleotides using standard radioimmunoassay techniques. We determined both cGMP and cAMP content for each sample using commercially available kits (RPA 525 and RPA 509 Amersham Corp., Ill., USA). Both cGMP and cAMP values were normalized relative to vessel protein content and were expressed as picomoles per milligram vessel protein.

Given our interest in the relative effects of SNAP and nitroglycerin on the levels of cGMP and cAMP, we evaluated the sensitivity and specificity of the assays employed. Prior experience with commercially prepared cyclic nucleotide radioimmunoassay kits indicated an unacceptable level of cross-reactivity between the antibodies for cGMP and cAMP provided with some kits. We determined the specificity by overloading the assays for cGMP and cAMP with known amounts of cGMP (4,800 fmol) and cAMP (7,200 fmol), respectively. Therefore, the amounts of cGMP and cAMP added to test specificity represented considerable excesses over the levels normally seen. In each case under the conditions we employed, the assays selected for this study failed to recognize ('undetectable') the added competing cyclic nucleotide. Standard curves for both assays covered the range of 1.5–96 fmol per 75-µl sample, and thus encompassed the range of values of cGMP and cAMP seen in our samples.

Under the conditions we employed, the assays consistently and reproducibly quantified low levels (1.5 fmol per 75-μl sample) of both cyclic nucleotides in our samples. Intra-assay duplicate errors were 6% or less and interassay errors averaged less than 8%. χ^2 values for our standard curves were consistently close to 1.0. The protein assay against which the cyclic nucleotide values were normalized routinely gave duplicate errors of <5% and standard curve r^2 values of 0.995 or better. Thus, we believe that the values of cGMP and cAMP measured in this study were accurate and specific.

Statistics

All data are expressed as mean ± standard error of the mean, and 'n' refers to the number of animals used in a given experimental group. For all data sets, we evaluated the homogeneity of variance assumption among subsets (homoscedasticity) using Bartlett's test. Where homoscedasticity was verified across groups under the same treatment (nitroglycerin or SNAP), we used either one-way or two-way analyses of variance (ANOVA): maturational age and artery were the factors. There were two ANOVA levels for both maturational age (newborn and adult) and artery type (posterior communicating and middle cerebral). From each ANOVA, there were up to 3 statistical results: (1) age effect; (2) artery effect, and (3) interaction effect. For all ANOVA analyses, we calculated individual post hoc differences between treatments of a given vessel type using Duncan's multiple range test. For data sets where homoscedasticity was not present, we performed single one-time comparisons between corresponding newborn and adult arteries using a Behren's Fisher analysis with pooled weighted variance. Statistical significance implies $p < 0.05$, unless otherwise stated.

Results

From 20 newborn lambs, we obtained 292 artery segments, and from 28 adult sheep, a total of 442. Artery wall thicknesses and diameters were similar to values previously published for ovine arteries by our laboratory [22]. Maximum contractile tensions averaged 1.01 ± 0.09 and 1.75 ± 0.14 g in newborn and adult middle cerebral arteries, respectively. Corresponding values in posterior communicating arteries averaged 1.45 ± 0.11 and 2.45 ± 0.19 g.

Procotol 1: Concentration-Response Relations

The cumulative concentration-relaxation relations for newborn (n = 7) and adult (n = 8) cerebral arteries are shown in figure 1 for both nitroglycerin and SNAP. Overall, relaxation magnitudes were greater in newborn than in adult arteries for both relaxants in both artery types. In the posterior communicating arteries, relaxation magnitudes for nitroglycerin averaged 94.5 ± 1.9% (newborn) and 76.1 ± 5.1% (adult), and for SNAP, they averaged 97.1 ± 1.2% (newborn) and 80.1 ± 3.5% (adult). In the middle cerebral arteries, relaxation magnitudes for nitroglycerin averaged 97.3 ± 1.1% (newborn) and 80.6 ± 4.0% (adult), and for SNAP, they averaged 96.4 ± 2.5% (newborn) and 80.8 ± 1.6% (adult). Magnitudes of relaxation did not vary significantly with either artery or relaxant type.

Sensitivity to nitroglycerin, as indicated by the pD_2 values of the concentration-response relations, was significantly greater in newborn (6.69 ± 0.17) than adult (5.86 ± 0.12) posterior communicating arteries and also in newborn (6.23 ± 0.15) compared with adult (5.05 ± 0.18) middle cerebral arteries. Similarly, sensitivity to SNAP was also significantly greater in newborn (6.58 ± 0.17) than adult (6.12 ± 0.17) middle cerebral arteries. However, sensitivity to SNAP was similar in newborn (7.01 ± 0.38) and adult (7.12 ± 0.17) posterior communicating arteries. Except for SNAP in the adult arteries, sensitivities to both relaxants were significantly greater in posterior communicating than middle cerebral arteries. In adult but not newborn arteries, sensitivities to SNAP were significantly greater than sensitivities to nitroglycerin.

Protocol 2: Rates of Relaxation to Nitroglycerin and SNAP

The 10 μ*M* concentrations of nitroglycerin and SNAP used to produce rapid relaxation were greater than or equal to the EC_{90} concentration in all arteries. Relaxation responses to this concentration of either nitroglycerin or SNAP were generally complete within 120 s in all arteries (fig. 2a). The rate coefficients for relaxation did not vary significantly with age for either relaxant in either artery type. Artery-to-artery differences in rates of relaxation were observed only in adult arteries, where rates were greater in the posterior communicating than in the middle cerebral arteries for both relaxants (fig. 2b). In addition, rates of relaxation were significantly greater for SNAP than for nitroglycerin in all groups.

Protocol 3: Cyclic Nucleotide Responses to Nitroglycerin and SNAP

To enable calculation of intracellular concentrations of cGMP and cAMP, we calculated the ratio of cellular protein to intracellular water in our arteries using the formula:

$$\%P/\%W_i \cdot (200 - \%W_t)/\%W_t$$

where %P equaled the percent dry weight protein, $\%W_i$ equaled the percent intracellular water, and $\%W_t$ equaled the total percent water. Both %P and $\%W_t$ were measured directly, as previously described [22], and the value used for $\%W_i$ was that measured previously in the same artery types used in the present study [24]. When the above for-

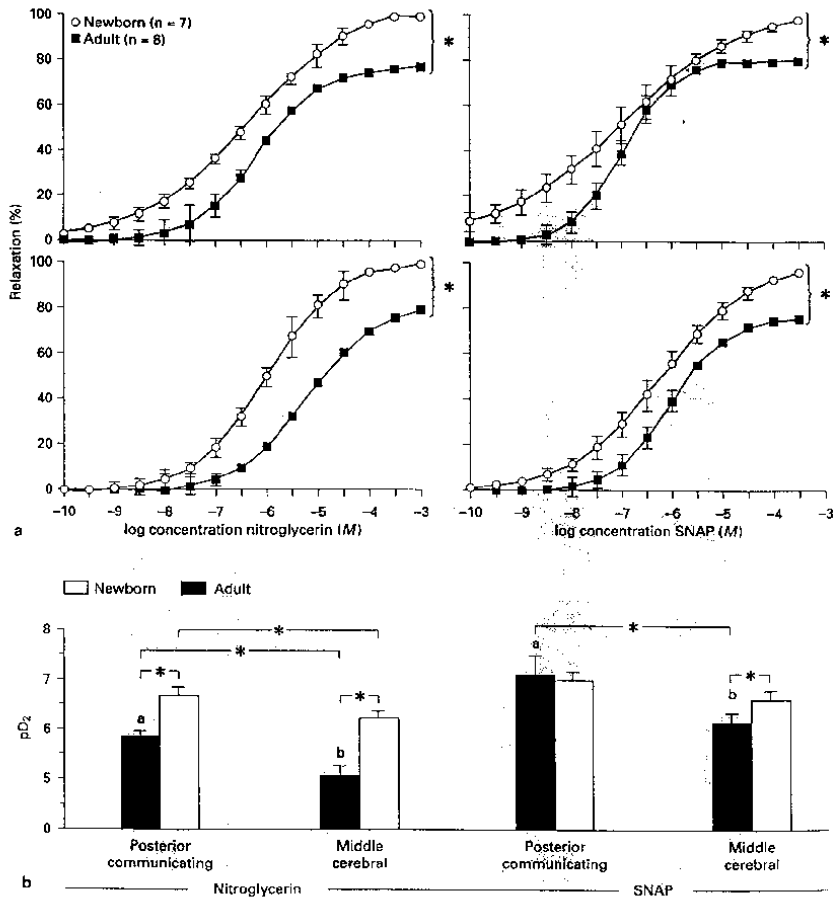

Fig. 1. Concentration-relaxation relations for nitroglycerin and SNAP in newborn and adult cerebral arteries. **a** Concentration-response relations obtained for nitroglycerin-induced (right panels) and SNAP-induced (left panels) relaxation of contractile tension induced by 10 μM serotonin with 20 μM histamine. **b** pD_2 ($-\log ED_{50}$ concentration) values for the concentration-response relations shown in **a**. Relaxation responses were calculated as percentages of initial contractile tone. All values are shown as means and standard errors. For the results of statistical comparisons, significant differences between nitroglycerin and SNAP are denoted by 'a' for posterior communicating arteries and by 'b' for middle cerebral arteries. Asterisks indicate significant differences at $p < 0.05$ between the values indicated.

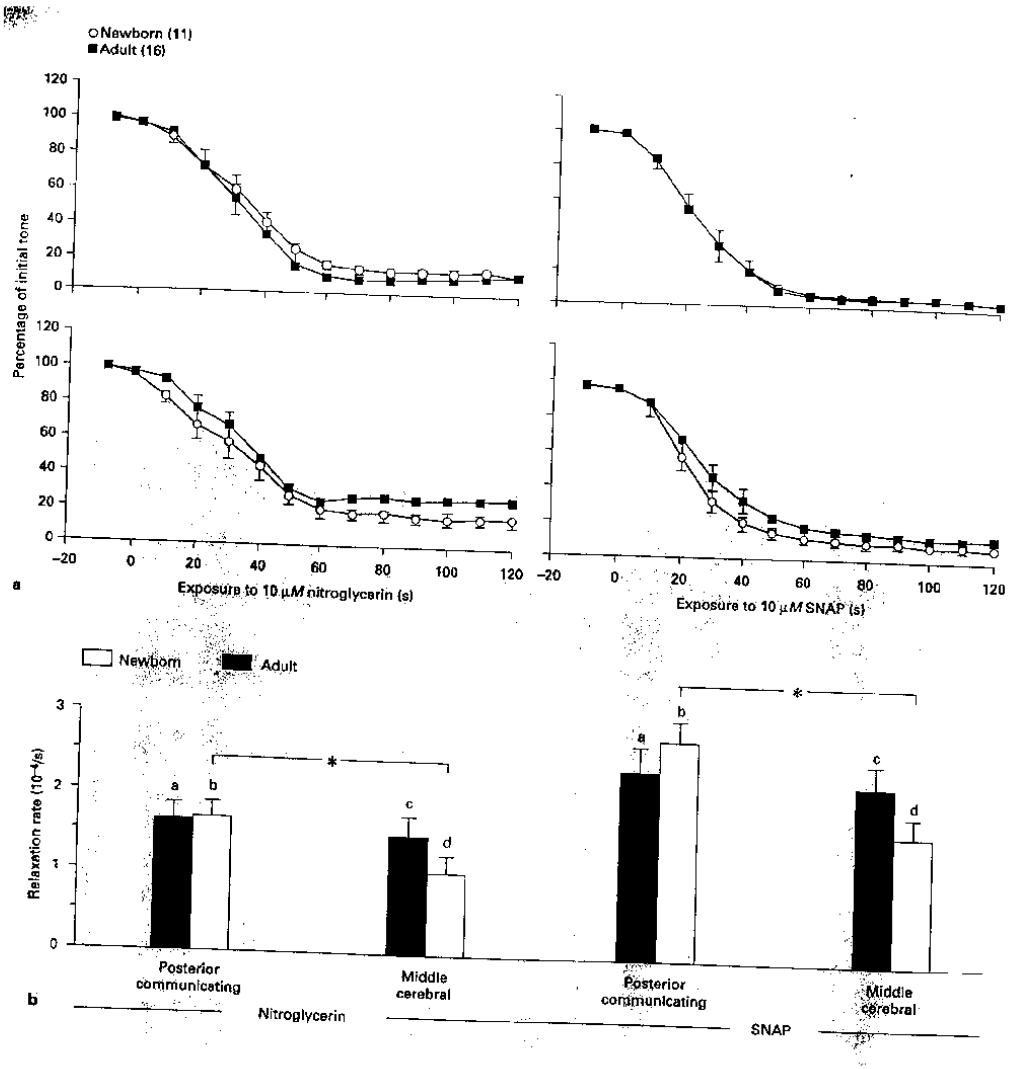

Fig. 2. Effects of 10 μM nitroglycerin and SNAP on relaxation rates in newborn and adult ovine cerebral arteries. **a** Time courses of responses of contractile tensions to either 10 μM nitroglycerin or 10 μM SNAP. The contractile tensions were expressed as percentages of initial contractile tone induced by 10 μM serotonin with 20 μM histamine. **b** Rate coefficients for relaxation (percentage of relaxation per minute) in each group, obtained by fitting the relaxation-time data to a monoexponential decay model. All values are given as means and standard errors. For the results of statistical comparisons, significant differences between nitroglycerin and SNAP are denoted by the letters 'a' to 'd'; columns with the same letter significantly differ from each other. Asterisks indicate significant differences at $p < 0.05$ between the values indicated. For newborns, n = 11 and for adults n = 16.

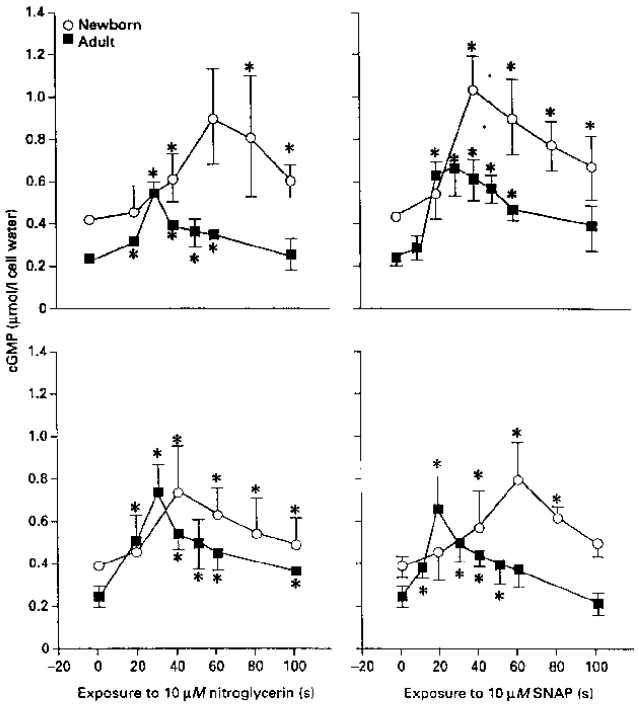

Fig. 3. Effect of maturation and artery type on cGMP responses to nitroglycerin and SNAP. Newborn and adult posterior communicating (upper panels) and middle cerebral (lower panels) arteries were precontracted with 10 µ*M* serotonin and 20 µ*M* histamine, and then exposed to either 10 µ*M* nitroglycerin (left panels) or 10 µ*M* SNAP (right panels). The cGMP levels were measured by radioimmunoassay in segments flash-frozen 10–100 s after exposure to each relaxant. Notice that the baseline values were significantly greater in newborn than corresponding adult arteries. All values are given as means and standard errors for 11 newborns and 16 adults. Asterisks indicate values significantly greater than corresponding baseline controls at time zero.

mula was used to estimate intracellular cyclic nucleotide concentrations in units of micromoles/liter cell water, basal cGMP and cAMP levels were significantly higher in newborn than in adult cerebral arteries regardless of artery type (fig. 3, 4; table 1). The ratios of basal cAMP to cGMP concentrations tended to be greater in adult (2.1) than in newborn (1.4) posterior communicating arteries, but were similar in adult (1.6) and newborn (1.6) middle cerebral arteries.

In all arteries, administration of either nitroglycerin or SNAP produced rapid increases in cGMP that peaked between 20 and 60 s and then returned toward baseline levels (fig. 3). In all cases, peak cGMP values were attained earlier in adult (20–30 s) than in corresponding newborn (40–60 s) arteries; the newborn time courses were shifted to the right relative to those of the adults. Except in nitroglycerin-treated middle cerebral arteries, peak cGMP values were significantly higher in newborn than adult arteries. Average peak cGMP concentrations (in micromoles/liter cell water) are given in table 1.

Administration of either nitroglycerin or SNAP also produced some significant increases in cAMP levels (fig. 4). In general, however, the peak cAMP responses appeared much later (40–60 s) than corresponding peaks of cGMP (20–30 s). In newborn arteries, only nitroglycerin-treated posterior communicating and SNAP-treated middle cerebral arteries exhibited significant increases in cAMP, whereas both nitroglycerin and SNAP produced significant cAMP increases in both adult artery types. When compared on the basis of fold increases above baseline, there were no significant age-related differences in cAMP responses among any of the experimental groups. Similarly, there were no significant differences in cAMP responses associated with either artery or relaxant type.

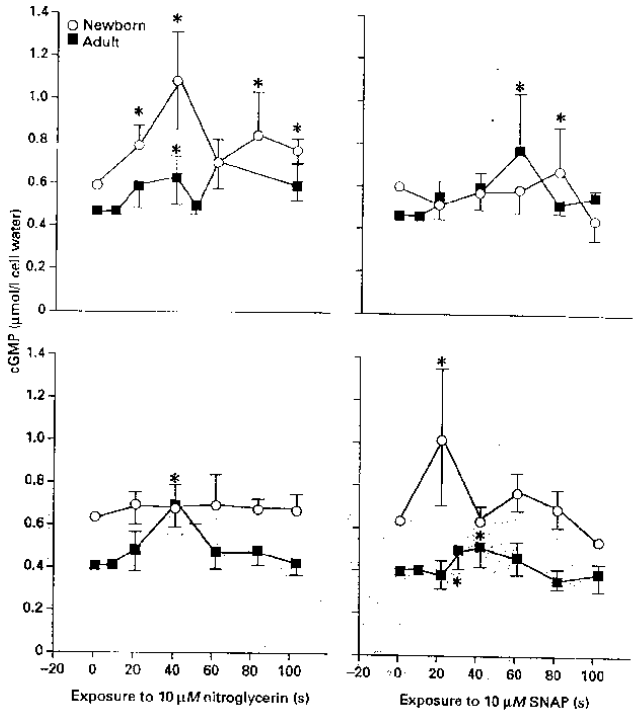

Fig. 4. Effect of maturation and artery type on cAMP responses to nitroglycerin and SNAP. After precontraction with 10 μM serotonin and 20 μM histamine, newborn and adult posterior communicating (upper panels) and middle cerebral (lower panels) arteries were exposed to either 10 μM nitroglycerin (left panels) or 10 μM SNAP (right panels). At different times of exposure to the relaxants, the arteries were flash-frozen in liquid nitrogen and then assayed for cAMP levels. Notice that baseline values were significantly greater in newborn than corresponding adult arteries. All values are given as means and standard errors obtained from 11 newborns and 16 adults. Asterisks indicate values significantly greater than corresponding baseline controls at time zero.

Table 1. Effects of age and artery type on basal and peak cyclic nucleotide responses to nitroglycerin (NG) and SNAP

Age	Artery	TX	BL cGMP	Peak cGMP	BL cAMP	Peak cAMP
Newborn	PC	NG	0.427 ± 0.045	0.901 ± 0.226*	0.595 ± 0.054	1.086 ± 0.237*
		SNAP		1.033 ± 0.145*		0.677 ± 0.203
	MCA	NG	0.388 ± 0.060	0.749 ± 0.198*	0.618 ± 0.054	0.677 ± 0.136
		SNAP		0.803 ± 0.163*		1.005 ± 0.315*
Adult	PC	NG	0.222 ± 0.024	0.534 ± 0.055*	0.458 ± 0.076	0.692 ± 0.119*
		SNAP		0.648 ± 0.127*		0.765 ± 0.267*
	MCA	NG	0.242 ± 0.037	0.726 ± 0.145*	0.391 ± 0.054	0.668 ± 0.087*
		SNAP		0.652 ± 0.156*		0.495 ± 0.083*

Shown above are the baseline (BL) and peak cGMP values (μmol/l cell water) attained following exposure to 10 μM NG or 10 μM SNAP in posterior communicating (PC) and middle cerebral arteries (MCA). Baseline values were averaged across treatment groups, and thus a single value is shown. Notice that the corresponding baseline cAMP values are significantly greater than baseline cGMP values, and basal levels of both cyclic nucleotides are significantly greater in newborn than adult arteries. Asterisks denote significant differences between peak values and corresponding basal values. All values are given as the means and standard errors for 11 newborns and 16 adults.

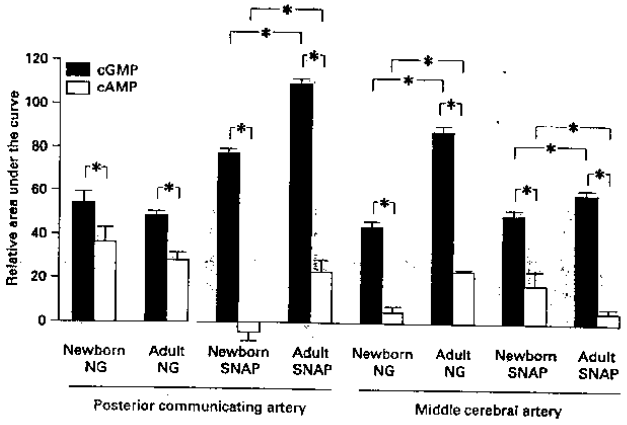

Fig. 5. Effect of maturation and artery type on the relative responses of cGMP and cAMP to NO. Shown above are the averaged values of the integrated areas under the time-concentration curves shown in figures 3 and 4. All cyclic nucleotide responses were normalized relative to the corresponding baseline values of individual experiments at time zero before arteries were exposed to nitroglycerin (NG) or SNAP, and are expressed as percentage areas above baseline. Asterisks indicate significant statistical differences at $p < 0.05$.

To compare the simultaneous responses in cGMP and cAMP, we integrated the areas above baseline and beneath the time-dependent cyclic nucleotide responses using Simpson's approximation (fig. 5). The integration interval extended between 0 and 100 s of exposure to either SNAP or nitroglycerin. In all groups, the areas under the curve were greater for newborn than adult arteries, and were greater for cGMP than for cAMP responses. Altogether, the data illustrate important parallel changes in cGMP and cAMP that occur in an age-, artery-, and relaxant-dependent manner.

Discussion

Despite numerous studies of the effects of NO synthase inhibitors on vascular reactivity, reports of the direct effects of NO on immature arteries are scarce, particularly for arteries of the cerebral circulation. The present study addresses this deficit and offers several new observations. First, the efficacy and potency of NO donors were generally greater in newborn than adult cerebral arteries denuded of endothelium. Second, rates of relaxation, and presumably rates of NO release, were faster for equimolar concentrations of SNAP than for nitroglycerin in both newborn and adult arteries denuded of endothelium. Third, basal concentrations were greater for cAMP than for cGMP, and both were greater in newborn than adult cerebral arteries denuded of endothelium. Fourth, in denuded adult cerebral arteries, NO-induced increases in cGMP occurred faster but relaxation developed more slowly than in denuded newborn cerebral arteries. Fifth, responses to NO donors involved significant parallel changes in cGMP and cAMP, the characteristics of which were age, artery, and agent specific.

Many previous studies suggest that early postnatal maturation alters patterns of NO-induced relaxation in the cerebral circulation. For example, the magnitude of endothelium-dependent vasodilatation is depressed in immature relative to mature cerebral arteries [25], a finding due perhaps to reduced expression of the eNOS enzyme in immature cerebrovascular endothelial cells [26]. In contrast, expression of soluble guanylate cyclase, the main target enzyme for NO in vascular smooth muscle, is more abundantly expressed in immature than mature cerebral arteries [27], and at least in basilar arteries, cGMP responses to NO appear enhanced in immature compared with mature cerebral arteries [5]. At the same time, total phosphodiesterase activity also appears enhanced in immature relative to mature cerebral arteries [18]. How the relative effects of maturation on the multiple mechanisms mediating NO-induced relaxation integrate to determine overall reactivity remains unclear, particularly because very few studies have simultaneously examined arteries of more than a single age. Equally important, it is clear that artery size can be an important

determinant of reactivity to NO and NO donors [28], and thus results obtained in large cerebral arteries cannot simply be extrapolated to the smaller arteries of the cerebral circulation. Further complicating understanding of NO-mediated vasodilatation is the fact that the great majority of published studies of this pathway in the neonate have used only inhibitors of NO synthesis rather than direct application of NO or NO donors [29–31].

The present studies clearly indicate that postnatal maturation attenuated reactivity to NO in denuded cerebral arteries (fig. 1). Consistent with reports that both the sensitivity and efficacy of cGMP were enhanced in immature compared with mature cerebral arteries [17], the efficacy of exogenous NO was enhanced in neonatal compared with adult cerebral arteries denuded of endothelium regardless of artery type or the source of NO. In contrast to NO efficacy, however, sensitivity to NO varied significantly between different artery types and sources of NO (fig. 1b). For NO spontaneously released from SNAP, sensitivity was similar in all groups except the denuded adult middle cerebral arteries where it was depressed. This difference in sensitivity, in the absence of a difference in efficacy, suggests that either the metabolism of NO is upregulated, or the kinetics of its ability to activate soluble guanylate cyclase are downregulated, as a consequence of maturation in middle cerebral but not posterior communicating arteries. This difference may simply reflect age- and artery-specific differences in soluble guanylate cyclase abundance [27], but could also reflect age-related differences in other enzymes mediating either NO oxidation or cGMP-mediated vasorelaxation [16, 17, 20, 21]. From a broader perspective, these results emphasize that the cerebrovascular sensitivity and efficacy of NO are independently regulated in an age- and artery-specific manner.

Compared with NO released from SNAP, sensitivity to NO released from nitroglycerin was depressed in both denuded middle cerebral and denuded posterior communicating arteries of the adult. These SNAP-nitroglycerin differences, however, were absent in denuded newborn arteries, suggesting that enzymatically mediated rates of NO release from nitroglycerin [9–11] may have been upregulated enough not to be rate-limiting for relaxation in the immature arteries. Consistent with this possibility, sensitivity to nitroglycerin was significantly greater in newborns than adults for both artery types examined (fig. 1b). In addition, sensitivity to NO was independent of the source of NO in all newborn arteries. As for NO released from SNAP, sensitivity to NO released from nitroglycerin was greater in denuded posterior communicating than in denuded middle cerebral arteries for both the adult and the newborn, which strengthens the suggestion that sensitivity to NO is highly artery specific, reflects functional specialization, and is greater in the larger posterior communicating arteries that serve primarily as conduit arteries at the base of the brain, than in the smaller, more distal and more nutritive middle cerebral arteries.

In parallel with the observed artery-to-artery differences in sensitivity to nitroglycerin, rates of relaxation to nitroglycerin were also significantly greater in denuded posterior communicating than in denuded middle cerebral arteries of the adult but not the newborn (fig. 2). Perhaps more importantly, rates of relaxation were significantly greater for SNAP than for nitroglycerin within each age-artery combination (fig. 2b). Given that the release of NO from nitroglycerin, but not from SNAP, requires enzyme action [9–11], the slower rate of relaxation observed in response to nitroglycerin most probably reflects the delay required for this reaction. Given that rates of relaxation to nitroglycerin decreased significantly during postnatal maturation in denuded middle cerebral but not denuded posterior communicating arteries, the data reinforce the view that sensitivity to NO is highly artery specific and reflects functional specialization. Because rates of relaxation to both SNAP and nitroglycerin exhibited no significant age-related differences, despite age-related differences in sensitivity to NO, the combined data suggest that rates of relaxation may be more dependent upon the efficacy than the sensitivity to NO, and in turn, may be independent of the well-established age-related differences in both the abundance of soluble guanylate cyclase [27], and reactivity to cGMP [17]. Thus, rates of relaxation to NO appear more dependent on common characteristics of contractile protein kinetics, than on age-related dynamic differences in the NO-cGMP relaxation pathway.

A key determinant of relaxant responses to NO is the basal level of cGMP within the smooth muscle cells. The greater the basal concentration of cGMP, the smaller the mass of cGMP that must be synthesized to attain the threshold concentration necessary for activation of protein kinase G, which in turn phosphorylates multiple intracellular proteins that together govern the relaxation response [32]. Consistent with studies of cGMP responses in other arteries [5], basal levels of cGMP were significantly greater in newborns than adults for both endothelium-denuded artery types examined (table 1). In addition, basal levels were also greater for cAMP than for cGMP, and the ratio of cAMP to cGMP increased with age, as reported by Kostromin et al. [33] for spleen T-lym-

phocytes. These changes suggest that the basal cyclic nucleotide ratios of synthesis to hydrolysis increase with postnatal age, and more so for cAMP than for cGMP [18, 33]. From a functional perspective, the data suggest that immature cerebral arteries require less total cGMP synthesis for activation of protein kinase G, and that their higher concentrations of both cAMP and cGMP increase the likelihood for functionally significant interactions between these two molecules [19].

Even though basal cGMP levels were greater in denuded newborn arteries, rates of increase in cGMP following exposure to NO were greater in denuded adult arteries (fig. 3). Correspondingly, peak cGMP concentrations were attained sooner in denuded adult than in denuded newborn arteries, regardless of the source of NO. The magnitudes of the peak cGMP concentrations, however, were significantly greater in denuded neonatal than in denuded adult posterior communicating arteries, but not in denuded middle cerebral arteries. This latter finding agrees well with the observed artery-to-artery differences in NO sensitivity and relaxation rates (fig. 1, 2) and suggests that peak cGMP concentration, relaxation rate, and NO sensitivity are all highly correlated at the vascular smooth muscle level. Because the rate of rise of cGMP in response to NO reflects the ratio of the synthetic capacity of soluble guanylate cyclase relative to the hydrolytic activity of phosphodiesterase, the data suggest that either the kinetics of cGMP synthesis are upregulated or that cGMP hydrolysis is downregulated in denuded adult relative to denuded newborn cerebral arteries. Given that soluble guanylate cyclase kinetics are similar in denuded neonatal and denuded adult cerebral arteries [27], and that total soluble guanylate cyclase and phosphodiesterase activities are greater in denuded neonatal compared with denuded adult arteries [18], age-related differences in phosphodiesterase activity are the most likely explanation for the observed age-related differences in the rates of cGMP responses to NO. The observed differences between denuded middle cerebral and denuded posterior communicating artery responses further suggest that soluble guanylate cyclase and phosphodiesterase activities are regulated differently in conduit arteries such as the posterior communicating arteries than in the more distal nutritive arteries such as the middle cerebral arteries. These findings further predict that phosphodiesterase inhibitors should vasodilate newborn more than adult arteries, and posterior communicating more than middle cerebral arteries [16, 19, 20].

Whereas peak cGMP concentrations were not attained until at least 40 s or more of exposure to NO (fig. 3), relaxation responses were typically apparent in less than 20 s (fig. 2). This finding suggests that the concentrations of cGMP necessary to activate protein kinase G were much less than the peak concentrations observed, which ranged from 0.53 to 1.03 μmol/l cell water (table 1). The data are thus highly consistent with published estimates for the apparent K_a values (concentration required for half-maximal activation) of purified vascular protein kinase G for cGMP, which range from 0.29 to 0.44 μ*M* [34]. This agreement not only validates the methods used in the present study to calculate intracellular cyclic nucleotide concentrations, but also demonstrates that the estimates of protein kinase G affinity for cGMP obtained in vitro are reasonable predictors of the interactions between cGMP and PKG in situ.

Unlike cGMP, which consistently exhibited transient increases in response to NO, cAMP responses to NO were highly variable and generally exhibited much smaller increases relative to baseline (fig. 4). Whereas peak cGMP ranged from 93 to 200% above baseline in response to NO, the corresponding peak values of cAMP ranged from only 10 to 83% (table 1). In absolute units, peak cGMP increases averaged 0.44 μmol/l, whereas cAMP increases averaged only 0.24 μmol/l. Still, many of the increases observed in cAMP were significant, and tended to occur later than the increases observed for cGMP. Together, these findings establish that exposure to NO produced simultaneous changes in cGMP and cAMP.

To explore possible correlations between the cyclic nucleotide responses to NO, we calculated the relative areas beneath the time-concentration profiles for each cyclic nucleotide. In all cases, the fold increases observed were greater for cGMP than for cAMP (fig. 5). In direct contrast to the peak responses (fig. 3), these increases were greater for denuded adult than for denuded newborn arteries for SNAP in both artery types, and for nitroglycerin in the denuded middle cerebral arteries. This latter finding again suggests that the dynamics of the responses to cyclic nucleotides vary with age and artery type, and further support the suggestion that the ratios of cGMP synthesis to hydrolysis are greater in denuded adult than in denuded newborn cerebral arteries. Equally important, we observed that for each significant difference in the cGMP area observed between corresponding denuded newborn and denuded adult arteries, there was also a parallel significant difference in the cAMP area (fig. 5). This latter finding implies a possible correlation between cAMP and cGMP responses to NO in these arteries. Although the mechanisms responsible for correlations between cAMP and cGMP responses to NO were not direct-

ly tested in the present study, it seems unlikely that by-products of both SNAP and nitroglycerin might have directly stimulated adenylate cyclase activity. Such effects are not supported by any literature and thus seem highly unlikely. Alternatively, it is possible that Type II and Type III phosphodiesterases, both of which are present to a variable extent in cerebral arteries [19], may have been involved. Through activation of these phosphodiesterases, cGMP could stimulate parallel decreases or increases in cAMP activity, respectively [35, 36]. Differences in the relative effects of NO on cGMP and cAMP levels in the various artery-treatment combinations could thus easily be explained by corresponding differences in the relative abundances of the phosphodiesterase subtypes. Given this possibility, further detailed studies of the effects of maturation on phosphodiesterase subtype expression and activity in cerebral arteries appear well justified.

On overview, cerebrovascular reactivity to NO was attenuated during postnatal maturation due to decreases in both the sensitivity to and efficacy of NO that reflect maturational decreases in soluble guanylate cyclase abundance and possible increases in NO turnover. Rates of relaxation were influenced by the source of NO, and were faster for NO spontaneously released from SNPA than for NO enzymatically released from nitroglycerin, regardless of age or artery type. Rates of relaxation were also influenced by artery type, and were faster in denuded posterior communicating than denuded middle cerebral arteries of the adult but were independent of artery type in the newborn, indicating that maturation slowed the mechanisms mediating NO-induced relaxation in the more distal cerebral arteries. Peak cGMP concentrations, relaxation rates, and NO sensitivity were all well correlated and basal levels of both cyclic nucleotides were elevated in immature arteries, indicating that basal ratios of synthesis to hydrolysis were greater in fetal than adult arteries denuded of endothelium. Interestingly, NO-induced increases in cGMP were accompanied by heterogeneous rises in cAMP, suggesting the possibility of cGMP-induced stimulation of Type I phosphodiesterase and/or inhibition of Type III phosphodiesterase. In turn, variable involvement of phosphodiesterases in the cyclic nucleotide responses to NO could potentially explain many of the artery- and age-specific responses observed. Clearly, cerebrovascular responses to NO involve multiple factors, many of which vary significantly with postnatal age and/or artery type. Although the precise mechanisms governing the abundance and activity of the regulatory enzymes involved remain unknown, the present results encourage careful consideration of the cerebrovascular effects of NO when administering NO therapeutically, particularly in the neonate.

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