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Effects of Maturation on Cyclic GMP Metabolism in Ovine Carotid Arteries

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ABSTRACT

Previous studies suggest that elevated basal levels of cGMP in newborn arteries may help explain why vascular resistance is lower in newborns than adults. To explore the reasons why basal cGMP is higher in neonatal arteries, the present studies examined rates of cGMP synthesis and degradation in newborn and adult ovine common carotid arteries. The measurements were performed in both intact and homogenized arteries, and results were normalized relative to cell water to estimate intracellular concentrations and minimize errors due to compositional differences between newborn and adult arteries. Steady state levels of cGMP measured under baseline conditions averaged 0.11 ± 0.02 μM in adult arteries and 0.59 ± 0.11 μM in newborn arteries. These resting cGMP levels were unaffected by endothelium removal. Under baseline conditions, steady state rates of cGMP synthesis (μmol of cGMP/L of cell water/min) were higher in newborn (0.31 ± 0.06) than in adult (0.15 ± 0.04) arteries. Maximal rates of cGMP degradation (μmol of cGMP/L of cell water/min) measured in artery homogenates were also much higher in preparations of newborn (106 ± 6) than of adult (78 ± 6) arteries. Together, these data suggest that the reason resting cGMP concentrations were higher in newborn than in adult arteries was due at least in part to a higher basal rate of cGMP synthesis in the newborn. Estimates of apparent Km values for PDE were also greater in newborn (2.9 μM) than in adult (1.5 μM) preparations, suggesting that age-related differences in the Km for PDE may also contribute to the elevated basal concentration of cGMP observed in the newborn. (***Pediatr Res* 39: 25–31, 1996**)

Abbreviations

cGMP, guanosine 3′,5′-cyclic monophosphate
PDE, cGMP-specific phosphodiesterases
SNAP, *S*-nitroso-*N*-acetyl-penicillamine
IBMX, 3-isobutyl-1-methylxanthine
TCA, trichloroacetic acid
EDRF, endothelium-derived relaxing factor

In the fetus, arterial pressure is low as is total peripheral resistance. With the transition from fetal to newborn life, many of the influences which depress fetal vascular resistance, such as high carbon dioxide tension, low oxygen tension, and high prostaglandin E levels, are mitigated and thus contribute to higher vascular resistance in the newborn (1). Not all factors favoring low vascular resistance, however, dissipate at birth as quickly as blood gas tensions and prostaglandin levels. For example, basal levels of cerebrovascular cGMP remain elevated in the newborn relative to the adult for at least 1 wk after birth (2). The reasons why these vasodilatory influences persist in newborn arteries remain unknown.

Because persistently low vascular resistance may contribute to neonatal cardiovascular and cerebrovascular complications (3, 4), it is of clinical importance to understand the mechanisms favoring low vascular resistance in the newborn. With regard to vascular cGMP, elevated basal levels could result either from elevated synthesis, or depressed degradation, relative to that of the adult. Elevated synthesis, in turn, could result from elevated basal release of nitric oxide from the vascular endothelium, but probably not from greater vascular sensitivity to nitric oxide because previous studies have shown that NO sensitivity is similar in newborn and adult arteries (2). Alternatively, elevated basal levels of vascular cGMP could result from lower levels of total phosphodiesterase activity in newborn relative to adult arteries.

Although considerable recent effort has been directed toward understanding vascular cGMP metabolism, very few studies have examined this topic in relation to maturation. To address this deficit, the present studies examined cGMP metabolism in newborn and adult ovine common carotid arteries. To circumvent the requirements for large amounts of tissue typical of many phosphodiesterase assays, we developed a method for estimating phosphodiesterase activity based on the effects of phosphodiesterase inhibitors on rates of cGMP accumulation in intact arteries. This approach made possible accurate measurements of rates for both synthesis and degradation of cGMP in

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small newborn arteries, and also preserved vascular compartmentalization which may be an important factor governing overall cGMP metabolism (5, 6). For comparison, parallel measurements using artery homogenates were also conducted.

METHODS

All procedures and protocols used in the present studies were approved by the Animal Research Committee of Loma Linda University and followed all guidelines put forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

We obtained common carotid arteries from young nonpregnant adult sheep (age 18–24 mo) and newborn lambs (age 3–5 d). Tissue from adult animals was obtained from a local slaughterhouse and kept packed in ice until dissection. Tissue from newborn animals was obtained from lambs brought into the facility, and killed with a lethal injection of sodium pentobarbital on the day of the experiments. Up to 8 ring segments were taken from each animal; from 25 newborn lambs we harvested 180 artery segments, and from 34 adult sheep, a total of 224. All reported values of *n* refer to the number of animals, not the number of segments.

The initial treatment of all arteries was identical and has been described in detail previously (7). Briefly, we cleaned the arteries of adipose and connective tissue and cut them into ring segments 3 mm in length. Except where noted, each segment was mechanically denuded of its endothelium using a roughened blunt hypodermic needle followed by a gentle flush with isotonic Krebs solution, as previously described (8, 9). Tissue segments that were used for enzyme activity assays were flash frozen in liquid nitrogen and stored at −80°C until assayed. All other vascular ring segments were mounted on paired wires between a force transducer (Kulite BG-10) and a post attached to a micrometer used to vary resting tension. In a Krebs-bicarbonate solution containing (in mM) 122 NaCl, 25.6 $NaHCO_3$, 5.56 dextrose, 5.17 KCl, 2.49 $MgSO_4$, 1.60 $CaCl_2$, 0.114 ascorbic acid, and 0.027 disodium EDTA, continuously bubbled with 95% O_2, 5% CO_2 and maintained at 38.5°C (normal ovine core temperature), we slowly and repeatedly stretched the freshly mounted arteries until the optimum baseline tensions of 1 g (7) remained stable for at least 30 min. We then contracted the arteries with an isotonic potassium Krebs solution containing 122 mM K^+ and 31 mM Na^+. After peak tensions were reached, we washed the arteries with normal sodium Krebs solution and allowed them to reequilibrate at baseline tension for another 30 min. Treatment of the artery segments thereafter varied with each protocol. During all experiments, we continuously digitized, normalized, and recorded contractile tensions using an on-line computer.

Using freshly mounted arteries, we conducted three experimental protocols. First, we determined the basal cGMP concentrations for both the newborn and adult in endothelium intact and denuded arteries. Second, we determined the basal rate of cGMP synthesis in endothelium denuded arteries. And third, we determined the cGMP time courses for both newborn and adult in PDE inhibited and uninhibited preparations. Using tissue which had not been mounted, we also determined the rate of cGMP disappearance in crude homogenates for each age group.

Protocol 1: Basal cGMP determination. Artery segments from each animal were arranged in pairs and one member of each pair was denuded of endothelium and the other remained intact, as previously described (9, 10). After mounting and equilibrating the artery pairs, we contracted each segment with isotonic 122 mM potassium and then repeatedly washed the segments with normal sodium-Krebs solution until resting baseline tension was reattained. We then froze the artery segments in liquid nitrogen. The frozen vessels were subsequently homogenized and assayed for protein and cGMP as described below.

Protocol 2: Determination of optimum IBMX concentration. To determine the concentration of IBMX (a wide-spectrum phosphodiesterase inhibitor) necessary to inhibit phosphodiesterase activity in our preparations, we measured cGMP accumulation over 12 min in uncontracted adult carotid segments in the presence of varying concentrations of IBMX (0, 3, 30, and 300 μM). IBMX was dissolved in 0.1% DMSO, and control segments were treated with vehicle only. Artery segments were frozen after 0, 4, 8, or 12 min of exposure to IBMX. The resulting cGMP values were plotted against time and compared with rates of cGMP accumulation in control segments without IBMX.

Protocol 3: Basal rates of cGMP synthesis. Measurements of basal rates of cGMP synthesis were based on paired differences in cGMP accumulation between control arteries and arteries treated with IBMX. Artery segments from each animal were denuded of endothelium, mounted in tissue baths, and arranged in two sets consisting of four artery segments each. All arteries were contracted with isotonic potassium-Krebs solution, then washed with normal sodium-Krebs solution and allowed to return to baseline tension. We then exposed the first set of arteries to the optimum concentration of IBMX determined above. The arteries were then flash frozen in liquid nitrogen after 0, 1, 2, and 4 min of exposure to IBMX. The second set of arteries served as controls and were flash frozen at the same intervals. All arteries were subsequently homogenized and assayed for protein and cGMP as described below.

Protocol 4: Effects of IBMX on cGMP accumulation responses to SNAP. To determine the time course of cGMP accumulation, we prepared and mounted six segments from each animal. All segments were denuded of endothelium, contracted with isotonic potassium-Krebs, then contracted a second time with 1 μM serotonin. In preliminary studies, 1 μM serotonin alone had no effect of cGMP levels. When contractile responses to serotonin had stabilized, we added SNAP (an exogenous source of nitric oxide) to a final concentration of 10 μM to each bath, and then froze the arteries after 0, 20, 40, 60, 80, or 100 s of exposure to SNAP. To estimate the time course of cGMP synthesis alone, we repeated these measurements in arteries preincubated with the optimum concentration of IBMX before the treatment with serotonin and SNAP. The frozen vessels were subsequently homogenized and assayed for protein and cGMP as described below.

Protocol 5: PDE activity in Crude Homogenates. We prepared crude homogenates from artery segments taken from the

same animals used in protocols 1–3, above. We homogenized approximately 25 mg (wet weight) of common carotid in 2 mL of 40 mM Tris-HCl (pH 7.5), containing 1.6 mM $CaCl_2$, 2 mM $MgCl_2$, and 1 mM dithiothreitol buffer at 4°C (11). The homogenization buffer also contained a mixture of protease inhibitors; 76.8 nM aprotinin, 83 mM benzamidine, 1 mM iodoacetamide, 1.1 μM leupeptin, 7 μM pepstatin A, and 0.23 mM phenylmethanesulfonyl fluoride (12). After glass-on-glass homogenization, the homogenates were centrifuged at 3000 × *g* for 30 min. An aliquot of the resulting supernate was assayed for Tris-HCl-soluble proteins as described below. We divided the remaining supernate into six 250-μL aliquots and placed them into tubes kept at 4°C. To remove any endogenous cGMP from the preparation, we individually warmed each tube in a 37°C water bath for a minimum 30 min "clearing period" (to allow complete hydrolysis of endogenous cGMP), before the addition of substrate [3000 pmol cGMP per tube in a 0.05 M sodium acetate buffer at pH 5.8 containing sodium azide (Amersham Corp., Arlington Heights, IL)]. Versions of this protocol without the clearing period produced varying starting levels of cGMP. The initial concentration of cGMP was approximately 10-fold greater than reported values of Km for PDE. After the addition of cGMP, each tube was allowed to incubate for t = 0, 5, 10, 15, 20, or 25 min. At the end of each incubation period, we added 2 mL of 6% TCA to each tube. The samples were then centrifuged, ether washed, lyophilized, and assayed for remaining cGMP. Control artery segments were "cleared" of endogenous cGMP as described above, then incubated with 300 μM IBMX for 15 min before the addition of cGMP. Thereafter, control preparations were treated as described above.

Cyclic Nucleotide and Protein Determinations. Frozen artery segments were stored at −80°C until assay, at which time they were individually homogenized in 1 mL of ice-cold 6% TCA using a motor-driven ground glass pestle and mortar (Lurex, Vineland, NJ). After centrifuging the homogenates for 60 min at 3000 × *g*, the resulting pellet was used for protein determination and the decanted supernates for subsequent cGMP assay.

Determination of protein content was identical to that previously described by Pearce *et al.* (7), except for samples from the crude homogenate preparation (see below). For each protein sample resulting from TCA precipitation, we resuspended the pellet in 1.0 M NaOH at 37°C for 60 min. This method of extraction is designed to exclude connective tissue and structural proteins, as previously shown by others (13, 14). After resuspension, the samples were centrifuged again for 60 min at 3000 × *g*. Aliquots of the resulting supernates were then neutralized with an equal volume of 0.9 M HCl. To avoid protein aggregation, we further diluted the samples with 125 mM urea at a 5:1 ratio of urea to sample. The protein was then quantified using the Bio-Rad's Coomassie Brilliant Blue protein dye (catalog no. 500-0006). BSA served as the reference for the standard curves, which were determined with each set of unknowns. As has been previously shown, this assay produces protein values which are both consistent and uniform in the vessel type studied (7).

The aliquots of Tris-HCl-soluble proteins from the crude homogenate preparation did not undergo NaOH resuspension or neutralization. These samples were diluted directly with 125 mM urea at the same 5:1 ratio before the addition of the Bradford dye. All other steps were as described above for the Tris-precipitated samples.

The supernates obtained after the TCA precipitation and centrifugation were washed with water-saturated diethyl ether a minimum of three times to remove TCA. Any remaining ether was then allowed to evaporate, and aliquots of the aqueous phase were then lyophilized and stored at 4°C until assayed. We determined the cGMP content for each sample using a commercially available RIA kit (RPA 525; Amersham Corp.) as previously described (2).

Data Analysis and Statistics. All cGMP values were normalized relative to intracellular water content to give units of apparent intracellular concentration (μmol of cGMP/L of cell water). By normalizing to cell water content, rather than raw protein, we were able to express cGMP in physiologically more relevant units of apparent intracellular concentration, thus avoiding maturational changes in cellular protein encountered when comparing the newborn to the adult (7). For this normalization, we first determined the ratio of intracellular water to base-soluble protein. In a separate series of measurements, the ^{57}Co-EDTA space was measured as described by Brading and Jones (15), and total water was determined by dehydration. The difference between the ^{57}Co-EDTA space and total water was taken as intracellular water volume, which was then expressed for each age group relative to protein content measured using the method described above in the same artery segments used for water distribution analysis. This ratio (in units of μl of water/μg of protein) was then used to convert measurements of cGMP in units of pmol/μg of protein into units of pmol/μl of intracellular water. The experiments used to quantify intracellular water to protein ratios have been described in detail elsewhere (16).

Basal rates of cGMP synthesis were calculated as the maximum slope of the relation between cGMP concentration and time of exposure to IBMX observed during the first four minutes of exposure to IBMX. The rates of disappearance of cGMP from crude homogenates was calculated for each animal as the slope of the relation between cGMP and time over the first 15 min of incubation. Similarly, rates of cGMP accumulation were calculated in whole arteries as the slope of the relation between cGMP and time, between 20 and 60 s of exposure to SNAP.

Estimated values of apparent Km for PDE were calculated using a rearrangement of the Michaelis-Menton equation which gives Km as a function of V_{max}, velocity, and substrate concentration:

$$\mathrm{Km} = [S] \cdot (V_{max} - V)/V$$

In this relation, [S] was taken as the concentration of cGMP in untreated arteries at 20 s of exposure to 10 μM SNAP (see Fig. 3), V was taken as the calculated rate of degradation shown in Figure 3, and V_{max} was taken as the rate of degradation obtained in the homogenate studies (see Fig. 4).

Throughout this report, all values are given as the mean and SEM. All reported values of n refer to the number of animals, and not the number of segments. For all statistical comparisons, corresponding newborn and adult variances were compared using an F ratio test. In cases where newborn and adult variances were not significantly different, significant differences between means were calculated using a t test. In cases where newborn and adult variances were significantly different, differences between means were calculated using a Behren's-Fisher analysis with a pooled variance.

RESULTS

Basal cGMP concentrations. As indicated in Figure 1, the basal cGMP concentration in the adult common carotid with intact endothelium (0.11 ± 0.02 μmol of cGMP/L of cell water) was not significantly different from that in the paired endothelium-denuded segment (0.11 ± 0.01 μmol of cGMP/L of cell water). Similarly, newborn values of basal cGMP concentration were not significantly different in common carotid segments with (0.59 ± 0.11 μmol of cGMP/L of cell water) or without (0.57 ± 0.07 μmol of cGMP/L of cell water) intact endothelium. For both intact and denuded segments, newborn basal cGMP levels were significantly higher than in adults.

Optimum IBMX concentration. All concentrations of IBMX examined in adult carotid preparations produced increases in cGMP content which were similar in time course. At 300 μM, IBMX produced peak values within 4 min (0.98 ± 0.18 μmol of cGMP/L of cell water), and these did not change significantly between 4 and 12 min of exposure to IBMX (8 min, 1.01 ± 0.3; 12 min, 1.02 ± 0.14 μmol of cGMP/L of cell water). Given that 300 μM IBMX was within the range of concentrations reported to inhibit PDE activity in other preparations (17–20), this was the concentration we used throughout this study. The observation that cGMP levels did not fall significantly in the presence of 300 μM IBMX in either newborn or adult artery preparations can be taken as further evidence that this concentration effectively inhibited all PDE activity in both age groups (Fig. 2).

Basal rates of cGMP synthesis. When endothelium-denuded artery segments were treated with 300 μM IBMX, cGMP levels slowly accumulated over 4 min in both age groups. The calculated basal rates of cGMP accumulation in the IBMX-treated arteries were significantly greater in newborn (0.31 ± 0.06 μmol of cGMP/L of cell water/min) than in adult (0.15 ± 0.04 μmol of cGMP/L of cell water/min) segments. Basal rates of synthesis in segments not treated with IBMX were not significantly different from zero in ether age group over the same time period.

Effects of IBMX on cGMP accumulation responses to SNAP. Treatment of whole endothelium-denuded artery segments with 10 μM SNAP rapidly increased cGMP levels to peak values within 60–80 s (Fig. 2). Peak levels in adult segments averaged 1.09 ± 0.15 μmol of cGMP/L of cell water, and this value was not significantly different from observed in newborn segments (1.12 ± 0.22 μmol of cGMP/L of cell water). After peak levels were reached, cGMP returned toward baseline levels within 100 s in both age groups. As indicated by the integrated area beneath the cGMP time curves, the total cGMP synthesized in the newborn (100.2 μM/s) was higher than in the adult (66.9 μM/s).

When whole endothelium-denuded artery segments were pretreated with 300 μM IBMX, the addition of 10 μM SNAP rapidly increased cGMP levels to plateau values within 100 s. Once attained, plateau values of cGMP were stable for at least 12 min. Plateau values averaged 2.26 ± 0.39 of μmol cGMP/L of cell water in adult segments and 5.88 ± 0.93 μmol of cGMP/L of cell water in newborn segments; the adult value was significantly less than observed in the newborn.

Using the cGMP time course data shown in Figure 2, we estimated the rates of change in cGMP synthesis and degradation during the linear phase of cGMP accumulation between 20 and 60 s. The rates of cGMP *synthesis* were taken as the observed rates of cGMP accumulation in IBMX-treated arter-

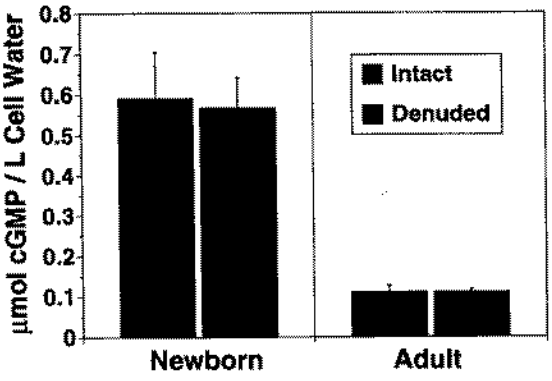

Figure 1. Effects of maturation and endothelium on basal cGMP. GAMP levels were determined by RIA in mounted whole arteries and normalized relative to intracellular water. All values are given as means ± SEM. n = 12 for adults and 10 for newborns in endothelium-intact segments. n = 15 for adults and 8 for newborns in endothelium-denuded segments. Endothelium removal had no significant effect in either age group. Both intact and denuded newborn values were significantly greater than corresponding adult values.

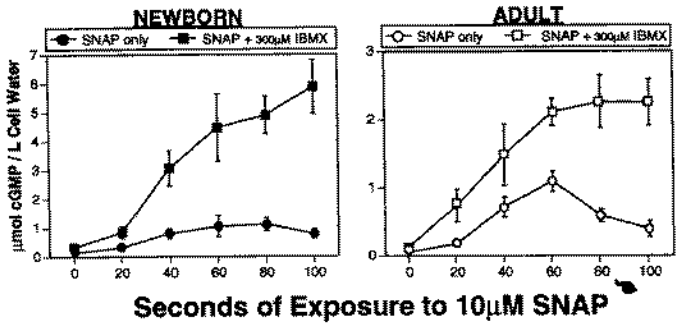

Figure 2. Effects of IBMX on the time course of cGMP accumulation after SNAP. Multiple segments from a single artery were frozen at varying times after exposure to 10 μM SNAP in the presence (*squares*) or absence (*circles*) of 300 μM IBMX. cGMP levels were normalized relative to intracellular water volume and are given as means ± SEM. Note that the vertical axes have different scales. The integrated area beneath the control time course was 100.2 μM/s in the newborn and 66.9 μM/s in the adult. n = 14 in the newborn and 10 in the adult for control segments. n = 9 in the newborn and 8 in the adult for IBMX-treated segments. The maximum cGMP concentration attained in IBMX-treated segments was significantly greater in newborn than in adult segments.

ies. As shown in Figure 3, these rates averaged 6.18 ± 1.27 μmol of cGMP/L of cell water/min in newborn segments, which was significantly greater than observed in adult segments (3.29 ± 0.96 μmol of cGMP/L of cell water/min). The rates of change observed in the untreated artery segments were assumed to reflect the *net difference* between the rates of cGMP synthesis and degradation. These values were not significantly different in newborn and adult segments, and averaged 0.89 ± 0.22 and 0.83 ± 0.19 μmol of cGMP/L of cell water/s, respectively. The rates of cGMP *degradation* were calculated as the differences between corresponding rates of cGMP accumulation observed in the IBMX-treated arteries (synthesis only), and the untreated arteries (synthesis minus degradation). These calculated values of cGMP degradation were significantly higher in newborn (5.29 ± 1.11 μmol of cGMP/L of cell water/s) than in adult (2.47 ± 0.67 μmol of cGMP/L of cell water/s) common carotid segments.

PDE activity in crude homogenates. Rates of cGMP disappearance measured in crude homogenates were measured between 0 and 15 min of addition of 3000 pmol of cGMP. Although measurements of cGMP were also taken at 20 and 25 min, the relation between cGMP and time started to become nonlinear after 20 min, and thus the latter time points were not used. Between 0 and 15 min, the rates of cGMP disappearance, which we took as estimates of maximal cGMP phosphodiesterase activity, were significantly greater in newborn (−105.69 ± 5.97 μmol of cGMP/L of cell water/min) than in adult (−77.46 ± 6.01 μmol of cGMP/L of cell water/min) preparations (Fig. 4).

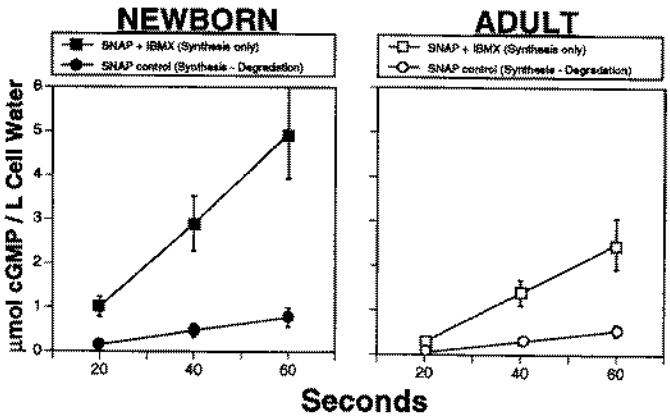

Figure 3. Rates of cGMP accumulation and degradation in whole arteries. The rates of change in cGMP were calculated between 20 and 60 s for the data shown in Figure 2. The rates of change observed in IBMX-treated segments (*squares*) were taken as estimates of synthesis and averaged 6.18 ± 1.27 (n = 9) and 3.29 ± 0.96 (n = 8) μmol of cGMP/L of cell water/min for the newborn and adult segments, respectively. The corresponding rates of cGMP accumulation observed in the control segments (*circles*) were 0.89 ± 0.22 (n = 14) and 0.83 ± 0.19 (n = 10) μmol of cGMP/L of cell water/min for the newborn and adult segments, respectively. Rates of degradation were taken as the differences in slope between the IBMX-treated and the control segments. These values averaged 5.29 ± 1.11 and 2.47 ± 0.67 μmol of cGMP/L of cell water/min for the newborn and adult segments, respectively. All values for slope are given as mean ± SEM. The calculated rates for both synthesis and degradation were significantly greater in newborn than in adult arteries.

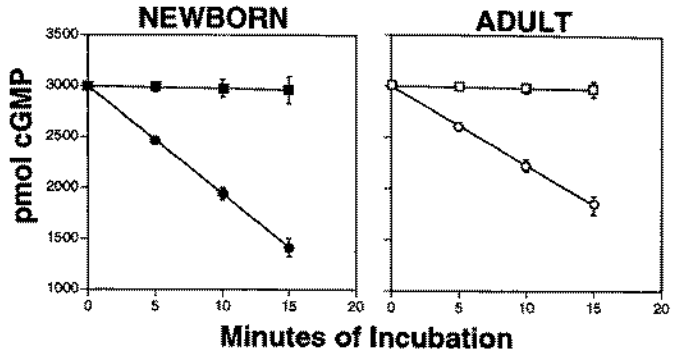

Figure 4. Rates of cGMP degradation in crude homogenates. Rates of disappearance of cGMP were measured in crude homogenates in the presence (*squares*) and absence (*circles*) of 300 μM IBMX. Initial concentrations of cGMP were 3000 pmol in all experiments, and cGMP values were normalized relative to the intracellular water volume of the tissues used to prepare the homogenates. Rates of degradation, as calculated from the slope values, averaged −105.69 ± 5.97 (n = 9) and −77.46 ± 6.01 (n = 11) μM/L of cell water/min for the newborn and adult preparations, respectively. All values are given as mean ± SEM of the percent initial values. For control measurements (IBMX + cGMP, indicated by *circles*), n = 5 for both newborn and adult preparations. The rate of cGMP disappearance was significantly greater in newborn than in adult homogenates.

DISCUSSION

Whereas it is widely accepted that vascular resistance is much lower in newborns than adults (1), our knowledge of the factors responsible for this age-related difference remains incomplete. In light of the well defined ability of cGMP to promote vasodilatation (21), observations that basal levels of cGMP are elevated in newborn relative to adult arteries (2) suggest that this second messenger may in some way be involved. This suggestion, in turn, leads to the question: "How and why is cGMP elevated in newborn arteries?"

As for any compound, the basal level of cGMP is directly determined by the balance between its rates of synthesis and degradation under basal conditions. Although the rate of cGMP synthesis could potentially be elevated in newborn arteries by a correspondingly elevated release of EDRF (nitric oxide), a direct activator of soluble guanylate cyclase, our finding that endothelium removal had little effect on basal cGMP levels in either newborn or adult arteries (Fig. 1) argues against the importance of this mechanism. This finding suggests that either: *1*) there is no significant basal release of EDRF in static, wire-mounted ovine carotids or *2*) that the effects of basal EDRF release are compensated for in some way by age-related differences in phosphodiesterase activity. Results in other artery preparations (22), including preliminary studies in our own laboratory under varying conditions, favor the latter interpretation. More importantly, this finding demonstrates that age-related differences in basal cGMP are independent of the endothelium and are instead governed by age-related differences in cGMP metabolism within the arterial smooth muscle.

To directly assess age-related differences in vascular cGMP metabolism, we estimated basal rates of cGMP synthesis in endothelium denuded preparations. These measurements were based on differences in basal rates of cGMP accumulation due to the presence or absence of IBMX, a wide-spectrum PDE inhibitor, at a concentration verified to inhibit total PDE activ-

ity in our preparation. The rate of rise in cGMP measured in this fashion was undoubtedly influenced by multiple factors including the rate of diffusion of IBMX into the smooth muscle cells, the rate of onset of IBMX inhibition of PDE, and most importantly, the rate of cGMP synthesis. Given that artery thicknesses were similar in both age groups, and the rates of rise of cGMP from basal levels to peak concentrations within the first 4 min of exposure to IBMX was highly linear in both age groups, the data suggest that onset of action of IBMX was relatively rapid, and that the main difference between newborn and adult rates of cGMP accumulation was due primarily to age-related differences in the rates of cGMP synthesis. This approach revealed that basal cGMP synthesis was greater in newborn than in adult common carotid arteries. Whereas the endothelium may influence basal guanylate cyclase activity in some preparations (21, 23, 24), the present findings further demonstrate age-related differences in guanylate cyclase activity that are independent of endothelial influences.

Whereas age-related differences in basal rates of cGMP synthesis and degradation have important implications for resting vascular tone, these relations may change during active vasodilatation secondary to guanylate cyclase activation (25). To explore this possibility, we examined rates of cGMP synthesis and degradation during maximal activation of guanylate cyclase by SNAP, a nitric oxide-releasing agent. As previously shown (2), SNAP produced transient increases in cGMP which varied with age (Fig. 2). Because the mean areas beneath these cGMP-time curves were significantly greater, and peak values were attained later, in newborn than in adult arteries, the results suggest that the turnover of cGMP during activation of guanylate cyclase varied with age. Consistent with this interpretation, peak values of cGMP did not vary with age in control segments, but were markedly greater in newborn than adult segments treated with IBMX. This latter result further suggests that both guanylate cyclase and phosphodiesterase activities were greater during guanylate cyclase activation in newborn than in adult arteries.

To quantify age-related differences in guanylate cyclase and phosphodiesterase activities during guanylate cyclase activation, we calculated the rates of cGMP accumulation in control and IBMX treated arteries during the early linear phase of the cGMP response to nitric oxide (20–60 s, see Fig. 3). Assuming that rates of accumulation approximate synthesis in IBMX-treated arteries, and that the difference between rates of cGMP accumulation in control and IBMX treated arteries approximate rates of cGMP degradation, we confirmed that rates for both synthesis and degradation were significantly higher in newborns than adults during maximal guanylate cyclase activation. In contrast, rates of cGMP accumulation in untreated controls were closely equivalent in newborn and adult arteries. This similarity emphasizes that rates of cGMP synthesis and degradation were closely matched in both age groups although the absolute values for synthesis and degradation varied markedly with age. Of additional importance was the observation that relative to rates of synthesis in adult arteries, synthesis in newborn arteries was greater under both basal and activated conditions.

A key factor in the relation between cGMP synthesis and degradation is the V_{max} for phosphodiesterase. Thus, we decided to examine the effects of maturation on V_{max} by measuring maximal rates of cGMP disappearance in crude artery homogenates. As expected, we found that maximal rates of cGMP disappearance were far greater in crude homogenates than in intact tissues. We also found that maximal rates of cGMP disappearance were markedly greater in newborn than in adult preparations (Fig. 4), and that in both age groups, these rates were far greater than estimates of maximal rates of cGMP accumulation measured in intact arteries. Given these observations, it remains possible that maximal PDE activity is far in excess of the maximal capacity for cGMP synthesis, and thus PDE may act as a "housekeeping" enzyme whose activity is indirectly determined by the rate of cGMP accumulation in intact arteries. However, much of this phosphodiesterase activity may be compartmentalized (5, 6), and thus age-related differences in compartmentalization may also contribute to the age-related differences in cGMP metabolism we observed.

The finding that maximal PDE activity was greater in newborn than adult carotid arteries could be explained by a greater tissue concentration of phosphodiesterase, and/or a different isozyme profile. Because Km values vary among different PDE isozymes, age-related differences in isozyme profiles should be reflected by differences in lumped Km values. To estimate lumped Km values, we used a rearrangement of the Michaelis-Menton equation which combined our measures of basal cGMP concentration ([S]), basal rates of cGMP synthesis (V), and maximal rates of cGMP degradation in homogenates (V_{max}) to calculate Km (see "Methods"). This approach provided Km values of 2.9 μM cGMP in newborn arteries, and 1.5 μM cGMP in adult arteries. The adult values calculated in this manner compared well with those previously reported in the literature for cGMP specific phosphodiesterase isozymes separated on a low resolution DEAE-cellulose column (27–29). Consistent with other indirect observations (30–33), our data suggests age-related differences in PDE isozyme profile. Although the relative importance of and causes for these differences remain uncertain, the PDE isozymes involved probably include types I and V which are the predominant cGMP metabolizing PDEs in vascular smooth muscle (34).

Overall, the present studies indicate that maturation involves important changes in many of the factors which govern vascular cGMP metabolism. These factors affect both basal and activated rates of cGMP synthesis and degradation, and probably also reflect differences in PDE isozyme profile. Because elevated cGMP levels may contribute to the lower hydraulic vascular resistance characteristic of neonates, and may also be of pathophysiologic consequence during episodes of transient hypertension which are often associated with intracranial small artery rupture, further studies of these age-related differences appear warranted. Fortunately, a growing diversity of pharmacologic tools is simplifying the study of these enzymes, both *in vitro* and *in vivo* (26, 35, 36), and should in the near future help elucidate the diverse and important roles guanylate cyclase and phosphodiesterase play in functional cardiovascular maturation.

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