Developmental Changes in Sodium Nitroprusside and Atrial Natriuretic Factor Mediated Relaxation in the Guinea Pig Aorta

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ABSTRACT. Sodium nitroprusside (SNP), a nonreceptor mediated stimulant of soluble guanylate cyclase, and atrial natriuretic factor, a receptor-dependent stimulator of particulate guanylate cyclase, mediate relaxation responses by increasing intracellular cGMP. This *in vitro* study was designed to compare the ontogeny of relaxation responses to SNP and atrial natriuretic factor in the guinea pig thoracic aorta. Aortic rings from fetuses at 55–60 d gestation (term = 68 d), 1- to 3-d-old newborn, and 12-wk-old adult Hartley guinea pigs were mounted in an organ bath, bathed in Krebs's solution, and connected to a force-displacement transducer to measure isometric tension. Relaxation responses to SNP and atriopeptin III were studied with the vessels at optimal resting tension and after pre-constriction with an EC₈₅ concentration of norepinephrine. SNP-mediated relaxation showed a significant increase in sensitivity with development among the three age groups ($p < 0.05$). Methylene blue, an inhibitor of soluble guanylate cyclase, produced no inhibition of relaxation to SNP in fetal aortae, significantly decreased responses along the straight portion of the concentration-response curve in newborn aortae ($p < 0.05$), and significantly shifted the concentration-response curve to the right ($p < 0.05$) in adult aortae; but did not prevent vessels from relaxing almost 100% in any age group. However, atriopeptin III-mediated responses were similar in the three age groups and were unaffected by methylene blue. These results suggest that 1) sensitivity to SNP increases with age from fetal through adult life; 2) relaxation mediated by atriopeptin III is similar during development; 3) methylene blue does not affect SNP mediated relaxation in fetuses but progressively decreases sensitivity to SNP in newborns and adults; and 4) methylene blue does not affect atriopeptin III-mediated relaxation in any age group. (*Pediatr Res* 27: 392–395, 1990)

Abbreviations

ANF, atrial natriuretic factor
SNP, sodium nitroprusside

APIII, atriopeptin III
NE, norepinephrine
MB, methylene blue

cGMP is an intracellular mediator of relaxation in vascular smooth muscle (1–3). cGMP production in the vascular smooth muscle occurs by direct stimulation of soluble guanylate cyclase by agents such as nitrites, nitrates, and endothelium-derived relaxing factor (3) or by receptor dependent stimulation of particulate guanylate cyclase by ANF (4).

ANF can be secreted by the fetal mammalian heart (5) resulting in measurable plasma levels of ANF in sheep (6–8), rats (5), and humans (9). Furthermore, it has been demonstrated that the ANF hormonal system is functional during fetal life (5, 9). Recent studies suggest that the hemodynamic response to ANF may evolve during maturation (6). Although it is known that isolated vessels located centrally consistently relax in the presence of ANF (10), little is known regarding the ontogeny of ANF-mediated vascular relaxation from fetal into adult life.

However, increasing attention is now focused on the role of cGMP-mediated processes in regulation of vascular tone (3) and this interest has extended to the ontogeny of these processes (5, 6, 11–13). Karaki *et al.* (12), working with thoracic aortic strips of rabbits, demonstrated an age-dependent postnatal decrease in relaxation responses to SNP. In contrast, Varille *et al.* (13) found similar relaxation responses to intrarenal infusion of ANF in fetal and newborn sheep.

These previous studies suggest that ontogenic differences may exist with respect to cGMP-mediated vascular relaxation. Thus, this study was designed to compare *in vitro* the ontogeny of relaxation responses to SNP, a nonreceptor-mediated stimulant of soluble guanylate cyclase, and ANF, a receptor-dependent stimulator of particulate guanylate cyclase in fetal, newborn, and adult guinea pig thoracic aorta.

MATERIALS AND METHODS

Thoracic aortae of Hartley guinea pigs from three age groups were studied: fetuses of either sex at 55 to 60 d gestation (72 ± 12 g) (term = 68 d), 1- to 3-d-old newborn males (105 ± 12 g), and 12-wk-old adult males (665 ± 94 g). Newborns and adults were sedated with 25 mg/kg ketamine hydrochloride (Vetalar, Parke-Davis, Morris Plains, NJ) and euthanized with 1 g/kg and 500 mg/kg, respectively, of thiopental sodium (Pentothal, Abbott Laboratories, Irving, TX). Pregnant animals were sedated with ketamine hydrochloride (25 mg/kg) and euthanized with 1 g/kg

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of thiopental sodium. Fetuses were immediately delivered and euthanized with 1 g/kg thiopental sodium. Thoracic aortae were removed and placed in cold Krebs's solution (mM): NaCl 118, KCl 4.7, CaCl₂ 2.2, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25, disodium EDTA 0.025, glucose 11; cleaned of connective tissue, cut into rings 3- to 4-mm wide, mounted on two fine stainless steel wires passed through the lumen and connected to a Grass FT.03 force transducer (Grass, Quincy, MA) coupled to a Gould 2600S pen recorder (Gould, Cleveland, OH) for continuous measurement of isometric force. Aortae were suspended in a 25 mL organ bath of Krebs's solution (37°C; pH 7.4), gassed with 95% O₂-5% CO₂ and equilibrated for 60 min with a bath change every 15 min. Vessels were stretched to their optimal tension as determined by maximal force developed to an EC₅₀ (concentration producing half maximal contraction) concentration of NE. Preparations were washed and optimal resting tension reattained and stabilized prior to and between experimental procedures.

The first series of experiments was designed to determine relaxation responses to cumulative concentrations of SNP (10⁻⁹-10⁻⁵ M) and APIII (10⁻¹⁰-10⁻⁷ M) in vessels preconstricted with an EC₈₅ concentration of NE. APIII is an active form of ANF extracted from rats (14) and shown in preliminary experiments to be active in guinea pigs. Constrictor responses to NE were allowed to stabilize before addition of cumulative concentrations of either SNP or APIII.

The second series of experiments was designed to determine the effects of MB, an inhibitor of soluble guanylate cyclase (2), on SNP- and APIII-mediated relaxation. Vessels were incubated with 10 μM MB for 20 min before preconstriction with NE and subsequent cumulative addition of SNP or APIII. Experiments were paired, with a vessel segment from the same animal serving as a control to study relaxation responses without exposure to MB.

This study was approved by the Animal Care and Use Committee, Tripler Army Medical Center. Procedures on the guinea pigs were in accordance with National Institutes of Health policies and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1985).

Drugs. The following drugs were used: NE hydrochloride, acetylcholine chloride, SNP (Sigma Chemical Co., St. Louis, MO), APIII (Peninsula Labs Inc., Belmont, CA), and MB (C.I. 52015, Hartman-Leddon Co., Philadelphia, PA). Solutions of NE, acetylcholine, and SNP were prepared in distilled water on the day of the experiment and kept on ice. Stock solutions of APIII (concentration 0.5 mg/mL) were made in 50 mM acetic acid and stored at -20°C. Fresh aliquots were made up in distilled water daily and kept on ice. Stock solution (10 μM) of MB was made in distilled water and stored at 4°C. Each dose was administered in 100-μL aliquots and drug concentrations are expressed as final molarity in the bath.

Statistical analysis. EC₅₀ values were derived and compared by simultaneous analysis of physiologic dose-response curves as described by DeLean *et al.* (15) and previously used by us (16, 17). The ALLFIT program (Biomedical Computing Technology Information Center, Vanderbilt Medical Center, Nashville, TN) adapted for an Apple computer by Martin H. Teicher, Department of Psychiatry, Harvard Medical School was employed for this analysis. ALLFIT is based on the logistic function

$$Y = \frac{a - d}{1 + (x/c)^b} + d$$

where x and y are dose and response, respectively and a, b, c, and d are the four fitted parameters; a = response at zero dose, b = slope factor, c = ED₅₀, and d = response at infinite dose. Goodness of fit of individual dose response curve was evaluated by an F ratio test and a run test; *p* < 0.05 indicates that shared parameters are significantly different from each other. Two-way ANOVA for repeated measures with Duncan's multiple range *post hoc* tests (18) was performed to determine differences in

relaxation responses, factoring for drugs and developmental stages. Paired *t* test was used to analyze data from experiments in which vessels were pretreated with MB. A *p* < 0.05 was considered significant. All values are expressed as mean ± SEM.

RESULTS

Optimal resting tension was 0.56 ± 0.02, 1.8 ± 0.06, and 3.04 ± 0.06 g in fetal, newborn, and adult guinea pigs, respectively. Newborns were slightly more sensitive to NE (EC₅₀ = 0.41 ± 0.01 μM) compared to fetuses (EC₅₀ = 1.9 ± 0.05 μM) and adults (EC₅₀ = 1.1 ± 0.03 μM) (*p* < 0.05).

Effect of SNP on thoracic aortic relaxation. SNP, a nonreceptor-mediated stimulator of soluble guanylate cyclase, produced complete relaxation of NE induced constriction in all the groups (Fig. 1). Furthermore, there was progressive and statistically significant age dependent shift of the relaxation curve to the left (Fig. 1), manifested by a progressive decrease in EC₅₀ values with maturation (Table 1).

Effect of APIII on thoracic aortic relaxation. In contrast, APIII, a receptor-dependent stimulator of particulate guanylate cyclase, produced similar relaxation responses after NE-induced constriction (Fig. 2) with no difference in EC₅₀ values among fetal, newborn and adult guinea pigs (Table 1).

Effect of MB on SNP- and APIII-mediated relaxation responses. An age-dependent effect of MB was observed for SNP-mediated relaxation. MB pretreatment did not affect SNP mediated relaxation in fetal aortae (Fig. 3A). However, in newborn aortae (Fig. 3B), a significant decrease in SNP-mediated relaxation was observed after MB treatment along the straight portion of the concentration response curve. Moreover, in adult aortae (Fig. 3C), MB pretreatment further decreased the ability of SNP to relax NE preconstricted aortae. However, MB pretreatment did not affect APIII mediated relaxation in any of the three age groups (Fig. 4).

Fig. 1. Effect of sodium nitroprusside on EC₈₅ norepinephrine contraction. *n* = number of animals. Values are means ± SEM.

Table 1. EC₅₀ values for SNP and APIII in guinea pig thoracic aorta preconstricted with an EC₈₅ dose of NE (mean ± SEM)

Age	SNP (nM)	APIII (nM)
Fetus	160 ± 4.0*	4.1 ± 0.06
Newborn	99 ± 2.4†	4.4 ± 0.07
Adult	26 ± 0.7	5.1 ± 0.08

* *p* < 0.05, fetus compared to newborn and adult.

† *p* < 0.05, newborn compared to adult.

Fig. 2. Effect of APIII on EC₈₅ norepinephrine contraction. n = number of animals. Values are means $\pm$ SEM.

DISCUSSION

This study demonstrates a significant age-dependent increase in SNP-mediated relaxation of NE-induced constriction *in vitro* among fetal, newborn, and adult guinea pig aortae, suggesting an age-dependent maturation of intracellular mechanisms mediating SNP responses. In contrast, Karaki *et al.* (12) observed a postnatal decrease in relaxation mediated by SNP, both in passively stretched and actively constricted rabbit thoracic aortic strips. Species differences may in part explain discrepancies between results of our study and that of Karaki *et al.* (12).

Results herein also demonstrate age dependent differences in SNP-mediated responses in the presence of MB, a selective inhibitor of soluble guanylate cyclase (2). To our knowledge, this is the first report demonstrating such an age-dependent response and suggests a maturational change involving SNP stimulated activation of soluble guanylate cyclase and cGMP-mediated relaxation from fetal to adult life in the guinea pig thoracic aorta. Alternately, the effects of MB may in part be mediated by autooxidation products of MB that have been shown to generate superoxide *in vitro* (19). However, the effects of superoxide generation in the aorta of developing guinea pigs are not known. Finally, differences in the penetration of MB into tissue seems an unlikely reason to explain our age-dependent differences because fetal aortae were thinner than adult aortae.

Our results support previous findings that SNP-mediated vascular relaxation in adult animals is inhibited by MB (20, 21). However, Kreye (22) was unable to demonstrate inhibition of SNP-mediated relaxation by MB in rabbit aortic strips. Otsuka *et al.* (23) observed that although incubation of rat vessels with 10 μ M MB completely inhibited subsequent SNP-induced increases in cGMP, SNP still induced complete relaxation of these vessels. Marshall *et al.* (19) studied cerebral arterioles of anesthetized cats, and found no effect on SNP-induced dilation by MB. Finally, Karaki *et al.* (24) have suggested that SNP has multiple sites of action although the cellular mechanism(s) stimulated by SNP remains incompletely understood and species dependent. Taken together, results of our study provide indirect evidence for age-dependent differences in mechanisms involved in mediating SNP relaxation and support the speculation that SNP may mediate relaxation responses by "cGMP-dependent" and "cGMP-independent" mechanisms (22-24). Further studies are necessary to investigate the ontogeny of SNP-mediated control of intracellular accumulation of cGMP.

Current evidence suggests that the site of administration and duration of infusion of ANF *in vivo* influences hemodynamic responses (13) by activation of compensatory mechanisms (6, 13, 25) yielding at times, completely opposing hemodynamic effects. The isolated vessel technique used herein avoids potential

Fig. 3. Effect of MB on sodium nitroprusside mediated relaxation. A, fetal (55- to 60-d gestation, term = 68 d); B, newborn (1-3 d old); C, adult (12 wk old) vessels, respectively. All vessels contracted with EC₈₅ norepinephrine. n = number of animals. Values are means $\pm$ SEM. Estimated EC₅₀ values: A without MB 175 nM, with MB 198 nM; B without MB 105 nM, with MB 157 nM; and C without MB 25 nM, with MB 66 nM.

confounding effects of circulating vasoactive substances, central neural control, and local metabolic control present *in vivo* to allow a more direct evaluation of agonist response. However, this isolated vessel technique does not allow evaluation of overall response on a vascular bed and its distal circulation.

Results of our study demonstrate APIII-mediated relaxation responses from 55- to 60-d gestation, providing further evidence that the ANF receptor complex is functional during fetal life (5,

Fig. 4. Effect of MB on APIII-mediated relaxation. *A*, fetal (55- to 60-d gestation, term = 68 d); *B*, newborn (1-3 d old); *C*, adult (12 wk old) vessels, respectively. All vessels contracted with EC_{85} norepinephrine. n = number of animals. Values are means $\pm$ SEM. Estimated EC_{50} values: *A*, without MB 3.4 nM, with MB 4.7 nM; *B*, without MB 3.8 nM, with MB 4.6 nM; and *C*, without MB 5.6 nM, with MB 5.7 nM.

6, 13). In addition, the sensitivity to APIII is similar from fetal to adult life, suggesting that ANF receptors are functionally mature from at least late 3rd trimester in the guinea pig thoracic aorta. In support of this hypothesis Varille *et al.* (13) observed similar renal vasodilatory effects of ANF in fetal and newborn sheep. Finally, our results demonstrate that MB pretreatment did not affect relaxation responses to APIII, supporting previous studies demonstrating that ANF stimulates particulate rather than soluble guanylate cyclase (4, 26). In contrast, Ohlstein and

Berkowitz (27) demonstrated inhibition of atriopeptin II-induced relaxation in rabbit thoracic aorta by MB. Reasons why results of these studies differ are difficult to explain.

In summary, our study demonstrates that in guinea pig thoracic aortae: 1) sensitivity to SNP increases with age from fetal through adult life; 2) relaxation mediated by APIII is similar during development; 3) MB does not affect SNP-mediated relaxation in fetuses but progressively decreases sensitivity to SNP in newborns and adults; and 4) MB does not affect APIII-mediated relaxation in any age group. Taken together, these results suggest that the cGMP-dependent mechanism of SNP action may be age dependent and that the overall vasodilatory response to ANF receptor stimulation is "mature" during late gestation in fetal guinea pigs.

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