Guananyl cyclase mediates ANP-induced vasoconstriction of murine splenic vessels

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Andrew, Peter S., and Susan Kaufman. Guanylyl cyclase mediates ANP-induced vasoconstriction of murine splenic vessels. Am J Physiol Regul Integr Comp Physiol 284: R1567–R1571, 2003.—We have previously shown that ANP causes differential constriction of the splenic vasculature of the rat (veins greater than arteries), which may be inhibited by blocking the production of cGMP with A7195. In this paper, we report experiments done on vessels derived from guanylyl cyclase (GC)-A knockout mice. Small splenic arteries (150-μm diameter) and veins (250-μm diameter) were dissected from male GC-A-deficient 129sv mice or age-matched wild-type controls and mounted in a wire myograph. In the wild-type mice, ANP exhibited higher potency in the veins than in the arteries (EC50 values wild-type mice: artery, 8 ± 3 × 10−9 M, n = 5 vs. vein, 6 ± 4 × 10−10 M, n = 5; P < 0.05). The concentration-response curve for ANP-induced vasoconstriction was also shifted leftward in denuded compared with intact arteries (EC50 values: denuded artery: 5 ± 3 × 10−10 M, n = 5 vs. intact artery, 8 ± 3 × 10−9 M, n = 5; P < 0.05), i.e., the denuded vessels were more reactive. By contrast, ANP caused no significant change in tension from baseline in intact splenic arteries, intact splenic veins, or denuded splenic arteries derived from the GC-A-deficient mice. Although these vessels did show normal concentration-dependent increases in tension to phenylephrine. We conclude that ANP causes vasoconstriction in the splenic vasculature by an endothelium-independent mechanism, mediated via guanylyl cyclase.

METHODS

The experiments described in this study were examined by the local Animal Welfare Committee and were found to be in compliance with the guidelines issued by the Canada Council on Animal Care.

Male GC-A-deficient (knockout) 129sv strain mice (~30 g body wt) were obtained courtesy of Dr. D. L. Garbers, Univ. of Texas Southwestern Medical Center, Dallas, TX. Male 129sv strain mice (~30 g body wt), from which the knockout mice were derived, were obtained from Charles River, St. Foy, Quebec, Canada. The mice were killed for at least 1 wk before experimental procedures, exposed to light of 12:12-h light-dark cycle, in a humidity- and temperature-controlled environment, and maintained on a 0.3% sodium diet and water ad libitum.

Vessel preparation. The mice were killed by cervical dislocation. The spleen and its associated vascular arcade were harvested and cut into small pieces (~1 mm across). Each piece of tissue was placed on a sterile Teflon strip and mounted in a 3-ml tracing chamber containing Krebs solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 11.5 mM NaHCO3, 1.2 mM KH2PO4, and 5.4 mM glucose) and equilibrated at 37°C with 95% O2 and 5% CO2. The chamber was placed in a stable temperature water bath and maintained at 37°C. The Krebs solution was aerated with 95% O2 and 5% CO2 at 100 ml/min and bubbled through the solution to keep the pH stable at 7.4. The solution was continuously stirred with a magnetic stirrer to maintain the temperature at 37°C. The chamber was maintained at a constant temperature of 37°C. The solution was continuously stirred with a magnetic stirrer to maintain the temperature at 37°C. The solution was continuously stirred with a magnetic stirrer to maintain the temperature at 37°C.

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rapidly removed through a midline laparotomy and placed in ice-cold HEPES-buffered phosphate saline solution (HEPES-PSS). First-order splenic arteries (~150-μm diameter) and veins (~250-μm diameter) were dissected free from surrounding adipose tissue, cut into ~2-mm lengths, and mounted on an isometric tension myograph system (Kent Scientific, Litchfield, CA). The blocks were positioned in an organ bath with 5 ml of HEPES-PSS solution kept at 37°C. Matched vessel segments (artery, vein) were mounted in parallel organ baths, and changes in isometric force were recorded on a data-acquisition system (Windaq, DATAQ Instruments, Akron, OH). For assessing endothelium-independent vasoreactivity of splenic arteries, the endothelium was removed by passing a human hair through the lumen of the vessel before mounting.

Resting length-tension curve. After mounting, vessels were allowed to stabilize for 30 min in HEPES-PSS buffer under no tension, during which time the buffer solution was changed at 10-min intervals. This was followed by a preconditioning stretch of ~0.6 mN, after which vessels were rested at 0.1–0.2 mN/mm and allowed to stabilize in HEPES-PSS buffer for a further 10 min. This was followed by generation of a resting length-tension curve. From Laplace’s law, the circumference that an artery would have at a transmural pressure of 100 mmHg (L100) or the circumference that a vein would have at a transmural pressure of 5 mmHg (L5) was calculated from the exponential curve fit of tension generated vs. internal vessel circumference.

Preliminary studies on murine splenic arteries (n = 7) and veins (n = 7) indicated that the point on the passive-active tension characteristics curve obtained at 0.8L100 and 0.8L5 provided the maximum active tension with least passive tension for arteries and veins, respectively. Vessels were set to their determined optimal tension and allowed to stabilize for 30 min in HEPES-PSS buffer, with buffer changed at 10-min intervals, before generation of a concentration-response curve.

Solutions and drugs. The HEPES-PSS solution, which was maintained at a pH of 7.4, contained (in mM) 142 sodium chloride, 4.7 potassium chloride, 1.17 magnesium sulfate, 1.56 calcium chloride, 1.18 potassium phosphate, 10 HEPES, and 5.5 glucose. Stock solutions of L-phenylephrine hydrochloride (Sigma Chemical, Ontario, Canada) were prepared in distilled water at concentrations of 10^{-5} and 10^{-4} M. ANP (Phoenix Pharmaceuticals, Mountain View, CA) was prepared in distilled water at concentrations of 10^{-6}, 10^{-5}, and 10^{-4} M. Appropriate dilutions of all stocks were obtained using HEPES-PSS. Acetyl-β-methylcholine chloride (Sigma Chemical) was prepared in distilled water at a concentration of 10^{-2} M.

Protocol. A series of cumulative concentration-response curves to phenylephrine (1 × 10^{-8}–1 × 10^{-3} M) was generated, and the EC50 concentration was determined for each vessel. After stabilization, a series of cumulative concentration-response curves to ANP (1 × 10^{-12}–1 × 10^{-6} M) was generated, and the EC50 concentration was determined for each vessel. Maximal tension generated (mN/mm) at a particular ANP concentration was recorded for each vessel. This was followed by generation of a concentration-response curve to phenylephrine. Vessels were treated for 5-min periods at each cumulative concentration of vasoactive agent; preliminary experiments confirmed that the maximal vasoconstrictive response at each concentration was achieved by this time. After exposure to phenylephrine, acetyl-β-methylcholine chloride (organ bath concentration 10^{-4} M) was added to the vessel to assess the integrity of the vascular endothelium. Intact vessels exhibited >50% vasorelaxation to acetyl-β-
methylycholine chloride (10^{-4} M). Denuded splenic arteries exhibited <10% vasorelaxation to acetyl-β-methylcholine chloride (10^{-4} M). At the end of the experiment, the vessels were exposed to high K+ to confirm that they were still viable.

Statistical analysis. The significance of differences in concentration-dependent vasoactivity was analyzed by two-way repeated-measures ANOVA, followed by the Student-Newman-Keuls post hoc analysis to identify the individual points of significance. The significance of differences in maximal tension (at 10^{-6} M ANP concentration) between intact arteries and veins, between intact and denuded arteries, and between vessels from wild-type vs. GC-A-deficient (knockout) mice, for both phenylephrine and ANP, were determined by using a two-way repeated-measures ANOVA. Data are presented as means ± SE. Significance was accepted at P < 0.05.

RESULTS

Wild-type mice. In vessels derived from wild-type mice, ANP caused a concentration-dependent vasoconstriction in arteries and in veins (Fig. 1, A and B). The veins were slightly more sensitive (higher potency) than the arteries to the vasoconstrictor actions of ANP (EC50 values: artery, 8 ± 3 × 10^{-9} M, n = 5 vs. vein, 6 ± 4 × 10^{-10} M, n = 5; P < 0.05). Removal of the endothelium shifted the concentration-response curve to the left (Fig. 2A) (EC50 value denuded arteries: 5 ±
3 × 10^{-10} M; P < 0.05). All vessels showed a concentration-dependent increase in tension in response to phenylephrine (P < 0.05); intact splenic arteries (n = 5) produced more tension than intact splenic veins (n = 5) (Fig. 3A) (P < 0.05). There was no significant difference in the response to phenylephrine between intact and denuded splenic arteries (n = 5) (Fig. 3B) (P > 0.05).

**GC-A deficient mice.** ANP caused no significant change in tension from baseline in intact splenic arteries (n = 5), intact splenic veins (n = 5), or denuded splenic arteries (n = 5) derived from GC-A-deficient (knockout) mice (Fig. 1, A and B, and Fig. 2B) (P < 0.05). However, all these vessels did show a significant concentration-dependent increase in tension in response to phenylephrine (Fig. 3C) (P < 0.05). The denuded splenic arteries were more responsive to phenylephrine than were the intact vessels (P < 0.05; 2-way repeated measures ANOVA: denuded vs. intact), although there was no significant difference in the maximal responses (intact: 0.85 ± 0.13 mN/mm, denuded: 1.01 ± 0.12 mN/mm) (Fig. 3D).

**DISCUSSION**

Although there have been reports that ANP has vasoconstrictor actions on the renal, splenic, and mesenteric vasculature (11, 28, 32–34), this current study provides the first direct in vitro evidence for ANP-induced vasoconstriction of isolated small vessels. ANP caused vasoconstriction of splenic vessels derived from 129sv (wild type) mice (Fig. 1). The vasoconstriction...
occurred via an endothelium-independent mechanism, because removal of the endothelium did not attenuate the response. To the contrary, constriction was enhanced in the denuded vessels (Fig. 2A). We conclude that this was due to release of a vasodilatory factor(s) from the endothelium, which countered the constrictive activity of the ANP. ANP-induced vasoconstriction was absent in splenic vessels derived from GC-A-deficient (knockout) mice (Fig. 1, A and B, and Fig. 2B). Our results suggest that ANP causes vasoconstriction in the splenic vasculature by an endothelium-independent mechanism, mediated via GC.

The GC-A-deficient vessels did not respond to ANP, whereas they contracted normally to phenylephrine with similar maximal responses to those observed in vessels from the wild-type mice (Fig. 3, A and C). The maximal responses to ANP of arteries and veins derived from wild-type mice were also very similar (Figs. 1 and 3A). Although the maximal response of the null GC-A mice to phenylephrine was not altered by removal of the endothelium, the concentration-response curve was slightly shifted to the left (Fig. 3D). It is conceivable that long-term changes associated with the higher blood pressure of the gene-disrupted mice could contribute to this phenomenon, although their hypertension is not severe (homozygotes 97 mmHg vs. wild-type 78 mmHg) (31). The physiological significance, and underlying mechanism, of this finding thus remains to be established.

ANP increases fluid extravasation from the splenic circulation by raising intrasplenic microvascular pressure (28). This is achieved by preferentially increasing postcapillary resistance (28). We previously found ANP-induced vasoconstriction of the splenic vasculature to be inhibited by A71915, a GC-A receptor antagonist (28), i.e., ANP appeared to cause vasoconstriction through activation of membrane-bound GC. ANP-induced vasoconstriction has also been reported in the mesenteric and renal vasculatures (11, 32–34), although the mechanism has never been elucidated. We have demonstrated in this current study that ANP-induced vasoconstriction, at least in the spleen, is indeed via a GC-mediated pathway. The vessels we used were small (150- to 250-μm diameter) and would have contributed to resistance to blood flow. Moreover, we would argue that the vasoactivity of these vessels is similar to that exhibited by the true pre-postcapillary vessels of the spleen, vessels that are far too small to mount in a myograph.

There are seven currently identified particulate GC isoforms (GC-A to GC-G) (18). Based on their ligand specificities, particulate GC isoforms have been classified as natriuretic peptide receptors, intestinal peptide-binding receptors, or orphan receptors (18). The GC-A isoform is linked to the NPRA. The development of a genetic model that eliminates the genes coding for the GC-A receptor (10, 16, 26) has enabled us to investigate the initial pathway for ANP-induced vasoconstriction in isolated blood vessels. These GC-A-deficient (knockout) mice exhibit a salt-resistant form of elevated blood pressure (17). It has also been shown, using these mice, that the ANP/GC-A signaling pathway is an essential component for acute natriuresis in response to isonotic saline volume expansion (16, 17). Moreover ANP, which normally induces vasorelaxation in aortic rings (27), fails to dilate vessels derived from GC-A-deficient mice, thus confirming that vasorelaxation is indeed mediated through the GC-A receptor (16). Indeed, the authors conclude that GC-A is the sole receptor for the acute effects of ANP on vascular tone. Although one can never ignore the fact that there may be other deficits in these genetically manipulated models, this particular mouse (the GC-A knockout) has been extensively studied. The characterization of this model, as well as that of other GC gene disruptions, has been recently reviewed (31).

Our results with splenic vessels derived from GC-A-deficient (knockout) mice demonstrate that ANP-induced vasoconstriction is also abolished in the absence of GC-A (Fig. 1, A and B). GC activation, which occurs in response to diverse signals such as nitric oxide (NO) and peptide ligands, initiates the conversion of the cytosolic purine nucleotide GTP to cGMP (18). Intracellular cGMP regulates cellular physiology by activating protein kinases, directly gating specific ion channels, or altering intracellular cyclic nucleotide concentrations through regulation of phosphodiesterases (18, 23, 30). ANP increases intracellular cGMP in a concentration- and time-dependent fashion in a number of cells and tissues (18). Typically, increases in cGMP in vascular smooth muscle cells initiate mechanisms that cause a decrease in intracellular Ca²⁺ (18, 30) and subsequent vasorelaxation (8). However, there is evidence that, in the absence of cGMP-dependent protein kinase, cGMP may initiate the release of Ca²⁺ from intracellular stores and cause vascular smooth muscle contraction (5, 13, 22, 29). We suggest that this could be the mechanism underlying ANP-induced vasoconstriction of the splenic vasculature. This pathway appears to be peculiar to membrane-bound GC-A because NO, which acts via soluble GC, dilates the splenic vessels as would be expected (3). However, the greater sensitivity of the veins than the arteries to the constrictive activity of ANP could be related to ANP-induced NO biosynthesis (7), because the splenic arteries are more responsive to NO than are the veins (3), i.e., NO-mediated modulation of the constrictive activity of ANP may be greater in the arteries.

In conclusion, this study has demonstrated that ANP induces vasoconstriction, not only in the rat splenic vasculature (28), but also in splenic vessels derived from mice. The constriction was independent of the endothelium but was counterbalanced by an endothelium-derived vasodilatory response. Splenic vessels derived from GC-A-deficient (knockout) mice did not exhibit any response to ANP, suggesting that the signal transduction pathway mediating ANP-induced vasoconstriction is GC-A activation.

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REFERENCES