Cyclic Guanosine Monophosphate Mediates Vascular Relaxation Induced by Atrial Natriuretic Factor

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SUMMARY The biochemical mechanism of action of synthetic atrial natriuretic factor (atriopeptin II) was studied in vascular smooth muscle of the rabbit thoracic aorta. Atriopeptin II caused a time-dependent and concentration-dependent increase in tissue levels of cyclic guanosine monophosphate that corresponded in these same tissues with vascular relaxation. The elevation of arterial cyclic guanosine monophosphate levels preceded the onset of vascular relaxation. Atriopeptin II did not alter vascular levels of cyclic adenosine monophosphate. The presence of a functionally intact vascular endothelium was not necessary for atriopeptin II to elicit vascular relaxation. Atriopeptin II-induced vascular relaxation and elevation of cyclic guanosine monophosphate levels were inhibited by the guanylate cyclase inhibitor methylene blue. These data suggest cyclic guanosine monophosphate mediates vascular relaxation produced by atriopeptin II. (Hypertension 7: 306-310, 1985)

KEY WORDS • atrial natriuretic factor • atriopeptin II • cyclic guanosine monophosphate • vascular relaxation • vascular endothelium

Atriopeptin II (AP II) is an endogenous natriuretic peptide hormone that has recently been recognized as an important endocrine mediator of renal and cardiovascular homeostasis. Atriopeptin II and close chemical analogues were originally identified, isolated, and purified from mammalian atria, and recent vigorous efforts have succeeded in creating synthetic hormone, as well as the cDNA clone. Atriopeptin II is a 23 amino acid peptide that displays potent natriuretic, diuretic, and vascular relaxant activity. Despite the important advances toward the elucidation of the nature of AP II, relatively little is known about the biochemical mechanisms by which AP II elicits its natriuretic, diuretic, or vascular relaxant activity.

Although the molecular mechanisms of hormone and drug action in smooth muscle contraction and relaxation have not been fully delineated, it is generally believed that cyclic nucleotides are mediators of vascular smooth muscle relaxation. Hormones such as catecholamines and prostaglandins of the E and I series elicit vascular smooth muscle relaxation through the generation of cyclic adenosine monophosphate (cyclic AMP). In contrast, nitric oxide–releasing vasodilators, such as sodium nitroprusside and nitroglycerin, elicit their vascular relaxant effect by increasing cellular levels of cyclic guanosine monophosphate (cyclic GMP). In contrast to agents such as acetylcholine, these agents do not require the presence of an intact vascular endothelium to produce vascular relaxation.

The present study was conducted to elucidate the molecular mechanism by which synthetic atrial natriuretic factor mediates vascular smooth muscle relaxant activity. The effects of AP II and atriopeptin I (AP I), were evaluated for their ability to influence vascular cyclic nucleotide levels in arterial rings during the process of active vascular relaxation.

Methods

New Zealand White rabbits weighing 2 to 3 kg were killed by cervical fracture and exsanguinated. Thoracic aorta were rapidly excised and carefully cleaned of connective tissue and adherent fat. The isolated arteries were cut into rings approximately 4 mm in length and suspended vertically in organ bath chambers (25 ml) containing Krebs-bicarbonate solution of the following composition (in mM): NaCl, 112; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄ - 7H₂O, 1.2; CaCl₂ - 2H₂O, 2.5; NaHCO₃ - 25.0; dextrose, 11.0; and ascorbate, 1.1.
Tissue baths were maintained at 37°C and continuously bubbled with 95% O₂ containing 5% CO₂. Tissues were allowed to equilibrate for 2 hours, during which time the Krebs solution was changed every 20 minutes. Resting tension of the vascular rings was maintained at 1 g during the equilibration period. Isometric tensions were recorded with a Beckman R-611 dynograph (Beckman Instruments, Inc., Palo Alto, CA) with Grass FT 03 force-displacement transducers (Grass Instrument Co., Quincy, MA).

After equilibration, tissues were contracted with norepinephrine (NE, 2 × 10⁻⁸-5 × 10⁻⁸ M). At peak contraction (4 minutes) arterial rings were frozen by clamps precooled in liquid N₂ or were administered drug. Tissues were then clamp-frozen exactly 4 minutes later or at the precise time interval indicated after the administration of drug. Isometric tensions were recorded until tissues were clamp-frozen. Frozen rings were stored at −85°C for subsequent determination of cyclic nucleotide content. Vascular relaxation was calculated as percentage of decrease of NE-induced tone.

Endothelial integrity was assessed as described by Furchgott and Zawadski. Endothelium was damaged by gently rubbing the intimal surface of the artery with a fine glass probe. Aortic rings with damaged endothelium never relaxed to acetylcholine. Confirmation that these procedures correlated with removal of the vascular endothelium was obtained by scanning electron microscopy.

Frozen vascular tissues were homogenized in 6% trichloroacetic acid and samples were centrifuged. Supernatant fractions were extracted with 5 volumes of ether (4 times), and a portion of the extract was acetylated. Aliquots of the acetylated extract were radioimmunoassayed for cyclic nucleotides. Cyclic nucleotide values were expressed as picomoles per gram wet weight (pmol/g wet wt).

The following drugs were used: l-norepinephrine bitartrate, acetylcholine chloride, ascorbic acid, and methylene blue (Sigma Chemical Co., St. Louis, MO); nitroglycerin (El Lilly & Co., Indianapolis, IN); AP I and AP II (Peninsula Laboratories, Belmont, CA).

Statistical significance for cyclic nucleotide levels was determined by Dunnet’s multiple range test, and values were considered significant when p < 0.05. Vascular relaxation results are expressed as means ± SEM, and statistical significance was determined by Student’s t test and was considered significant when p < 0.05.

Results

Our laboratory began to probe for the mechanism whereby AP II elicits its vascular relaxation by determining if the vascular endothelium influenced synthetic AP II’s vascular relaxant activity. Synthetic AP II was a potent relaxant of the rabbit aorta in the presence or absence of the vascular endothelium (Figure 1). Thus, the similarity between the failure of endothelial removal to influence either the vascular relaxant response of AP II or nitroglycerin suggested to us the possibility of similar intracellular mechanisms of action. We therefore investigated whether cyclic GMP was playing a role in AP II-mediated vascular relaxation.

Administration of AP II caused a concentration-dependent increase in cyclic GMP levels in NE-contracted rabbit thoracic aortic rings (see Table 1). In this series of experiments, AP II caused approximately a fivefold increase in cyclic GMP levels at the highest concentration tested. In contrast, AP II did not alter arterial cyclic AMP levels. Nitroglycerin also significantly increased cyclic GMP levels (p < 0.05) in these tissues by approximately fivefold without significantly affecting cyclic AMP.

Atriopeptin I is a 21 amino acid peptide differing...
from AP II in that it lacks two amino acids (Phe-Arg). Atriopeptin I is natriuretic but does not relax blood vessels. In our studies the lack of aortic relaxation was confirmed; however, in these tissues AP I had no effect on either cyclic AMP or cyclic GMP (see Table 1). Atriopeptin I thus differs substantially from its close chemical congener AP II.

To rule out the possibility of interference with the cyclic GMP radioimmunoassay, AP II was run through the extraction and assay procedure. No alteration of the standard curve for cyclic GMP was seen. In addition, AP II added directly to the radioimmunoassay reaction tubes did not affect the standard curve for cyclic GMP.

AP II elevated cyclic GMP levels independent of the presence of the vascular endothelium. A series of experiments demonstrated that AP II (10^{-8} M) elevated cyclic GMP levels (pmol/g wet wt) from 16.3 ± 4 to 106 ± 28 in endothelium intact tissues, in contrast to endothelium damaged tissues, which were elevated from 5.3 ± 0.5 to 36 ± 3. Basal levels of cyclic GMP were lower in aortic rings with damaged endothelium, and AP II caused less accumulation of cyclic GMP in these tissues. The overall percent increase in cyclic GMP levels in response to AP II was approximately the same in both endothelium intact and damaged vascular rings, 5.5-fold and 5.8-fold respectively.

A time course for the accumulation of cyclic GMP in rabbit aortic rings was performed and is shown in Figure 2. Administration of AP II increased cyclic GMP levels, and the increase corresponded with the vascular relaxation. Cyclic GMP levels peaked approximately 2 minutes after the addition of AP II, and the increase was maintained for at least 10 minutes.

Interestingly, 15 seconds after the administration of AP II vascular relaxations had not started, whereas cyclic GMP levels had increased approximately by 70%.

Methylene blue, an inhibitor of guanylate cyclase,23,24 has previously been shown to inhibit nitroglycerin-induced vascular relaxation and the associated increase in cyclic GMP levels. In the present studies, methylene blue (10^{-5} M) shifted the dose-response curve for AP II-mediated vascular relaxation to the right by eightfold (Figure 3). Methylene blue had no effect on the vascular relaxation induced by isoproterenol or adenosine (data not shown).

Methylene blue also inhibited the generation of cyclic GMP induced by AP II. In a series of experiments, AP II (10^{-8} M) elevated cyclic GMP levels from a control value of 15 ± 2 pmol/g wet wt to 79 ± 10 pmol/g wet wt; however, in the presence of 10^{-5} M methylene blue the elevation of cyclic GMP was only to 51 ± 7 pmol/g wet wt. Methylene blue (10^{-5} M) by itself lowered basal levels of cyclic GMP from 18 ± 4 to 5 ± 1 pmol/g wet wt.

**Discussion**

The data in the present study clearly demonstrate that AP II is a potent non-endothelial-dependent vascular relaxant that elevates vascular levels of cyclic GMP. Atriopeptin II produced a time-dependent and concentration-dependent accumulation of cyclic GMP that temporally correlated with relaxation. Furthermore, methylene blue not only inhibited the vascular relaxation induced by AP II but also blunted the accompanying increase in cyclic GMP levels.

There appeared to be a temporal relationship between the increase in arterial levels of cyclic GMP and the vascular relaxation induced by AP II. As illustrated
in Figure 2, the increase in cyclic GMP preceded the onset of vascular relaxation. This observation is consistent with the suggestion that a causal relationship exists between AP II-induced cyclic GMP accumulation and vascular relaxation. The results of this investigation are in analogous agreement with other studies that strongly supported an involvement of cyclic GMP in vascular smooth muscle relaxation elicited by nitric oxide–releasing vasodilators.

Atriopeptin I was inactive as a vascular relaxant in the isolated rabbit thoracic aortic ring. This observation is in agreement with that of Currie et al., who first demonstrated that AP I possessed natriuretic and diuretic activity and relaxed intestinal smooth muscle (chick rectum) but not vascular smooth muscle. We have extended these observations by investigating AP I’s ability to influence vascular cyclic nucleotide levels. As shown in Table 1, AP I did not alter arterial levels of either cyclic GMP or cyclic AMP. These data support the hypothesis that the vascular relaxant activity of AP II is mediated by cyclic GMP because if AP I had increased cyclic GMP levels but not induced relaxation, then the vascular relaxant activity of AP II would have been dissociated from its accompanying increase in cyclic GMP levels.

Atriopeptin II’s pattern of vasodilator activity, that is, its ability to elicit vascular relaxation independent of the vascular endothelium and to increase cyclic GMP, is similar to that of the nitric oxide–releasing vasodilators, such as nitroglycerin and sodium nitroprusside. Winquist et al. have recently demonstrated that a synthetic 26 amino acid fragment of atrial natriuretic factor is a potent and nonselective vasodilator with a profile of activity similar to that of sodium nitroprusside. They suggested that atrial natriuretic factor and sodium nitroprusside may share a common mechanism of action. Our data also suggest similarities between the pharmacology of AP II and the nitric oxide–releasing vasodilators. Like nitroglycerin and sodium nitroprusside, AP II elicits vascular relaxation independent of the vascular endothelium and elevates vascular levels of cyclic GMP.

Rapoport and colleagues have demonstrated that removal of the vascular endothelium reduces basal levels of cyclic GMP in blood vessels. Although the absolute increase in vascular cyclic GMP levels induced by AP II was greater in endothelium intact tissues, the percent increase in cyclic GMP levels was approximately the same in both endothelium intact and damaged aortic rings. This finding suggests that there is the possibility of separate and distinct pools of cyclic GMP in blood vessels. There is apparently a pool of cyclic GMP in the vascular endothelium that does not contribute to vascular smooth muscle relaxation because removal of the endothelium does not affect vascular relaxation. What role cyclic GMP is playing in the endothelium is presently unclear, and studies showing the presence of guanylate cyclase in the vascular endothelium are lacking.

The effects of the nitric oxide–releasing vasodilators are thought to be mediated through the activation of the soluble form of the enzyme guanylate cyclase and the subsequent formation of cyclic GMP. It is at this point that disparities between these vasodilators and AP II appear. While it is true that AP II increased arterial levels of cyclic GMP, the mechanism does not appear to be the same as the nitric oxide–releasing vasodilators. Initial experiments have provided no evidence for AP II stimulation of the purified soluble form of guanylate cyclase (Ohlstein EH, Berkowitz BA, Ignarro LJ, unpublished observations, 1984). This finding is in sharp contrast to the nitric oxide–releasing vasodilators, which are potent stimulators of the soluble form of the enzyme but relatively weak on the particulate form. Thus, future efforts are best directed to the effects of AP II on particulate guanylate cyclase and cyclic GMP selective phosphodiesterase. If AP II is a phosphodiesterase inhibitor, then it is extremely specific for cyclic GMP because, as demonstrated in Table 1, AP II did not affect arterial levels of cyclic AMP.

Methylene blue, an inhibitor of guanylate cyclase, was used to test the hypothesis that AP II-induced increases in cyclic GMP were caused by an activation of guanylate cyclase. Our studies revealed that methylene blue inhibited both AP II-induced vascular relaxation and the increase in arterial cyclic GMP levels. In view of the inability of AP II to directly stimulate the soluble form of guanylate cyclase, these data must be interpreted cautiously. We are not aware if methylene blue inhibits the particulate form of guanylate cyclase. If methylene blue is an inhibitor of particulate guanylate cyclase and AP II is a stimulator of this enzyme then this question may be thusly resolved. Methylene blue was not a nonspecific depressant of vascular smooth relaxation because it did not affect relaxation induced by isoproterenol or adenosine; however, we cannot rule out indirect or other effects of methylene blue on vascular smooth muscle.

If atrial natriuretic factor is a direct stimulator of an enzyme that catalyzes the formation of cyclic GMP or an inhibitor of cyclic GMP metabolism has yet to be firmly established. Perhaps atrial natriuretic factor interacts at a membrane bound receptor that causes the release of a mediator (i.e., lipid peroxide or free radical) that activates cytosolic guanylate cyclase, thereby indirectly stimulating the enzyme. If such was the case, then one would not expect stimulation of this enzyme in cell-free systems. This would be analogous to acetylcholine increasing arterial cyclic GMP levels in vascular tissue with an intact vascular endothelium. Acetylcholine does not stimulate guanylate cyclase directly, but rather causes the release of an endothelium-derived relaxing factor, which in turn is thought to stimulate guanylate cyclase. If AP II causes the release of a mediator that stimulates soluble guanylate cyclase, it probably is not an endothelium-derived relaxing factor because the relaxation induced by AP II is independent of the presence of a functionally intact vascular endothelium.

The ability to measure cyclic nucleotides in the same tissue undergoing vascular relaxation is a particularly...
useful correlate of these physiological and biochemical processes. Vascular mechanisms in the thoracic aortic wall are not always truly reflective of mechanisms controlling vascular resistance. The effects of AP II on cyclic GMP at the resistance level have yet to be demonstrated. Nevertheless, several intriguing questions merit further study: (1) Are the marked effects of AP II on cyclic GMP caused by stimulation of a particulate form of the enzyme guanylate cyclase? (2) Are there specific cyclic GMP caused by stimulation of a particulate form of the enzyme guanylate cyclase? (3) Does cyclic GMP mediate the natriuretic effects of AP II? Clearly the present suggestion that AP II is a "candidate" endogenous hormone that stimulates endothelium-independent vascular relaxation mediated by cyclic GMP is a unique and promising mechanistic hypothesis for future study.

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