Role of Nitric Oxide–cGMP Pathway in Adrenomedullin-Induced Vasodilation in the Rat

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Abstract—We previously reported that adrenomedullin (AM), a potent vasodilator peptide discovered in pheochromocytoma cells, stimulates nitric oxide (NO) release in the rat kidney. To further investigate whether the NO-cGMP pathway is involved in the mechanisms of AM-induced vasodilation, we examined the effects of E-4021, a cGMP-specific phosphodiesterase inhibitor, on AM-induced vasorelaxation in aortic rings and perfused kidneys isolated from Wistar rats. We also measured NO release from the kidneys using a chemiluminescence assay. AM (10^{-10} to 10^{-7} mol/L) relaxed the aorta precontracted with phenylephrine in a dose-dependent manner. Denudation of endothelium (E) attenuated the vasodilatory action of AM (10^{-7} mol/L AM: intact (E+) $-25.7\pm5.2\%$ versus denuded (E−) $-7.8\pm0.6\%$, $P<0.05$). On the other hand, pretreatment with 10^{-8} mol/L E-4021 augmented AM-induced vasorelaxation in the intact aorta ($-49.0\pm7.9\%$, $P<0.05$) but not in the denuded one. E-4021 also enhanced acetylcholine (ACh)-induced vasorelaxation in the rat intact aorta (10^{-7} mol/L ACh $-36.6\pm8.4\%$ versus 10^{-8} mol/L E-4021+10^{-7} mol/L ACh $-62.7\pm3.1\%$, $P<0.05$). In perfused kidneys, AM-induced vasorelaxation was also augmented by preincubation with E-4021 (10^{-9} mol/L AM $-15.4\pm0.6\%$ versus 10^{-8} mol/L E-4021+10^{-9} mol/L AM $-23.6\pm1.2\%$, $P<0.01$). AM significantly increased NO release from rat kidneys (ΔNO: $+11.3\pm0.8$ fmol $\cdot$ $min^{-1} \cdot g^{-1}$ kidney at 10^{-9} mol/L AM), which was not affected by E-4021. E-4021 enhanced ACh-induced vasorelaxation (10^{-9} mol/L ACh $-9.7\pm1.7\%$ versus 10^{-8} mol/L E-4021+10^{-9} mol/L ACh $-18.8\pm2.9\%$, $P<0.01$) but did not affect ACh-induced NO release from the kidneys. In the aorta and the kidney, 10^{-4} mol/L of N^G-nitro-L-arginine methyl ester, an NO synthase inhibitor, and 10^{-5} mol/L of methylene blue, a guanylate cyclase inhibitor, reduced the vasodilatory effect of AM. These results suggest that the NO-cGMP pathway is involved in the mechanism of AM-induced vasorelaxation, at least in the rat aorta and kidney. **(*Hypertension*. 1999;33:689-693.)**

Key Words: adrenomedullin ■ nitric oxide ■ cyclic GMP ■ endothelium ■ phosphodiesterase inhibitors ■ rats

Adrenomedullin (AM) is originally isolated from the pheochromocytoma cells by assaying the activity to increase platelet cAMP.[1] AM increases intracellular cAMP in cultured vascular smooth muscle cells (VSMCs)[2,3] and mesangial cells.[4] It is well established that the increase in intracellular cAMP of VSMC is associated with endothelium-independent vasorelaxation. On the other hand, Shimekake et al[5] observed that AM increased cGMP in rat aortic strips and that this effect was suppressed by pretreatment with N^G-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase (NOS) inhibitor. This finding suggests that the nitric oxide (NO)–cGMP pathway may be involved at least in part in the mechanisms of AM-induced vasodilation. We have also reported that AM decreased renal vascular resistance in the rat isolated perfused kidney.[6] This vasodilation was associated with a dose-dependent increase in NO release, which was measured with a chemiluminescence method.

Numerous studies other than the reports noted above have been conducted to explore the mechanisms for AM-induced vasodilation. However, it is still controversial whether it is endothelium-dependent[6–8] or -independent.[9,10] Most studies evaluated the endothelium-dependency of AM-induced vasodilation with NOS inhibitors such as L-NMMA. However, because the NOS inhibitors sometimes increase baseline vascular tone, it is difficult to get a clear-cut conclusion. Therefore, some other evidence in addition to NOS inhibition may be required to demonstrate the mechanisms of AM-induced vasodilation.

Because cGMP is degraded and inactivated by phosphodiesterase (PDE) in VSMCs, cGMP-specific PDE inhibitors may enhance the effects of NO. Actually, Thusu et al[11] reported that zaprinast, a cGMP-specific type V PDE inhibitor, augmented the vasodilatory effect of a NO donor on

Received July 2, 1998; first decision August 13, 1998; revision accepted October 28, 1998.

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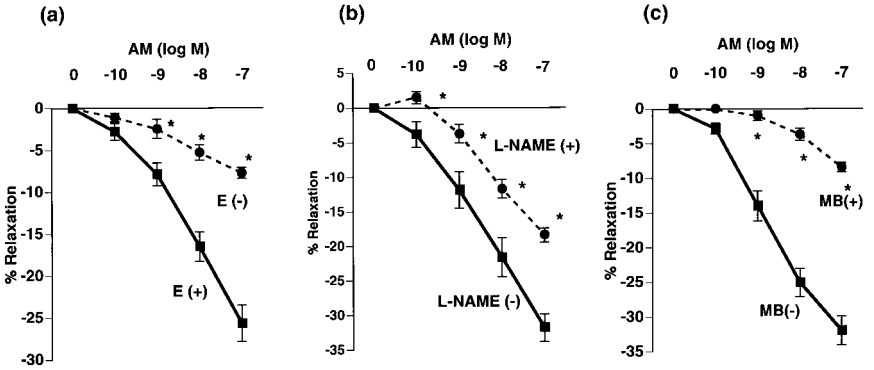

Figure 1. Line graphs showing the effects of endothelium-denudation (a), L-NAME (b), and methylene blue (MB; c) on AM-induced vasorelaxation in the rat aortic ring precontracted with norepinephrine. n=6 in each group. E(-) indicates endothelium-denuded, E(+), endothelium-intact. Values represent mean±SE. *$P<0.05$ vs E (+) in (a); *$P<0.05$ vs L-NAME (-) in (b); *$P<0.05$ vs MB (-) in (c).

isolated pulmonary arteries. Cohen et al[12] also demonstrated that cGMP-specific PDE inhibition reduced pulmonary artery resistance in conscious pulmonary hypertensive rats.

In the present study, to further explore whether the NO-cGMP pathway is involved in AM-induced vasodilation, we examined the effects of E-4021 [1-(6-chloro-4-(3,4-methylbenzyl) amino-quinazoline-2-yl) piperidine-4-carboxylate], a cGMP-specific type V PDE inhibitor[13] on AM-induced vasodilation in rat aortic rings and isolated perfused kidneys.

Methods

Organ Chamber Experiments

Vascular responses of the thoracic aorta from 12-week-old male Wistar rats were tested in organ chambers according to a technique previously described.[14] Briefly, the thoracic aorta was excised from the rat, and aortic rings (4 mm in length) were mounted in organ chambers filled with 25 mL of an oxygenated Krebs-Ringer bicarbonate solution at 37°C. Isometric tension was recorded with a force transducer (Oriental).

The aortic rings were precontracted with L-norepinephrine, and responses to AM (10^{-10} to 10^{-7} mol/L) at 70% of maximal contraction obtained in each individual ring (n=6 each) were studied in the presence or absence of the vascular endothelium. The endothelium of the aortic ring was removed by gentle rubbing with a stainless steel needle. To evaluate the role of the NO-cGMP pathway, the responses to AM were tested in the presence of N^G-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, methylene blue, a guanylate cyclase inhibitor, or E-4021. We also evaluated the effects of E-4021 on acetylcholine (ACh)-induced vasorelaxation (10^{-9} to 10^{-5} mol/L), which is mediated by cGMP. Endothelium-independent relaxation was tested with 10^{-4} mol/L papaverine. Relaxation in aortic rings was expressed as a percent decrease in tension.

Isolated Perfused Kidney

Male Wistar rats weighing 319±7 g were anesthetized intraperitoneally with 30 mg/kg pentobarbital, then the right kidney was isolated and perfused as previously described.[15] In brief, after an abdominal incision, we punctured the mesenteric artery with an 18 gauge double lumen needle and positioned the tip in the right renal artery. Perfusion was then started at 5 mL/min with a Krebs-Henseleit buffer, and the kidney was isolated without ischemia. The buffer was saturated with 95% O_2-5% CO_2 at 37°C and contained 10^{-6} mol/L phenylephrine to maintain perfusion pressure at about 100 mm Hg (106±4 mm Hg). Renal perfusion pressure (RPP) was monitored at the renal artery through a double-lumen needle connected to a pressure transducer. The renal vein was also cannulated to drain the perfusate into the NO assay system.

Measurement of NO Release

NO concentration in the perfusate was measured with a chemiluminescence assay.[15,16] The venous effluent was introduced into a rotatory mixer for thorough mixing with a chemiluminescence probe of 10 mmol/L H_2O_2, 18 μmol/L recrystallized luminol, 2 mmol/L potassium carbonate, and 150 mmol/L desferrioxamine. The mixture of the perfusate and probe then entered a chemiluminescence detector. The chemiluminescent signal was measured continuously and recorded on a standard pen recorder. The NO signal was calibrated using a NO solution.

After a 60-minute equilibrium period, 10^{-8} mol/L E-4021 was infused through a 3-way stopcock. Ten minutes later, vehicle and 10^{-11} to 10^{-8} mol/L of AM were consecutively added to the E-4021–containing buffer at 10-minute intervals. To evaluate the effects of NOS inhibition, AM-induced renal vasorelaxation was tested in the presence of 10^{-4} mol/L L-NMMA infusion. To confirm the specificity of the effect of E-4021, we examined the effects of E-4021 on $10^{-9.5}$ to 10^{-7} mol/L ACh– and 10^{-8} to 10^{-6} mol/L salbutamol–induced renal vasodilation, which are believed to be mediated at least in part by NO-cGMP and cAMP, respectively. We compared the effects of AM, Ach, and salbutamol in the presence of E-4021 on RPP and NO release with those in the absence of E-4021. NO release was normalized by kidney weight (1.42±0.04 g) and expressed as fmol · min^{-1} · g kidney wt^{-1}.

Calculations and Statistical Analysis

Results of the experiments are given as the mean±SEM. Data were analyzed by ANOVA for repeated measures. Effects of agents were assessed by Dunnett's test. Differences of $P<0.05$ were considered statistically significant.

Results

In the aortic rings, 10^{-10} to 10^{-7} mol/L of AM caused a dose-dependent vasorelaxation. This effect of AM was significantly attenuated by endothelium denudation (Figure 1a), which abolished the vasorelaxant effect of ACh. In the presence of 10^{-4} mol/L of L-NAME, AM-induced relaxation was also significantly attenuated (Figure 1b). Furthermore, 10^{-7} mol/L of methylene blue inhibited AM-induced vasorelaxation at 10^{-9} to 10^{-7} mol/L (Figure 1c). Methylene blue and L-NAME inhibited the effects of AM by 40% and 74%, respectively. On the other hand, 10^{-8} mol/L E-4021 significantly augmented the vasorelaxation induced by AM by 91% at 10^{-7} mol/L AM (Figure 2a). The effects of E-4021 on ACh-induced vascular relaxation, which is known as a cGMP-dependent response, were also tested in the aortic rings (Figure 2b). E-4021 significantly enhanced ACh-induced vasorelaxation by about 30% at the dose of 10^{-9} to 10^{-6} mol/L.

In the isolated perfused kidney, E-4021 per se caused a dose-dependent decrease in RPP between 10^{-8} and 10^{-6} mol/L. RPP, which was maintained about 100 mm Hg by 10^{-6} mol/L phenylephrine, was significantly decreased, by as

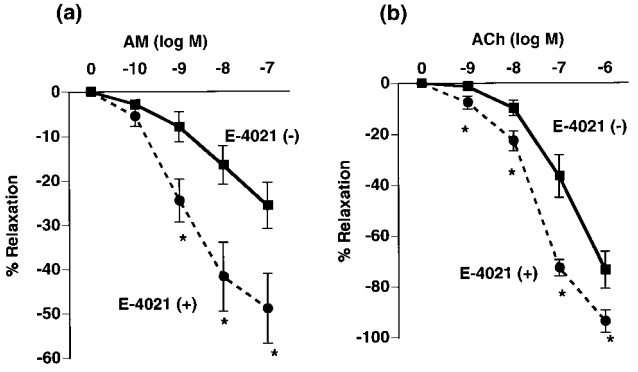

Figure 2. Line graphs showing the effects of E-4021 on AM-induced (a) and ACh-induced (b) vasorelaxation in rat aortic rings precontracted with norepinephrine. n=6 in each group. Values represent mean±SE. *$P<0.05$ vs E-4021 (-).

much as 30% during the infusion of 10^{-6} mol/L E-4021 (data not shown). Therefore, we used 10^{-8} mol/L E-4021 as pretreatment because this concentration of E-4021 caused only a 5% reduction in baseline RPP. As shown in Figure 3, ACh caused potent vasodilation and NO release in a dose-dependent manner. E-4021 potentiated these effects at either concentration. However, NO release was not influenced by E-4021. Although salbutamol showed a potent vasodilatory action, it did not cause release of NO at all. The PDE inhibitor altered neither salbutamol-induced vasodilation nor NO release (Figure 4). Figure 5 demonstrates the effects of E-4021 and L-NAME on the renal vasorelaxation induced by AM. AM decreased RPP in a dose-dependent manner between

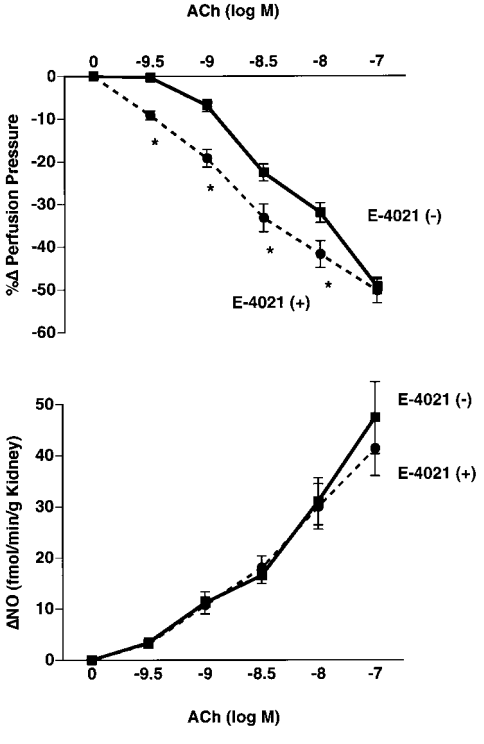

Figure 3. Line graphs showing the effects of E-4021 on ACh-induced vasorelaxation and NO release in rat isolated perfused kidneys. n=5 in each group. Values represent mean±SE. *$P<0.05$ vs E-4021 (-).

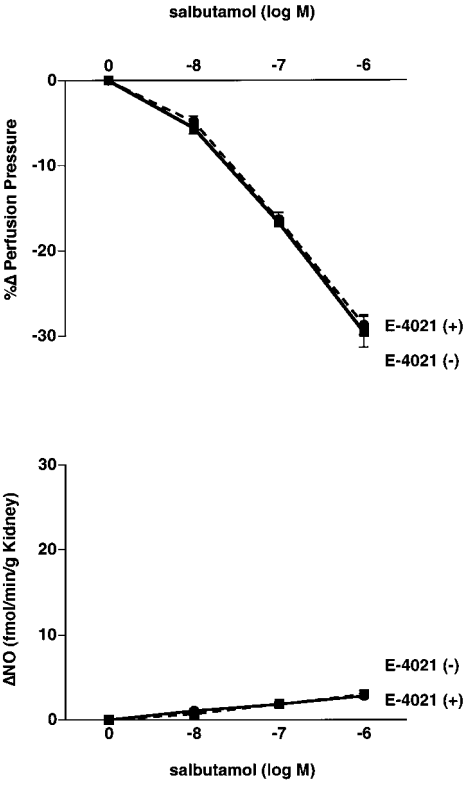

Figure 4. Line graphs showing the effects of E-4021 on salbutamol-induced vasorelaxation and NO release in rat isolated perfused kidneys. n=5 in each group. Values represent mean±SE.

10^{-11} and 10^{-8} mol/L. This renal vasorelaxation was associated with NO release, although the degree was smaller than that caused by ACh. AM-induced vasodilation was significantly augmented by pretreatment with E-4021. However, NO release induced by AM was not affected by E-4021. On the other hand, NOS inhibition by L-NAME decreased both renal vasodilation and NO release at any dose of AM.

Discussion

AM is a potent vasodilator peptide particularly in the renal vessels. As mentioned before, cAMP has been considered to be a primary second messenger of AM.[1] In fact, Ishizaka et al[3] and Eguchi et al[2] reported that AM increased cAMP in cultured rat VSMCs in a dose-dependent manner. AM also increases cAMP in renal tubular cells,[17] mesangial cells,[4] and endothelial cells.[18] In VSMCs, increases in cAMP result in activation of protein kinase A, increasing in turn Ca^{2+} efflux through the Ca^{2+} pump. Kureishi et al[19] showed that AM decreased high K-induced contraction and $[Ca^{2+}]_i$ in porcine coronary arteries.

However, we have suggested another possible mechanism for the vasodilatory action of AM. To examine the possible involvement of the NO-cGMP pathway in AM-induced vasorelaxation, we examined the effects of a cGMP-specific PDE inhibitor on vasorelaxation induced by AM in the aorta and renal vessels of rats. E-4021 has been reported to be a cGMP-specific type V PDE inhibitor.[12,13] Cohen et al[12] showed that PDE inhibition by E-4021 increased the cGMP

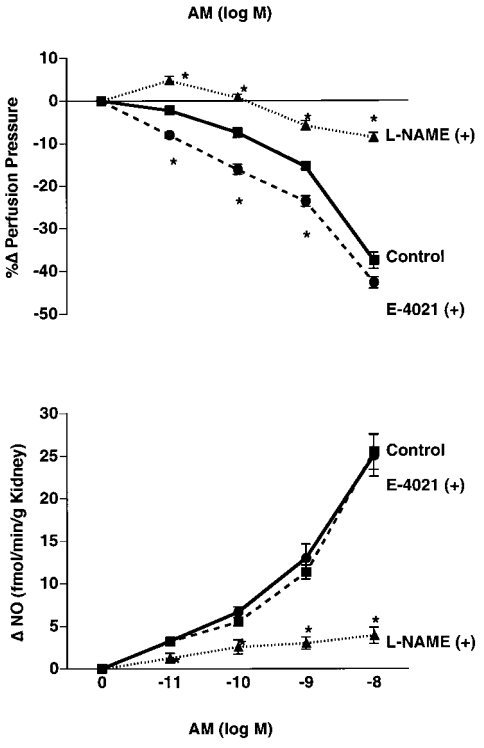

Figure 5. Line graphs showing the effects of E-4021 and L-NMMA on AM-induced vasorelaxation and NO release in rat isolated perfused kidneys. n=5 in each group. Values represent mean±SE. *$P<0.05$ vs control.

level in the perfusate and reduced hypoxic vasoconstriction in the isolated perfused lungs of rats with pulmonary hypertension. In the present study, AM-induced vasorelaxation was augmented by E-4021. As shown in Figures 3 and 5, there were no differences in the vasodilator responses to the maximal dose of AM or ACh between control and E-4021–treated kidneys. In our system of the isolated perfused kidney precontracted with phenylephrine, the maximal responses to endothelium-dependent vasodilators are about 50% in terms of reductions of RPP. The responses to the maximal dose of AM or ACh used in this study almost reached this level. Therefore, in this condition, the addition of E-4021 might not augment the vasodilatory responses to AM or ACh. This stimulatory effect of the PDE inhibitor on AM-induced renovascular relaxation was also observed in aortic rings. These observations suggest that a cGMP-mediated mechanism is involved in AM-induced vasorelaxation. Furthermore, vascular relaxation by AM was attenuated by denudation of the endothelium, a guanylate cyclase inhibitor, or a NOS inhibitor. It is well established that when guanylate cyclase is stimulated by NO, cGMP is increased in VSMCs.[20] These findings show that AM-induced vasorelaxation is, at least in part, NO-mediated in the thoracic aortas and renal arteries of rats.

However, the controversy as to whether AM-induced relaxation is endothelium-dependent or not still persists. Miura et al[7] reported that renal vasodilation caused by intra-arterial administration of AM in dogs was suppressed by N^G-nitro-L-arginine and that an excessive amount of L-arginine restored this suppression. On the other hand, Gardiner et al[21] reported that AM-induced vasodilation in the hindquarter was only slightly inhibited by L-NAME. Heaton et al[22] also observed that L-NAME did not antagonize the vasodilatory effect of AM in pulmonary vessels of the rat. It is possible that the endothelium-dependency of the action of AM depends on differences in vascular beds.

The mechanism of NO release by AM is not clear in this study. However, because the vasodilatory response to AM occurred rapidly (less than 15 seconds), the type of NOS involved in AM-induced relaxation may be constitutive NOS. In addition, we previously showed an increase in $[Ca^{2+}]_i$ transient of cultured bovine carotid endothelial cells in response to AM.[6] The activity of endothelial constitutive NOS depends on intracellular Ca^{2+} and calmodulin concentration.[23,24] Shimekake et al[5] also demonstrated that AM increased intracellular Ca^{2+} and cGMP in cultured bovine aortic endothelial cells. It is possible that the increase in $[Ca^{2+}]_i$ induced by AM activates endothelial constitutive NOS and increases NO release from the vascular endothelium. On the other hand, it has been reported that there is a site for phosphorylation by cAMP-dependent protein kinase on endothelial NOS.[24] In addition, cAMP directly activates inducible NOS. However, it is unlikely that the increase in cAMP directly stimulates NO release from endothelial cells. Although the structures of the putative receptors for AM and calcitonin gene–related peptide have been demonstrated,[25,26] their intracellular signaling or distribution has not been clarified. Some receptors such as calcitonin receptors activate adenylate cyclase and phospholipase C.[27,28] If AM receptors with such characteristics abundantly exist on the vascular endothelial cells, AM may increase $[Ca^{2+}]_i$ and thereby NO release. In addition to the direct effect of NO on VSMCs, it is possible that endothelium-derived NO potentiates or stimulates other receptors or channels to promote further relaxation in response to AM. Bolotina et al demonstrated that NO directly activates Ca-dependent K channels in VSMCs.[29] Furthermore, NO reduces the production of vasoconstrictive substances (eg, endothelin) through a cGMP-dependent mechanism.[30]

In conclusion, the vasodilatory effect of AM is at least in part endothelium-dependent. Not only the cAMP-related mechanism but also the NO-cGMP pathway may be involved in AM-induced vasorelaxation in the rat aorta and kidney. Because vascular endothelial cells and smooth muscle cells synthesize AM, AM may contribute to the regulation of vascular tone through NO-cGMP signaling mechanisms.

Acknowledgments

This study was supported in part by Grants-in-Aid 07557055 and 09281206 from the Japanese Ministry of Education, Culture and Science, Japan. The authors thank Eisai Co (Tokyo, Japan) for generously providing E-4021 for this study.

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Hypertension. 1999;33:689-693
doi: 10.1161/01.HYP.33.2.689
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563