Original Article

Endothelial Responses of the Aorta from Adrenomedullin Transgenic Mice and Knockout Mice

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Adrenomedullin (AM) is a potent vascular wall-derived vasorelaxing peptide which induces the release of nitric oxide (NO). To explore the role of endogenous AM in vascular function, we examined the effects of acetylcholine (ACh), AM, and AM receptor antagonists [AM (22–52), and calcitonin gene-related peptide (CGRP) (8–37)] on the isometric tension of aortic rings isolated from AM transgenic (TG) and knockout (KO) mice and wild type littermates (WT). ACh and AM caused a dose-dependent reduction of the isometric tension of aortic rings, but the degree of vasodilatation was smaller in TG than in KO or WT (% tension [10^{-6} mol/l ACh]: KO $-69 \pm 10\%$, WT $-39 \pm 8\%$, TG $-29 \pm 1\%$, $p < 0.01$). On the other hand, N^G -nitro-L-arginine methyl ester, an NO synthase inhibitor, induced greater vasoconstriction in TG (% tension 10^{-5} mol/l: KO $+78 \pm 16\%$, WT $+99 \pm 27\%$, TG $+184 \pm 20\%$, $p < 0.01$), whereas E-4021, a cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase inhibitor, caused greater vasodilation in TG mice. Both AM antagonists increased tension in TG to a greater extent than in KO or WT mice (% tension [10^{-6} mol/l CGRP (8–37)]: KO $+24 \pm 5\%$, WT $+51 \pm 6\%$, TG $+75 \pm 7\%$, $p < 0.01$). Endothelial denudation of the aorta diminished the vasoconstriction caused by the AM antagonists. In conclusion, the amounts of AM expressed in the aortic endothelium influenced baseline NO release. AM antagonists increased vascular tone in WT as well as in TG, suggesting that endogenous AM plays a physiological role in the regulation of aortic tone. (*Hypertens Res* 2003; 26 (Suppl): S79–S84)

Key Words: adrenomedullin, nitric oxide, cyclic guanosine monophosphate, endothelium

Introduction

Adrenomedullin (AM) is a vascular hormone; the major source of circulating AM is the vascular wall, and the vessels are also the major target of this peptide (1, 2). The effects of AM are thought to be exerted through both endothelium-independent and -dependent mechanisms (1, 3, 4). It has been

reported that the secretion of AM is increased in some cardiovascular diseases, including sepsis, heart failure, renal failure and hypertension (5–11). These conditions are commonly accompanied by drastic changes in systemic vascular resistance. Since AM exerts a vasodilatory action, it is possible that increases in AM play a causative or compensatory role in these disease conditions.

We recently developed AM transgenic (TG) (12) and

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knockout (KO) mice (13). The former shows decreased blood pressure, while heterozygotes of the latter show elevated blood pressure. In a subsequent work, we analyzed the effects of endogenous AM on renal vascular tone using AM TG and KO mice. The results showed that the baseline renal vascular tone changed in parallel with the production rate of AM (14). Furthermore, endogenous AM had a protective effect on renal ischemia/reperfusion injury. These beneficial effects of AM were shown to be attributable, at least in part, to endothelium-derived NO (14).

In the present study, to explore the role of endogenous AM in the regulation of aortic tone, we examined the aortic responses of AM TG and KO mice to AM and AM receptor antagonists.

Methods

Animals

All mouse studies were performed in concordance with the University of Tokyo guidelines for animal experiments. AM TG mice and KO mice were established as previously reported (12, 13). TG mice were established using the AM gene with the promoter region of the preproendothelin-1 gene, resulting in overexpression of AM in the vascular wall, particularly in the endothelium, and 2- to 5-fold increases in AM expression in the aorta (12). AM KO mice were established by replacing exons 1 to 4 with the neomycin-resistant gene. Because AM KO homozygotes (AM^{-/-} mice) could not survive, the heterozygotes (AM^{+/-} mice), in which the AM content is only half of that in wild type (WT) mice (13), were used as AM KO mice.

Measurement of Aortic Tension

Vascular responses of the thoracic aorta from 12-week-old, male mice were tested in organ chambers. The thoracic aorta was excised from TG ($n = 8$), KO ($n = 10$) and WT ($n = 10$) mice and then dissected free from connective tissue under a magnifying glass. Aortic rings (5 mm in length) were mounted horizontally between two stirrups in organ chambers filled with 20 ml of an oxygenated Krebs-Ringer bicarbonate solution at 37°C. One stirrup was connected to an anchor and the other to a force transducer (Oriental Co., Tokyo) to record isometric tension.

The aortic rings were precontracted with 10^{-6} mol/l L-nor-epinephrine, and responses to acetylcholine (ACh) and AM were studied in the presence or absence of the vascular endothelium. The endothelium of the aortic ring was removed by gentle rubbing with a twist of cotton. The responses to L-NAME arginine methyl ester (L-NAME), an NO synthase (NOS) inhibitor, AM (22–52), the calcitonin gene-related peptide (CGRP) (8–37), AM receptor antagonists, or E-4021, a phosphodiesterase (PDE) inhibitor, were studied in the same manner. E-4021 is a type V PDE inhibitor which has been

reported to selectively inhibit cyclic guanosine monophosphate (cGMP)-specific PDE (15). Relaxation in aortic rings was expressed as a percent decrease in tension.

To confirm the involvement of NO in AM-induced vasodilation, we examined the effects of AM in endothelial NO synthase (eNOS) KO mice (16) (Jackson Lab., Bar Harbor, USA). As described previously, the thoracic aortas were carefully dissected from 15-week-old eNOS KO mice and age-matched control mice ($n = 5$, each). The aortic rings were mounted with and without the endothelium. Following a 60-min equilibrium period, ACh and AM were administered.

Drugs and Chemicals

Laboratory reagents and chemicals used to prepare Krebs-Henseleit solution were purchased from Wako Pure Chemicals (Osaka, Japan). AM, AM (22–52) and CGRP (8–37) were from the Peptide Institute (Osaka, Japan). E-4021 was a gift from Eisai Co., Ltd. (Tokyo). All other chemicals were from Sigma-Aldrich Japan (Tokyo).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM. Statistical comparisons were made by analysis of variance followed by the Student-Neumann-Keuls test. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

As shown in Fig. 1, ACh decreased aortic tension in a dose-dependent manner, but endothelial denudation abolished the response to ACh in the three groups of mice aortas. The degree of vasodilation caused by ACh was smaller in TG mice than in WT or KO mice. Figure 2 shows that AM also decreased aortic tension, and this decrease was similar to that by ACh. The responses of the aorta from TG mice to AM were attenuated, compared to those of WT or KO mice. Endothelial denudation did not abolish the effects of AM, but it significantly decreased the vasodilation caused by AM in these mice.

Figure 3 illustrates the effects of AM antagonists on aortic tension. Both AM (22–52) and CGRP (8–37) induced vasoconstriction in a dose-dependent fashion. These antagonists increased the tension in TG mice to a greater extent than in WT and KO mice. Furthermore, the denuded aortas responded neither to AM (22–52) nor to CGRP (8–37). As shown in Fig. 4, L-NAME increased the aortic tension in a dose-dependent fashion while E-4021 decreased aortic tension. The responses were greater in TG mice. The effects of L-NAME and E-4021 on aortic tension were significantly diminished by endothelial denudation.

Finally, the effects of ACh and AM were examined in the aorta of eNOS KO mice. In control mice, both ACh and AM

Fig. 1. Effects of acetylcholine (ACh) on aortic tension in adrenomedullin transgenic (TG), wild-type (WT) and knockout (KO) mice in the presence [E(+)] and absence [E(-)] of endothelium. * $p < 0.05$, † $p < 0.01$ vs. WT.

Fig. 2. Effects of adrenomedullin (AM) on aortic tension in AM transgenic (TG), wild-type (WT) and knockout (KO) mice in the presence [E(+)] and absence [E(-)] of endothelium. * $p < 0.05$, † $p < 0.01$ vs. WT.

decreased aortic tension in a dose-dependent manner (Fig. 5). However, ACh did not dilate the aorta of eNOS KO mice in the presence or absence of the endothelium. Although AM reduced aortic tension, the degree of reduction was significantly smaller in eNOS KO mice than in controls. Endothelial denudation reduced AM-induced vasodilation in control mice, but did not affect the aortic responses to AM in eNOS KO mice.

Discussion

In the present study the effects of ACh and AM on aortic tension were attenuated in AM TG mice. This phenomenon was also observed in the renal vasculature isolated from AM TG mice (14). The vasodilatory effects of ACh and AM were decreased substantially after endothelial denudation of the aortic rings. This suggested that both ACh and AM are endothelium-dependent vasodilators also in the mice aortas. In AM TG mice, the findings that L-NAME induced greater vasoconstriction and E-4021 induced greater vasodilation suggest that endothelium-derived NO release from the aorta of AM TG mice was increased under the basal conditions,

3a

3b

Fig. 3. Effects of adrenomedullin (AM) receptor antagonists, AM (22–52) (3a) and CGRP (8–37) (3b) on aortic tension in AM transgenic (TG), wild-type (WT) and knockout (KO) mice in the presence [E(+)] and absence [E(-)] of endothelium. * $p < 0.05$, † $p < 0.01$ vs. WT.

4a

4b

Fig. 4. Effects of L-NAME arginine methyl ester (L-NAME) (4a) and the cGMP-specific phosphodiesterase inhibitor E-4021 (4b) on aortic tension in adrenomedullin transgenic (TG), wild-type (WT) and knockout (KO) mice in the presence [E(+)] and absence [E(-)] of endothelium. * $p < 0.05$ vs. WT.

Fig. 5. Effects of acetylcholine (ACh) (5a) and adrenomedullin (AM) (5b) on aortic tension in endothelial NO synthase-knockout mice (eNOS KO) in the presence [E(+)] and absence [E(-)] of endothelium. * $p < 0.05$, † $p < 0.01$ vs. WT.

whereas that from AM KO mice was decreased. On the other hand, the responses to both ACh and AM were attenuated in AM TG mice. This finding cannot be explained by the down-regulation of AM receptors alone. AM TG mice may somehow show hyporesponsiveness to the NO-cGMP pathway under condition of stimulation by ACh or AM. A similar blunted response to ACh has been observed in eNOS TG mice (17, 18). In eNOS KO mice, the effects of ACh were completely diminished while those of AM were partially suppressed. The resistant artery of the eNOS KO mice still responds to ACh. It has been suggested that the remainder of the ACh-induced vasodilation in eNOS KO mice may be due to the endothelium-derived hyperpolarizing factor (EDHF) (19). With regard to the vasodilation induced by AM in eNOS KO mice, the involvement of EDHF is possible, but it is more likely that AM exerts an endothelium-independent mechanism in the aorta *via* cyclic adenosine monophosphate (cAMP). We have found that AM-induced NO release is

attributable to the activation of phosphatidyl inositol-3 kinase/Akt, resulting in phosphorylation of eNOS (20).

AM is considered a member of CGRP superfamily (1). In fact, AM and CGRP share common receptors: the calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs). Three types of RAMPs have been identified: the CRLR and RAMP1 complex is specific for CGRP, while the CRLR and RAMP2 or RAMP3 complex is specific for AM (21). However, the selectivity of these complexes for each peptide is not very high. In fact, the effects of CGRP and AM have been substantially blocked by CGRP (8–37) in the rat mesenteric artery (22) and renal artery (3). Furthermore, the expression of AM receptors is species- and tissues-dependent (23). In the present study both CGRP (8–37) and AM (22–52) increased the aortic tension in AM TG mice as well as in WT mice. This suggests that endogenous AM may exert a tonic inhibition on vascular contraction. We have already reported a similar phenomenon in the isolated perfused kidneys from AM TG, KO and WT mice. Both antagonists could block the action of not only AM but also CGRP. Therefore, endogenous AM and/or CGRP may play a role in the regulation of vascular tone. However, the effects of the antagonists were abolished after endothelium denudation, suggesting that the antagonists-induced vasoconstriction might have been due to endothelium-derived substances. Since CGRP exists in the perivascular area, it is unlikely that CGRP contributes to the effects of the antagonists. Endogenous peptides, in this case AM, may exert these effects on the vessels *via* the endothelium, most likely through NO.

In the present study, endothelial denudation substantially decreased the AM-induced vasodilatory response of the mice aorta, suggesting that this response is dependent on the endothelium. We previously reported that denudation attenuated AM-induced relaxation of the rat aorta by about 50% (4). We surmised that the remaining AM-induced vasodilation was due to an endothelium-independent mechanism, such as an increase in cAMP. However, it remains unclear whether or not the vasodilatory effect of AM in the rat is endothelium-dependent. Matsunaga *et al.* (24) have shown that removal of the endothelium or pretreatment with L-NAME almost completely inhibited AM-induced vasodilatation in the rat aorta. On the other hand, it has been reported that AM did not exert an endothelium-dependent vasodilatory effect in the vasculature of the lung (25) or the hind limbs of rats (26). AM-induced vasorelaxation in the kidney has been associated with increases in NO release in both mice (14) and rats (4). Further studies will be needed to elucidate these apparently diverse mechanisms of the involvement of AM in vasodilation.

The role of endogenous AM is still unclear. To explore this, three lines of AM KO mice have been independently established (13, 27, 28). In each of these three lines, AM^{-/-} mice were stillborn. We reported that in AM^{-/-} mice the maturation of the vascular structure was altered, resulting in

bleeding of the fetuses and death on the 13.5th day of the embryonic period (13). Thus, AM is considered to play an important role in vasculogenesis. AM^{+/-} mice, on the other hand, appear to grow normally but show evidence of hypertension. However, Shimosawa *et al.* (28) did not find any differences in blood pressure between their AM^{+/-} and control mice. This discrepancy may be explained by the presence/absence of the gene encoding for the pro-adrenomedullin N-terminal 20 peptide (PAMP); however, determination of the exact mechanisms for this discrepancy may require further study. We have previously reported that renal vascular tone is increased in AM^{+/-} mice. In the present study AM receptor antagonists increased the aortic tension of not only AM transgenic mice but also WT mice, suggesting that endogenous AM plays a physiological role in the regulation of aortic tone.

Chu *et al.* (29) showed that CGRP (8–37) inhibited the interleukin-1 β (IL-1 β)-induced increase in skin blood flow. In skin lesions, IL-1 β increased AM mRNA, suggesting that CGRP (8–37) may antagonize the effect of endogenous AM. Furthermore, Nishikimi *et al.* (30) reported that *in vivo* administration of CGRP (8–37) increased blood pressure and decreased urinary flow in rat models of malignant hypertension. These findings suggest that, at least in certain pathological conditions, increases in AM may participate in the regulation of vascular tone. Further studies will be needed to clarify the role of AM in pathophysiological conditions in humans.

In conclusion, overexpression of AM decreased aortic tone in the present study, while deficiency of AM increased it. These findings and the fact that AM receptor antagonists enhanced aortic constriction indicate that endothelium-derived AM may play a significant role in the regulation of aortic tone.

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