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Review

Adrenomedullin receptors: pharmacological features and possible pathophysiological roles☆

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Abstract

Three receptor activity modifying proteins (RAMPs) chaperone calcitonin-like receptor (CLR) to the cell surface. RAMP2 enables CLR to form an adrenomedullin (AM)-specific receptor that is sensitive to AM-(22–52) (AM_1 receptor). RAMP3 enables CLR to form an AM receptor sensitive to both calcitonin gene-related peptide (CGRP)-(8–37) and AM-(22–52) (AM_2 receptor), though rat and mouse AM_2 receptors show a clear preference for CGRPα-(8–37) over AM-(22–52). RAMP1 enables CRL to form the CGRP-(8–37)-sensitive $CGRP_1$ receptor, which can also be activated by higher concentrations of AM. Here we review the available information on the pharmacological features and possible pathophysiological roles of the aforementioned AM receptors.

Keywords: Adrenomedullin; Antagonist; Calcitonin-like receptor; Heterodimer; Receptor activity modifying protein; Transfection

1. Introduction

Adrenomedullin (AM) and calcitonin gene-related peptide (CGRP) belong to the calcitonin (CT) superfamily of regulatory peptides and are comprised of 52 and 37 amino acids, respectively [5,38]. CGRP is expressed in α- and β-forms (CGRPα and CGRPβ, respectively), which differ by one amino acid in rats and by three in humans [6,54,76]. CGRPα is generated by alternate tissue-specific splicing of the calcitonin gene [38]; CGRPβ is not derived from the calcitonin gene, despite its high sequence homology with CGRPα [6]. Although the sequence identity between the AM and CGRP peptides is only 30%, the two structures required for biological activity are totally conserved: a C-terminal amid and a ring structure comprised of six amino acids linked by a disulfide bridge between cysteine residues at positions 16 and 21 of AM and 2 and 7 of CGRP [92]. Both peptides and their specific and common binding sites are widely distributed among peripheral tissues and in the central nervous system, enabling them to exert a wide variety of biological effects, including potent vasorelaxation [21,92]. To evaluate the pharmacological characteristics of AM and CGRP, many laboratories have used the $CGRP_1$ receptor antagonist CGRP-(8–37) [13] and the AM receptor antagonist AM-(22–52) [19], both of which contain no disulfide bond.

In 1998, McLatchie et al. [50] identified and cloned human receptor activity modifying protein 1 (RAMP1; 148 amino acids), which enhances the activity of endogenous CGRP receptors in *Xenopus* oocytes. cDNAs encoding human RAMP2 and -3 (175 and 148 amino acids, respectively) were then cloned by expressed sequence tag analysis [50], and eventually all three isoforms were also cloned in rat and mouse [58,71]. RAMP1 was found to serve as an accessory protein involved in the transport of an orphan receptor, calcitonin-like receptor (CLR), to the cell surface, and when co-transfected into mammalian cells with RAMP2 or -3, CLR was found to function as an AM receptor. All three CLR/RAMP complexes exhibit a 1:1 stoichiometry [33,50]. And although they share less than 30% sequence identity, they all exhibit a common structure that includes a large extracellular N-terminal domain, a single membrane-spanning domain, and a very short cytoplasmic domain [79], and all three CLR/RAMP heterodimeric receptors mediate intracellular cAMP production and Ca^{2+} mobilization [41]. We recently identified the individual RAMP domains responsible for agonist binding to CLR/RAMP heterodimers, and their

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deletion yielded a group of dominant-negative (DN) RAMP mutants [42–44].

Like CLR, calcitonin (CT) receptor belongs to the Class II (or Class B) family of G protein-coupled receptors, members of which share a number of structural features and are all activated by peptide ligands [74]. CT receptor shares ~55% overall amino acid sequence identity with CLR, though the transmembrane domains are almost 80% identical [74]. The best characterized splice variants of human CT receptor differ depending on the presence ($CT_{(b)}$ receptor) or absence ($CT_{(a)}$ receptor) of 16 amino acids in the first intracellular loop [74]. In that regard, co-transfection of COS-7 cells with a RAMP plus the most common variant, the $CT_{(a)}$ receptor, leads to formation of a 1:1 dimer at the cell surface [16] and augmentation of responses evoked by amylin or CGRP [16,47,56,85]. AM can also elicit cAMP production via $CT_{(a)}$ receptor/RAMP1, -2 or -3 overexpressed in human embryonic kidney (HEK)-293 cells, though the responses are at least 10 to 100-fold weaker than those elicited by amylin or CGRPα [45,46]. In this review, we will summarize the pharmacological characteristics of the AM receptors associated with RAMP accessory proteins and their possible pathophysiological roles.

2. Factors influencing CLR/RAMP transfection

Before transfecting CLR or a RAMP accessory protein into mammalian cells, the cells' endogenous gene expression and function should be adequately characterized because native receptor components can modify the binding to and function of overexpressed CLR/RAMP heterodimers. In addition, we have found that the outcomes of transfection studies may vary due to such factors as the source, background and passage of the cells used [15], the growth hormones present in the medium, transfection efficacy, inconsistencies in the animal species and materials used [31], and the position of ligand iodination [46].

Because of their high transfection efficacy, HEK-293 and monkey COS-7 cells have been frequently used in a variety of transfection studies. In some HEK-293 cell lines that endogenously express RAMP1, transfection of hCLR led to significant increases in CGRP-evoked cAMP production [20,59]. Another HEK-293 cell line was shown to endogenously express low levels of RAMP2, but in that case transfection of hCLR or hRAMP1 did not augment cAMP production stimulated by CGRPα or AM, indicating that the RAMP2 was totally inactive [42,44,59]. Thus, the functionality of endogenous RAMPs likely affects the efficacy of transfected CLR [1,20,44].

Like HEK-293 cells, monkey COS-7 cells were derived from kidney, and the finding that transfection of either CLR or RAMP1 alone increased CGRP binding by ~30% suggested that these cells endogenously express CLR and one or more RAMPs ([31], unpublished observation). Indeed, we were able to detect both CLR and RAMP2 mRNAs in these cells using RT-PCR with primers for human cDNAs ([59], unpublished observation). Moreover, an RT-PCR analysis carried out by Tilakaratne et al. [85] showed that the yield of RAMP2 was lower than that of RAMP1 or RAMP3; apparently, however, none of the RAMP proteins was functional. Another consideration is that Choksi et al. [15] showed that in SK-N-MC neuroblastoma cells, which endogenously express CLR, RAMP1 and -2 (RAMP1 > RAMP2), basal expression of receptor proteins, CGRP binding and CGRP-evoked cAMP production were all markedly diminished with continuous cell passage. This raises the possibility that some data obtained from general-purpose cultured cells, including HEK-293 and COS-7 cells, may not reflect the native cellular background due to their high passage.

Finally, Hay et al. [31] showed the importance of carrying out pharmacological characterizations using CLR and RAMP cDNAs from the same animal species. In addition, it is desirable to use cell lines, agonists, antagonists and radioligands that are derived from the same species whenever possible. Ideally, moreover, the cell lines used for these transfection studies should not endogenously express RAMPs, CLR or CT receptors.

3. Recombinant AM_1 and AM_2 receptors

The pharmacological characteristics of AM_1 and AM_2 receptor subtypes (CLR/RAMP2 and CLR/RAMP3, respectively) were well summarized by Muff et al. ([35], for review see Ref. [57]). So we will summarize them only briefly here. In HEK-293 and COS-7 cells co-transfected with RAMP2 and human (h)-, rat (r)-, mouse (m)- or bovine (b)CLR, the rank order of inhibition of ^{125}I-AM binding was as follows: AM > AM receptor antagonist > CGRP-(8–37) > CGRPα. Amylin and CT were without effect. The CGRPα/AM EC_{50} ratios for cAMP production in HEK-293 and COS-7 cells transfected with these receptors are ~40- to 150 and >180, respectively. The lower selectivity for AM seen in some HEK-293 cells could be due in part to the presence of endogenous functional hRAMP1 or hCT receptor. In HEK-293 cells co-expressing hRAMP2 and h- or bCLR, hAM-(22–52) antagonized hAM-stimulated cAMP production with IC_{50}'s of 125–400 nM; hCGRPα-(8–37) was >20-fold weaker [3,59]. rAM-(20–50) inhibited rAM- and rCGRPα-evoked cAMP production with similar K_i's, whereas rCGRPα-(8–37) failed to antagonize either response [35]. In COS-7 cells co-transfected with mCLR and mRAMP2, 1 μM rAM-(20–50) raised the EC_{50} for rAM and rCGRPα 11- and >14-fold, respectively, and the corresponding K_i was 131 nM for rAM-evoked cAMP production [35], while 1 μM rCGRPα-(8–37) raised the K_i for rAM-evoked responses to 380 nM [35]. Although the $CGRP_1$ receptor antagonist CGRPα-(8–37) also weakly antagonized AM-evoked cAMP production in mammalian cultured cells transfected with CLR/RAMP2, the antagonist

Table 1
Pharmacological features of recombinant CLR/RAMP heterodimers

Receptor subtype	Agonist specificity		Antagonist potency for AM-evoked responses	
	AM	CGRP α	hAM-(22–52)or rAM-(20–50)	CGRP-(8–37)
CLR/RAMP1 ($CGRP_1$ receptor)		<	≪	
CLR/RAMP2 (AM_1 receptor)		≫	≫	
CLR/RAMP3 (AM_2 receptor)				
Humans, pigs, cows		≫	>	
Rats, mice		>	≪	

had no effect on responses evoked by 200 nM rAM in yeast cells transfected with hCLR/hRAMP2, which endogenously express no CLR/RAMP system [51].

Thus, the CLR/RAMP2 heterodimer defines the AM_1 receptor, which recognizes AM over CGRP with a selectively of two to three orders of magnitude, and which is more effectively antagonized by either hAM-(22–52) or rAM-(20–50) than by CGRP-(8–37) (Table 1).

The available data on AM binding to the AM_2 receptor and the resultant signaling are somewhat limited. In HEK-293 or COS-7 cells expressing h-, m-, b- or pAM_2 receptor, inhibition of ^{125}I-AM binding (IC_{50} = 1.4–4.6 nM) by AM was comparable to that seen with AM_1 receptors [35,57]. Again, the IC_{50} values observed with AM_1 and AM_2 receptors were comparable to those for AM-evoked cAMP production via either receptor. hAM-(22–52) and hCGRPα-(8–37) inhibited ^{125}I-AM binding to the hAM_2 receptor with equal potency (IC_{50} = 16–38 nM and 12–35 nM, respectively), and hCGRPα was only ~4-fold less potent. This differs from the profile obtained with rodent AM_2 receptor expressed in COS-7 cells, where rAM = rCGRPα-(8–37) > rAM-(20–50) = rCGRPα [35]. When co-transfected with hRAMP3 into HEK-293 cells, p- and bCLR were even more selective for AM over CGRPα, and hCGRPα-(8–37) was insensitive. In HEK-293 cells co-transfected with hRAMP3 and h-, p- or bCLR, hCGRPα was 30- to 100-fold less potent than hAM with respect to evoked cAMP production, which is similar to results obtained with AM_1 receptors. By contrast, in COS-7 cells co-expressing mRAMP3 and r- or mCLR, CGRPα was only 12- to 16-fold less potent than AM, and amylin and CT were inactive. In HEK-293 cells expressing bCLR/hRAMP3, hAM-(22–52) antagonized hAM-stimulated cAMP production with an IC_{50} of 85 nM; that hCGRPα-(8–37) was 20-fold less potent [3] is consistent with the inhibitory effects of both antagonists on bAM_1 receptor. In COS-7 cells expressing mCLR/mRAMP3, 1 μM rCGRPα-(8–37) increased the EC_{50}'s of rAM and rCGRPα 10- and 25-fold, respectively, giving K_i's of 127 nM and 47 nM [35]. On the other hand, 1 μM rAM-(20–50) raised the EC_{50} of rAM and rCGRPα 4- and 12-fold, respectively, giving K_i's of 480 nM and 120 nM [35]. In yeast cells expressing hCLR/hRAMP3, AM-(22–52) but not CGRP-(8–37) antagonized rAM-mediated responses [51].

Thus, unlike the pharmacological features of the AM_1 receptor, those of the AM_2 receptor, differ among species (Table 1). In h-, p- and bAM_2 receptors, AM antagonists are more potent than CGRP-(8–37), which is not so different from that seen with AM_1 receptors. By contrast, r- and mAM_2 receptors recognize AM over CGRP with a selectivity of one order of magnitude, and are more effectively antagonized by CGRP-(8–37) than by AM antagonists.

4. A recombinant $CGRP_1$ receptor able to respond to AM

In HEK-293 cells co-transfected with hRAMP1 and h-, p- or bCLR, ^{125}I-CGRP binding was inhibited by CGRPα with an IC_{50} of 0.1–2.2 nM [2,3,24,50]. Likewise, CGRPβ showed a high-affinity (IC_{50} = 0.01–0.3 nM) for these receptors. Although the $CGRP_1$ receptor antagonist CGRP-(8–37) lacks the ring structure required for CGRP signaling, it was capable of binding to CLR/RAMP1 heterodimers with an affinity comparable to that of CGRPα. By contrast, AM was 25- to 500-fold less potent than CGRPα, and the AM receptor antagonist AM-(22–52) was largely inactive. When COS-7 or Drosophia Schneider 2 cells were transfected into r- or mCLR plus m- or hRAMP1, CGRPα, CGRPβ and CGRP-(8–37) all strongly inhibited ^{125}I-CGRP binding with very similar potencies (IC_{50} = 1.2–7 nM) [4,9,34,35]. In those cases, AM was ~20- to 40-fold less potent than CGRPα, and AM-(22–52) was totally inactive. Taken together, these results indicate that, for the CLR/RAMP1 dimer, binding affinities determined as a function of competitive inhibition of ^{125}I-CGRP binding is as follows: CGRPα = CGRPβ = CGRP-(8–37) > AM ≫ hAM-(22–52) or rAM-(20–50).

When hRAMP1 and h-, p- or bCLR were co-transfected into HEK-293 cells, CGRPα and CGRPβ augmented cAMP production with similar potencies (EC_{50} = 0.3–7.8 nM) [2,3,24,41,42], and AM was only ~2- to 8-fold less potent than CGRPα [2,3,24,42]. In COS-7 cells transfected with r- or mCLR plus m- or hRAMP1, AM was ~100- to 300-fold less potent than CGRPα, though the EC_{50}'s of CGRPα and CGRPβ (0.1–0.9 nM) were about the same as that seen in HEK-293 CLR/RAMP1 transfectants [9,34,35]. There has been only a single study in which functional expression of hCLR/hRAMP heterodimeric receptors was evaluated using a null system, the yeast *Saccharomyces cerevisiae* [51]. In control cells, neither CGRP nor AM (1 μM) elicited β-galactosidase activity, but after co-transfection of hCLR and hRAMP1, both hCGRPα and hAM elicited β-galactosidase activity, and hAM was ~20–30-fold less potent than hCGRPα. Taken together, these results indicate that the rank order of agonists eliciting functional responses in CLR/RAMP1 transfectants is as follows: CGRPα

= CGRPβ > AM, which is consistent with the profile of inhibition of ^{125}I-CGRP binding.

There have been several reports on the effect of CGRP-(8–37) on AM-mediated responses [15,35,59]. In HEK-293 cells co-transfected with hCLR and hRAMP1, hCGRPα-(8–37), but not hAM-(22–52), dose-dependently inhibited cAMP production evoked by 10 nM hAM or 1 nM hCGRPα with estimated IC_{50}'s of ~30 nM and ~200 nM, respectively [59]. Comparable results were obtained in HEK-293 cells transfected with bCLR/hRAMP1; IC_{50}'s for hCGRPα-(8–37)-mediated inhibition of hAM and hCGRPα responses were 35 nM and 110 nM, respectively [3]. In hCLR/hRAMP1-transfected yeast cells, hCGRPα-(8–37) blocked the response to 2 μM rAM and 100 nM hCGRPβ with IC_{50}'s of ~1 and 4 μM, respectively, whereas hAM-(22–52) had no effect [51]. In COS-7 cells co-transfected with mCLR and mRAMP1, EC_{50}'s for rAM- and rCGRPα-evoked cAMP production were increased 209- and 100-fold in the presence of 1 μM rCGRPα-(8–37), but 1 μM rAM-(20–50) was without effect; the calculated K_i's for rCGRPα-(8–37) were 12 nM and 5 nM for rCGRPα and rAM-evoked cAMP production, respectively [35]. Thus, a $CGRP_1$ receptor antagonist can inhibit AM responses ~3- to 7-fold more effectively than CGRP responses in CLR/RAMP1 transfectants.

The findings summarized above indicate that, irrespective of its animal species, AM can interact with $CGRP_1$ receptors and that AM responses mediated via this receptor are antagonized by CGRPα-(8–37) but not by a selective AM receptor antagonist (Table 1). Still, although higher concentrations of AM can activate this receptor, there is no evidence that the $CGRP_1$ receptor functions as a CGRP-(8–37)-sensitive AM receptor in vivo.

5. Possible CT-(8–32)-sensitive AM receptors

Recent studies have shown that CT receptors can also interact with RAMPs, thereby forming heterodimeric receptors that are highly sensitive to amylin and CGRP [16,45–47,56,85]. Of the human CT receptor isoforms, $CT_{(a)}$ receptor is the more strongly expressed and the more widely distributed.

Co-expression of human $CT_{(a)}$ receptor with any hRAMP in HEK-293 cells led to significant increases in hAM-evoked cAMP production (EC_{50} > 1–5 nM) [45], though hAM remained at least 10 to 100-fold less potent than hCGRPα [45,46]. Salmon (s)CT-(8–32), but not hCGRPα-(8–37) or hAM-(22–52), antagonized hAM-evoked cAMP production via human $CT_{(a)}$ receptor/RAMP1, -2 or -3 with IC_{50}'s of 40–100 nM, but had little effect on AM responses mediated via the CT receptor alone. By contrast, 1 μM sCT-(8–32) had no effect on hCGRPα- or hAM-evoked cAMP production in cells expressing CLR/RAMP1, -2 or -3 [45]. Thus, AM can also activate sCT-(8–37)-sensitive receptors comprised of human $CT_{(a)}$ receptor with any RAMP in HEK-293 cells. Whether this receptor behaves as a functional AM receptor in vivo remains unclear, however.

6. Endogenous AM receptors in tissues and cell lines

CGRP and AM have been reported to share a number of pharmacological features; for example, many effects of AM are apparently blocked by CGRPα-(8–37). In basilar arteries isolated from Wistar-Kyoto and stroke-prone spontaneously hypertensive rats, relaxations elicited by AM and CGRP were markedly inhibited by 1 μM hCGRP-(8–37) [65]; likewise, treatment with 100 nM CGRP-(8–37) inhibited relaxations induced by either AM or CGRP in isolated canine retinal arteries [69], and 1 μM CGRP-(8–37) inhibited AM- and CGRPα-evoked relaxations in porcine coronary arteries [97]. The effects of AM, which was 4- to 100-fold less potent than CGRP, were all endothelium-independent.

There have been several reports that CGRPα-(8–37), but not AM-(22–52), blocks AM-evoked cellular responses. For instance, in neonatal cardiac myocytes and non-myocytes, AM elicited concentration-dependent increases in cAMP, and CGRP was about 20–60 times more potent than AM [64]. CGRP-(8–37) dose-dependently blocked cAMP production induced by AM or CGRP in both cell types, whereas AM-(22–52) at concentrations up to 1 μM did not [64]. In non-myocytes, the estimated IC_{50}'s for CGRP-(8–37)-mediated inhibition of cAMP production elicited by 10 nM AM and 1 nM CGRP were ~1 and ~10 nM, respectively [64]. Tomoda et al. [86] demonstrated that in rat non-myocytes, which abundantly express RAMP1 but express only low levels of RAMP2 and CLR, AM was ~100-fold less potent than CGRP with respect to stimulation of cAMP production, and that a CGRP receptor antagonist dose-dependently reduced IL-6 secretion stimulated by AM plus IL-1β, but an AM receptor antagonist did not. These findings suggest that endogenous co-expression of RAMP1 with CLR may yield a receptor common to both CGRP and AM.

On the other hand, AM was ~100-fold more potent than CGRPα with respect to evoked cAMP production in human and rabbit aortic endothelial cells [37,55], human aortic smooth muscle cells [37] or rat cerebral microvessels [40], all of which endogenously express AM_1 receptors comprised almost exclusively of CLR and RAMP2. In rabbit aortic endothelial cells, hAM-(22–52) inhibited AM-evoked cAMP production with a K_i of 3 nM [55]. Moreover, Kobayashi et al. [40] demonstrated that in rat cerebral microvessels AM-(22–52) (1 μM), but not CGRP-(8–37) (1 μM), blocked AM (10 nM)-evoked cAMP production with an IC_{50} of 1.8 μM. In this case, ^{125}I-AM binding was inhibited by AM and AM-(22–52) with IC_{50}'s of 0.3 and 7.6 nM, respectively; CGRP and CGRP-(8–37) were much less potent (IC_{50} > 100 nM). These results are compatible with those obtained with NG108-15 neuroblastoma × glioma hybrid cells, which are believed to endogenously express AM_1 re-

ceptor [50] and various CLR/RAMP2-transfected cells [57]. Such AM-specific effects were also observed in mesangial cells [72,73], mesenteric arteries [26], cerebral parenchymal microvessels [83], iris sphincter [98], oculus [17] and testicular peritubular myoid cells [78]. Among them, rat mesangial cells were recently found to express CLR as well as all three RAMPs (RAMP2 > RAMP1 = RAMP3) [67].

Because RAMPs are so ubiquitously expressed [50,58, 71,79], their combined presence make interpreting some AM-mediated responses difficult. For instance, in porcine coronary arteries, where both RAMP1 and RAMP2 are expressed (RAMP1 > RAMP2), 1 μM CGRPα-(8–37) inhibited AM-induced vasodilation, whereas 1 μM AM-(22–52) had little effect [29]. And although human coronary arteries express equal levels of RAMP2 and RAMP1, the vasorelaxant effect of AM was ~100-fold less potent than that of CGRPα and was antagonized by CGRPα-(8–37) but not by AM-(22–52) [30]. This may be explained by the finding that the interaction between CLR and RAMP1 predominates over that between CLR and RAMP2 [9,34]. On the other hand, based on data obtained in rabbit aortic endothelial cells, it has been suggested that RAMP3 has great affinity for CLR [55]. In isolated rat uterus, where all three RAMPs are highly expressed at equal levels, 1 μM CGRPα-(8–37) or AM-(22–52) totally blocked the inhibitory effects of AM on bradykinin-induced periodic contractions [95]. In that case, CGRPα-(8–37)- and AM-(22–52)-sensitive AM_2 receptors may predominate among the three AM receptors present. It remains unclear, however, whether these phenomena reflect competition among RAMPs for interaction with CLR, interaction among RAMPs themselves (RAMP homo- or heterodimerization) or both.

To our knowledge, there has been only one previous observation of CT-(8–32)-sensitive AM responses that were insensitive to hCGRP-(8–37) and hAM-(22–52) [18]. In that case, CT receptor was endogenously expressed in human T47D breast cancer cells, and the observed EC_{50} values for AM and CT were 132 and 0.5 nM, respectively. Whether T47D cells also express RAMP is unknown, however.

Champion et al. [12] demonstrated that in the hindlimb vascular bed of the cat, neither CGRP-(8–37) nor AM-(22–52) antagonized the vasodilatory effect of AM, but they suppressed CGRP-mediated responses equally. In another study, moreover, AM-induced relaxation of mouse aortic rings was only slightly (3-fold) shifted by AM-(22–52), and not at all by CGRP-(8–37), and both antagonists failed to inhibit CGRP-mediated vasorelaxation [7]. However, those studies provided no information as to whether CT induces vasorelaxation and CT-(8–32) inhibits AM-mediated responses. In general, the vasodilation elicited by CT is substantially weaker than that elicited by CGRP or AM [8]. It is thus unlikely that the vasorelaxant effects of AM are mediated via three AM receptors comprised of RAMP1, -2 or -3 plus CLR or CT receptors. Instead, another AM receptor may be present there.

7. Assessment of endogenous CLR expression

An earlier Northern blot analysis revealed that, in humans, the mRNA encoding CLR was predominantly expressed in the lung, less so in heart, and not at all in brain and other peripheral tissues [1]. Later, RT-PCR analysis showed CLR mRNA to be present in a variety of human tissues and cells, including cerebral arteries [77], adrenal cortex [84], uterus [63], hairy skin [27], SK-N-MC neuroblastoma cells [15,50], KG1C oligodendroglial cells [90] and coronary artery endothelial and smooth muscle cells [60], and that at least one RAMP isoform was also expressed. L6 myoblastic cells also showed no CLR signals in Northern blot analyses, despite the presence of sufficient CLR protein to measure high-affinity binding of AM and CGRP [50]. Apparently, the fact that CLR mRNA may be undetectable by Northern analysis does not exclude its presence. Furthermore, in situ hybridization did not reveal rCLR signals in the cerebellum or spleen, despite the presence of densely distributed CGRP binding sites [23]; the authors suggested the existence of another CGRP receptor. In yet another study, however, CLR mRNA was readily detectable in rat cerebellum and spleen by Northern analysis [11]. In that case, the CLR mRNA correlated significantly with RAMP2 mRNA ($R = 0.94$, $P = 0.016$) in eight rat tissues, including cerebellum and spleen, and ^{125}I-AM binding tended to correlate with CLR mRNA ($R = 0.93$, $P = 0.11$) and RAMP2 mRNA ($R = 0.95$, $P = 0.14$), but not RAMP3 mRNA ($R = 0.32$, $P = 0.40$) [11].

There also have been several recent reports showing widespread distribution of CLR-like immunoreactivity (CLR-LI) in human organs, including lung, heart ventricle and kidney [27,28,63]. CLR-LI was also detected in the endothelium of all blood vessels and tended to be more strongly detected in venous than arterial vessels, with the exception of the pulmonary artery and vein [28]. In human middle cerebral arteries, levels of CLR mRNA were independent of artery diameter, but levels of RAMP1 and RAMP2 mRNAs tended to increase with increases in artery size and decreases in responsiveness to CGRP and AM [77]. In that case, CLR protein levels should also be evaluated in arteries of varying size. Nonetheless, it appears that CLR is widely expressed along with RAMP, leading to formation of functional AM receptors in vivo.

8. Expression of mRNAs encoding CLR, RAMPs and AM under various pathologic conditions

As shown in Table 2, changes in the gene expression of the three RAMP isoforms do indeed vary under different pathological conditions. In patients with pregnancy-induced hypertension, for example, the level of RAMP2 mRNA was increased in the fetal membrane, but was reduced, along with CRLR mRNA, in umbilical artery and uterus—i.e., the expression of RAMP2 mRNA was negatively correlated with

Table 2
Alterations of mRNAs encoding CLR, RAMPs and AM under various pathologic conditions

Pathology	Materials		CLR	RAMP1	RAMP2	RAMP3	AM	References
Hypertension	Women (+pregnancy)	Umbilical artery	↓	ND	↓	ND	↑	[48]
		Uterine muscle	↓	ND	↓	ND	ND	[48]
	Rat (+phenylephrine)	PVN/NTS	ND	ND	↓	ND	ND	[82]
	SHR-SP	Left ventricle	↑	ND	↑	ND	↑	[91]
Heart failure	Rat (+myocarial infarction)	Left ventricle	ND	ND	↑	ND	↑	[68]
		Atrium	↑	ND	↑	ND	↑	[87]
		Ventricle	↑	ND	↑	ND	↑	[87]
		Kidney	→	ND	→	ND	→	[87]
	Rat (+aortic banding)	Left ventricle	↑	ND	↑	↑	↑	[70]
	Rat (+aortocaval shunt)	Left ventricle	↑	ND	↑	→	↑	[70]
		Kidney	→	ND	→	→	↑	[96]
Cardiomyopathy	Rat (+isoproterenol)	Heart	ND	ND	↑	ND	↑	[75]
Renal failure	Rat (+5/6 nephrectomy)	Kidney	↓	ND	→	↓	→	[88]
Nephropathy	Rat(+ureteral obstruction)	Obstructed kidney	↑	↑	↑	→	→	[58]
Diabetes	Rat (+streptozotocin)	Kidney	ND	ND	↑	ND	↑	[32]
Salt loading	Rat (+8% NaCl)	Adrenal gland	↑	↑	↑	→	↑	[10]
		Kidney	↑	→	→	↑	↑	[10]
Sepsis	Rat (+lipopolysaccharide)	Lung/Spleen/Thymus	↓	↓	↓	↑	ND	[71]
Others (in vitrto)	Human neuroblastoma cell (+hypoxia)		ND	→	↓	(–)	↑	[39]
	Human CAEC (+C-reactive protein)		→	→	→	(–)	↓	[60]
	Human CASMC (+C-reactive protein)		→	→	→	(–)	↓	[60]
	Human CASMC (+tumor necrosis factor-α		↓	↓	↓	(–)	↑	[61]
	HumanCASMC(+dexamethasone)		↑	↑	→	ND	↑	[25]
	Rat cardiomyocyte (+endothelin I)		↑	→	↓	↑	↑	[52]
	Rat cardiomyocyte (+angiotensin II)		→	↑	→	↑	↑	[53]
	Rat mesangial cell (+PDGF)		ND	ND	→	↑	↑	[67]

ND: not determined; CLR: calcitonin-like receptor; RAMP: receptor activity modifying protein, adrenomedullin; SHR-SP: spontaneously hypertensive stroke-prone rat; PVN: paraventricular nucleus; NTS: nucleus tractus solitarius; CAEC: coronary artery endothelial cell; CASMC: coronary artery smooth muscle cell; PDGF: platelet-derived growth factor.

the systolic and diastolic pressures in both tissues [48]. The reduced expression of AM_1 receptor mRNA in umbilical artery may have contributed to the increased resistance in the umbilical circulation seen in these cases, though it remains unclear whether the increased AM downregulates its own receptors.

There is also a recent study showing a similar correlation between systemic blood pressure and levels of RAMP2 mRNA [82]. Phenylephrine-induced blood pressure elevation decreased expression of RAMP2 mRNA in the paraventricular nucleus (PVN) and the nucleus tractus solitarius (NTS) [82], whereas nitroprusside-induced hypotension increased RAMP2 mRNA expression in the NTS and decreased AM expression in the PVN [82]. Alterations in the levels of RAMP2 in these autonomic nuclei may affect the ability of central AM to regulate sympathetic activity. On the other hand, in the spontaneously hypertensive stroke-prone rat (SHR-SP), CLR, RAMP2 and AM mRNAs were all upregulated in the hypertrophied left ventricle (LV), and decreasing blood pressure using captopril or trichlormethiazide resulted in reduced expression of all the upregulated genes [91]. In addition, the fact that AM inhibits angiotensin II-induced hypertrophy of cultured rat cardiomyocytes suggests the increased expression of AM_1 receptor may ameliorate the LV hypertrophy [89].

During heart failure induced by myocardial infarction in rat, expression of AM and RAMP2 or RAMP3 mRNA was increased in both the nonischemic and ischemic regions of the LV [68,87], and treatment with the endothelin receptor antagonist bosentan prevented the increase in RAMP2 mRNA [68]. The induction of the AM signaling system may be beneficial, as early infusion of AM (for a week) powerfully inhibited ventricular remodeling after myocardial infarction in rats [62]. In rat cardiac overload models, both pressure overload (by aortic banding) and volume overload (by aortocaval shunt) led to increased expression of AM and AM_1 receptor mRNAs, but unlike pressure overload, volume overload did not enhance RAMP3 mRNA expression [70]. Thus, LV AM_1 and AM_2 receptors might play different roles during cardiac overload. In addition, AM protected rat myocardium from isoproterenol-induced hypertrophy and necrosis [36], and RAMP2 mRNA was upregulated in the myocardium of isoproterenol-treated rats [75].

Although AM exerts both diuretic and natriuretic effects [21], renal expression of AM_1 or AM_2 receptor mRNA was unchanged during heart failure [87,96]. Expression of CLR and RAMP3 mRNAs was downregulated in the remnant kidney after 5/6 nephrectomy, which is a rat model of acute renal failure, while RAMP2 mRNA was unchanged [88]. Despite no change in AM mRNA expression, CLR,

RAMP1 and RAMP2 mRNAs, but not RAMP3 mRNA, were upregulated in kidneys with ureteral obstruction [58]. The increase in AM_1 receptor may provide protection against proliferative or fibrotic changes in the obstructed kidney. Streptozocin-induced diabetic rats showed upregulated expression of AM and RAMP2 in hypertrophied glomeruli and in afferent arterioles and increased urinary excretion of nitric oxide (NO_2^- and NO_3^-) due partly to AM stimulation [32]. In this model, adenovirus-mediated AM gene transfer improved cardiac function and prevented renal damage [32]. Chronic salt loading led to increased expression of AM and mRNA CLR in the adrenal glands and kidneys of the rat, without significant elevation of blood pressure [10]. In that case, RAMP1 and RAMP2 mRNAs were increased in adrenal glands, while RAMP3 mRNA was increased in kidneys [10]. In addition to its diuretic and natriuretic effects, AM can also inhibit angiotensin II- or potassium-induced aldosterone secretion from adrenal glands [93,94]. After salt loading, therefore, some secondary mediators may differentially regulate the expression of AM receptor components to restore water and electrolyte balance.

During sepsis, levels of both circulating and local AM were markedly increased in humans and rats [49,66]. By contrast, expression of CLR and the three RAMP mRNAs were markedly downregulated in many tissues [71]. This is most likely due to the marked increases in the level of various agonists, cytokines or both. It is noteworthy that in lung, spleen and thymus the upregulation of RAMP3 mRNA was accelerated during the late stage of sepsis, suggesting that RAMP3 may be involved in immune function.

There have been several in vitro studies showing the effects of various agents on AM receptor components. In cultured human neuroblastoma cells, hypoxia induced downregulation of RAMP2 mRNA with no effect on RAMP1 mRNA [39], though the mechanisms remain unclear. C-reactive protein (CRP) dose-dependently decreased AM release from human coronary artery endothelial and smooth muscle cells (HCAECs and HCASMCs), but did not affect expression of CLR, RAMP1 and RAMP2 mRNA or AM-evoked cAMP production in either cell type [60]. In HCASMCs, low concentrations of TNF-α downregulated CLR, RAMP1 and RAMP2 mRNAs, thereby decreasing AM-evoked cAMP production, while higher concentrations of TNF-α elicited secretion of small amounts of AM from the cells [61]. It is, therefore, likely that the increased AM contributes little to the downregulation of AM receptors.

Aside from vasorelaxation, the most powerful effect of AM is the inhibition of the vascular oxidative stress known to contribute significantly to the development of atherosclerosis [80,81]. This suggests that downregulation of AM by CRP, together with downregulation of AM receptors by TNF-α, contributes to the progression of coronary atherosclerosis. By contrast, dexamethasone (≥10 nM) increased CLR and RAMP1 mRNAs, but not RAMP2 mRNA, in HCASMCs [25], though no effect on AM_1 receptor function was reported. Endothelin I and angiotensin II differentially regulated the expression of CLR and all three RAMP mRNAs in rat cardiomyocytes, resulting in increases in AM-evoked cAMP production [52,53]. In addition, AM has been shown to decrease platelet-derived growth factor (PDGF)-induced mesangial cell proliferation [14], and Nowak et al. clearly showed that in mesangial cells PDGF stimulated expression of RAMP3 mRNA and protein, as well as AM-stimulated adenylate cyclase activity [67]. The increase in RAMP3 mRNA was dependent on mRNA stability, but not on transcription, and was mitogen-activated protein kinase (MAPK) kinase (MEK)- and p38 MAPK-dependent [67]. They also demonstrated that transfection of RAMP2 and RAMP3 enhances AM-mediated inhibition of mesangial cell hypertrophy. By contrast, AM promoted migration and invasion of human umbilical vein endothelial cells, which express endogenous AM_1 and AM_2 receptors [22]. Interestingly, both phenomena were strongly inhibited by pretreatment with anti-CLR/anti-RAMP2 or anti-CLR/anti-RAMP3 antibodies, all of which were raised against amino acids in the respective extracellular N-terminal domains [22]. Such blocking antibodies should be useful for clarification of the pathophysiological roles of endogenous AM receptor subtypes.

9. Concluding remarks and future perspectives

Co-expression of CLR and RAMP2 or RAMP3 can produce functional AM receptors (Table 1), but only CLR/RAMP2 comprises an AM-specific receptor that is particularly sensitive to AM-(22–52)—i.e., this heterodimer defines the AM_1 receptor subtype. CLR/RAMP3 defines the AM_2 receptor, which cross-reacts with CGRP at lower concentrations and is more sensitive to CGRP-(8–37) than is the AM_1 receptor. With the r- or m AM_2 receptor, in particular, AM-evoked responses are more effectively blocked by CGRP-(8–37) than by AM-(22–52), though this is not observed with the h-, p- or bAM_2 receptor. Overexpressed $CGRP_1$ receptor (CLR/RAMP1) can also respond to AM at higher concentrations, and the responses are blocked by CGRP-(8–37) but not by AM-(22–52) (Table 1), though there has been no evidence that $CGRP_1$ receptor can also act as a CGRP-(8–37)-sensitive AM receptor in vivo. Thus, in tissues where only $CGRP_1$ and AM_1 receptors are present, combined use of the both antagonists will be useful for defining these receptor subtypes. But in cases where the AM_2 receptor is also present, the antagonist selectivity will be impaired.

Actually, RAMPs commonly coexist in native tissues and cells [74,79]. We recently identified three dominant-negative RAMP mutants able to inhibit endogenous CLR/RAMP function [42–44]. However, the coexistence of multiple RAMP isoforms means that these mutants cannot selectively inhibit the corresponding RAMPs, most likely due to competitive interactions among RAMPs. The identification of specific, selective negative-regulators for CLR/RAMPs will be needed to clarify the role of individual AM receptors

under various pathophysiological conditions and to examine whether other AM receptors may be present in some vascular beds [7,12].

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References

[1] Aiyar N, Rand K, Elshourbagy NA, Zeng Z, Adamou JE, Bergsma DJ, et al. A cDNA encoding the calcitonin gene-related peptide type 1 receptor. J Biol Chem 1996;271:11325–9.
[2] Aiyar N, Disa J, Pullen M, Nambi P. Receptor activity modifying proteins interaction with human and porcine calcitonin receptor-like receptor (CRLR) in HEK-293 cells. Mol Cell Biochem 2001;224:123–33.
[3] Aiyar N, Disa J, Ao Z, Xu D, Surya A, Pillarisetti K, et al. Molecular cloning and pharmacological characterization of bovine calcitonin receptor-like receptor from bovine aortic endothelial cells. Biochem Pharmacol 2002;63:1949–59.
[4] Aldecoa A, Gujer R, Fischer JA, Born W. Mammalian calcitonin receptor-like receptor/receptor activity modifying protein complexes define calcitonin gene-related peptide and adrenomedullin receptors in *Drosophila* Schneider 2 cells. FEBS Lett 2000;471:156–60.
[5] Amara SG, Jonas V, Rosenfeld MG, Ong ES, Evans RM. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 1982;298:240–4.
[6] Amara SG, Arriza JL, Leff SE, Swanson LW, Evans RM, Rosenfeld MG. Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 1985;229:1094–7.
[7] Ashton D, Hieble P, Gout B, Aiyar N. Vasodilatory effect of adrenomedullin in mouse aorta. Pharmacology 2000;61:101–5.
[8] Born W, Fischer JA. Calcitonin gene products: molecular biology, chemistry, and actions. Handbook Exp Pharmacol 1993;107:569–616.
[9] Buhlmann N, Leuthauser K, Muff R, Fischer JA, Born W. A receptor activity modifying protein (RAMP)2-dependent adrenomedullin receptor is a calcitonin gene-related peptide receptor when coexpressed with human RAMP1. Endocrinology 1999;140:2883–90.
[10] Cao YN, Kitamura K, Kato J, Kuwasako K, Ito K, Onitsuka H, et al. Chronic salt loading upregulates expression of adrenomedullin and its receptors in adrenal glands and kidneys of the rat. Hypertension 2003;42:369–72.
[11] Chakravarty P, Suthar TP, Coppock HA, Nicholl CG, Bloom SR, Legon S, et al. CGRP and adrenomedullin binding correlates with transcript levels for calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) in rat tissues. Br J Pharmacol 2000;130:189–95.
[12] Champion HC, Santiago JA, Murphy WA, Coy DH, Kadowitz PJ. Adrenomedullin-(22–52) antagonizes vasodilator responses to CGRP but not adrenomedullin in the cat. Am J Physiol 1997;272:R234–42.
[13] Chiba T, Yamaguchi A, Yamatani T, Nakamura A, Morishita T, Inui T, et al. Calcitonin gene-related peptide receptor antagonist human CGRP-(8–37). Am J Physiol 1989;256:E331–5.
[14] Chini EN, Choi E, Grande JP, Burnett JC, Dousa TP. Adrenomedullin suppresses mitogenesis in rat mesangial cells via cAMP pathway. Biochem Biophys Res Commun 1995;215:868–73.
[15] Choksi T, Hay DL, Legon S, Poyner DR, Hagner S, Bloom SR, et al. Comparison of the expression of calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) with CGRP and adrenomedullin binding in cell lines. Br J Pharmacol 2002;136:784–92.
[16] Christopoulos G, Perry KJ, Morfis M, Tilakaratne N, Gao Y, Fraser NJ, et al. Multiple amylin receptors arise from receptor activity-modifying protein interaction with the calcitonin gene product. Mol Pharmacol 1999;56:235–42.
[17] Clementi G, Floriddia ML, Prato A, Marino A, Drago F. Adrenomedullin and ocular inflammation in the rabbit. Eur J Pharmacol 2000;400:321–6.
[18] Disa J, Dang K, Tan KB, Aiyar N. Interaction of adrenomedullin with calcitonin receptor in cultured human breast cancer cells T47D. Peptides 1998;19:247–51.
[19] Eguchi S, Hirata Y, Iwasaki H, Sato K, Watanabe TX, Inui T, et al. Structure-activity relationship of adrenomedullin, a novel vasodilatory peptide, in cultured rat vascular smooth muscle cells. Endocrinology 1994;135:2454–8.
[20] Elshourbagy NA, Adamou JE, Swift AM, Disa J, Mao J, Ganguly S, et al. Molecular cloning and characterization of the porcine calcitonin gene-related peptide receptor. Endocrinology 1998;139:1678–83.
[21] Eto T. A review of the biological properties and clinical implications of adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP), hypotensive and vasodilating peptides. Peptides 2001;22:1693–711.
[22] Fernandez-Sauze S, Delfino C, Mabrouk K, Dussert C, Chinot O, Martin PM, et al. Effects of adrenomedullin on endothelial cells in the multistep process of angiogenesis: involvement of CRLR/RAMP2 and CRLR/RAMP3 receptors. Int J Cancer 2004;108:797–804.
[23] Fluhmann B, Lauber M, Lichtensteiger W, Fischer JA, Born W. Tissue-specific mRNA expression of calcitonin receptor-like receptor during fetal and postnatal development. Brain Res 1997;774(1/2):184–92.
[24] Fraser NJ, Wise A, Brown J, McLatchie LM, Main MJ, Foord SM. The amino terminus of receptor activity modifying proteins is a critical determinant of glycosylation state and ligand binding of calcitonin receptor-like receptor. Mol Pharmacol 1999;55:1054–9.
[25] Frayon S, Cueille C, Gnidehou S, de Vernejoul MC, Garel JM. Dexamethasone increases RAMP1 and CRLR mRNA expressions in human vascular smooth muscle cells. Biochem Biophys Res Commun 2000;270:1063–7.
[26] Fujioka H, Okamura T, Toda N. Inhibition by adrenomedullin of amine release from adrenergic nerves in dog mesenteric arteries. Eur J Pharmacol 1999;385:155–61.
[27] Hagner S, Haberberger RV, Overkamp D, Hoffmann R, Voigt KH, McGregor GP. Expression and distribution of calcitonin receptor-like receptor in human hairy skin. Peptides 2002;23:109–16.
[28] Hagner S, Stahl U, Knoblauch B, McGregor GP, Lang RE. Calcitonin receptor-like receptor: identification and distribution in human peripheral tissues. Cell Tissue Res 2002;310:41–50.
[29] Hasbak P, Sams A, Schifter S, Longmore J, Edvinsson L. CGRP receptors mediating CGRP-, adrenomedullin- and amylin-induced relaxation in porcine coronary arteries. Characterization with 'Compound 1' (WO98/11128), a non-peptide antagonist. Br J Pharamacol 2001;133:1405–13.
[30] Hasbak P, Opgaard OS, Eskesen K, Schifter S, Arendrup H, Longmore J, et al. Investigation of CGRP receptors and peptide pharmacology in human coronary arteries. Characterization with a nonpeptide antagonist. J Pharmacol Exp Ther 2003;304:326–33.
[31] Hay DL, Howitt SG, Conner AC, Doods H, Schindler M, Poyner DR. A comparison of the actions of BIBN4096BS and CGRP8-37 on CGRP and adrenomedullin receptors expressed on SK-N-MC, L6, Col 29 and rat 2 cells. Br J Pharmacol 2002;137:80–6.
[32] Hiragushi K, Wada J, Eguchi J, Matsuoka T, Yasuhara A, Hashimoto I, et al. The role of adrenomedullin and receptors in glomerular hyperfiltration in streptozocin-induced diabetic rats. Kidney Int 2004;65:540–50.

[33] Hilairet S, Belanger C, Bertrand J, Laperriere A, Foord SM, Bouvier M. Agonist-promoted internalization of a ternary complex between calcitonin receptor-like receptor, receptor activity-modifying protein 1 (RAMP1), and β-arrestin. J Biol Chem 2001;276:29575–81.
[34] Husmann K, Sexton PM, Fischer JA, Born W. Mouse receptor-activity-modifying proteins 1, -2 and -3: amino acid sequence, expression and function. Mol Cell Endocrinol 2000;162:35–43.
[35] Husmann K, Born W, Fischer JA, Muff R. Three receptor-activity-modifying proteins define calcitonin gene-related peptide or adrenomedullin selectivity of the mouse calcitonin-lik receptor in COS-7 cells. Biochem Pharmacol 2003;66:2107–15.
[36] Jun Y, Dong Z, Zi CY, Qing T, Tao ZY, Yun SX, et al. The protective role of adrenomedullin on myocardium necrosis induced by isoproterenol. Chin Pharmacol Bull 1996;12:530–3.
[37] Kamitani S, Asakawa M, Shimekake Y, Kuwasako K, Nakahara K, Sakata T. The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. FEBS Lett 1999;448:111–4.
[38] Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993;192:553–60.
[39] Kitamuro T, Takahashi K, Totsune K, Nakayama M, Murakami O, Hida W, et al. Differential expression of adrenomedullin and its receptor component, receptor activity modifying protein (RAMP) 2 during hypoxia in cultured human neuroblastoma cells. Peptides 2001;22:1795–801.
[40] Kobayashi H, Minami S, Yamamoto R, Masumoto K, Yanagita T, Uezono Y, et al. Adrenomedullin receptors in rat cerebral microvessels. Mol Brain Res 2000;81:1–6.
[41] Kuwasako K, Shimekake Y, Masuda M, Nakahara K, Yoshida T, Kitaura M, et al. Visualization of the calcitonin receptor-like receptor and its receptor activity-modifying proteins during internalization and recycling. J Biol Chem 2000;275:29602–9.
[42] Kuwasako K, Kitamura K, Ito K, Uemura T, Yanagita Y, Kato J, et al. The seven amino acids of human RAMP2 (86–92) and RAMP3 (59–65) are critical for agonist binding to human adrenomedullin receptors. J Biol Chem 2001;276:49459–65.
[43] Kuwasako K, Kitamura K, Onitsuka H, Uemura T, Nagoshi Y, Kato J, et al. RAMP domains involved in adrenomedullin binding specificity. FEBS Lett 2002;519:113–6.
[44] Kuwasako K, Kitamura K, Nagoshi Y, Cao YN, Eto T. Identification of the human receptor activity-modifying protein 1 domains responsible for agonist binding specificity. J Biol Chem 2003;278:22623–30.
[45] Kuwasako K, Kitamura K, Nagoshi Y, Eto T. Novel calcitonin-(8–32)-sensitive adrenomedullin receptors derived from co-expression of calcitonin receptor with receptoractivity-modifying proteins. Biochem Biophys Res Commun 2003;301:460–4.
[46] Kuwasako K, Cao Y-N, Nagoshi Y, Tsuruda T, Kitamura K, Eto T. Characterization of the human calcitonin gene-related peptide receptor subtypes associated with receptor activity-modifying proteins. Mol Pharmacol 2004;65:207–13.
[47] Leuthauser K, Gujer R, Aldecoa A, McKinney RA, Muff R, Fischer JA, et al. Receptor-activity-modifying protein 1 forms heterodimers with two G-protein-coupled receptors to define ligand recognition. Biochem J 2000;351:347–51.
[48] Makino Y, Shibata K, Makino I, Kangawa K, Kawarabayashi T. Alteration of the adrenomedullin receptor components gene expression associated with blood pressure in pregnancy-induced hypertension. J Clin Endocrinol Metab 2001;86:5079–82.
[49] Matsui E, Kitamura K, Yoshida M, Kato J, Asada Y, Sumiyoshi A, et al. Biosynthesis and secretion of adrenomedullin and proadrenomedullin N-terminal 20 peptide in a rat model of endotoxin shock. Hypertens Res 2001;24:543–9.
[50] McLatchie LM, Fraser NJ, Main MJ, Wise A, Brown J, Thompson N, et al. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. Nature 1998;393:333–9.
[51] Miret JJ, Rakhilina L, Silverman L, Oehlen B. Functional expression of heteromeric calcitonin gene-related peptide and adrenomedullin receptors in yeast. J Biol Chem 2002;277:6881–7.
[52] Mishima K, Kato J, Kuwasako K, Ito K, Imamura T, Kitamura K, et al. Effects of endothelin on adrenomedullin secretion and expression of adrenomedullin receptors in rat cardiomyocytes. Biochem Biophys Res Commun 2001;287:264–9.
[53] Mishima K, Kato J, Kuwasako K, Imamura T, Kitamura K, Eto T. Angiotensin II modulates gene expression of adrenomedullin receptor components in rat cardiomyocytes. Life Sci 2003;73:1629–35.
[54] Morris HR, Panico M, Etienne J, Tippins J, Girgis SI, MacIntyre I. Isolation and characterization of human calcitonin gene-related peptide. Nature 1984;308:746–8.
[55] Muff R, Leuthauser K, Buhlmann N, Foord SM, Fischer JA, Born W. Receptor activity modifying proteins regulate the activity of a calcitonin gene-related peptide receptor in rabbit aortic endothelial cells. FEBS Lett 1998;441:366–8.
[56] Muff R, Buhlmann N, Fischer JA, Born W. An amylin receptor is revealed following co-transfection of a calcitonin receptor with receptor activity modifying proteins-1 or -3. Endocrinology 1999;140:2924–7.
[57] Muff R, Born W, Fischer JA. Adrenomedullin selectivity of calcitonin-like receptor/receptor activity modifying proteins. Hypertens Res 2003;26:S3–8.
[58] Nagae T, Mukoyama M, Sugawara A, Mori K, Yahata K, Kasahara M, et al. Rat receptor-activity-modifying proteins (RAMPs) for adrenomedullin/CGRP receptor: cloning and upregulation in obstructive nephropaty. Biochem Biophys Res Commun 2000;270:89–93.
[59] Nagoshi Y, Kuwasako K, Ito K, Uemura T, Kato J, Kitamura K, et al. The calcitonin receptor-like receptor/receptor activity-modifying protein 1 heterodimer can function as a calcitonin gene-related peptide-(8–37)-sensitive adrenomedullin receptor. Eur J Pharmacol 2002;450:237–43.
[60] Nagoshi Y, Kuwasako K, Cao Y-N, Kitamura K, Eto T. Effects of C-reactive protein on atherogenic mediators and adrenomedullin in human coronary artery endothelial and smooth muscle cells. Biochem Biophys Res Commun 2004;314:1057–63.
[61] Nagoshi Y, Kuwasako K, Cao YN, Imamura T, Kitamura K, Eto T. Tumor necrosis factor-α downregulate adrenomedullin receptors in human coronary artery smooth muscle cells. Peptides, 2004;25:1115–1121.
[62] Nakamura R, Kato J, Kitamura K, Onitsuka H, Imamura T, Cao YN, et al. Adrenomedullin administration immediately after myocardial infarction ameliorates progression of heart failure in rats. Circulation, in press.
[63] Nikitenko LL, Brown NS, Smith DM, MacKenzie IZ, Bicknell R, Rees MCP. Differential and cell-specific expression of calcitonin receptor-like receptor and receptor activity modifying proteins in the human uterus. Mol Hum Reprod 2001;7:655–64.
[64] Nishikimi T, Horio T, Yoshihara F, Nagaya N, Matsuo H, Kangawa K. Effect of adrenomedullin on cAMP and cGMP levels in rat cardiac myocytes and nonmyocytes. Eur J Pharmacol 1998;353:337–44.
[65] Nishimura Y, Suzuki A. Relaxant effects of vasodilator peptides on isolated basilar arteries from stroke-prone spontaneously hypertensive rats. Clin Exp Pharmacol Physiol 1997;24:157–61.
[66] Nishio K, Akai Y, Murao Y, Doi N, Ueda S, Tabuse H, et al. Increased plasma concentration of adrenomedillin correlate relaxation of vascular tone in patients with septic shock. Crit Care Med 1997;25:953–7.
[67] Nowak W, Parameswaran N, Hall CS, Aiyar N, Sparks HV, Spielman WS. Novel of a adrenomedullin receptor by PDGF: role of receptor activity modifying protrin-3. Am J Physiol Cell Physiol 2002;282:C1322–31.
[68] Oie E, Vinge LE, Yndestad A, Sandberg C, Grogaard HK, Attramadal H. Induction of a myocardial adrenomedullin signaling system during ischemic heart failure in rats. Circulation 2000;101:415–22.

[69] Okamura T, Ayajiki K, Kangawa K, Toda N. Mechanisms of adrenomedullin-induced relaxation in isolated canine retinal arteries. Invest Ophthalmol Vis Sci 1997;38:56–61.
[70] Onitsuka H, Imamura T, Ito K, Kuwasako K, Yamakawa H, Hirano S, et al. Differential gene expression of adrenomedullin receptors in pressure- and volume-overloaded heart—role of angiotensin II. Peptides, 2004;25:1107–14.
[71] Ono Y, Okano I, Kojima M, Okada K, Kangawa K. Decreased gene expression of adrenomedullin receptor in mouse lung during sepsis. Biochem Biophys Res Commun 2000;271:197–202.
[72] Osajima A, Uezono Y, Tamura M, Kitamura K, Mutoh Y, Ueta Y, et al. Adrenomedullin-sensitive receptors are preferentially expressed in cultured rat mesangial cells. Eur J Pharamacol 1996;315:319–25.
[73] Osajima A, Kato H, Uezono Y, Sura T, Okazaki M, Oishi Y, et al. Adrenomedullin inhibits transmural pressure induced mesangial cell proliferation through activation of protein kinase A. Nephron 1999;83:352–7.
[74] Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, et al. International union of pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. Pharmacol Rev 2002;54:233–46.
[75] Qi YF, Shi YR, Bu DF, Pang YZ, Tang CS. Changes of adrenomedullin and receptor activity modifying protein 2 (RAMP2) in myocardium and aorta in rats with isoproterenol-induced myocardial ischemia. Peptides 2003;24:463–8.
[76] Rosenfeld MG, Mermod JJ, Amara SG, Swanson LE, Sawchenko PE, Rivien J, et al. Production of a novel neuropeptide encoded by calcitonin gene via tissue-specific RNA processing. Nature 1983;304:129–35.
[77] Sams A, Knyihar-Csillik E, Engberg J, Szok D, Tajti J, Bodi I, et al. CGRP and adrenomedullin receptor population in human cerebral arteries: in vitro pharmacological and molecular investigation in different artery sizes. Eur J Pharmacol 2000;408:183–93.
[78] Santiemma V, Rossi F, Guerrini L, Markouizou A, Pasimeni G, Palleschi S, et al. Adrenomedullin inhibits the contraction of cultured rat testicular peritubular myoid cells induced by endothelin-1. Biol Reprod 2001;64:619–24.
[79] Sexton PM, Albiston A, Morfis M, Tilakaratne N. Receptor activity modifying proteins. Cell Signal 2002;13:73–83.
[80] Shimosawa T, Shibagaki Y, Ishibashi K, Kitamura K, Kangawa K, Kato S, et al. Adrenomedullin, an endogenous peptide, conteracts cardiovascular damage. Circulation 2002;105:106–11.
[81] Shimosawa T, Ogihara T, Matsui H, Asano T, Ando K, Fujita T. Deficiency of adrenomedullin induces insulin resistance by increasing oxidative stress. Hypertension 2003;41:1080–5.
[82] Stachniak THE, Krukoff TL. Receptor activity modifying protein 2 distribution in the rat central nervous system and regulation by changes in blood pressure. J Neuroendocrinol 2003;15:840–50.
[83] Takao M, Tomita M, Tanahashi N, Kobari M, Fukuuchi Y. Transient vasodilatory effects of adrenomedullin on cerebral parenchymal microvessels in cats. Neurosci Lett 1999;268:147–50.
[84] Thomson LM, Kapas S, Carroll M, Hinson JP. Autocrine role of adrenomedullin in the human adrenal cortex. J Endocrinol 2001;170:259–65.
[85] Tilakaratne N, Christopoulos G, Zumpe E, Foord SM, Sexton PM. Amylin receptor phenotypes derived from human calcitonin receptor/RAMP coexpression exhibit pharmacological differences dependent on receptor isoform and host cell environment. J Pharmacol Exp Ther 2000;294:61–72.
[86] Tomoda Y, Kikumoto K, Isumi Y, Katafuchi T, Tanaka A, Kangawa K, et al. Cardiac fibroblasts are major production and target cells of adrenomedullin in the heart in vivo. Cardiovasc Res 2001;49:721–30.
[87] Totsune K, Takahashi K, Mackenzie HS, Murakami O, Arihara Z, Sone M, et al. Increased gene expression of adrenomedullin and adrenomedullin-receptor complexes, receptor-activity modifying protein (RAMP)2 and calcitonin-receptor-like receptor (CRLR) in the hearts of rats with congestive heart failure. Clin Sci 2000;99:541–6.
[88] Totsune K, Takahashi K, Mackenzie HS, Arihara Z, Satoh F, Sone M, et al. Adrenomedullin and its receptor complexes in remnant kidneys of rats with renal mass ablation: decreased expression of calcitonin receptor-like receptor and receptor-activity modifying protein-3. Peptides 2001;22:1933–7.
[89] Tsuruda T, Kato J, Kitamura K, Kuwasako K, Imamura T, Koiwaya Y, et al. Adrenomedullin: a possible autocrine or paracrine inhibitor of hypertrophy of cardiomyocytes. Hypertension 1998;31:505–10.
[90] Uezono Y, Nakamura E, Ueda Y, Shibuya I, Ueta Y, Yokoo H, et al. Production of cAMP by adrenomedullin in human oligodendroglial cell line KGIC: comparison with calcitonin gene-related peptide and amylin. Mol Brain Res 2001;97:59–69.
[91] Wang X, Nishikimi T, Akimoto K, Tadokoro K, Mori Y, Minamino N. Upregulation of ligand, receptor system, and amidating activity of adrenomedullin in left ventricular hypertrophy of severely hypertensive rats: effects of angiotensin-converting enzyme inhibitors and diuretic. J Hypertens 2003;21:1171–81.
[92] Wimalawansa SJ. Amylin, calcitonin gene-related peptide, calcitonin, and adrenomedullin: a peptide superfamily. Crit Rev Neurobiol 1997;11:167–239.
[93] Yamaguchi T, Baba K, Doi Y, Yano K. Effects of adrenomedullin on aldosterone secretion by dispersed rat adrenal zona glomerulosa cells. Life Sci 1995;56:379–87.
[94] Yamaguchi T, Baba K, Doi Y, Yano K, Kitamura K, Eto T. Inhibition of aldosterone production by adrenomedullin, a hypotensive peptide, in the rat. Hypertension 1996;28:308–14.
[95] Yanagita T, Yamamoto R, Sugano T, Kobayashi H, Uezono Y, Yokoo H, et al. Adrenomedullin inhibits spontaneous and bradykinin-induced but not oxytocin- or prostaglandin $F_2\alpha$-induced periodic contraction of rat uterus. Br J Pharmacol 2000;130:1727–30.
[96] Yoshihara F, Nishikimi T, Okano I, Horio T, Yutani C, Matsuo H, et al. Alterations of intrarenal adrenomedullin and its receptor system in heart failure rats. Hypertension 2001;37:216–22.
[97] Yoshimoto R, Mitsui-Saito M, Ozaki H, Karaki H. Effects of adrenomedullin and calcitonin gene-related peptide on contractions of the rat aorta and porcine coronary artery. Br J Pharmacol 1998;123:1645–54.
[98] Yousufzai SYK, Ali N, Abdel-Latif AA. Effects of adrenomedullin on cyclic AMP formation and on relaxation in iris sphincter smooth muscle. Invest Ophtalmol Vis Sci 1999;40:3245–53.

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Adrenomedullin induces matrix metalloproteinase-2 activity in rat aortic adventitial fibroblasts

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Abstract

Background. The delicate balance of the extracellular matrix (ECM) determines the stiffness of the vascular wall, and adventitial fibroblasts are involved in ECM formation by synthesizing and degrading matrix proteins. In the present study, we examined the effect of the bioactive peptide adrenomedullin (AM) on activity and expression of matrix metalloproteinases (MMPs) in cultured aortic adventitial fibroblasts.

Methods and results. In cultured adventitial fibroblasts isolated from aorta of adult Wistar rats, 10^{-6} mol/L angiotensin II (Ang II) significantly ($p < 0.05$) down-regulated MMP-2 activity as determined by in vitro gelatin zymography. In contrast, 10^{-7} mol/L synthetic rat AM significantly ($p < 0.05$) stimulated zymographic MMP-2 activity by 23%, increasing intracellular cAMP, and AM abolished the action of Ang II, augmenting the MMP-2 activity. Similarly, Ang II down-regulated MMP-2 protein expression assessed by Western blotting, whereas AM increased it. Furthermore, 8-bromo-cAMP, an analogue of cAMP, mimicked the effect of AM, and H-89, an inhibitor for protein kinase A (PKA), significantly decreased the basal and AM-induced MMP-2 activity.

Conclusion. This study provides a new insight into the biological action of AM and its intracellular signaling system of cAMP/PKA stimulating the matrix degrading enzyme MMP-2, suggesting an important role for this molecule in modulating ECM deposition in the adventitial layer.

Keywords: Adrenomedullin; Vascular remodeling; Collagen; Adventitia; Matrix metalloproteinase

Vascular remodeling, defined as a change of vessel size, is initially a physiological adaptation to physical stimuli, such as blood flow, shear stress, and wall tension, to preserve the luminal size of the vessels [1,2]. However, the remodeling process becomes maladaptative in diseased situations such as atherosclerosis or vascular injury, leading to inappropriate extracellular matrix (ECM) reorganization. The delicate balance of ECM synthesis and degradation determines the level of ECM deposition in the vascular wall. A group of Ca^{2+}- and Zn^{2+}-dependent endopeptidases, matrix metalloproteinases (MMPs), contribute to the vascular remodeling by degrading several ECM proteins [3]. MMPs are present in the vasculature: MMPs-1, -14 in endothelial cells, MMPs-1, 2, 3, 9, 14 in smooth muscle cells [3], and MMPs-2, -9 in adventitial fibroblasts [4]. The adventitial layer of vascular wall has recently been recognized to have a significant role in the process of vascular remodeling [5], and either the systemic or local

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renin–angiotensin system plays a pivotal role in vascular fibrosis by stimulating proliferation and collagen production of adventitial fibroblasts [6,7].

Enhanced ECM deposition makes the vascular wall stiffer, subsequently increasing peripheral resistance [2], therefore pharmacological intervention to ameliorate ECM deposition might be attractive in maintaining a distensibility of the vasculature. The bioactive peptide adrenomedullin (AM), initially isolated from human pheochromocytoma [8], has been shown to be produced by the cardiovascular tissues where it exerts multiple actions mainly via the intracellular cyclic AMP (cAMP) [9]. For example, AM has been found to inhibit cellular proliferation and collagen synthesis of cardiac fibroblasts in vitro [10,11]. Based upon the action of AM on the fibroblasts in the heart, we hypothesized that AM plays an important role in the vascular remodeling by affecting the ECM turnover. In the present study, we conducted in vitro experiments to examine whether AM affects expression and activity of the matrix degrading enzyme MMPs in cultured fibroblasts isolated from rat aortic adventitia.

Methods

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2003-023).

Materials. Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum were from Gibco-BRL. Synthetic rat AM was purchased from Peptide Institute (Osaka, Japan), H-89 was from Seikagaku (Tokyo, Japan), and other reagents were from Sigma or otherwise indicated in the text.

Cell culture. Adventitial fibroblasts of aorta were prepared according to the method of Gu and Brecher with our modification [12]. Aortic tissues resected aseptically from 10-week-old male Wistar rats were placed in ice-cold DMEM/F-12 containing 10% fetal bovine serum and antibiotics. Loosely adhering connective tissue was rapidly removed from the aorta, and the luminal surface was opened by a longitudinal cut. Endothelial cells were removed by gently rubbing the lumen with the blunt side of dissecting scissors, and the medial layer was peeled off with the use of two forceps. The remaining tissues, predominantly adventitia, were cut into segments ~2 mm^3 and placed in DMEM/F-12 solution for subsequent enzymatic digestion with 0.12% trypsin and 0.03% collagenase. The cells were then incubated in 10 cm collagen type 1-coated culture plates. After achieving confluence, they were harvested with trypsin and used for experiments at passage 3–5.

The cultured cells were incubated in DMEM/F12 medium with 10% fetal bovine serum, followed by the incubation with serum-free medium containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite for 24 h. Thereafter, the cells were incubated with fresh serum-free medium described above in the presence or absence of angiotensin II (Ang II), synthetic rat AM or 8-bromo-cAMP (8-Br-cAMP). In another set of experiments, H-89, a specific protein kinase A (PKA) inhibitor, was added 30 min prior to AM administration. The isolated cells were positive for vimentin, α-smooth muscle actin, but negative for von-Willebrand factor, desmin, smooth muscle-myosin heavy chain and caldesmon, indicating that they are activated adventitial fibroblasts.

In vitro gelatin zymography. Conditioned media of adventitial fibroblasts (2 μg protein) were concentrated and used for the zymographic gelatinase activity assay as described before [13]. In brief, samples mixed with Laemmli sample buffer were loaded onto 10% Tris–glycine gels with 0.1% gelatin (Invitrogen) in Tris–glycine sodium dodecyl sulfate running buffer. The gels were then washed with renaturing buffer, followed by incubation with zymogen developing buffer for 24 h. They were stained with 0.5% (wt/vol) Coomassie brilliant blue overnight and then with destaining buffer. Lytic bands corresponding to the pro- and active-forms of MMP-2 were analyzed as a total MMP-2 activity with NIH image software v. 1.63.

Western blot. Denatured protein extracts (30 or 60 μg) from cultured adventitial fibroblasts were subjected to sodium dodecyl sulfate–polyacrylamide gel as previously described [13]. The separated proteins were electrically transferred onto polyvinylidene difluoride membranes (Bio-Rad). Equal protein loading was verified by staining the gels with Coomassie brilliant blue. After blocking the non-specific background with 5% skim milk, polyvinylidene difluoride membranes were incubated with anti-MMP-2 polyclonal antibody (AB809, Chemicon, 1:1000), anti-tissue inhibitor of MMP-2 (TIMP-2) polyclonal antibody (AB801, Chemicon, 1:1000) or anti-MMP-14 monoclonal antibody (Daiichi Fine Chemical F-84, 10 μg/mL) followed by incubation with horseradish peroxidase-coupled secondary antibody. Immunoreactive bands were visualized by the ECL Plus detection kit (Amersham), and the intensity of the bands was analyzed densitometrically (Chemi Doc Documentation System, Bio-Rad).

Assays for cAMP and AM. Intracellular cAMP concentration in the adventitial fibroblasts was measured as previously described [10]. In brief, the fibroblasts were incubated in Hanks' balanced salt solution containing 20 mmol/L *N*-[2-hydroxyethyl]piperazine-3-isobutyl-1-methylxanthine for 10 min at 37 °C. AM was then added at the indicated concentrations. The reaction was terminated by aspirating the medium and adding cold 6% trichloroacetic acid. Cells were extracted with water-saturated ethyl ether, and cAMP was measured by radioimmunoassay. To evaluate AM secretion from the adventitial fibroblasts, conditioned media were collected at indicated time points, and rat AM immunoreactivity in the media was measured with commercially available immunoradiometric assay kits (Shionogi, Japan).

Statistical analysis. All data are expressed as means ± SEM. Results of the Western blot and zymography are expressed as ratios relative to the respective control. Comparisons between groups were assessed with one-way ANOVA followed by Fisher's test. A statistical significance was accepted at $p < 0.05$.

Results

Effects of Ang II and AM on MMP-2 activity and protein

Fig. 1A illustrates the effects of Ang II and AM on zymographic MMP-2 activity in the cultured aortic adventitial fibroblasts. One μmol/L of Ang II significantly ($p < 0.05$) decreased zymographic MMP-2 activity in these cells. Conversely, 10^{-7} mol/L synthetic rat AM significantly increased MMP-2 activity by 23% ($p < 0.05$), and AM abolished the action of Ang II, augmenting MMP-2 activity, at 10^{-7} and 10^{-8} mol/L. MMP-9 is another metalloproteinase having an important role in the adventitia [4], but the band corresponding to MMP-9 activity of 92 kDa was too faint to be quantified in control fibroblasts and those stimulated by either Ang II or AM. Fig. 1B illustrates the effects of Ang II and AM on MMP-2 protein expression. Similar to the zymographic finding, 10^{-6} mol/L Ang II down-regulated protein expression of MMP-2, while 10^{-7} mol/L AM increased the MMP-2 expression whether or not Ang II was added.

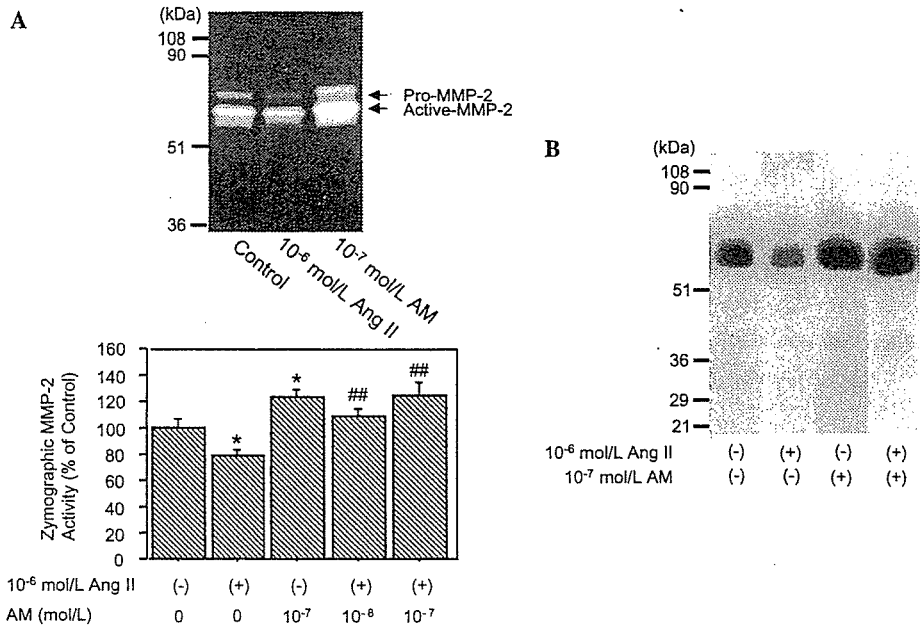

Fig. 1. (A) Effects of Ang II and AM on zymographic MMP-2 activity in cultured adventitial fibroblasts. Adventitial fibroblasts were incubated with or without 10^{-6} mol/L Ang II in the absence or presence of indicated concentration of synthetic rat AM for 24 h. Values are shown as means ± SEM ($n = 7$–8). The upper panel shows a representative zymographic picture. $^{*}p < 0.05$, vs. control cells; $^{\#\#}p < 0.01$, vs. cells incubated with Ang II. (B) Effects of Ang II and AM on protein expression of MMP-2 in adventitial fibroblasts. Identical results were obtained in three independent experiments.

Roles of cAMP/PKA signaling on zymographic MMP-2 activity

Figs. 2A and B illustrate the effect of AM on intracellular cAMP content in cultured adventitial fibroblasts. One μmol/L AM significantly stimulated cAMP production, peaking at 10 min (Fig. 2A), and this effect was dose-dependent (Fig. 2B). Figs. 2C and D illustrate the effects of the cAMP analogue 8-Br-cAMP and the specific PKA inhibitor H-89 on zymographic MMP-2 activity. 8-Br-cAMP mimicked the effect of AM stimulating MMP-2 activity (Fig. 2C), while H-89 significantly decreased the MMP-2 activity of the cells incubated with or without 10^{-7} mol/L AM (Fig. 2D).

AM secretion from adventitial fibroblasts

Adventitial fibroblasts time-dependently secreted AM into the media up to 48 h under the serum-free conditions. Rat AM concentrations in the conditioned media were: 6 h, 1.4 ± 0.1; 24 h, 2.0 ± 0.3; and 48 h, 5.5 ± 0.3 fmol/10^5 cells ($n = 6$).

Discussion

Balance between ECM synthesis and degradation determines its abundance in the vasculature. Although hemodynamic stimuli largely regulate vascular remodeling in normal arteries, humoral factors such as Ang II, inappropriately stimulating collagen synthesis, seem to be of importance in the pathological situation [1]. MMPs reorganize the vessel structure by degrading the ECM proteins and a significant role for MMPs in vascular remodeling has been suggested [3]. For example, the plasma level of MMP-1 was reduced in hypertensive patients [14], and MMPs-1, 3, and 9 were up-regulated in the atherosclerotic lesion [15,16]. Constitutively produced by vascular endothelial and smooth muscle cells, MMP-2 degrades gelatin, type I, IV, V, VII collagen, and elastin [17,18]. Here we report for the first time that the bioactive peptide AM increased protein level and enzymatic activity of MMP-2 in cultured aortic adventitial fibroblasts of rats. Hernandez-Barrantes et al. [19] showed that MMP-2 is regulated by MMP-14, a membranous type of MMP, and by the tissue inhibitor of MMP (TIMP)-2. We examined expressions of these proteins by Western blotting, but neither AM nor Ang II affected the MMP-14 protein expression and TIMP-2 was undetectable in the fibroblasts (data not shown).

Renin–angiotensin system activation has been shown to be involved in the process of vascular remodeling. Angiotensinogen is expressed in the adventitia [20], and angiotensin converting enzyme was found to be induced in the injured artery [21]. Further, mast cell mainly distributed in the adventitia has been reported to be an additional source of renin [22]. These findings suggest an existence of the local renin–angiotensin system in vascular adventitia. Recent reports have shown that MMP-2 activity is down-regulated in the

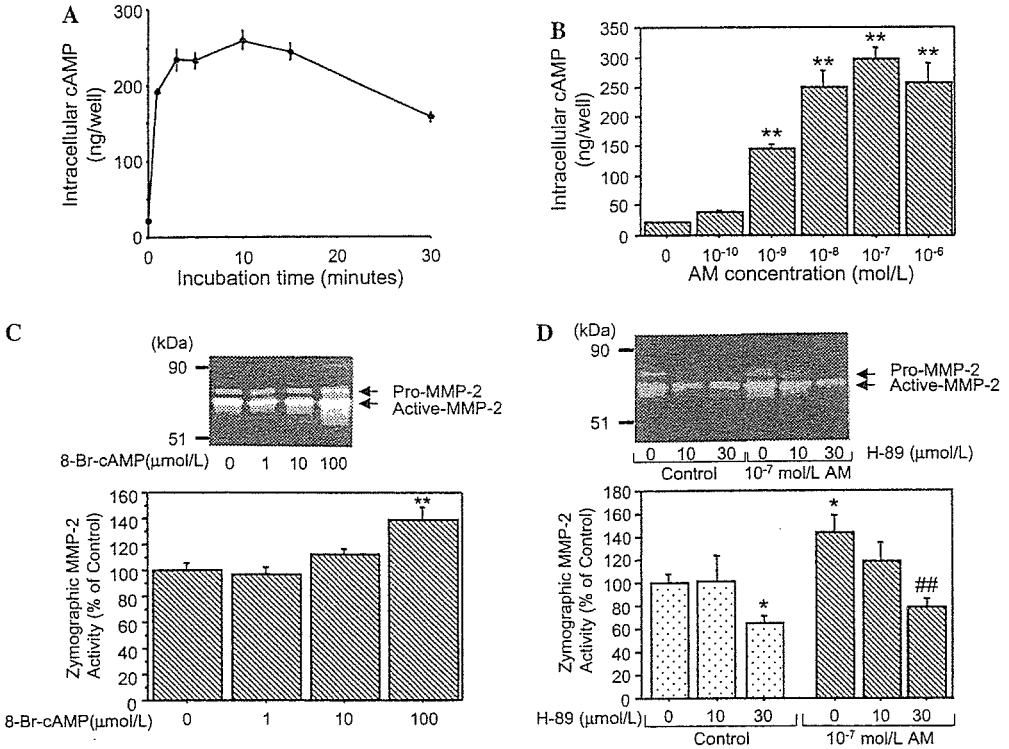

Fig. 2. (A,B) Time course of cAMP elevation by 10^{-6} mol/L rat synthetic AM (A) and dose-dependent effect of AM on cAMP elevation at 10 min (B) in adventitial fibroblasts. (C,D) Effects of 8-Br-cAMP (C) and H-89 (D) on zymographic MMP-2 activity in adventitial fibroblasts. The upper panels show representative zymographic pictures. Values are shown as means ± SEM, and the sample numbers were four in (A) and (B) and six in (C) and (D), respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, vs. control fibroblasts; $^{\#\#}p < 0.01$, vs. AM-treated cells without H-89.

vasculature of rats and humans with diabetes mellitus [23,24], where the renin–angiotensin system has been shown to be activated; though, the role of AM in the vascular adventitia remains unknown. In the present study, we examined the effects of Ang II and AM on the ECM metabolism in the cultured adventitial fibroblasts. When assessed by [^{3}H]proline incorporation into the cells, AM failed to inhibit the Ang II-induced de novo collagen synthesis (data not shown). However, AM increased not only basal levels of MMP-2 protein expression and its enzymatic activity but also those reduced by Ang II.

AM was initially isolated during experiments monitoring cAMP elevation in rat platelets [8], and many of the actions of AM have been shown to be mediated by intracellular cAMP [9–11]. In the present study, the cAMP analogue 8-Br-cAMP mimicked the effect of AM, stimulating zymographic MMP-2 activity, and PKA inhibition with H-89 attenuated its activity. These results suggest that the cAMP/PKA signaling system is involved in the AM-induced activation and up-regulation of MMP-2 in the aortic adventitial fibroblasts. This is comparable with the finding by Maioli et al. [25] who reported that parathyroid hormone-related peptide stimulated MMP-2 activity by intracellular cAMP accumulation without affecting collagen synthesis in human skin fibroblasts.

It has been well documented that AM is produced by the vascular wall, particularly in endothelial and smooth muscle cells [26]. Immunohistochemical staining for AM was also observed in the adventitia of rat femoral artery [27], and consistent with this, we found that cultured aortic adventitial fibroblasts also secreted AM in this study. According to our unpublished observation, intravenous AM infusion attenuated collagen accumulation in the adventitia following ballooning injury in rat carotid artery, suggesting an important role of AM as an anti-fibrotic factor in the vascular remodeling. Taken together, renin–angiotensin system activation increases ECM formation by stimulating collagen synthesis and by decreasing MMP-2 activity in the adventitia, making the vascular wall stiffer; while AM may reduce vascular stiffness by enhancing action of the matrix degradation enzyme MMP-2, thus antagonizing the action of Ang II in the adventitial layer. However, this hypothesis should be tested further by in vivo studies.

In summary, this study provides a new insight into the biological action of AM in vascular remodeling, suggesting that AM may modulate ECM metabolism by augmenting the MMP-2 action in the adventitia.

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References

[1] M.R. Ward, G. Pasterkamp, A.C. Yeung, C. Borst, Arterial remodeling. Mechanisms and clinical implications, Circulation 102 (2000) 1186–1191.

[2] H.D. Intengan, E.L. Schiffrin, Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis, Hypertension 38 (2001) 581–587.

[3] Z.S. Galis, J.J. Khatri, Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly, Circ. Res. 90 (2002) 251–262.

[4] W.J. Cai, S. Koltai, E. Kocsis, D. Scholz, S. Kostin, X. Luo, W. Schaper, J. Schaper, Remodeling of the adventitia during coronary arteriogenesis, Am. J. Physiol. Heart Circ. Physiol. 284 (2003) H31–H40.

[5] S. Sartore, A. Chiavegato, E. Faggin, R. Franch, M. Puato, S. Ausoni, P. Pauletto, Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant, Circ. Res. 89 (2001) 1111–1121.

[6] K.T. Weber, Y. Sun, E. Guarda, Structural remodeling in hypertensive heart disease and the role of hormones, Hypertension 23 (1994) 869–877.

[7] D.K. Kim, J.E. Huh, S.H. Lee, K.P. Hong, J.E. Park, J.D. Seo, R.W. Lee, Angiotensin II stimulates proliferation of adventitial fibroblasts cultured from rat aortic explants, J. Korean Med. Sci. 14 (1999) 487–496.

[8] K. Kitamura, K. Kangawa, M. Kawamoto, Y. Ichiki, S. Nakamura, H. Matsuo, T. Eto, Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma, Biochem. Biophys. Res. Commun. 192 (1993) 553–560.

[9] K. Kitamura, K. Kangawa, T. Eto, Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions, Microsc. Res. Tech. 57 (2002) 3–13.

[10] T. Tsuruda, J. Kato, K. Kitamura, M. Kawamoto, K. Kuwasako, T. Imamura, Y. Koiwaya, T. Tsuji, K. Kangawa, T. Eto, An autocrine or a paracrine role of adrenomedullin in modulating cardiac fibroblast growth, Cardiovasc. Res. 43 (1999) 958–967.

[11] T. Horio, T. Nishikimi, F. Yoshihara, H. Matsuo, S. Takishita, K. Kangawa, Effects of adrenomedullin on cultured rat cardiac myocytes and fibroblasts, Eur. J. Pharmacol. 382 (1999) 1–9.

[12] M. Gu, P. Brecher, Nitric oxide-induced increase in p21 (Sdi1/Cip1/Waf1) expression during the cell cycle in aortic adventitial fibroblasts, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 27–34.

[13] T. Tsuruda, G. Boerrigter, B.K. Huntley, J.A. Noser, A. Cataliotti, L.C. Costello-Boerrigter, H.H. Chen, J.C. Burnett Jr., Brain natriuretic peptide is produced in cardiac fibroblasts and induces matrix metalloproteinases, Circ. Res. 91 (2002) 1127–1134.

[14] C. Laviades, N. Varo, J. Fernández, G. Mayor, M.J. Gil, I. Monreal, J. Díez, Abnormalities of the extracellular degradation of collagen type I in essential hypertension, Circulation 98 (1998) 535–540.

[15] Z.S. Galis, G.K. Sukhova, M.W. Lark, P. Libby, Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques, J. Clin. Invest. 94 (1994) 2493–2503.

[16] S.T. Nikkari, K.D. O'Brien, M. Ferguson, T. Hatsukami, H.G. Welgus, C.E. Alpers, A.W. Clowes, Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis, Circulation 92 (1995) 1393–1398.

[17] F.G. Spinale, Matrix metalloproteinases: regulation and dysregulation in the failing heart, Circ. Res. 90 (2002) 520–530.

[18] R.M. Senior, G.L. Griffin, C.J. Fliszar, S.D. Shapiro, G.I. Goldberg, H.G. Welgus, Human 92- and 72-kilodalton type IV collagenases are elastases, J. Biol. Chem. 266 (1991) 7870–7875.

[19] S. Hernandez-Barrantes, M. Toth, M.M. Bernardo, M. Yurkova, D.C. Gervasi, Y. Raz, Q.A. Sang, R. Fridman, Binding of active (57 kDa) membrane type 1-matrix metalloproteinase (MT1-MMP) to tissue inhibitor of metalloproteinase (TIMP)-2 regulates MT1-MMP processing and pro-MMP-2 activation, J. Biol. Chem. 275 (2000) 12080–12089.

[20] L.A. Cassis, K.R. Lynch, M.J. Peach, Localization of angiotensinogen messenger RNA in rat aorta, Circ. Res. 62 (1988) 1259–1262.

[21] J.L. Zhuo, P. Froomes, D. Casley, J.J. Liu, C. Murone, Y.S. Chai, B. Buxton, F.A. Mendelsohn, Perindopril chronically inhibits angiotensin-converting enzyme in both the endothelium and adventitia of the internal mammary artery in patients with ischemic heart disease, Circulation 96 (1997) 174–182.

[22] R.B. Silver, A.C. Reid, C.J. Mackins, T. Askwith, U. Schaefer, D. Herzlinger, R. Levi, Mast cells: a unique source of renin, Proc. Natl. Acad. Sci. USA 101 (2004) 13607–13612.

[23] S. Jesmin, I. Sakuma, Y. Hattori, A. Kitabatake, Role of angiotensin II in altered expression of molecules responsible for coronary matrix remodeling in insulin-resistant diabetic rats, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 2021–2026.

[24] V. Portik-Dobos, M.P. Anstadt, J. Hutchinson, M. Bannan, A. Ergul, Evidence for a matrix metalloproteinase induction/activation system in arterial vasculature and decreased synthesis and activity in diabetes, Diabetes 51 (2002) 3063–3068.

[25] E. Maioli, V. Fortino, C. Torricelli, B. Arezzini, C. Gardi, Effect of parathyroid hormone-related protein on fibroblast proliferation and collagen metabolism in human skin, Exp. Dermatol. 11 (2002) 302–310.

[26] T. Eto, J. Kato, K. Kitamura, Regulation of production and secretion of adrenomedullin in the cardiovascular system, Regul. Pept. 112 (2003) 61–69.

[27] M. Yamasaki, J. Kawai, T. Nakaoka, T. Ogita, A. Tojo, T. Fujita, Adrenomedullin overexpression to inhibit cuff-induced arterial intimal formation, Hypertension 41 (2003) 302–307.

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Antifibrotic effect of adrenomedullin on coronary adventitia in angiotensin II-induced hypertensive rats

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Abstract

Objective: The extracellular matrix (ECM) determines the structural integrity of the heart and vasculature, participating in cardiovascular remodeling. We previously reported that adrenomedullin (AM) inhibited cellular proliferation and protein synthesis of cardiac fibroblasts; however, the precise mechanisms of AM actions as an antifibrotic factor remain unknown. The purpose of this study was to examine the biological actions of AM against the profibrotic factor angiotensin II (Ang II) in coronary adventitia.

Methods and results: Rats with hypertension induced by Ang II infusion were administered 0.06 μg/kg/min recombinant human AM subcutaneously for 14 days. The AM infusion significantly ($p<0.05$) reduced the Ang II-induced increase of coronary adventitial fibroblasts expressing Ki-67 and α-smooth muscle actin (α-SMA) in the left ventricle, by 65%, and 62%, respectively, without affecting systolic blood pressure, left ventricle/body weight, or cross-sectional area of myocardial fibers. Collagen deposition of coronary arteries was reduced by the AM infusion (−24%, $p<0.01$), and these effects of AM were accompanied by significant reductions in gene expression of type 1 collagen (−49%, $p<0.05$) and transforming growth factor-β1 (TGF-β1) (−55%, $p<0.01$). In cultured cardiac fibroblasts, 10^{-7} mol/L AM exerted an inhibitory effect on TGF-β1-induced α-SMA expression ($p<0.01$) that was mimicked by 8-bromo-cAMP and attenuated by the protein kinase A inhibitor H-89.

Conclusion: AM decreased Ang II-induced collagen deposition surrounding the coronary arteries, inhibiting myofibroblast differentiation and expressions of ECM-related genes in rats. The present findings further support the biological action of AM as an antifibrotic factor in vascular remodeling.

Keywords: Extracellular matrix; Fibrosis; Hypertension; Peptide hormone; Remodeling

1. Introduction

Cardiac fibrosis is an important clinical disorder leading to deleterious consequences for myocardial function such as systolic and diastolic heart failure [1]. Particularly, thickening of the adventitia surrounding intramyocardial coronary arteries, where extracellular matrix (ECM) first accumulates in response to systemic hypertension, has been thought to reduce oxygen and nutrient supply to the *myocardium*, resulting in deterioration of ventricular function [2]. Emerging concepts of vascular remodeling underline the importance of the ECM scaffold in the vessel wall. The activated adventitial fibroblasts, known as myofibroblasts characterized by α-smooth muscle actin (α-SMA) expression, play important roles in the pathological vascular remodeling [3,4]. Therefore, both understanding of the regulation of fibroblast activation and the

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development of effective pharmacological intervention to manipulate fibroblast function are necessary to attenuate adverse remodeling.

A body of evidence suggests that the renin–angiotensin–aldosterone system is an important factor in progression of myocardial and vascular fibrosis accompanied by upregulation of transforming growth factor-β1 (TGF-β1) [5–7]. TGF-β1 induces a phenotypic change of fibroblasts to myofibroblasts in hypertensive heart disease, coronary restenosis following angioplasty, and in the healing process after myocardial infarction [8,9]. Blockage of TGF-β1 signaling was indeed reported to prevent fibroblast proliferation and diastolic cardiac dysfunction [10].

Adrenomedullin (AM), initially isolated from human pheochromocytoma [11], has been reported to have multiple functions in the cardiovascular system [12]. We and others have previously reported that AM inhibited proliferation and collagen synthesis induced by angiotensin II (Ang II) in cardiac fibroblasts of neonatal rats in vitro [13,14], suggesting a possible role of AM in attenuating cardiovascular remodeling. However, the precise mechanism by which AM acts as an antifibrotic factor in vivo remains to be elucidated.

Based upon previous studies, we hypothesized that activation of adventitial fibroblasts would result in coronary matrix remodeling in rats infused with Ang II and that pharmacological intervention with AM would lead to attenuation of perivascular fibrosis by modulating fibroblast function. Our aim in this study was to examine the biological action of AM against the profibrotic factor Ang II in coronary adventitia of rats.

2. Methods

2.1. Animals experiments

Eight-week-old male Wistar rats (Charles River Japan) weighing 200 to 250 g were housed in a temperature- and light-controlled room (25±1 °C; 12/12-h light/dark cycle) with normal rat chow and water given ad libitum. The rats were divided into three groups: control group (n=5) and two Ang II-infused groups with (n=11) or without (n=15) AM treatment. They were implanted with miniosmotic pumps (Alzet model 2002) under pentobarbital sodium anesthesia, that released either saline or 250 ng/kg/min Ang II for 14 days. In the Ang II-infused groups, another pump was implanted to infuse saline or 0.06 μg/kg/min of recombinant human AM (Shionogi & Co., Japan). The dose of AM used in this study was determined by referring to our previous observation, in which cardiac remodeling after myocardial infarction was significantly inhibited without affecting systemic blood pressure [15]. Blood pressure was measured while awake at least 9 times by tail-cuff plethysmography (Softron, BP-98A), and the mean value was recorded. At day 14, the rats were killed by decapitation and trunk blood was collected for measuring AM concentration. Plasma levels of human and rat AM were determined with commercially available immunoradiometric assay kits (Shionogi & Co., Japan). After removing atria and right ventricle of the heart, left ventricle was frozen in liquid nitrogen or fixed in 10% formalin and was embedded in paraffin wax.

The present study was performed in accordance with the Animal Welfare Act and with approval of the University of Miyazaki Institutional Animal Care and Use Committee (2003-023). This investigation confirmed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Histology and immunohistochemistry

Ventricular tissues, fixed in 10% formalin and embedded in paraffin, were sectioned at 2 μm thickness. After deparaffinization with xylene and graded alcohol, slides were immersed in 3% H_2O_2 in methanol to block endogenous peroxidase activities, thereafter incubated with 5% skim milk to reduce the nonspecific background. The section slides were then stained with either anti-monoclonal α-SMA antibody (Clone 1A4, DAKO) at a dilution of 1:200, or antipolyclonal TGF-β1 antibody (sc-146, Santa Cruze) at 1:100 at 4 °C. After the overnight reaction with antibodies, the slide sections were incubated with EnVision+ (DAKO) for 30 min, visualized with 0.05% 3, 3′-diaminobenzidine containing hydrogen peroxide, and counterstained with hematoxylin. For detection of Ki-67 antigen, a nuclear protein expressed in proliferating cells, tissue sections were autoclaved at 121 °C for 10 min in 10 mmol/L citrate buffer (pH 6.0) prior to incubation with primary antibody (Clone MIB-5, DAKO) at a dilution of 1:25. For the detection of collagen, slides were incubated with 0.1% picrosirius red (Direct Red 80, Sigma) dissolved in saturated picric acid for 10 min as described before [15]. The specificity of the antibody for TGF-β1 was confirmed by substitution of nonimmune rabbit serum and the absorption test as described before [16].

2.3. Morphology and cell counting

Morphological evaluation and cell counting of coronary arteries sectioned at the middle portion in the left ventricle were performed by a single observer in a blind manner. Each section immunostained with the antibody against either Ki-67 or α-SMA was scanned at a magnification of ×200, and the number of positive cells surrounding the coronary artery was determined. At least five images of fibrosis areas surrounding the blood vessel were randomly selected from each slide, and examined using an image

analysis system (Axio Vison 2.05 Carl ZEISS, Munchen, Germany) to calculate ratios of the perivascular fibrosis area to the total vascular area. To evaluate the interstitial fibrosis, collagen volume fraction in the interstitial space between myocardial fibers was determined by calculating the ratio of collagen area to the selected myocardial tissue area as previously described [15]. To measure the *cardiocyte* size, cross-sectional area of *myocardial fiber* was measured at the level of nuclei in at least 10 *cardiocytes* as described before [16]. Longitudinal- or oblique-sectioned *cardiocytes* were excluded for the analysis.

2.4. Gene expression

Gene expressions for TGF-β1 and type 1 collagen in total RNA isolated from left ventricle were measured by using real time-quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems) as previously described [17]. cDNA reverse transcribed from total RNA was amplified with the following oligonucleotide probes labeled with 6-carboxy-fluorescencein as reporter fluorescence and 6-carboxy tetramethyl-rhodamine as quencher fluorescence: TGF-β1 [18], TACGCCTGAGTGGCTGTCTTTTGA (nucleotide 985–1008); type 1 collagen [19], ACTGGAGACAGAGGA-CCGCGTGGAC (nucleotide 103–127); 18*S* ribosomal RNA [20], TGCCGACGGGCGCTGACC (nucleotide 176–193) and with the following pairs of oligonucleotides: TGF-β1 [18], TTCCTGGCGTTACCTTGGT (nucleotide 943–961, forward primer) and GCCACTGCCGGACAACT (nucleotide 1018–1034, reverse primer); type 1 collagen [19], TGCTGCTTGCAGTAACGTCG (nucleotide 32–51, forward primer) and TCAACACCATCTCTGCCTCG (nucleotide 148–167, reverse primer); 18*S* rRNA [20], CTTTGGTCGCTCGCTCCTC (nucleotide 118–136, forward primer) and CTGACCGGGTTGGTTTTGAT (nucleotide 229–248, reverse primer). The PCR products electrophoresed were observed at the expected molecular sizes, and the gene expression levels were normalized relative to that of 18*S* rRNA.

2.5. Cell culture

Cultured cardiac fibroblasts of neonatal rats were prepared as previously described [13]. After achieving confluence in the DMEM/F12 medium with 10% FBS, the cells were incubated with serum-free medium containing 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite for 24 h. The medium was then exchanged for fresh serum-free medium described above and incubated with or without synthetic rat AM (Peptide Institute, Osaka, Japan), recombinant human TGF-β1 or 8-bromo-cAMP (Sigma, MO, USA). In another series of experiment, H-89 (Seikagaku, Tokyo, Japan), a specific protein kinase A inhibitor, was added to culture medium at least 30 min before the incubation with AM or TGF-β1.

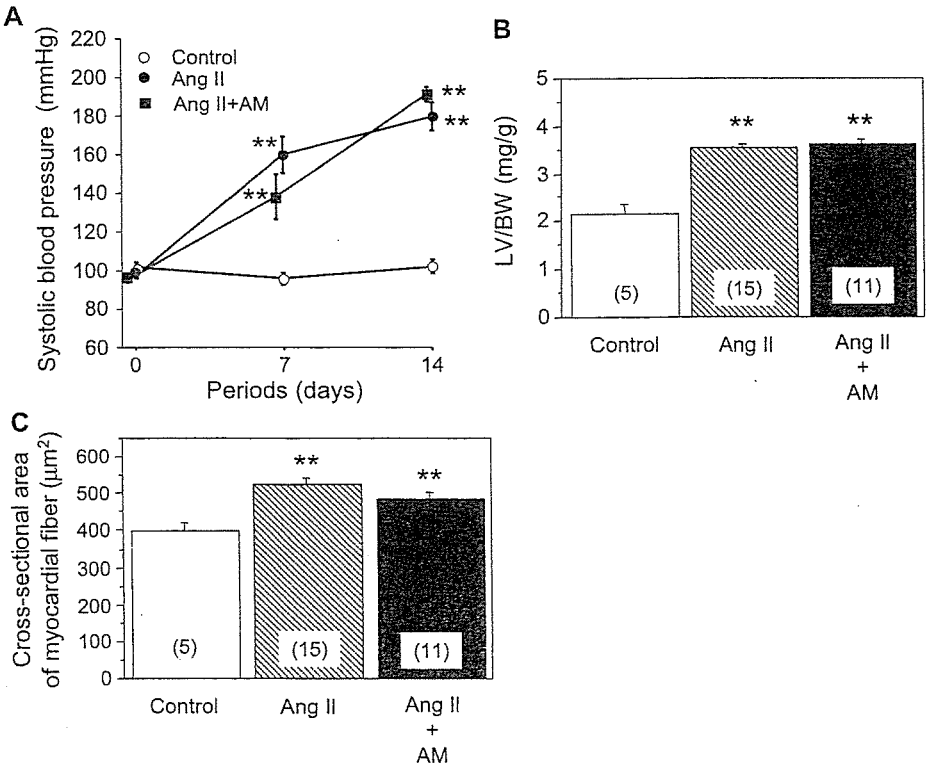

Fig. 1. Effects of Ang II and co-administration of AM on systolic blood pressure (A), left ventricular weight/body weight (LV/BW) (B), and *cross-sectional area of myocardial fiber* (C). Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined. $^{**}p<0.01$, compared to controls.

2.6. Western blot

Denatured protein extract (5 μg) from the cultured cardiac fibroblasts was subjected to sodium dodecyl sulfate–polyacrylamide gel as previously described [21]. The separated proteins were electrically transferred onto polyvinylidene difluoride (PVDF) membranes (BIO-RAD). Equal protein loading was verified by staining the gels with Coomassie brilliant blue. After blocking the non-specific background with 5% skim milk, PVDF membranes were incubated with the anti-α-SMA monoclonal antibody at a dilution of 1:1000, followed by incubation with horseradish peroxidase-coupled second antibody. Immunoreactive bands were visualized by the ECL Plus detection kit (Amersham), and intensities of the bands were analyzed densitometrically (Chemi Doc™ Documentation System, BIO-RAD).

2.7. Statistical analysis

All data are expressed as means±S.E.M. Comparisons between groups were assessed with one-way ANOVA followed by the Fisher's test. A statistical significance was accepted at $p<0.05$.

3. Results

3.1. Systolic blood pressure, left ventricle/body weight, and cardiocyte size

Fig. 1A illustrates the effects of Ang II and AM on systolic blood pressure. Continuous, subcutaneous Ang II infusion significantly ($p<0.01$) increased systolic blood pressure at days 7 and 14, and the co-administration of AM

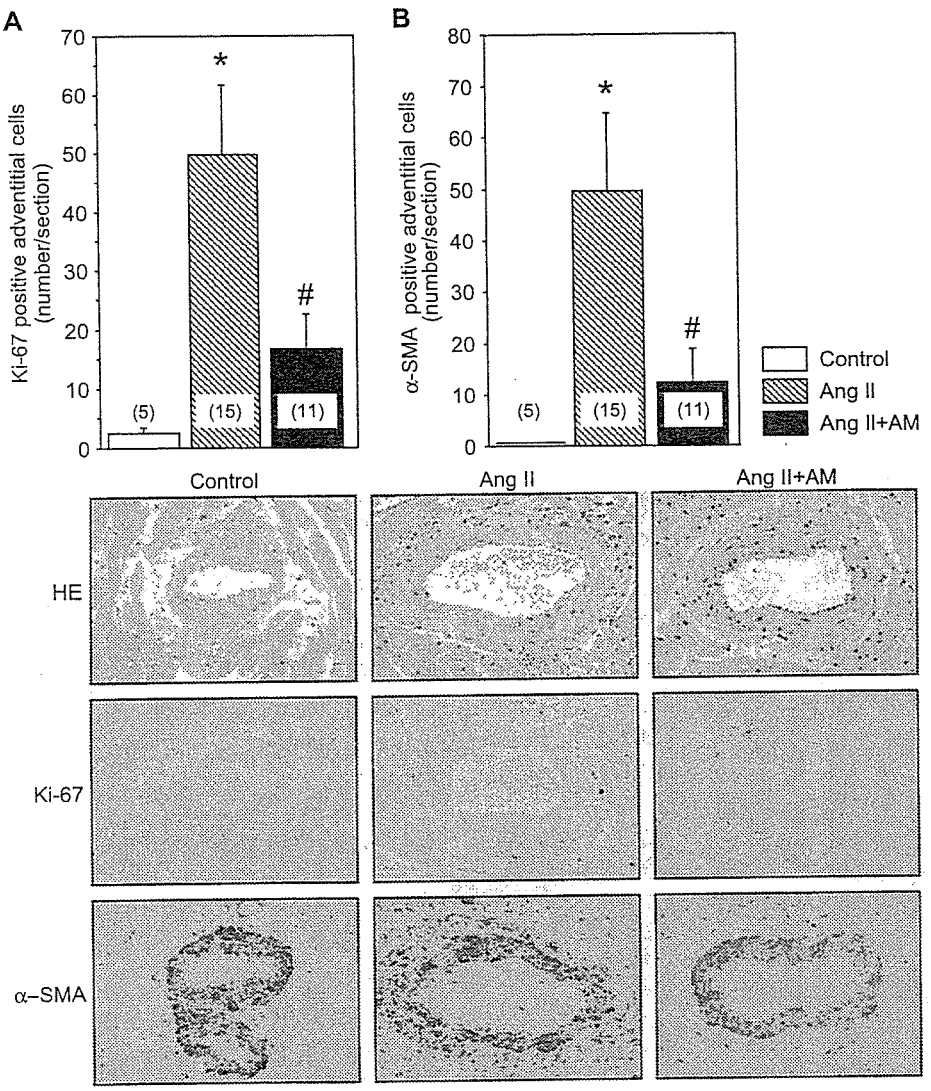

Fig. 2. Effects of Ang II and AM on the number of adventitial fibroblasts expressing Ki-67 antigen (A) and of those positive for α-SMA (B). The bottom panels show the representative histological sections stained with hematoxylin eosin (HE), anti-Ki-67, and α-SMA antibodies. Values are shown as means±S.E.M. Parentheses indicate the number of rats examined. *$p<0.05$, compared to controls; #$p<0.05$, compared to Ang II group.

with Ang II did not affect systolic blood pressure significantly. In addition, Ang II significantly ($p<0.01$) increased the left ventricle/body weight (LW/BW) and *cross-sectional area of myocardial fiber*, compared to control at day 14, without a significant difference in LV/BW or *cardiocyte* size between the Ang II and Ang II+AM groups (Fig. 1B and C).

3.2. Fibroblast proliferation and myofibroblast differentiation

Fig. 2A and B illustrate the effects of Ang II and AM on staining for Ki-67 antigen and α-SMA in the perivascular area of coronary arteries. Ang II significantly ($p<0.01$) increased the number of fibroblasts expressing Ki-67 antigen, a marker for proliferating fibroblasts, and this increase was significantly ($p<0.05$) inhibited by the co-administration of AM at day 14 (Fig. 2A). Similarly, the Ang II-induced increase in number of the fibroblasts expressing α-SMA, a marker for myofibroblast differentiation, was significantly ($p<0.05$) reduced by AM (Fig. 2B).

3.3. Type 1 collagen gene expression and adventitial area

Fig. 3A illustrates the effects of Ang II and AM on type 1 collagen mRNA expression. The Ang II infusion significantly ($p<0.05$) increased type 1 collagen expression in the left ventricle, and the co-administration of AM significantly ($p<0.05$) attenuated its expression by 49% at day 14. The effects of Ang II and AM on the adventitial area surrounding the coronary arteries are shown in Fig. 3B as composite data and in Fig. 3C as representative pictures. Ang II significantly ($p<0.01$) increased perivascular fibrosis at day 14, and the co-administration of AM significantly ($p<0.01$) decreased it. Similarly, the Ang II infusion significantly increased interstitial fibrosis of the left ventricular myocardium (+130%, $p<0.01$), while AM inhibited this Ang II effect (−54%, $p<0.01$).

3.4. TGF-β1 expression

As shown in Fig. 4A, Ang II significantly ($p<0.01$) increased TGF-β1 gene expression in the left ventricle, while the co-administration of AM significantly ($p<0.01$) attenuated its expression by 55%. Fig. 4B illustrates the distribution of TGF-β1 immunoreactivity in the coronary arteries. TGF-β1 immunoreactivity was intensely stained in the adventitial fibroblasts, as well as in vascular smooth muscle cells and *myocardial fibers* of the Ang II-treated rats, while those cells were faintly stained in the control and AM-treated rats.

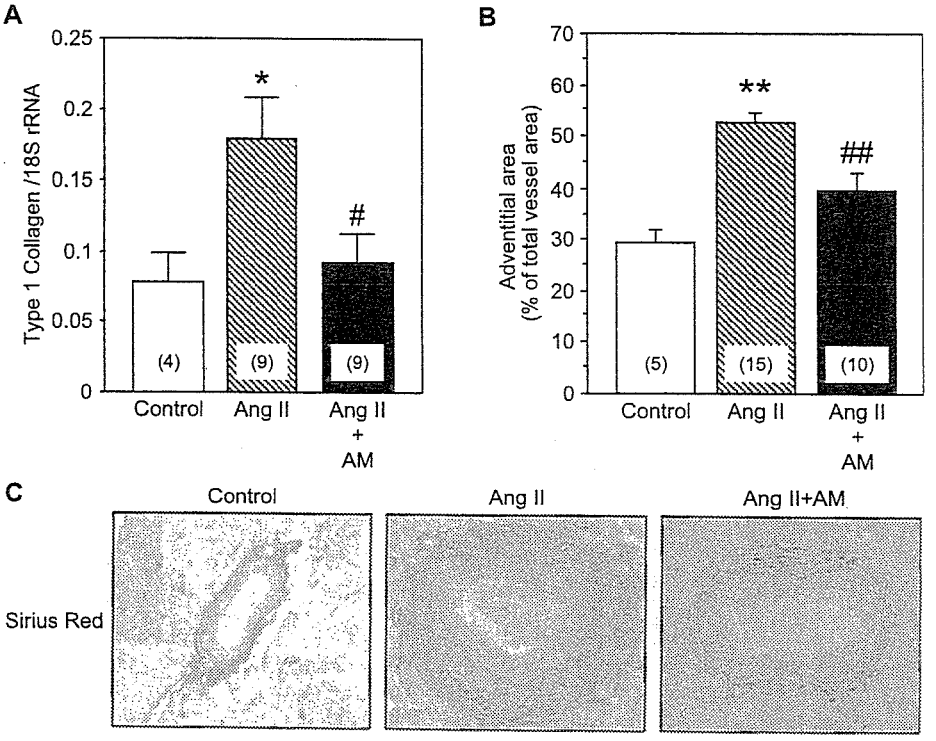

Fig. 3. Effects of Ang II and AM on gene expression of type 1 collagen (A) and on adventitial area determined by sirius red staining (B). The bottom panels (C) show the representative pictures for sirius red staining. Values are shown as means±S.E.M. Parentheses indicate the numbers of rats examined. *$p<0.05$, **$p<0.01$, compared to controls; $^{\#}p<0.05$, $^{\#\#}p<0.01$, compared to Ang II.