Review

Adrenomedullin-RAMP2 System in Vascular Endothelial Cells

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Vascular endothelial cells play key roles in maintaining vascular and organ homeostasis. Adrenomedullin (AM), originally identified as a vasodilating peptide, is now recognized to be a pleiotropic molecule involved in both circulatory homeostasis and the pathogenesis of cardiovascular diseases. We have reported that knockout mice deficient in AM or receptor activity-modifying protein 2 (RAMP2), an AM-receptor accessory protein, show vascular endothelial cell deformities that are embryonically lethal. To directly clarify the pathophysiological functions of the vascular AM-RAMP2 system, we generated vascular endothelial cell-specific RAMP2 knockout mice. Using these mice, we found that the AM-RAMP2 system is a key determinant of vascular integrity and homeostasis from prenatal stages through adulthood. This review highlights the functions of AM-RAMP2 in vascular endothelial cells.

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Key words: Adrenomedullin (AM), Receptor activity-modifying protein (RAMP), Vascular endothelial cell, Vascular integrity, Homeostasis

Introduction

Atherosclerosis is the most frequently occurring cause of chronic organ dysfunction and shortens the healthy life span of a large numbers of individuals. Various acquired and congenital factors can accelerate the progression of organ dysfunction; however, the primary cause is still a matter of considerable debate. Generally, organ dysfunction begins slowly; however, it is progressive and intractable in its chronic phase^{1,2}. From a functional viewpoint, chronic organ dysfunction is caused by the dysregulation of physiological homeostasis and its repair processes. The vascular system, in particular, plays a crucial role in organ homeostasis because it is essential for organ and tissue construction, supplies oxygen and nutrients, and mobi-

lizes inflammatory cells to regions of injury^{3,4}. Ischemic diseases are caused by reductions in the blood supply due to atherosclerosis or thrombosis. On the other hand, while regeneration of the vasculature is a hoped-for treatment of ischemic diseases, dysregulated angiogenesis can exacerbate malignant tumors, diabetic retinopathy, and chronic inflammatory diseases. It was also recently demonstrated that vascular senescence and failure are closely associated with heart disease, cerebrovascular disease, cancer, and chronic inflammation⁵. In addition, the vascular endothelial system can be considered the largest system in the body, given its length and area, and the vascular endothelial cells, through their active secretion of bioactive molecules, play central roles in vascular homeostasis⁶⁻⁸. Revealing the mechanisms underlying the functional integrity of the vascular endothelial system could serve as the basis for novel approaches to therapy.

Adrenomedullin (AM) is a bioactive peptide first identified in human pheochromocytoma⁹. Although AM is now known to be secreted from various organs and tissues, it is mainly produced by vascular endothelial cells and serves numerous physiological functions.

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AM initially attracted attention as a vasoactive peptide that induced vasodilation and lowered blood pressure⁹. In subsequent studies, however, AM was shown to also have anti-inflammatory, anti-apoptotic, and anti-oxidative properties, to name a few¹⁰⁻¹². In addition, plasma AM levels are elevated in patients with hypertension, congestive heart failure, or myocardial infarction^{11, 13}, suggesting that AM is involved in these diseases in some way.

We previously reported that AM knockout (AM^{-/-}) mice die in utero at mid-gestation because of abnormalities in their vasculature structure¹⁴. From that result, it was apparent that AM is indispensable for proper vascular development. Heterozygous AM knockout (AM^{+/-}) mice grow to adulthood and exhibit no obvious abnormality at first glance. However, upon injury to the cardiovascular system, subsequent cardiac hypertrophy and fibrosis, renal failure, and arteriosclerosis are all worse in AM^{+/-} mice than control mice. By contrast, we also observed that AM exerts organ-protective and anti-arteriosclerotic effects, and that blood vessel-specific AM transgenic mice are resistant to organ damage and arteriosclerosis¹⁵⁻¹⁷. On the basis of these observations, AM is now thought of as a pleiotropic bioactive molecule, and its clinical application is much anticipated^{12, 18}.

Calcitonin gene related-peptide (CGRP) is in the same peptide family as AM and shares with AM a seven transmembrane domain G protein-coupled receptor, called calcitonin receptor-like receptor (CLR)^{19, 20}. The specificity of CLR for AM and CGRP is thought to be regulated by receptor activity-modifying proteins (RAMPs), which are membrane proteins having a single membrane-spanning domain. Three RAMP subtypes (RAMP1, 2, and 3) have been identified. By interacting with RAMP1, CLR acquires high affinity for CGRP; by interacting with RAMP2 or RAMP3, CLR acquires high affinity for AM.

Despite its potential for therapeutic application, use of AM as a therapeutic drug is limited by its short half-life in the blood stream. However, we noted that the expression of RAMP2 was particularly high in vascular endothelial cells in embryonic AM^{-/-} mice just before they die. This prompted us to speculate that RAMP2 is the primary determinant of AM functionality in blood vessels. Consistent with that idea, we previously observed that homozygous RAMP2 knockout (RAMP2^{-/-}) mice die mid-gestation because of vascular abnormalities with systemic edema and bleeding, just as AM^{-/-} mice do²¹. Moreover, another group reported the effect of RAMP2 overexpression using transgenic mice, which overexpress RAMP2 in their vascular smooth muscle cells. These mice showed ele-

vated vasodilatory action against AM administration²². On the basis of these findings, we have worked to clarify the physiological functions of the AM-RAMP2 system in the vasculature by generating and utilizing a series of genetically engineered mice. In this review, we focus on the AM-RAMP2 system of vascular endothelial cells.

Functional Biology of AM in Endothelial Cells

AM is a member of the calcitonin (CT) peptide superfamily, which includes CT, CGRP, amylin (AMY), and intermedin¹¹. AM is secreted in a wide array of organs and tissues and is involved in a variety of biological functions^{12, 23, 24}, including exertion of protective effects against cardiovascular damage, possibly through inhibition of oxidative stress. Plasma AM levels are increased in conditions such as essential hypertension, renal failure, and congestive heart failure, and the magnitude of the elevation is in proportion to the severity of the disease^{11, 25, 26}. The role of AM in endothelial cell growth and survival has also been reported^{12, 27}. AM upregulates the expression of both vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS)^{16, 28}, and it induces phosphorylation/activation of Akt and extracellular signal-regulated kinase (ERK)1/2. It also induces tube formation in human umbilical vein endothelial cells (HUVECs) an effect inhibited by pretreatment with a phosphoinositide 3-kinase (PI3K) inhibitor or a ERK1/2 inhibitor, and it exerts angiogenic effects through the activation of Akt, MAPK, and focal adhesion kinase in endothelial cells^{12, 29}.

AM is known to regulate vascular permeability *in vitro* and *in vivo*^{21, 30}, in part by preventing endothelial contraction and intercellular gap formation. Molecules that increase intracellular cAMP within endothelial cells strengthen barrier function and reduce endothelial permeability^{30, 31}. AM exerts its effects by increasing the levels of intracellular cAMP via its receptors^{19, 32}. In addition, small GTPases, particularly Rac1 and RhoA, play crucial roles in the regulation of endothelial cell barrier function by regulating the formation of cortical actin and stress fibers, respectively³¹. Elevation of cAMP leads to Rac1 activation, which in turn strengthens endothelial barrier function by enhancing cortical actin ring formation³³. AM-stimulated endothelial cells exhibit cortical actin formation, which disappears upon treatment with a Rac1 inhibitor³⁴.

Fig. 1. E-RAMP2^{-/-} mice show high perinatal mortality with systemic edema and the severe deformation of endothelial cells (cited from reference³⁴).

(A) Appearance of perinatal control and E-RAMP2^{-/-} mice. E-RAMP2^{-/-} mice showed severe systemic edema. (B, C) Electron micrographs of aortas from control and E-RAMP2^{-/-} mouse embryos. E-RAMP2^{-/-} endothelial cells showed deformities and were partially detached (arrows). Scale bars = 5 μ m.

Receptor System Used by AM and its Family Peptides

The main body of the AM receptor is CLR, a 7-transmembrane domain G protein-coupled receptor. CLR associates with one of the three RAMP subtypes, which determines the affinity of CLR for its ligands^{19, 20}. CT receptor and CLR interact with multiple ligands from the CT peptide family, the members of which are CT, CGRP, AM, AMY, and intermedin. CT family peptides have diverse and overlapping physiological actions that include decreasing bone resorption (CT), vasodilation (CGRP and AM) and decreased nutrient intake (AMY), to name just three^{11, 35}. A new member, intermedin (also called AM2), which also interacts with the CLR-RAMP complex, was recently added to the family^{36, 37}. The CT receptor does not require RAMP to bind and respond to CT, but it can associate with RAMPs. This results in a set of receptors that typically have a high affinity for AMY and varied affinity for CGRP¹¹.

RAMP expression is also modulated by various agents in cell culture and in animal models of human disease³⁸. For example, marked changes induced in the cardiovascular and renal systems provide evidence of an important role for dynamic RAMP regulation in those systems. Studies suggest that regulation of RAMPs can modulate the pathophysiology of conditions linked to RAMP-interacting G protein-coupled receptors. For example, human single nucleotide polymorphism (SNP) study described the relationship between CLR and essential hypertension³⁹. Another human

study revealed a relationship between RAMP1 SNPs and the incidence of cerebral infarction⁴⁰ and the mutation of the human RAMP3 gene has also been reported⁴¹.

Targeting Genes in the Endothelial AM-RAMP2 System in Mice

Genetically engineered knockout mice have proven to be a powerful tool with used to identify and manipulate various mechanisms and pathways and have the potential to shed light on novel targets for therapy. We showed that RAMP2^{-/-} mice have a lethal phenotype similar to that of AM^{-/-} mice, suggesting that the AM-RAMP2 system is specifically involved in vascular development²¹. To directly clarify the physiological functions of the vascular AM-RAMP2 system, we used the Cre-loxP system to generate vascular endothelial cell-specific AM and RAMP2 knockout mouse lines, including vascular endothelial cell-specific RAMP2^{-/-} (E-RAMP2^{-/-}) mice, drug-inducible E-RAMP2^{-/-} (DI-E-RAMP2^{-/-}) mice, and vascular endothelial cell-specific AM^{-/-} (E-AM^{-/-}) mice, as well as aged conventional RAMP2 heterozygous knockout (RAMP2^{+/-}) mice³⁴. Using these mice, we were able to determine both the acute and chronic effects of RAMP2 deletion and to demonstrate the contribution made by the AM-RAMP2 system to the maintenance of vascular integrity and organ homeostasis.

Whereas conventional RAMP2^{-/-} embryos die mid-gestation (around 14.5 dpc)^{21, 42}, E-RAMP2^{-/-} embryos survive until later in development, though

Fig. 2. E-RAMP2^{-/-} mice show progressive organ damage (cited from reference³⁴).

(A) Livers showed cirrhosis-like changes in 6-month-old E-RAMP2^{-/-} mice.

(B) Cardiac enlargement was seen in 6-month-old E-RAMP2^{-/-} mice.

(C-E) Histological abnormalities in kidneys from E-RAMP2^{-/-} mice. (C) Section of whole E-RAMP2^{-/-} kidney showing an enlarged renal pelvis. (D) Section of renal cortex from an E-RAMP2^{-/-} kidney showing polycystic changes. Scale bars = 100 μ m. (E) Enlargement of the glomerular area in an E-RAMP2^{-/-} kidney. Scale bars = 50 μ m.

most die during the perinatal period. Perinatal stage E-RAMP2^{-/-} mice exhibit systemic edema (**Fig. 1A**) as well as vascular abnormalities, including the malformation of aortic endothelial cells and partial detachment of endothelial cells from the basement membrane (**Fig. 1B, C**). In E-RAMP2^{-/-} embryos, the lesion is limited in the endothelial cells, which likely accounts for their longer survival. Nonetheless, most E-RAMP2^{-/-} mice die because of edema, reflecting the endothelial abnormality and resultant vascular leakage. Clearly, endogenous RAMP2 is essential for endothelial cell viability and vascular integrity. In contrast to E-RAMP2^{-/-} neonates, E-AM^{-/-} neonates appear healthy and present no overtly pathophysiological phenotypes³⁴. This is likely because AM is also secreted from cells other than endothelial cells, and circulating and/or paracrine AM from these other sources would compensate for the endothelial AM deficiency during development.

Among the 5% of E-RAMP2^{-/-} mice that survive until adulthood, RAMP2 expression in aortic endothelial cells is 20% of that in the littermate con-

trols. Within the surviving E-RAMP2^{-/-} adults, the aortic smooth muscle layers are disarrayed, and the endothelial cells are detached from the basement membrane and are severely deformed. These phenomena suggest congenital endothelial cell abnormalities induce a secondary postnatal disorder affecting other vascular component cells. Furthermore, E-RAMP2^{-/-} adults show marked accumulation of inflammatory cells along the blood vessels within major organs, and the chronic vascular damage due to this inflammation likely accelerates vascular senescence⁵. Although the neonates were normal, E-AM^{-/-} adults showed similar organ damage. Consistent with that idea, 2-year-old RAMP2^{+/-} mice also show inflammatory cell infiltration of their main organs. More interestingly, organ damage developed spontaneously in E-RAMP2^{-/-} adults. E-RAMP2^{-/-} adults developed organ damage that included cirrhosis-like changes to the liver (**Fig. 2A**), cardiac enlargement with fibrosis (**Fig. 2B**), hydronephrosis, renal polycystic changes, and glomerulosclerosis (**Fig. 2C-E**). Taken together, these findings suggest endothelial RAMP2 deficiency is a primary

cause of subsequent vascular inflammation, fibrosis, and chronic organ dysfunction.

We also generated a line of DI-E-RAMP2-/- mice, in which we were able to induce endothelial cell-specific RAMP2 gene deletion on demand. DI-E-RAMP2-/- mice exhibit marked systemic edema caused by increased vascular permeability. DI-E-RAMP2-/- mice did not show edema before the induction of Cre recombinase. However, after the 5 day administration of tamoxifen to induce Cre recombinase, DI-E-RAMP2-/- mice showed visible edema from day 5, mainly in the lower half of the body. The edema gradually extended to the upper half of the body and face, and significant body weight gain compared with control mice was detected from 1 week. When we examined the function of the AM-RAMP2 system during the early stages of retinal angiogenesis⁴³, we found that DI-E-RAMP2-/- mice retinas showed abnormal vessel development, with delayed vessel progression in the periphery and excessive branching at the vascular front. Actin polymerization in DI-E-RAMP2-/- endothelial cells also appeared in disarray, and there was a loss of actin-bundle formation under the plasma membrane. When we analyzed the activation of Rac1 and RhoA in DI-E-RAMP2-/- endothelial cells, we found that levels of the activated form of Rac1 (Rac1-GTP) were significantly reduced, whereas the activated form of RhoA (RhoA-GTP) was increased. These observations suggest the AM-RAMP2 system regulates the Rac1-GTP/RhoA-GTP ratio and thus cortical actin formation, and that a defect in this system disrupts actin formation and endothelial barrier function. With DI-E-RAMP2-/- mice, we were thus able to clarify both the acute and chronic effects of RAMP2 deletion and demonstrate that RAMP2 is essential for endothelial cell viability, vascular integrity, and homeostasis.

Apart from its maintenance of endothelial function, AM also reportedly possesses lymphatic endothelial activity. It was reported that secondary lymphedema induced in the hind-limb skin incision model was more severe in AM+/- mice, and the edema was prevented when circulating AM levels were restored by the systemic delivery of exogenous peptide⁴⁴. In addition, drug-induced knockout of CLR has been reported to cause chronic edema and dermal lymphatic insufficiency in adult mice⁴⁵. These reports suggest that AM-CLR signaling is important for the maintenance of normal lymphatic vessels; however, in both AM and CLR knockout mice, all of the signaling mediated by RAMPs (RAMP1-3) are functionally deleted, as either the ligand or receptor is absent.

Because RAMP2 associates with multiple G protein-coupled receptors beyond CLR, it is possible that

the vascular phenotypes of RAMP2-/- mice can be attributed to additional signaling pathways, and this notion is further supported by more severe phenotypes of RAMP2+/- mice compared with CLR+/- mice⁴⁶. To determine which RAMP is fundamentally important for the regulation of lymphatic vessels by AM, it was necessary to separately delete each RAMP isoform. Recently, we reported the functional differentiation of RAMP2 and RAMP3 by using each knockout mice. We found that RAMP3 knockout mice (RAMP3-/-) showed more severe post-operative lymphedema compared with wild-type mice, which was not observed in lymphatic endothelial cell-specific RAMP2-/-⁴⁷. Moreover, we previously reported that AM-/- mice and RAMP2-/- mice die in utero because of the same defect in vascular development²¹. In contrast, RAMP1 knockout mice and RAMP3 knockout mice were born normally^{48, 49}. We also found that aged RAMP2+/- mice spontaneously develop severe organ fibrosis with accelerated vascular senescence in spite of the compensatory upregulation of AM³⁴. These findings strongly support that RAMP2 is the key determinant of the vascular homeostasis regulated by AM.

Concluding Remarks and Future Perspectives

AM, originally identified as a vasodilating peptide, is now recognized to be a pleiotropic molecule involved in both circulatory homeostasis and the pathogenesis of cardiovascular diseases. Because of the wide range of its bioactivity, AM has been attracting attention for its potential clinical applications. But like other bioactive endogenous peptides, the clinical applicability of AM has limitations, the most obvious of which is its very short half-life in the blood, which makes its use impractical for the treatment of chronic diseases. However, it is noteworthy that we were able to modulate vascular AM function by manipulating RAMP2. This suggests RAMP2, in particular, to be a therapeutic target through which the vascular function of AM can be manipulated. Because RAMPs are low-molecular-weight proteins, the structural analysis and synthesis of specific agonists or antagonists are much more realistic for RAMPs than for CLR, a 7-transmembrane domain G protein-coupled receptor.

In sum, the vascular AM-RAMP2 system plays critical roles in the regulation of vascular integrity, including the maintenance of vascular structure, regulation of angiogenesis, and vasoprotection against vascular injury. Studies of AM and RAMP2 should bring about novel approaches to the treatment of diseases arising from vascular failure.

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Conflicts of Interest

None.

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