Vascular Endothelial Adrenomedullin-RAMP2 System Is Essential for Vascular Integrity and Organ Homeostasis

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Background—Revealing the mechanisms underlying the functional integrity of the vascular system could make available novel therapeutic approaches. We previously showed that knocking out the widely expressed peptide adrenomedullin (AM) or receptor activity-modifying protein 2 (RAMP2), an AM-receptor accessory protein, causes vascular abnormalities and is embryonically lethal. Our aim was to investigate the function of the vascular AM-RAMP2 system directly.

Methods and Results—We generated endothelial cell–specific RAMP2 and AM knockout mice (E-RAMP2−/− and E-AM−/−). Most E-RAMP2−/− mice died perinatally. In surviving adults, vasculitis occurred spontaneously. With aging, E-RAMP2−/− mice showed severe organ fibrosis with marked oxidative stress and accelerated vascular senescence. Later, liver cirrhosis, cardiac fibrosis, and hydronephrosis developed. We next used a line of drug-inducible E-RAMP2−/− mice (DI-E-RAMP2−/−) to induce RAMP2 deletion in adults, which enabled us to analyze the initial causes of the aforementioned vascular and organ damage. Early after the induction, pronounced edema with enhanced vascular leakage occurred. In vitro analysis revealed the vascular leakage to be caused by actin disarrangement and detachment of endothelial cells. We found that the AM-RAMP2 system regulates the Rac1-GTP/RhoA-GTP ratio and cortical actin formation and that a defect in this system causes the disruption of actin formation, leading to vascular and organ damage at the chronic stage after the gene deletion.

Conclusions—Our findings show that the AM-RAMP2 system is a key determinant of vascular integrity and homeostasis from prenatal stages through adulthood. Furthermore, our models demonstrate how endothelial cells regulate vascular integrity and how their dysregulation leads to organ damage. (Circulation. 2013;127:842-853.)

Key Words: aging ▪ arteriosclerosis ▪ edema ▪ endothelium ▪ vasculature

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Clinical Perspective on p 853

Vascular endothelial cells (ECs) and vasoactive molecules play key roles in the maintenance of vascular homeostasis.14 ECs actively secrete a variety of bioactive molecules, including nitric oxide, atrial natriuretic peptide, prostacyclin, and adrenomedullin (AM). Originally identified as a vasodilating peptide isolated from human pheochromocytoma,5 AM is now known to be widely secreted from a variety of organs and tissues, including ECs, and to be involved in a number of biological functions.6-13 Plasma levels of AM are elevated in patients with such cardiovascular diseases as hypertension and congestive heart failure.14,15 Moreover, it was recently...
reported that plasma AM levels are a highly sensitive marker of chronic kidney disease that may be predictive of its prognosis.\textsuperscript{16} We previously showed that homozygotic AM knockout (KO) (AM\textsuperscript{-/-}) mice die at midgestation as a result of edema and hemorrhage and clarified the critical role of AM in angiogenesis.\textsuperscript{25} Heterozygotic AM KO (AM\textsuperscript{+/+}) mice grow to adulthood with no apparent deficits but show accelerated cardiac hypertrophy, fibrosis, renal failure, and arteriosclerosis on cardiovascular injury. Given these observations, the clinical application of AM has been much anticipated\textsuperscript{18-22}; however, AM is a peptide with a short half-life in the bloodstream, which limits its usefulness for the treatment of chronic diseases.

To overcome that limitation, we have been focusing on the receptor system of AM. AM is a member of the calcitonin superfamily and acts via a G protein–coupled 7-transmembrane domain receptor, calcitonin receptor–like receptor.\textsuperscript{23,24} The specificity of calcitonin receptor–like receptor for its ligands is regulated by a group of 3 receptor activity–modifying proteins, RAMP1, -2, and -3. We have shown that homozygotic RAMP2 KO (RAMP2\textsuperscript{-/-}) mice die in utero. Interestingly, among the RAMP KO mice, RAMP2\textsuperscript{-/-} mice die of vascular abnormalities similar to those observed in AM\textsuperscript{-/-} mice.\textsuperscript{25} This suggests that RAMP2 is the key determinant of the vascular function of AM.

Our aim in the present study was to clarify the pathophysiological function of the vascular AM-RAMP2 system directly. To accomplish that, we generated and used 4 KO models: EC-specific (E)-RAMP2\textsuperscript{-/-} (E-RAMP2\textsuperscript{-/-}) embryos and mice expressing tamoxifen-inducible (DI)-E-RAMP2\textsuperscript{-/-}, and EC-specific (E)-AM\textsuperscript{-/-} (AM\textsuperscript{-/-}) embryos and mice expressing tamoxifen-inducible Cre-recombinase (Cre-ERT2) under the regulation of VE-cadherin promoter were crossed with floxed RAMP2 mice (RAMP2\textsuperscript{flox/flox}) to create vascular EC-specific RAMP2 conditional KO mice (E-RAMP2\textsuperscript{-/-}) and tamoxifen drug-inducible (DI)-E-RAMP2\textsuperscript{-/-}, and EC-specific (E)-AM\textsuperscript{-/-} (AM\textsuperscript{-/-}) mice. With these models, 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.

### Methods

#### Mouse Models

Vascular endothelial cadherin (VE-cadherin) Cre transgenic mice and mice expressing tamoxifen-inducible Cre-recombinase (Cre-ERT2) under the regulation of VE-cadherin promoter were crossed with floxed RAMP2 mice (RAMP2\textsuperscript{flox/flox}) to create vascular EC-specific RAMP2 conditional KO mice (E-RAMP2\textsuperscript{-/-}) and tamoxifen drug-inducible (DI) vascular EC-specific RAMP2 KO mice (DI-E-RAMP2\textsuperscript{-/-}), respectively.

#### Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction Analysis

The primers and probes used are listed in Table I in the online-only Data Supplement. For other experimental procedures, please refer to the online-only Data Supplement.

### Results

#### Generation of Vascular EC-Specific RAMP2 KO Mice

VE-cadherin Cre transgenic mice were crossed with floxed RAMP2 mice (RAMP2\textsuperscript{flox/flox}) to create vascular EC-specific RAMP2 conditional KO mice (E-RAMP2\textsuperscript{-/-}; Figure 1A). Conventional RAMP2\textsuperscript{-/-} embryos died at midgestation (=14.5 days after conception), as we reported previously.\textsuperscript{25} In contrast, EC-specific RAMP2\textsuperscript{-/-} (E-RAMP2\textsuperscript{-/-}) embryos survived until later in development, although most died during the perinatal period (Figure 1B). Among the RAMP2\textsuperscript{flox/flox} Cre\textsuperscript{ERT2} intercrosses, the estimated survival rate of E-RAMP2\textsuperscript{-/-} mice at 4 weeks of age was <5% (Table II in the online-only Data Supplement). Perinatal-stage E-RAMP2\textsuperscript{-/-} mice exhibited systemic edema (Figure 1C and 1D), as well as interstitial edema within the intestinal villi (Figure 1E) and lung (Figure 1F) and severe hemorrhagic changes within the liver (Figure 1G). As expected, E-RAMP2\textsuperscript{-/-} mice showed vascular abnormalities, including malformation of aortic ECs (Figure 1H) and partial detachment of ECs from the basement membrane (Figure 1I and 1J).

#### Abnormalities in the Vascular Structure of Surviving E-RAMP2\textsuperscript{-/-} Adults

In the 5% of E-RAMP2\textsuperscript{-/-} mice that survived until adulthood, RAMP2 expression in aortic ECs was 20% of that in their littermate controls (data not shown). The surviving E-RAMP2\textsuperscript{-/-} adults also exhibited thinning of the aortic wall and enlarged aortic diameters (Figure 1K and 1L). Both systolic and diastolic blood pressures were lower in E-RAMP2\textsuperscript{-/-} than control mice (Figure 1M). Electron microscopic observation revealed that the aortic smooth muscle layer was in disarray in E-RAMP2\textsuperscript{-/-} mice (Figure 1O) and that the ECs were detached from the basement membrane and severely deformed (Figure 1N). These morphological changes in the vascular cells may partially explain the enlargement of the aorta and reduction in blood pressure. Given these observations, we speculated that EC viability was diminished in E-RAMP2\textsuperscript{-/-} mice, which was confirmed by the severe disruption of angiogenesis seen in vivo aortic ring assays (Figure 1P).

#### Severe Vascular Inflammation and Organ Fibrosis in E-RAMP2\textsuperscript{-/-} Adults

Vasculitic lesions developed spontaneously in surviving E-RAMP2\textsuperscript{-/-} adults, beginning when they were 6 months old, and severe infiltration and accumulation of inflammatory cells were observed in blood vessels within the major organs, including the liver (Figure 2A), kidneys (Figure 2B), and lungs (Figure 2C). The infiltrating cells were CD3\textsuperscript{+} or CD8\textsuperscript{+}, which is indicative of T cells and macrophages, respectively (Figure 2D and 2E). In addition, the expression of inflammatory cytokines and macrophage markers was upregulated (Figure 2F).

We speculated that the vascular damage in the surviving adult E-RAMP2\textsuperscript{-/-} mice was the primary cause of the vasculitis. In that regard, it was recently suggested that vascular damage may be the cause of vascular senescence.\textsuperscript{26,27} To test whether these phenomena indicate accelerated senescence, we next evaluated aged conventional RAMP2 KO mice (RAMP2\textsuperscript{+/+}). At 2 years of age, RAMP2\textsuperscript{+/+} mice showed inflammatory cell infiltration of the main organs (Figure 2G–2I). Senescence-associated (SA)-β-gal staining, which is commonly used to assess accelerated aging, was more intense in the aortas of the aged RAMP2\textsuperscript{+/+} than WT mice (Figure 2J). Moreover, the SA-β-gal staining could be detected at a much younger age (6 months) in E-RAMP2\textsuperscript{-/-} mice (Figure 2K).

The presence of activated Akt and p53 in the aorta is another recognized marker of vascular senescence.\textsuperscript{28}
Levels of Akt and p53 activation were much greater in aged RAMP2+/− than WT mice (Figure 2L). No difference was seen between the levels of Akt and p53 activation in younger RAMP2+/− and WT mice, although the inflammatory cell adhesion markers intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 were already upregulated in the young RAMP2+/− animals. This is consistent with the idea that vascular senescence is preceded by vascular damage and inflammation.

More interestingly, organ damage developed spontaneously in E-RAMP2−/− adults. In particular, the livers of E-RAMP2−/− mice appeared cirrhotic by the time the animals were 6 months old (Figure 3A). Pathological analysis revealed that the cirrhosis-like changes were not caused by damage to parenchymal hepatocytes but by severe fibrosis along the vasculature within the liver, most was likely due to dysfunction of the sinusoidal ECs (Figure 3B and 3C). Cardiac enlargement with interstitial fibrosis was also seen in 6-month-old E-RAMP2−/− mice (Figure 3D and 3E). In addition, quantitative real-time polymerase chain reaction showed elevated expression of atrial natriuretic peptide, brain natriuretic peptide, and transforming growth factor-β, which is indicative of heart failure and fibrosis (Figure 3F). In addition, spontaneous hydronephrosis (Figure 3G) and polycystic changes (Figure 3H) were noted in the kidneys of E-RAMP2−/− mice, as were glomerular enlargement and sclerotic changes (Figure 3I). Electron microscopy revealed podocyte fusion, hyperplasia of the basal membrane, and partial dehiscence between the glomerular ECs and basal membrane (Figure 3J). Renal expression of transforming growth factor-β and collagen was also upregulated in E-RAMP2−/− mice (Figure 3K).

**Figure 1.** E-RAMP2−/− mice showed systemic edema and high perinatal mortality, whereas surviving adult mice showed vascular abnormalities. A, Strategy of conditional gene targeting of mouse receptor activity-modifying protein 2 (RAMP2). A RAMP2-floxed mouse was crossbred with a vascular endothelial (VE) cadherin promoter-driven Cre recombinase-expressing mouse (bottom) to generate vascular endothelial cell (EC)-specific RAMP2−/− mice (E-RAMP2−/−). B, E-RAMP2−/− survival curve. E-RAMP2−/− embryos generally survived at least until the perinatal period. C and D, E19.5 E-RAMP2−/− embryos showed systemic edema (C) with increased body weight (D). n=11 for both control and E-RAMP2−/−. Bars are means±SEM. **P<0.01. E through G, Hematoxylin and eosin staining of the intestine (E), lung (F), and liver (G) from E19.5 RAMP2flox/flox (Cont) and E-RAMP2−/− mice. Scale bars=50 mm (E), 400 mm (F), and 200 mm (G). H through J, Electron micrographs of aortas from E19.5 control and E-RAMP2−/−. E-RAMP2−/− ECs were detached (arrows). Scale bars=5 μm. K and L, Hematoxylin and eosin staining of aortas from adult control and E-RAMP2−/−. Scale bars=300 μm (K) and 50 μm (L). M, Blood pressures in adult control and E-RAMP2−/− mice. DBP indicates diastolic blood pressure; and SBP, systolic blood pressure. n=12 for control and n=9 for E-RAMP2−/−. Bars are means±SEM. **P<0.01. N and O, Electron micrographs of adult aortas. E-RAMP2−/− aortas showed detachment of ECs (N) and a disordered smooth muscle layer (O). Scale bars=5 μm. P, Ex vivo analysis of angiogenesis using adult aortic ring specimens after 14 days of culture in collagen gel.

**EC-Specific AM KO Mice Showed Vascular Inflammation and Glomerulosclerosis**

We next compared EC-specific AM KO (E-AM−/) with E-RAMP2−/−. In contrast to E-RAMP2−/− neonates, E-AM−/− neonates were healthy and presented no overtly pathological phenotypes (Table III in the online-only Data Supplement). We suggest that in E-AM−/− neonates, circulating and/or paracrine AM from sources other than ECs likely attenuates the phenotypes observed in E-RAMP2−/− neonates. In adults, however, the vascular inflammation and organ damage were similar to that seen in E-RAMP2−/− mice. E-AM−/− mice showed severe infiltration and accumulation of inflammatory cells around the vasculature (Figure 4A and 4B). They also showed glomerulosclerotic changes with mesangial expansion and upregulation of inflammatory molecules (Figure 4C–4E).
RAMP2 Deficiency Enhances Inflammatory Reactions in Both ECs and Leukocytes

We speculated that the observed vasculitic lesions began with the cellular adhesion of inflammatory cells to ECs. To test that idea, we initially examined the effect of endothelial RAMP2-deficiency on the interaction between ECs and macrophages in vitro. After isolating and culturing liver sinusoidal ECs (LSECs) from RAMP2+/− and WT mice, we evaluated the attachment of macrophages isolated from green fluorescent protein mice (provided by Dr Masahiro Sato of Kagoshima University). We found that green fluorescent protein–positive macrophages attached more readily to RAMP2+/− LSECs than to WT LSECs (Figure 5A and 5B) and that vascular cell adhesion molecule-1 expression was higher in RAMP2+/− LSECs than in WT cells (Figure 5C).

To then assess the effect of RAMP2-deficiency on the leukocytes, we crossed breeding DsRed+ (provided by Dr Yoh-Ichi Tagawa of the Tokyo Institute of Technology) with RAMP2+/− mice to generate a DsRed+RAMP2+/− mouse line. We found that green fluorescent protein–positive macrophages attached more readily to RAMP2+/− LSECs than to WT LSECs (Figure 5A and 5B) and that vascular cell adhesion molecule-1 expression was higher in RAMP2+/− LSECs than in WT cells (Figure 5C).

RAMP2 Deficiency Increases Oxidative Stress Whereas RAMP2 Overexpression Leads to Resistance to Senescence Under Conditions of Oxidative Stress in ECs

It has been reported that AM exerts antioxidant effects. In 6-month-old E-RAMP2−/− mice, we detected elevated levels of oxidative stress in the major organs. Levels of 4-hydroxy-2-nonenal immunostaining, which reveals the presence of peroxides of unsaturated fatty acids, were increased in E-RAMP2−/− (Figure 5K). Consistent with this finding, the expression of 3 NADPH oxidase subunits (p22-phox, p47-phox, and p67-phox) was upregulated in RAMP2−/− macrophages (Figure 5L). Thus, RAMP2 deficiency in ECs appears to exacerbate oxidative stress.

We next used EAhy926 ECs to generate EC lines stably overexpressing RAMP2. H2O2 treatment has been shown...
to induce EC senescence.\textsuperscript{29} We found that after incubation for 24 hours in the presence of H\textsubscript{2}O\textsubscript{2} plus AM, control cells were strongly stained by SA-β-gal, but cells overexpressing RAMP2 were resistant to staining (Figure 5M).

**Initial EC Change Elicited by RAMP2 Deletion in Drug-Inducible EC-Specific RAMP2 KO Mice**

As mentioned, most E-RAMP2\textsuperscript{−/−} mice died as neonates, and analysis using adult mice was limited. Therefore, we next generated a line of drug-inducible EC-specific RAMP2 KO (DI-E-RAMP2\textsuperscript{−/−}) mice (Figure 6A). With this model, we are able to selectively delete RAMP2 gene from the ECs of adult mice through administration of tamoxifen. In DI-E-RAMP2\textsuperscript{−/−}, endothelial RAMP2 expression was reduced to \(\approx 20\%\) of control, whereas adrenomedullin (AM) expression was upregulated to 200\% of control (Figure 6B). Interestingly, the tamoxifen-treated mice showed remarkable body weight gains with systemic edema (Figure 6C and 6D). The cause of the edema was enhanced vascular permeability and plasma leakage, as evidenced by the extravascular leakage of Evans Blue dye (Figure 6E). In a hind-limb ischemia model, which enables us to evaluate the postnatal angiogenic potency, we also found that blood flow recovery was delayed in DI-E-RAMP2\textsuperscript{−/−} (Figure 6F).

Next, we evaluated whether we could rescue the phenotype of DI-E-RAMP2\textsuperscript{−/−} by vascular overexpression of RAMP2 gene. We used the gene delivery method in the hind-limb ischemia model by electroporation of plasmid, which could overexpress RAMP2 under the control of VE-cadherin promoter (Figure I in the online-only Data Supplement). At first, we confirmed that the VE-cadherin promoter works only in vascular ECs; when VE-cadherin–enhanced green fluorescent protein plasmid was electroporated at the femoral region, the signal was detected only at the isolectin-positive ECs (Figure 6G). In addition, after 24 hours of electroporation of VE-cadherin-RAMP2 plasmid, we confirmed that RAMP2 expression was strongly induced in DI-E-RAMP2\textsuperscript{−/−} (Figure 6H). Actually, the RAMP2 gene delivery in DI-E-RAMP2\textsuperscript{−/−} kidneys was strongly induced in DI-E-RAMP2\textsuperscript{−/−} kidneys. In the E-RAMP2\textsuperscript{−/−} kidney, fusion of podocytes (top) and detachment of endothelial cells from the basement membrane (bottom) are apparent (arrows). Scale bars=1 μm. K, Quantitative real-time PCR analysis of control and E-RAMP2\textsuperscript{−/−} kidneys. Fibrotic markers were upregulated in E-RAMP2\textsuperscript{−/−} kidneys. n=4 for both control and E-RAMP2\textsuperscript{−/−}. Bars are means±SEM. *P<0.05. ANP indicates atrial natriuretic factor; BNP, brain natriuretic peptide; and TGF-β, transforming growth factor-β.

Using the DI-E-RAMP2\textsuperscript{−/−} line, we further evaluated the primary cause of the vascular failure. In electron micrographs of aortas obtained 2 weeks after RAMP2 deletion, ECs in DI-E-RAMP2\textsuperscript{−/−} mice appeared deformed and were detached from the basement membrane (Figure 6M). We speculated
that the cause was cytoskeletal abnormality within the ECs. Consistent with that idea, actin polymerization in DI-E-RAMP2−/− ECs appeared in disarray, and there was a loss of actin-bundle formation under the plasma membrane (cortical actin formation; Figure 6N). It has been reported that small GTPases, Rac1 and RhoA, play crucial roles in the regulation of the barrier function of ECs by regulating the formation of cortical actin and stress fibers.30 When we analyzed the activation of Rac1 and RhoA in DI-E-RAMP2−/− ECs, 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 (Figure 6O and 6P). On the other hand, AM treatment enhanced cortical actin formation in human umbilical vein ECs (Figure 6Q). Notably, the Rac1 inhibitor NSC23766 blocked the AM-induced enhancement of cortical actin and stress fibers. E-RAMP2−/− embryos, which die at midgestation, E-RAMP2−/− embryos survived until later stages of development. As we and other groups reported previously, in conventional RAMP2−/− embryos, deformity was detected in both the endothelium and the smooth muscle layer in blood and lymphatic vessels.25,31 In contrast, in the E-RAMP2−/− embryos used in this study, the lesion was limited in the ECs, which likely accounts for their longer survival. Nonetheless, most E-RAMP2−/− mice died as a result of edema, reflecting the endothelial abnormality and vascular leakage. Clearly, endogenous RAMP2 is essential for EC viability and vascular integrity.

About 5% of E-RAMP2−/− mice survived to adulthood. E-RAMP2−/− adults were apparently normal when young but showed EC deformity and reduced viability later. Interestingly, the cellular deformities in the adult animals were not limited to the endothelium but included the smooth muscle cell layer, which suggests that the congenital EC abnormalities induced secondary postnatal disorder of other vascular component cells. E-RAMP2−/− mice also had lower blood pressures than WT mice, which seems contradictory because AM is well known to be a vasodilator. We speculate that the reduction in blood pressure might be due to the observed morphological changes in the vascular cells. Another possibility is that our findings are consistent with the report that although AM is a vasodilator when injected peripherally, it is a vasoconstrictor when injected intracerebrally; that is, when endothelial RAMP2 deficiency affects both peripheral and central AM signaling, the expected central phenotype is observed.

E-RAMP2−/− adults also showed marked accumulation of inflammatory cells along the blood vessels within major organs, and the chronic vascular damage by such inflammation can accelerate vascular senescence.28,33–35 We found...
Figure 5. Receptor activity-modifying protein 2 (RAMP2) deficiency enhances endothelial cell (EC) inflammatory reactions and oxidative stress. A and B, Macrophage adhesion to ECs. Macrophage adhesion to liver sinusoidal ECs (LSECs) cultured from wild-type (WT) or RAMP2+/− mice was analyzed. RAMP2+/− LSECs showed greater adhesion, which was indicated by a higher cell count. Scale bars=100 μm. n=8 for both WT and RAMP2+/−. Bars are mean±SEM. *P<0.05. C, Quantitative real-time polymerase chain reaction (PCR) analysis of LSECs. n=6 for both WT and RAMP2+−. Bars are mean±SEM. **P<0.01; *P<0.05. D through G, To analyze the effect of RAMP2 deficiency on peripheral leukocytes and macrophages,uffy coat and peritoneal macrophages collected from DsRed/RAMP2+/+ or DsRed/RAMP2+/− mice were cultured with LSECs from WT mice. Attached DsRed+ cells were photographed (D and F) and counted (E and G). RAMP2+/− leukocytes (D and E) and macrophages (F and G) showed greater attachment than those of WT. Scale bars=100 μm. n=8 for both WT and RAMP2+/−. Bars are mean±SEM. **P<0.01; ***P<0.001. H and I, Effect of RAMP2 deficiency on phagocytosis of macrophages. Photographs (H) and dot plot (I) representing phagocytosis of FITC-labeled particles by macrophages (circles in the dot plot) are shown. Phagocytosis was not different between WT and RAMP2+/−. Scale bars=50 μm. J, Quantitative real-time PCR analysis of macrophages from RAMP2−/− and WT mice. n=8 in both WT and RAMP2−/−. Bars are mean±SEM. **P<0.01. K, Enhanced oxidative stress in E-RAMP2−/− mice. Immunostaining of unsaturated fatty acid peroxides was enhanced in E-RAMP2−/− liver and kidney. Particularly strong staining was detected around central veins in the liver and renal tubules in the kidney. Scale bars=200 μm. L, Quantitative real-time PCR analysis of NADPH-oxidase subunits in the kidney. n=6 for control (Cont) and n=3 for E-RAMP2−/−. Bars are means. *P<0.05. M, Suppression of oxidative stress–induced cellular senescence in ECs stably overexpressing (O/E) RAMP2. After 24 hours of treatment with H2O2 (200 μmol/L) and adrenomedullin (AM; 10−7 mol/L), senescence-associated β-galactosidase positivity was detected in the control EAhy926 endothelial cells but not RAMP2-overexpressing cells. Scale bars=100 μm. eNOS indicates endothelial nitric oxide synthase; IL, interleukin; and MCP-1, monocyte chemoattractant protein-1.
Figure 6. Analysis of vascular endothelial RAMP2 deficiency in adults using drug-inducible endothelial cell (EC)-specific RAMP2 knock-out mice (DI-E-RAMP2−/−). A, Strategy of drug-inducible conditional gene targeting of mouse RAMP2. A RAMP2-floxed mouse was crossbred with a vascular endothelial (VE)-cadherin promoter-driven, tamoxifen-inducible Cre recombinase transgenic mouse to generate drug-inducible EC-specific RAMP2−/− mice (DI-E-RAMP2−/−). B, Quantitative real-time polymerase chain reaction (PCR) analysis of liver sinusoidal ECs (LSECs) cultured primarily from DI-E-RAMP2−/− mice (2 weeks after treatment with tamoxifen or corn oil). In tamoxifen-treated mice, endothelial RAMP2 expression was reduced to ≈20% of control, whereas adrenomedullin (AM) expression was
that activation of aortic Akt and p53 and SA-β-gal staining, 2 indicators of vascular senescence, were enhanced in adult E-RAMP2−/− mice and that similar vasculitic lesions were present in aged conventional RAMP2−/− mice. The vascular lesions in E-RAMP2−/− mice led to organ damage, which included liver cirrhosis, cardiac enlargement with fibrosis, hydropnephrosis, polycystic changes in the kidney, and glomerulosclerosis. Taken together, these findings suggest that endothelial RAMP2 deficiency is the primary cause of subsequent vascular inflammation, accelerated senescence, fibrosis, and chronic organ dysfunction.

Unlike virus-related liver cirrhosis, in which the lesion starts within the hepatocytes, the cirrhotic changes seen in E-RAMP2−/− mice were caused by severe fibrosis that started in the vasculature. This is consistent with idea that vascular damage is the primary cause of this cirrhosis. In the E-RAMP2−/− kidney, both glomerular and interstitial changes were observed, along with epithelial cell damage (podocyte fusion) associated with glomerular EC deformity. And in the heart, cardiac enlargement and fibrosis led to heart failure. Thus, loss of EC viability and vascular integrity accelerated vascular senescence and exacerbated interstitial lesions, making it the primary cause of chronic organ dysfunction.

For this study, we also generated EC-specific AM KO mice (E-AM−/−). In contrast to E-RAMP2−/− neonates, the E-AM−/− neonates appeared healthy. It seems that the phenotype caused by knocking out the ligand during development is much milder than that caused by knocking out the receptor. The reason probably is that AM is secreted from cells other than ECs, and circulating and/or paracrine AM from these other sources compensate for the endothelial AM deficiency during development. In adults, however, the chronic reduction in AM signaling led to vascular inflammation and chronic organ damage in E-AM−/− mice, suggesting that aging is another important factor contributing to the emergence of the pathophysiological phenotype.

To better understand the mechanism by which endothelial RAMP2 deficiency could promote the aforementioned pathological features, we first characterized the inflammatory response triggered in RAMP2-deficient ECs. We recently reported that the expression of adhesion markers was downregulated by AM. In the absence of AM signaling in RAMP2-deficient ECs in the present study, these inflammatory adhesion molecules were upregulated, facilitating the attachment of macrophages. This inflammatory response, along with the partial detachment of ECs from the basement membrane, likely accelerates the attachment, transmigration, and accumulation of inflammatory cells within the vascular wall.
We also found high levels of oxidative stress, including elevation of NADPH oxidase levels, within the major organs of E-RAMP2−/− mice. It has been reported that AM exerts strong antioxidant effects, and in the present study, AM protected ECs stably overexpressing RAMP2 from cellular senescence induced by oxidative stress. This suggests that AM-RAMP2 signaling suppresses oxidative stress and that endothelial RAMP2 may be an effective target through which to regulate oxidative stress.

Because most E-RAMP2−/− mice die as neonates, analysis of adult animals has been limited. To overcome this limitation, we generated a drug-inducible EC-specific RAMP2 KO mouse (DI-E-RAMP2−/−). In this model, we can induce RAMP2 gene deletion in adults on demand and then analyze the changes induced by RAMP2 deletion. Interestingly, DI-E-RAMP2−/− mice exhibit marked systemic edema caused by increased vascular permeability. It is widely recognized that an increase in intracellular cAMP within ECs strengthens their barrier function and reduces endothelial permeability, both in vitro and in vivo. Inflammatory stimuli. In addition, small GTPases, especially Rac1 and RhoA, play crucial roles in the regulation of EC barrier function by regulating the formation of cortical actin and stress fibers, respectively. Elevation of cAMP leads to Rac1 activation, which in turn strengthens endothelial barrier function by enhancing cortical actin ring formation. We found that in DI-E-RAMP2−/− ECs, actin polymerization was disrupted and Rac1 activation was reduced. In contrast, AM-stimulated ECs showed cortical actin formation, which disappeared on treatment with a Rac1 inhibitor. Taken together, these findings suggest that in DI-E-RAMP2−/− ECs, downregulation of cAMP production and Rac1 activation reduces cortical actin formation, diminishing endothelial barrier function.

The relationship between the AM receptor and human disease has also been reported. A human single-nucleotide polymorphism study described the relationship between calcitonin receptor-like receptor with essential hypertension. A human study also revealed the relationship between RAMP1 single-nucleotide polymorphisms and the incidence of cerebral infarction. Some human mutations of RAMP3 gene have also been reported. On the other hand, there have been no reports on human RAMP2 gene mutation. As we have shown, RAMP2 deletion can cause embryonic lethality at midgestation, and this may be one reason that we cannot see human congenital disease. In this context, the EC-specific conditional KO mouse model in this study should be recognized as a model of vascular failure and relevant organ dysfunction and should not be recognized as a specific congenital disease model.

In this study, we were able to clarify both the acute and chronic effects of RAMP2 deletion in the adult and to demonstrate that the AM-RAMP2 system is essential for vascular integrity and organ homeostasis. The illustration in Figure 8 summarizes the series of phenomena stemming from RAMP2 deletion. Early after RAMP2 gene deletion, ECs show morphological changes resulting from actin filament abnormality (disappearance of the cortical actin ring and disarray of actin polymerization). The resultant EC deformation causes detachment, barrier dysfunction, enhanced vascular permeability, and edema, which in turn promote the attachment and infiltration of inflammatory cells. Chronic vascular inflammation induces vascular...
damage and accelerated senescence and enhances oxidative stress and organ fibrosis. Finally, the accumulated disorders cause chronic organ dysfunction with aging. The results obtained with these models demonstrate that vascular EC integrity is essential for organ homeostasis. We also suggest that RAMP2, through which we could modulate vascular integrity, is a potentially useful therapeutic target for the treatment of chronic organ dysfunction.

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Disclosures

None.

References


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**CLINICAL PERSPECTIVE**

Adrenomedullin (AM), originally identified as a vasodilating peptide, is now recognized to be a pleiotropic vasoactive molecule involved in both the pathogenesis of cardiovascular diseases and circulatory homeostasis. Because of its wide range of bioactivity, AM has been attracting attention for its potential clinical applications. On the other hand, the clinical applicability of AM, like that of other bioactive endogenous peptides, has limitations: AM has a very short half-life in the blood, which makes the use of AM impractical for the treatment of chronic diseases. It is noteworthy that we were able to modulate the vascular function of AM by modulating receptor activity-modifying protein 2 (RAMP2). RAMP2 in particular could be a therapeutic target by which to manipulate the vascular functions of AM. Because RAMPs are low-molecular-weight proteins, structural analysis and the synthesis of specific agonists or antagonists are much more realistic compared with the AM receptor calcitonin-receptor-like receptor, which belongs to 7-transmembrane domain G protein–coupled receptors. 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. In that context, studies of AM and RAMP2 should bring about novel approaches for the treatment of diseases derived from vascular failure. Conditional gene targeting models in this study, which enable the spatial and temporal modulation of the gene expression, could elucidate the detailed pathophysiological roles of the AM-RAMP2 system.
Vascular Endothelial Adrenomedullin-RAMP2 System Is Essential for Vascular Integrity and Organ Homeostasis

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Supplementary methods

Mouse models

RAMP2-/- mice were generated in our group1. Vascular endothelial cadherin (VE-cadherin) Cre transgenic mice2 and mice expressing tamoxifen-inducible Cre-recombinase (Cre-ERT2) under the regulation of VE-cadherin promoter3 were crossed with floxed RAMP2 mice (RAMP2flox/flox) to create vascular EC-specific RAMP2 conditional KO mice (E-RAMP2-/-) (Fig. 1a) and tamoxifen drug-inducible (DI) vascular EC-specific RAMP2 KO mice (DI-E-RAMP2-/-) (Fig. 6a), respectively. Tamoxifen (Sigma) was dissolved in corn oil (Sigma) to a concentration of 10 mg/ml, after which 1 mg was intraperitoneally injected into male mice (8 weeks old) daily for 5 days. Little toxicity was observed following the injection into wild-type (WT), RAMP2flox/flox, or Cre-ERT2 mice.

Mice with conditional KO of AM in ECs (E-AM-/-) were created by crossing floxed AM mice4 with transgenic mice expressing Cre recombinase under the control of the VE-cadherin promoter (Stock Number 006137, The Jackson Laboratory). All animal handling procedures were in accordance with a protocol approved by the Ethics Committee of Shinshu University and the CIBIR.

Cell isolation and culture

Primary adult mouse liver sinusoidal endothelial cells (LSECs) were isolated using a two-step collagenase perfusion and centrifugation method5. Inhibitors of Rac1 and ROCK used are NSC 23766 (SANTA CRUZ) and Y27632 (Wako).
A RAMP2 overexpressing (RAMP2 O/E) cell line was created using EAhy926 ECs (provided by Dr. Edgell of the University of North Carolina) as described previously. RAMP2 O/E and EAhy926 cells were treated with 200 µmol/L hydrogen peroxide ($H_2O_2$) (Sigma) for 24h and with $10^{-7}$ M AM (provided Dr. Kangawa of the National Cardiovascular Center Research Institute).

Buffy coat (combined leukocyte and platelet fractions) was isolated from blood samples collected from mice as described previously. Peritoneal macrophages were obtained from mice by injecting 2 ml of 5% thioglycolate (DIFCO) intraperitoneally. The elicited macrophages were harvested by peritoneal lavage 4 days after thioglycolate administration.

Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Kurashiki, Japan).

**Histology**

Each organ was fixed overnight in methanol or 4% paraformaldehyde, embedded in paraffin, and cut into 4-µm-thick sections for histological examination. The sections were then deparaffinized for hematoxylin/eosin, Masson’s trichrome, Sirius red and silver staining, and for immunohistochemistry. The antibodies used were rabbit anti-CD3 (A 0452, DAKO, Carpinteria, CA), rat anti-mouse F4/80 (Serotec) and rat anti-mouse 4-hydroxy-2-nonenal (4HNE) (NOF corporation). Whole aorta was stained for senescence-associated β-gal (SA-β-gal) as described previously.
**Transmission electron microscopy**

Specimens were fixed in 2.5% glutaraldehyde (pH7.2), embedded in epoxy resin (Epok) 812 (Oken shoji Co.), cut into ultrathin sections, double-stained with uranyl acetate and lead citrate, and examined in an electron microscope.

**Western blot analysis**

Western blot analysis was carried out using protein extracts from aorta. The lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-Akt, anti-phospho-Akt (p-Akt) (Cell Signaling Technology), anti-ICAM-1, anti-VCAM-1, anti-p53 and anti-β-actin (Santa Cruz Biotechnology) antibodies. The blots were then developed using a SNAP i.d. system (MILLIPORE, Billerica, MA).

**Quantitative real-time RT-PCR analysis**

Quantitative real-time RT-PCR was carried out using an Applied Biosystems 7300 real-time PCR System with SYBR green (Toyobo, Japan) or Realtime PCR Master Mix (Toyobo) and TaqMan probes (MBL). The primers and probes used are listed in Supplementary Table. 3. Values were normalized to mouse GAPDH (Pre-Developed TaqMan assay reagents, Applied Biosystems).

**Macrophage/leukocyte and LSEC adhesion assay**

For macrophage and LSEC adhesion assay, LSECs from WT and RAMP2+/− mice were seeded into 35-mm plates and incubated for 24 h, after which macrophages (10^5
cells) extracted from GFP+, DsRed/RAMP2 +/- or DsRed/RAMP2+/- mice were added. For leukocyte and LSEC adhesion assay, Buffy coat (10⁵ cells) extracted from DsRed/RAMP2+/- or DsRed/RAMP2+/- mice was added. After incubation for an additional 3 h, the cells were washed three times with PBS, and the macrophages were counted in 10 random fields/dish.

**Analysis of phagocytosis of macrophages**

In vitro phagocytic uptake studies were carried out using fluorescent microparticles (Polysciences, Inc.) in elicited macrophages according to the manufacturer’s protocol. In brief, 2 × 10⁵ of thioglycollate-elicited macrophages were mixed with 1 × 10⁶ opsonized fluorescent microparticles overnight and then washed with ice-cold PBS. Thereafter, phagocytosis was examined using a fluorescence microscope and analyzed by flow cytometry.

**Aortic ring assay**

The thoracic aorta was dissected from the posterior mediastinum and placed in serum-free EBM-2 endothelial basal medium (Cambrex). The vessel was then cut into 1-mm-long rings, which were subjected to 8 consecutive washes with serum-free EBM-2. The aortic rings were then embedded in thick collagen gel (Cellmatrix Type I-A; Nitta Gelatin) and cultured for 14 days, with or without recombinant hVEGF (50 ng/ml; R&D Systems).
**Rac1 and Rho pull-down assays**

The activation of Rac1 was assessed using a Rac1 activation kit (Enzo Life Science) according to the manufacturer’s instructions. The activation of Rho was assessed using a Rho Activation Assay kit (MILLIPORE) according to the manufacturer’s instructions.

**Vascular permeability assay**

Mice were anesthetized, after which 100 µl of 1% Evans Blue (Wako) were injected intravenously. Thirty minutes after injection, extravasation of the Evans Blue dye was visualized in back skin, ear and hindlimb.

**Unilateral hind-limb ischemia model**

DI-E-RAMP2/- mice and their controls were subjected to experimentally induced hind-limb ischemia produced by unilateral occlusion of the femoral artery, as described previously. Laser Doppler perfusion imaging (LDPI) (Omegazone, Omegawave, Inc., Tokyo, Japan) was used to evaluate the blood flow. The calculated perfusion was expressed as a ratio of the left (ischemic) to the right (normal) limb. Capillary density in the limbs at 11 days after surgery was assessed by fluorescence staining with isolectin GS-IB4 (Invitrogen). Interstitial edema area was calculated as the interval spaces within the muscles.

**Construction of Plasmid DNA**

Expression vector cassette, which contains a 2.53 kb 5’-flanking region of mouse
vascular endothelial (VE)-cadherin (pVE-Cont), was kindly provided by Dr. Huber (Supplementary Figure 1a). To generate RAMP2-gene delivery plasmid (pVE-mRAMP2), mouse RAMP2 ORF (mRAMP2) (0.57 kb) was inserted at the EcoRI site of the pVE-Cont (Supplementary Figure 1b). In this plasmid, the transcript of mRAMP2 cDNA is generated under the control of the VE-cadherin promoter. EGFP expression vector (pVE-EGFP) was also generated to confirm proper gene delivery (Supplementary Figure 1c).

**In vivo electroporation-mediated gene transfer**

Following the hind-limb ischemia operation, the mice were electroporated plasmids into their femoral regions. A volume of 20 μL of plasmid (2 mg/ml) was injected into the femoral muscle with a 29-gauge needle. Immediately after intramuscular injection of the plasmid, the muscle was held by an electrode and in vivo electroporation was performed with a pulse generator (BTX T820, BTX, San Diego, CA, USA, CUY 560-5, NEPA GENE, Chiba, Japan). The voltage, pulse length, and number of pulses of the electroporation were 100 V, 50 ms, and 3 pulses followed by 3 inverse pulses, respectively.

**Statistical analysis**

Statistical analysis was performed using Student's t test for data following an expected normal distribution and Mann Whitney U test where data was not normally distributed. Values are expressed as means ± SEM in Student's t test and means in Mann
Whitney U test. Values of *p<0.05 or **p<0.01 were considered significant.
Supplementary figure legends

Supplementary Table. 1
Result of the genotyping of pups from RAMP2\textsuperscript{flox/flox} Cre+/- intercrosses

Real numbers and estimated pup numbers of each genotype are shown. Estimated pup ratio of RAMP2\textsuperscript{flox/flox} Cre+ to RAMP2\textsuperscript{flox/flox} Cre- = 3:1 for the RAMP2\textsuperscript{flox/flox} Cre+/- intercrosses; therefore, the estimated number of RAMP2\textsuperscript{flox/flox} Cre+ = 207 (3 fold higher than the 69 of RAMP2\textsuperscript{flox/flox} Cre-). The estimated survival rate of RAMP2\textsuperscript{flox/flox} Cre+ pups (EC-specific RAMP2/- (E-RAMP2/-)) is calculated as 7/207 x 100 = 3.4%.

Supplementary Table. 2
Result of the genotyping of pups from AM\textsuperscript{flox/flox} Cre+/- with AM\textsuperscript{flox/flox} Cre-/- intercrosses

Unlike E-RAMP2/- pups, EC-specific adrenomedullin-/- (E-AM-/-) pups were all healthy and presented no overtly pathophysiological phenotype. In AM\textsuperscript{flox/flox} Cre+/- and Cre-/- intercrosses, the numbers of Cre+ and Cre- pups were nearly equal, which indicates that the absence of AM in the ECs does not influence birth rate.

Supplementary Table. 3
Primers and probes for quantitative real-time RT-PCR analysis

Supplementary Figure. 1
Plasmid DNA used for the gene delivery experiment

(a) Control plasmid, which contains a 2.53 kb 5’-flanking region of mouse vascular endothelial (VE)-cadherin (pVE-Cont).
(b) RAMP2-gene delivery plasmid (pVE-mRAMP2), in which mouse RAMP2 ORF (mRAMP2) (0.57 kb) was inserted at the EcoRI site of the pVE-Cont.

(c) EGFP expression plasmid (pVE-EGFP), in which EGFP ORF (0.74 kb) was inserted at the EcoRI site of the pVE-Cont.
References


Genotype of adult mouse from $\text{RAMP2}^{\text{flox/flow}} \text{Cre}^+/\text{-}$ male and female mouse intercrosses

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Supplementary Table.1
Genotype of adult mouse from AM\textsuperscript{flox/flox} Cre+/- and Cre-/- male and female mouse intercrosses

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**Supplementary Table.3**
Supplementary Figure 1