Novel Regulation of Cardiac Metabolism and Homeostasis by the Adrenomedullin-Receptor Activity-Modifying Protein 2 System

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Abstract—Adrenomedullin (AM) was identified as a vasodilating and hypotensive peptide mainly produced by the cardiovascular system. The AM receptor calcitonin receptor-like receptor associates with receptor activity-modifying protein (RAMP), one of the subtypes of regulatory proteins. Among knockout mice (−/−) of RAMPs, only RAMP2−/− is embryonically lethal with cardiovascular abnormalities that are the same as AM−/−. This suggests that the AM-RAMP2 system is particularly important for the cardiovascular system. Although AM and RAMP2 are highly expressed in the heart from embryo to adulthood, their analysis has been limited by the embryonic lethality of AM−/− and RAMP2−/−. For this study, we generated inducible cardiac myocyte-specific RAMP2−/− (C-RAMP2−/−). C-RAMP2−/− showed changes in mitochondrial structure and downregulation of mitochondria-related genes involved in oxidative phosphorylation, β-oxidation, and reactive oxygen species regulation. Furthermore, the heart failure was preceded by changes in peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), a master regulator of mitochondrial biogenesis. Metabolome and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) imaging analyses revealed early downregulation of cardiolipin, a mitochondrial membrane-specific lipid. Furthermore, primary-cultured cardiac myocytes from C-RAMP2−/− showed reduced mitochondrial membrane potential and enhanced reactive oxygen species production in a RAMP2 deletion–dependent manner. C-RAMP2−/− showed downregulated activation of cAMP response element binding protein (CREB), one of the main regulators of mitochondria-related genes. These data demonstrate that the AM-RAMP2 system is essential for cardiac metabolism and homeostasis. The AM-RAMP2 system is a promising therapeutic target of heart failure. (Hypertension. 2013;61:341-351.) ● Online Data Supplement

Key Words: cardiac failure ▪ cardiac myocytes ▪ metabolism ▪ mitochondria ▪ mouse mutants

Adrenomedullin (AM) was identified as a vasodilating and hypotensive peptide mainly produced by the cardiovascular system.1 AM is primarily secreted by vascular cells, and it is also secreted from kidney, lung, and heart.2 AM functions as a local autocrine/paracrine mediator; as well as a circulating hormone,3 exerting natriuretic,4 antioxidative,5,6 and other effects. AM levels in the blood are increased in hypertensive7,8 heart failure,9 and myocardial infarction,10 which suggest its involvement in cardiovascular disease. Homozygotic AM knockout (AM−/−) mice were dead in utero because of abnormal cardiovascular development.11 On the other hand, heterozygotic AM knockout (AM±) mice were apparently normal, although they exhibited higher blood pressure and cardiac hypertrophy when subjected to cardiovascular stress.6,12 Conversely, transgenic mice overexpressing AM have shown lower blood pressure and resistance to various forms of organ damage,13,14 suggesting that AM exerts organ protective as well as hypotensive effects.15-18

The AM receptor calcitonin receptor-like receptor is a 7-transmembrane domain G protein–coupled receptor19 that associates with an accessory protein, receptor activity-modifying protein (RAMP), which is composed of 160 amino acids and includes a single membrane-spanning domain. So far, 3 RAMP isoforms have been identified, but...
only RAMP2 homozygotic knockout (RAMP2−/−) is lethal in utero and reproduces the phenotypes observed in AM−/− mice.20 This suggests that AM signaling via the calcitonin receptor-like receptor/RAMP2 heterodimer (AM-RAMP2 system) is particularly important for the cardiovascular system. In a rat hypertension model induced by inhibition of nitric oxide synthase, AM and the AM receptor components were reported to be upregulated in the heart, probably as a compensatory response against cardiac hypertrophy.21 Although AM and RAMP2 are highly expressed in the heart from embryo to adulthood, analysis of the pathophysiological functions has been limited by the lethality of both the AM−/− and RAMP2−/− genotypes. We speculated that we can overcome this limitation by using cardiac myocyte-specific conditional gene targeting. For this study, we generated an inducible cardiac myocyte-specific RAMP2 knockout (C-RAMP2−/−) mouse, which enabled us to induce RAMP2 gene deletion in the adult and to directly assess the involvement of the AM-RAMP2 system in the pathophysiology of cardiovascular disease.

Methods
For experimental procedures not described herein, please refer to the online-only Data Supplement Methods section.
Animals
A mouse line exhibiting cardiac myocyte-specific RAMP2 deletion was generated by cross-breding RAMP2 flox mice20 with α- myosin heavy chain (MHC)-MerCreMer transgenic (Tg) mice.21. To induceCre recombinaction, RAMP2 flox/flox-αMHC-MerCreDer Tg/+ mice (male, 8–12 weeks old) were administered tamoxifen (Sigma) IP once a day for 5 days at a dose of 30 mg/kg per day followed by a 2-day interval without drug administration. For the study of chronic stage after the gene deletion, the treatment was followed by a 23-day interval without drug administration.

All of the experiments were performed in accordance with the Declaration of Helsinki and were approved by the Shinshu University Ethics Committee for Animal Experiments.

Isolation of Embryonic Cardiac Myocytes
Embryonic cardiac myocytes were isolated from embryos at E14.5 to E16.5. Cardiac myocytes from RAMP2 flox/flox-αMHC-MerCreDer Tg/+ embryos or αMHC-MerCreDer (without loxp) embryos were treated with 500 nmol/L of 4-OH-tamoxifen (SIGMA).

Isolation of Adult Cardiac Myocytes
Adult cardiac myocytes were isolated from RAMP2 flox/flox-αMHC-MerCreDer Tg/+ mice treated with tamoxifen or vehicle. Ventricular myocytes were enzymatically isolated from the heart of adult mice, as described previously.22

Ca2+ Imaging
Ca2+ imaging was performed as described previously.24 Fluorescence images were acquired with a laser scanning microscope 7 LIVE laser scanning microscope (Zeiss). Ca2+ transient was assessed from fluorescence changes in individual cardiac myocytes in the presence or absence of 10−7 mol/L AM.

Mitochondrial Function
Mitochondrial membrane potential was assessed using fluorescent tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) staining. Cardiac myocytes were stained with 100 nmol/L of TMRE for 15 minutes, after which the culture medium was replaced with fresh medium. Mitochondrial reactive oxygen species (ROS) production was assessed using MitoSOX Red (Molecular Probes) staining. The cells were then visualized using a Zeiss laser scanning microscopy 5 EXCITER fluorescence microscope system.

Analysis of Phospholipid Molecular Species
Phospholipids such as cardiolipin were extracted by the Bligh and Dyer method and analyzed according to the online-only Data Supplement Methods section.

A frozen section of the heart was placed on an indium tin oxide coated glass slide (Sigma-Aldrich) and stored at −80°C. 9-Aminoacridine at a concentration of 5 mg/mL, dissolved in 70% ethanol was used for matrix. Matrix deposition was performed by a chemical printer (CHIP-1000, Shimadzu Corporation) and dried up. Matrix-assisted laser desorption/ionization mass spectra were acquired using AXIMA Performance (Shimadzu) with a 337-nm nitrogen laser, operating in the reflector/negative mode.

Statistics
Quantitative values are expressed as the mean±SE. Student t test was used to determine significant differences between 2 groups. One-way ANOVA followed by Fisher protected least significant difference was used to determine significant differences between 3 groups. Dunnett test was used to determine significant differences between >3 groups. Values of P<0.05 were considered significant.

Results
C-RAMP2−/− Mice Exhibit Dilated Cardiomyopathy
To induce cardiac myocyte-specific deletion of RAMP2, RAMP2 flox/flox-αMHC-MerCreDer Tg mice were treated with tamoxifen. It has been reported that a high oral dose of tamoxifen (80 mg/kg body weight for 7 days; total dose, 560 mg/kg) can induce MerCreDer nuclear translocation and dilated cardiomyopathy in mice.22 To avoid the spontaneous occurrence of heart failure, we used a lower dose of tamoxifen (30 mg/kg body weight administered IP daily for 5 days; total dose, 150 mg/kg) and withdrawal for 2 days. After this procedure, the level of RAMP2 gene expression within whole-heart specimens (including both myocytes and nonmyocytes) was reduced to ~60% of control. We designated tamoxifen-treated RAMP2 flox/flox-αMHC-MerCreDer Tg mice as C-RAMP2−/−. We also confirmed that this dosage does not cause spontaneous heart failure in αMHC-MerCreDer Tg mice without the RAMP2-flox region (Figure S1 in the online-only Data Supplement).

C-RAMP2−/− hearts showed enlargement of ventricles (Figure 1A), with significant increases in the cardiothoracic ratio and heart weight/body weight ratio, although blood pressure was reduced (Figure 1B and 1C). Echocardiography showed C-RAMP2−/− hearts to have dilated left ventricles (Figure S1), with diminished systolic function (Figure 1D and Table 1).

Table 1. Echocardiographic Data of Control and C-RAMP2−/− Mice (Acute Phase of the Gene Deletion).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>C-RAMP2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDD, mm</td>
<td>3.81±0.09</td>
<td>4.35±0.29</td>
</tr>
<tr>
<td>LVDs, mm</td>
<td>2.56±0.11</td>
<td>3.82±0.48*</td>
</tr>
<tr>
<td>LVPPW, mm</td>
<td>0.73±0.03</td>
<td>0.74±0.07</td>
</tr>
<tr>
<td>LVPPVs, mm</td>
<td>1.07±0.04</td>
<td>0.93±0.10</td>
</tr>
<tr>
<td>EF, %</td>
<td>61.68±2.84</td>
<td>26.93±11.68*</td>
</tr>
<tr>
<td>FS, %</td>
<td>32.82±1.90</td>
<td>14.36±6.83*</td>
</tr>
</tbody>
</table>

EF indicates ejection fraction; FS, fractional shortening; LVDD, diastolic left ventricular dimension; LVDs, systolic left ventricular dimension; LVPPWs, systolic left ventricular posterior wall thickness; and LVPPVs, systolic left ventricular posterior wall thickness. n=6–7.

**P<0.05.

Table 2. Echocardiographic Data of Control and C-RAMP2−/− Mice (Chronic Phase of the Gene Deletion).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>C-RAMP2−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVDD, mm</td>
<td>3.93±0.11</td>
<td>4.20±0.16</td>
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<tr>
<td>LVDs, mm</td>
<td>2.75±0.06</td>
<td>3.14±0.12*</td>
</tr>
<tr>
<td>LVPPW, mm</td>
<td>0.77±0.02</td>
<td>0.77±0.04</td>
</tr>
<tr>
<td>LVPPVs, mm</td>
<td>1.00±0.03</td>
<td>0.99±0.05</td>
</tr>
<tr>
<td>EF, %</td>
<td>57.67±1.23</td>
<td>49.92±2.06*</td>
</tr>
<tr>
<td>FS, %</td>
<td>29.91±0.88</td>
<td>25.10±1.26*</td>
</tr>
</tbody>
</table>

EF indicates ejection fraction; FS, fractional shortening; LVDD, diastolic left ventricular dimension; LVDs, systolic left ventricular dimension; LVPPWs, systolic left ventricular posterior wall thickness; and LVPPVs, systolic left ventricular posterior wall thickness. n=5.

**P<0.05.
which suggests that C-RAMP2−/− mice experience dilated cardiomyopathy-like heart failure. Histological analysis revealed enlargement of the cardiac myocytes (Figure 1E and 1G). Electron microscopic observation revealed myofibril disarray and Z-line dislocation, as well as enhanced perivascular fibrosis (Figure 1F). In addition, quantitative reverse-transcription polymerase chain reaction (Figure 1H) showed that brain natriuretic peptide (BNP) and sarcoendoplasmic reticulum calcium ATPase 2 (SERCA2) expressions were upregulated and downregulated, respectively, in C-RAMP2−/− hearts. These alterations in gene expression are consistent with the dilated cardiomyopathy-like changes in C-RAMP2−/−.

We further analyzed the chronic phase of the gene deletion. We analyzed C-RAMP2−/− at 23 days after tamoxifen administration. At this stage, C-RAMP2−/− still showed dilatation of ventricles (Figure 2A) with enlargement of cardiac myocytes (Figure 2B and 2C). Echocardiography showed partial recovery of cardiac function; however, compared with control mice, C-RAMP2−/− still showed significantly reduced systolic function with left ventricular dilatation (Figure 2D and Table 2).

Increased Oxidative Stress in C-RAMP2−/− Hearts
We next attempted to identify the cause of the heart failure in C-RAMP2−/−. Immunostaining of 4-hydroxynonenal (HNE), a peroxidized lipid, showed that levels of oxidative stress were elevated in C-RAMP2−/− hearts (Figure 3A, center).

We orally administered the antioxidant 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) to analyze whether the elevated ROS is the primary cause of heart failure in C-RAMP2−/−. TEMPOL treatment reduced ROS levels in the hearts of C-RAMP2−/− (Figure 3A, right). Moreover, the enlargement of cardiac myocytes was reversed (Figure 3B). On the other hand, the altered expression of BNP and SERCA2 was unaffected (Figure 3C), which suggests that mechanisms other than ROS elevation are also involved in the onset of failure in C-RAMP2−/− hearts.

Downregulation of Mitochondrial Membrane-Specific Lipid in C-RAMP2−/− Hearts
To further clarify the mechanisms involved in the failure of C-RAMP2−/− hearts, we used metabolome analysis
to comprehensively evaluate the metabolic changes in C-RAMP2−/− hearts. The lipid analysis revealed a distribution shift from phosphatidylcholines to lysophosphatidylcholines (data not shown). Lysophosphatidylcholines derive from the partial degradation of phosphatidylcholines through the removal of 1 of their 2 fatty acids. We speculated that the enhanced oxidative stress in C-RAMP2−/− hearts might promote the degradation of phospholipids.

Furthermore, we found that several forms of cardiolipin were downregulated in C-RAMP2−/− hearts (Figure 4A). Cardiolipin is a mitochondrial inner membrane-specific lipid that binds to cytochrome C, anchoring it in the inner mitochondrial membrane, and is essential for ATP production. To confirm that this down-regulation was a result of myocyte abnormalities, we performed matrix-assisted laser desorption/ionization-Time-of-flight mass spectrometry imaging analysis, which enabled us to analyze the metabolome directly using ionized cells from frozen sections and to exclude fibrotic areas and blood vessels. Using this approach, we confirmed that cardiolipins were downregulated in myocytes (Figure 4B). Cardiolipins containing docosahexaenoic acid were downregulated beginning early (day 2) after RAMP2 deletion (Figure 4C). Taken together, these findings suggest mitochondrial changes involving the abnormal distribution of cardiolipin are the earliest events in the heart failure seen after RAMP2 deletion.

**Structural and Functional Mitochondrial Abnormalities in C-RAMP2−/− Hearts**

Consistent with the results above, electron microscopic observation showed the presence of mitochondrial changes after...
RAMP2 deletion (Figure 5A). The mitochondrial cristae formations were disrupted. In addition, mitophagy, the phenomena of removing damaged mitochondria, was observed in C-RAMP2−/− hearts (Figure 5A, right). The mitochondrial DNA content was not changed in C-RAMP2−/− hearts (Figure 5B), but quantitative reverse-transcription polymerase chain reaction showed that expression of mitochondria-related genes, including peroxisome proliferator-activated receptor-γ coactivator-1α, β (PGC-1α, β), peroxisome proliferator-activated receptor-α, estrogen-related receptor-α (in charge of mitochondria regulation), carnitine palmitoyl transferase 1α, 2, medium-chain acyl dehydrogenase (in charge of β-oxidation), ATP synthase F1 complex-β subunit, cytochrome C oxidase (in charge of oxidative phosphorylation), uncoupling protein 3, and superoxide dismutase 2 (in charge of mitochondria ROS regulation), were all downregulated in C-RAMP2−/− hearts (Figure 5C). This suggests that heart failure in C-RAMP2−/− is related to mitochondrial dysfunction rather than mitochondrial volume.

We next examined the time course of the gene expression in C-RAMP2−/− hearts. PGC-1α is one of the master regulators of mitochondrial biogenesis. Although we detected no cardiac dysfunction during the period of tamoxifen treatment (from day 0 to 5), gene expression of PGC-1α was transiently upregulated on day 3 and downregulated thereafter (Figure 5D). On the other hand, BNP expression was unaffected during days 0 to 5, and it was upregulated from day 7 onward. This suggests that mitochondrial changes occur before the development of heart failure.

**In Vitro Analysis of Cardiac Myocytes From C-RAMP2−/−**

To further confirm that mitochondrial dysfunction is the primary cause of heart failure but not secondary to the contractile dysfunction, we cultured primary cardiac myocytes isolated from RAMP2 flox/flox-αMHC-MerCreMer Tg embryos. We then treated the cells with 4-OH-tamoxifen and directly analyzed mitochondrial function. 4-OH-tamoxifen-treated C-RAMP2−/− myocytes showed weak TMRE staining, which indicates depolarization of the mitochondrial membrane (Figure 6A). In addition, Mito SOX red staining revealed mitochondrial ROS levels to be elevated in C-RAMP2−/− myocytes (Figure 6B). We also confirmed that 4-OH-tamoxifen treatment itself did not affect mitochondrial function, because 4-OH-tamoxifen-treated αMHC-MerCreMer without loxP cells showed no changes in TMRE or Mito SOX Red staining (right panels of Figure 6A and 6B). Gene expression of the mitochondrial regulatory factors PGC-1α and β was also suppressed in the C-RAMP2−/− myocytes (Figure 6C).

Furthermore, we analyzed adult cardiac myocytes. In TMRE and Mito SOX Red staining, cardiac myocytes isolated from C-RAMP2−/− showed weaker mitochondrial membrane potential and increased mitochondrial ROS production (Figure 6D). Next, to clarify robustness of the adult cardiac myocytes, we analyzed the Ca2+ transient of the isolated adult myocytes (transient cytosol Ca2+ elevation in response to electric pacing) using a Ca2+ imaging system. Peak twitch amplitude of the

**Figure 5.** Mitochondria in cardiac myocyte-specific receptor activity-modifying protein 2 knockout (C-RAMP2−/−) hearts exhibit structural and functional abnormalities. A, Transmission electron micrographs showing cardiac mitochondria. C-RAMP2−/− showed damaged mitochondria and appearance of mitophagy (arrow at right). Scale bar, 1 μm. B, Relative mitochondrial DNA/nuclear DNA ratios. Data are shown as the ratio when mean of the control group = 1. C, Relative cardiac gene expression of mitochondria-related molecules involved in overall mitochondrial regulation (peroxisome proliferator-activated receptor-γ coactivator [PGC]-1α, PGC-1β), peroxisome proliferator-activated receptor [PPAR]-α, and estrogen-related receptor [ERR]-γ), β-oxidation carnitine palmitoyl transferase (CPT)-1α, CPT-2, and medium-chain acyl dehydrogenase [MCAD]), oxidative phosphorylation (ATP synthase F1 complex β subunit and cytochrome C oxidase [COX IV]), and mitochondrial reactive oxygen species (ROS) regulation (uncoupling protein 3 [UCP3] and superoxide dismutase 2 [SOD2]). Expression levels in C-RAMP2−/− hearts were normalized to the control, which was assigned a value of 1. *P<0.05, **P<0.01, ***P<0.001. D, Relative cardiac expression of PGC-1α, BNP on the indicated days. Expression levels were normalized to the level on day 0, which was assigned a value of 1. *P<0.05 vs day 0; Dunnett test. Bars in B through D show means±SEM.
cytosol Ca$^{2+}$ fluorescent after the pacing was significantly lower in C-RAMP2−/− compared with control (Figure 6E). In addition, the decay speed of the transiently elevated Ca$^{2+}$ fluorescent was slowed, and the half-life of the decay tended to be longer in C-RAMP2−/− than control (Figure 6F). These results suggest that failing cardiac myocytes of C-RAMP2−/− exhibited abnormal Ca$^{2+}$ handling. With AM stimulation, the amplitude of the Ca$^{2+}$ transient was elevated in control cardiac myocytes; however, the response was blunted in C-RAMP2−/− myocytes (Figure 6G).

**CREB Activation Is Suppressed in C-RAMP2−/− Hearts**

AM was originally identified through its ability to increase intracellular cAMP levels. Therefore, to assess signaling pathways involved in the mitochondrial abnormalities in C-RAMP2−/− hearts, we first analyzed intracellular cAMP levels in cultured myocytes from C-RAMP2−/− hearts. cAMP was reduced in C-RAMP2−/− myocytes (Figure 7A). Moreover, Western blot analysis showed that the level of cAMP response element binding (CREB) protein activation was significantly reduced in C-RAMP2−/− hearts (Figure 7B and 7C).

Forskolin stimulates adenylyl cyclase directly, thereby increasing intracellular cAMP levels. In C-RAMP2−/− hearts, forskolin treatment partially recovered CREB phosphorylation (data not shown) and mediated repolarization of the mitochondrial membrane in cardiac myocytes (Figure 7D). When we tested its effects in vivo, we found that forskolin reduced ROS levels in C-RAMP2−/− hearts (Figure 7E). In addition, forskolin partially recovered the heart failure-related changes in BNP and sarcoendoplasmic reticulum calcium ATPase 2 expression (Figure 7F). Gene expression of various mitochondria-related factors was downregulated in C-RAMP2−/− compared with control. However, with forskolin treatment, some factors that were related to mitochondria regulation (PGC-1α, PGC-1β, peroxisome proliferator-activated receptor -α, and estrogen-related receptor-α) were significantly upregulated in C-RAMP2−/−. Other factors (CPT-2, medium-chain acyl dehydrogenase, ATP synthase F1 complex β subunit, cytochrome C oxidase, uncoupling protein 3, and superoxide dismutase 2) were also partially restored (Figure 7G).

**Discussion**

AM is a circulating peptide and its level in blood is increased in heart failure,9 perhaps as a compensatory response. Although it is known that AM is upregulated in response to various stresses, it is difficult to evaluate the role of AM within the heart using conventional knockout mice, because hemodynamic changes caused by systemic AM deficiency would likely affect cardiac function. Conditional gene targeting is a novel way to solve this problem; however, cardiomyocyte-specific AM knockout mice are also problematic, because AM is secreted from both myocytes and nonmyocytes in the heart, and the secreted AM may work between each other. In the present study, therefore, we chose to disrupt the AM receptor system in cardiac myocytes by generating an inducible conditionally RAMP2 gene-targeted mouse.

Figure 8 summarizes our findings for the AM-RAMP system in cardiac homeostasis. We found that cardiomyocyte-specific
RAMP2 deletion evoked dilated cardiomyopathy-like heart failure with cardiac dilatation in the adult heart. This heart failure was associated with high levels of oxidative stress. In previous studies using AM± mice, AM was shown to exert strong antioxidant effects in the heart through activation of endothelial nitric oxide synthase and regulation of NADPH oxidase. As expected, C-RAMP2−/− hearts showed increased oxidative stress, and the superoxide dismutase mimic TEMPOL reversed the

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Figure 7. cAMP and cAMP response element binding protein (CREB) activation are suppressed in cardiac myocyte-specific receptor activity-modifying protein (RAMP) 2 knockout (C-RAMP2−/−) hearts. A, Effect of RAMP2 deletion induced by 4-OH-tamoxifen (Tam) treatment on intracellular cAMP levels in primary cultured cardiac myocytes stimulated with 10−7 mol/L adrenomedullin (AM). Measurements were made using an enzyme immunoassay. B, Western blot analysis of CREB and phosphorylated CREB (p-CREB). C, Effect of RAMP2 deletion on CREB activation indicated by its phosphorylation ratio (pCREB/CREB). D, Tetramethylrhodamine ethyl ester (TMRE) staining of cardiac myocytes showing the forskolin-induced restoration of mitochondrial membrane potential after RAMP2 deletion. Scale bar, 10 μm. E, 4-Hydroxynonenal (HNE) immunostaining of heart sections. Forskolin treatment reversed reactive oxygen species (ROS) in C-RAMP2−/− hearts. Scale bar, 50 μm. F, Relative gene expression of heart failure–related molecules (brain natriuretic peptide [BNP] and sarcoendoplasmic reticulum calcium ATPase [SERCA] 2). *P<0.05, **P<0.01 vs control; Fisher protected least significant difference (PLSD). G, Relative gene expression of the mitochondria-related molecules. *P<0.05, **P<0.01, ***P<0.001 vs control. #P<0.05 vs C-RAMP2−/−; Fisher PLSD. Bars in A, C, F, and G show mean±SEM.
increases in ROS. However, because the heart failure caused by RAMP2 deletion was not fully reversed by the ROS reduction, we speculated that other critical factors were involved.

The mitochondria in C-RAMP2−/− hearts showed structural changes that were associated with reduced expression of various mitochondria-related molecules. Furthermore, lipid metabolome analysis and matrix-assisted laser desorption/ionization-time-of-flight mass (MALDI-TOF-MS) spectrometry imaging analysis, which are novel strategies enabling clarification of tissue-specific metabolic alterations, revealed that levels of cardiolipin, a mitochondrial inner membrane-specific lipid, were decreased beginning early after RAMP-2 deletion. Cardiolipin binds to enzymes involved in oxidative phosphorylation, anchoring them to the mitochondrial inner membrane, and regulating their activity. It is essential for mitochondrial function, and dysregulation of cardiolipin induces heart failure. To further confirm that mitochondrial dysfunction is the primary cause of heart failure but not secondary to the contractile dysfunction, we cultured primary cardiac myocytes isolated from RAMP2 flox/flox-α-MHC-MerCreMer Tg embryos. We then induced RAMP2 deletion in vitro and proved mitochondrial dysfunction and enhanced ROS production occurred in a RAMP2 deletion-dependent manner. We also confirmed poor Ca2+-handling in cardiac myocytes isolated from adult C-RAMP2−/−.

We found that the altered expression of BNP after RAMP2 deletion was preceded by altered expression of the transcriptional coactivator PGC-1α, which governs mitochondrial biology through broad regulation of genes in both the nuclear and mitochondrial genomes. Induction of PGC-1α is regulated by multiple signaling molecules, including p38 mitogen-activated protein kinase, Ca2+ calcineurin, AMP-activated protein kinase, Sirt1, and CREB. In the liver, CREB regulates PGC-1α by binding to the cAMP responsive element in the PGC-1α promoter and inducing expression of the gluconeogenic program. Downregulation of CREB is associated with aging, hypertension, insulin resistance, and vascular disease. Moreover, transgenic mice overexpressing a dominant-negative CREB in their hearts exhibit dilated cardiomyopathy-like heart failure with mitochondrial abnormalities. These reports suggest that regulation of PGC-1α by CREB plays a pivotal role in cardiovascular homeostasis. In the present study, we found that CREB activation was reduced in C-RAMP2−/− hearts and speculated that CREB suppression underlies the observed mitochondrial dysfunction and heart failure. Downstream effects mediated by the AM-RAMP2 system include cAMP production, Ca2+ mobilization, and activation of Akt and other signaling molecules. All of these molecules are involved in CREB activation, suggesting that RAMP2 deletion suppresses CREB by altering several signaling pathways.

Consistent with that idea, forskolin, which directly stimulates adenylyl cyclase and cAMP production, reversed the mitochondrial membrane depolarization and mitochondrial ROS elevation seen in cultures of primary C-RAMP2−/− cardiomyocytes. In vivo, forskolin ameliorated the oxidative stress and fibrosis in C-RAMP2−/− hearts, reversed the heart failure-related changes in BNP and SERCA2 expression, and restored expression of PGC-1α, estrogen-related receptor-α (ERR-α) and other mitochondrial genes. These data show that the AM-RAMP2 system is essential for appropriate regulation of cardiac function and homeostasis and that it acts via CREB and PGC-1 to affect mitochondrial ATP production, lipid metabolism, and ROS regulation.

**Perspectives**

AM is a peptide originally found as a vasodilating and hypotensive peptide secreted from various cells and tissues, including the cardiovascular system. In this study, we directly assessed the roles of the AM-RAMP2 system in cardiac myocytes and its pathophysiological significance in heart failure. These data show that the AM-RAMP2 system may be a promising therapeutic target of heart failure. On the other hand, the clinical applicability of AM, like that of other
bioactive endogenous peptides, has 2 serious limitations: AM has a very short half-life in the blood, and the cost of the recombinant protein is very high, which together make the use of AM impractical for treatment of chronic diseases. It is, therefore, noteworthy that we are able to affect the cardiac activity of AM by modulating RAMP2. In fact, we would expect that greater specificity can be achieved by targeting RAMP2 than by targeting calcitonin receptor-like receptor, which can also function as a receptor for α-calcitonin gene-related peptide, β-calcitonin gene-related peptide, amylin, and intermediin. In this study, we have shown that the AM-RAMP2 system is essential for cardiac homeostasis. Our findings could potentially provide the critical basis for developing medicines targeting the AM-RAMP2 system, which could directly promote both energy production and ROS suppression in cardiac myocytes.

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**Disclosures**

None.

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What Is New?

- By generating cardiac myocyte-specific RAMP2 deletion model mouse (C-RAMP2−/−), we directly proved the critical roles of AM-RAMP2 system in the heart.
- C-RAMP2−/− developed dilated cardiomyopathy-like heart failure.
- We found novel regulation of cardiac mitochondrial function by the AM-RAMP2 system.

What Is Relevant?

- AM is a promising therapeutic molecule for treating hypertensive diseases through its well-known vasodilative function. In this study, we proved that the AM-RAMP2 system also directly regulates cardiac metabolism and homeostasis.

Summary

AM-RAMP2 system is a promising therapeutic target for heart failure in addition to hypertension.
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Novel Regulation of Cardiac Metabolism and Homeostasis by the Adrenomedullin-RAMP2 System

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Short title: AM-RAMP2 in cardiac homeostasis

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Supplementary Methods

Echocardiography

Mice were set in a supine position. Two-dimensional (2D) guided M-mode echocardiography was performed using a Vevo 2100 imaging system (VisualSonics, Toronto, Canada). The heart at the level of the left ventricle (LV) papillary muscle was imaged in the 2D mode using a parasternal short-axis view at a depth setting of 13 mm.

Histological examination

Heart sections were fixed in 10% formalin neutral buffer, embedded in paraffin, and cut into 5 µm thick sections. Some samples were used for H&E staining. Immunohistochemical staining of 4-hydroxy-2-nonenal (4HNE) was performed using an anti-4-HNE antibody (NOF Corporation, Japan). A biotin-conjugated goat anti-mouse antibody, a Histofine MOUSESTAIN KIT (mouse on mouse system, Nichirei, Japan), and 3,3′-diaminobenzidine (DAB) were used to visualize labeling. To quantify cardiac interstitial fibrosis, images of Masson trichrome staining were captured with a digital camera and analyzed using Scion Image software.

Transmission electron microscopy

Specimens were fixed in 2% glutaraldehyde (pH 7.2) and 4% osmium tetroxide, embedded in epoxy resin (Epok) 812 (Oken Shoji, Japan), cut into ultrathin sections, double-stained with uranyl acetate and lead citrate, and examined in an electron microscope (JEM-1010; Jeol, Japan).

TEMPOL and Forskolin treatment

For TEMPOL experiments, mice were administered TEMPOL (10 mmol/L; Alexis Biochemicals) in drinking water for 7 days. Control mice received tap water. For the forskolin experiments, mice were administered forskolin (Calbiochem) intrapleurally once a day for 7 days at a dose of 0.4 mg/kg/day. Forskolin was dissolved in DMSO and then further diluted in PBS. Control mice received the vehicle. For in vitro analysis, cultured cardiac myocytes were treated with 20 nM forskolin.

Quantitative reverse transcriptase PCR analysis
Total RNA was extracted from tissues using Trireagent (Molecular Research Center, Inc.), after which the RNA was treated with DNA-free (Ambion) to remove contaminating DNA and subjected to reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcriptase PCR analysis was carried out using an ABI Prism 7300 Sequence Detection System (Applied Biosystems) with SYBER Green (Toyobo, Japan). Values were normalized to GAPD (TaqMan Ribosomal RNA Control Reagents VIC probe; Applied Biosystems). The primers and probes used are listed in Table S1.

**Analysis of phospholipid molecular species**

The mouse hearts were homogenized with chloroform/methanol (1:2), and phospholipids such as cardiolipin (CL) were extracted by the Bligh and Dyer method. The total lipid extracts were dried under a gentle stream of nitrogen gas and were re-dissolved in 1 mL of chloroform/methanol (1:1).

Lipid extracts were separated on a Develosil C30 (3.0 µm, 150 mm × 1.0 mm i.d.) column (Nomura Chemical Co. Ltd., Japan) using a liquid chromatography system consisting of two Shimadzu LC10ADvp micro-pumps, an SCL-10Avp controller (Shimadzu, Japan), an HTC PAL autosampler (CTC Analysis, Switzerland), and Chromelone software (Dionex, CA), and were measured by a full scan of precursor ions (MS1) within m/z 400–2000 in negative ion mode using an LTQ Orbitrap mass spectrometer (Thermo-Fisher Scientific, MA), as described previously with several modifications.

CL species were identified by the exact mass value (within 5 ppm accuracy) and LC retention time. MS/MS (MS2) and MS/MS/MS (MS3) measurements at a collision energy setting of 35% were performed as needed. An LTQ-Orbitrap analyzer was used for quantification of each precursor ion, and an LTQ-IT analyzer was used for MS3 measurement.

**On-Tissue MALDI-MS imaging analysis**

Mass spectra were obtained with an accelerating voltage of 20 kV, a mass range of 1–2,000 Da, and “pulsed extraction mass” set to 1,500 Da. The mass spectrometer was calibrated with spotted angiotensin II and 9-aminoacridine, which had a mass-to-charge ratio (m/z) of 1,044.52 and 193.07, respectively, in the negative mode.
Western blot analysis

Heart tissue was lysed in an ice-cold RIPA Lysis Buffer System (Santa Cruz) supplemented with PosSTOP phosphatase inhibitor (Roche Applied Science) and then sonicated. Samples of the resultant lysate (40-60 µg) were subjected to SDS-PAGE, and the resolved proteins were transferred to cellulose nitrate membranes (GE Healthcare). After blocking in 5% skim milk, the membranes were incubated with primary antibodies against CREB (Abcam) and phospho-CREB (Abcam), followed by appropriate secondary antibodies (Santa Cruz). The bound antibodies were visualized using chemiluminescent HRP substrate (MILLIPORE), and the chemiluminescence was analyzed using an Image Quant LAS 4000 system (GE Healthcare). Levels of CREB activation were determined based on the ratio of band intensities after blotting with antibodies specific for the phosphorylated and unphosphorylated proteins. For quantification, Western blot images were captured and analyzed using Scion Image software.

cAMP measurement

The cAMP content of cardiac myocytes was measured using a cAMP Biotrak Enzyme Immunoassay System (Amersham).

Isolation of embryonic cardiac myocytes

Collected embryonic heart was washed in PBS, minced, and incubated with 0.05% trypsin-EDTA (GIBCO) for 10 min at 37°C with agitation. During this period, the digestion buffer was replaced 3 times. The dispersed cells were filtered through 40-µm nylon mesh (BD Falcon) and then incubated in culture dishes for 60 min to remove non-myocytes. Unattached viable cells were collected and cultured on gelatin-coated dishes at 37°C in MEM supplemented with 10% FBS and penicillin/streptomycin. Using this protocol, we consistently obtained cell populations containing at least 80% cardiac myocytes.

Isolation of adult cardiac myocytes

A heart mounted on a Langendorff apparatus was digested with Ca²⁺-free Tyrode solution containing 0.80 mg/ml collagenase, 0.06 mg/ml protease (Sigma-Aldrich, Inc.),
1.20 mg/ml hyaluronidase (Sigma-Aldrich, Inc.), 0.03 mg/ml DNase I (F. Hoffmann-La Roche Ltd., Basel, Switzerland), and 0.50 mg/ml bovine serum albumin at 37°C for 2 min. Isolated ventricular myocytes were suspended in Ca²⁺-free Tyrode solution containing 1 mg/ml bovine serum albumin at room temperature, and the Ca²⁺ concentration was gradually increased to 1.8 mmol/L.

**Ca²⁺ imaging**

Ca²⁺ imaging was performed as previously described (Nakada et al, 2012, Biochem. J. 448: 221-231). Briefly, cardiac myocytes plated on laminin-coated glass bottom dishes were incubated with 5 µM Fluo-4-AM (Dojindo) plus 0.01% Cremophore EL (Sigma) and 0.02% bovine serum albumin (Sigma) in serum-free DMEM for 45 min at 37°C followed by de-esterification. Cardiac myocytes were superfused with the modified Tyrode solution (136.5 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.53 mM MgCl₂, 5.5 mM HEPES, and 5.5 mM glucose, pH 7.4) at room temperature and paced with 1-ms pulses of 50 V at 0.5 Hz. Fluorescence images were acquired with an LSM 7 LIVE laser scanning microscope (Zeiss). Ca²⁺ transient was assessed from the changes in fluorescence in individual cardiac myocytes in the presence or absence of 10⁻⁷ M AM.

**Mitochondrial DNA determination**

Heart tissue was collected and treated in 0.5 mL of 0.25 mg/mL proteinase K (Invitrogen) solution at 55°C for 3 h. The lysate was mixed in phenol/chloroform (Wako, Japan) for 1 h. The supernatant was transferred to a 2 mL Eppendorf tube containing 0.5 mL chloroform and mixed. After centrifugation (9000 g for 10 min), the supernatant was transferred to a 1.5 mL Eppendorf tube containing 1 mL ethanol. The tube was maintained at -20°C for 1 h and centrifuged (18,000 g, 10 min) to collect the DNA pellet. The DNA pellet was washed with 1 mL 70% ethanol, dried, and dissolved in 0.2 mL Tris-EDTA (TE) buffer.

Mitochondrial DNA (mtDNA): The nuclear DNA (nDNA) ratio was determined using a real-time PCR system (ABI Prism 7300 Sequence Detection System) with SYBER Green (Toyobo, Japan). The cytochrome c oxidase subunit 1 (COX1) gene of the mtDNA and the NDUFV1 nDNA gene was measured. The COX1 primers were 5-TGC TAG CCG CAG GCA TTA C-3 (forward primer) and 5-GGG TGC CCA AAG
AAT CAG AAC-3 (reverse primer). The NDUFV1 primers were 5-CTT CCC CAC TGG CCT CAA G-3 (forward primer) and 5-CCA AAA CCC AGT GAT CCA GC-3 (reverse primer). Values were normalized to NDUFV1.
Table S1  Primers used for quantitative reverse transcriptase PCR analysis.

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<tr>
<th>gene</th>
<th>sequence (5' to 3')</th>
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<tr>
<td>BNP</td>
<td>F TCCAGAGCAATTCAAGATGCA</td>
</tr>
<tr>
<td></td>
<td>R GTCTTTTCATTGCGCTTCC</td>
</tr>
<tr>
<td>SERCA2</td>
<td>F GGCAGGATGAGGATGTACAT</td>
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<tr>
<td></td>
<td>R CTGGGCTGAAAGGCTTAATTCC</td>
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<tr>
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<tr>
<td></td>
<td>R CGACACGGAGATTTAAGGAAGA</td>
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<tr>
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<tr>
<td></td>
<td>R GGCCAGAAGTCCCTTAGGATAG</td>
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<tr>
<td>PPAR-α</td>
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</tr>
<tr>
<td></td>
<td>R TTTGGAAAGAGAAAGGTGCA</td>
</tr>
<tr>
<td>ERR-α</td>
<td>F GTACTGCAGAGTGAGGATGGA</td>
</tr>
<tr>
<td></td>
<td>R TCTAGGACCAGGTCTCAGCAA</td>
</tr>
<tr>
<td>CPT-1α</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>CPT-2</td>
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<td>MCAD</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>R ATCCCCAGCAGCGGAATAA</td>
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Figure S1  Tamoxifen-treated $\alpha$MHC-MerCreMer Tg mice without the RAMP2-flox region. (A) Representative transthoracic M-mode echocardiogram. (B) Tamoxifen-treated mice (Tamox) showed no changes in the fractional shortening (FS), ejection fraction (EF), and systolic left ventricular dimension (LVDs) compared with control mice.