Induction of a Myocardial Adrenomedullin Signaling System During Ischemic Heart Failure in Rats

Erik Øie, MD; Leif Erik Vinge, MD; Arne Yndestad, MSc; Cecilie Sandberg, MSc; Haakon K. Grøgaard, MD; Håvard Attramadal, MD, PhD

Background—Increased plasma adrenomedullin (ADM) levels have been reported in congestive heart failure (HF). The present study was designed to investigate myocardial regulation of the different components of the ADM signaling system (ADM, ADM receptor, and receptor-activity–modifying protein-2, RAMP-2) during ischemic HF in rats and to identify the cells in the myocardium displaying ADM-like immunoreactivity (ADM-ir). Furthermore, the effects of endothelin (ET) receptor antagonism on expression of the myocardial ADM system during HF were investigated.

Methods and Results—Northern blot analysis revealed increased ADM mRNA expression in the nonischemic left ventricle, with maximal levels 28 days after induction of myocardial infarction (1.5-fold, \( P<0.05 \)) compared with the sham group. Parallel elevations of myocardial ADM receptor and RAMP-2 mRNA levels were also observed (2.3- and 1.5-fold increase, respectively; \( P<0.05 \)). In addition, high levels of ADM mRNA were seen in the ischemic region. Immunohistochemical analysis revealed a substantial increase of ADM-ir in microvascular endothelium and perivascular interstitial cells of myocardial tissue contiguous to the ischemic region. In addition, radioligand binding studies demonstrated a 1.6-fold increase of specific ADM binding sites in the failing left ventricle (\( P<0.05 \)). Intervention with the mixed ET\(_A\)/ET\(_B\) receptor antagonist bosentan (100 mg · kg\(^{-1}\) · day\(^{-1}\) PO) for 15 days prevented the increase of RAMP-2 mRNA.

Conclusions—The study demonstrates a concerted induction of several components of the myocardial ADM signaling system during postinfarction failure and that the vessels are the main source of myocardial ADM. Our observations indicate a role for ADM as an autocrine/paracrine factor during ventricular remodeling after myocardial infarction. (Circulation. 2000;101:415-422.)

Key Words: adrenomedullin ■ endothelin ■ myocardium ■ heart failure
secretion. However, several important aspects of a putative myocardial ADM system have not been studied. First, the regional regulation of ADM in relation to the ischemic myocardium in postinfarction failure is unknown. Second, it is not known whether the ADM receptor or RAMP-2 mRNA levels are coregulated with ADM in the failing heart. The present study was conducted to identify and localize cells in normal and failing myocardial tissue containing immunoreactive ADM and to investigate the regulation of ADM, ADM receptor, and RAMP-2 mRNAs in different regions of the heart after induction of MI. In addition, determination of ADM receptor binding sites in normal and failing myocardial tissues was performed. Furthermore, to elucidate to what extent ET-1 may induce the myocardial ADM signaling system, we addressed the effects of ET receptor antagonism on ADM and RAMP-2 mRNA expression in the left ventricle (LV) after induction of ischemic HF.

Methods

Experimental Procedure

We used the left coronary artery–ligated rat model of HF according to the method of Selye et al15 with minor modifications.16

The course of myocardial ADM, ADM receptor, and RAMP-2 mRNA expression was investigated at various time points after ligation of the left coronary artery during the development of HF. Rats were euthanized 2, 7, 28, 42, and 56 days after induction of MI, and myocardial tissue from the different chambers of the heart was sampled, snap-frozen in liquid nitrogen, and stored at −70°C. Sham-operated rats were euthanized at the same time points and served as controls. In rats that underwent ligation of the left coronary artery, only those with LV end-diastolic pressure (LVEDP) ≥10 mm Hg were considered to have HF and were included in the study.

The purpose of the second part of the experiments was to investigate the effects of ET receptor antagonism on myocardial ADM and RAMP-2 mRNA expression in the LV after induction of postinfarction failure. Rats were randomized to treatment with the mixed ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist bosentan (F. Hoffmann-La Roche; 100 mg·kg<sup>−1</sup>·day<sup>−1</sup>; n = 16) or vehicle (water; n = 17). The intervention was started 24 hours after induction of MI to minimize the possibility of a direct effect of bosentan on infantile heart. Bosentan or vehicle was administered by gavage once daily for 3 or 15 days. A group of sham-operated rats received no treatment (n = 10).

The animal experiments, procedures, and housing were in accordance with institutional guidelines and national legislation conforming to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of 18 March 1986.

Hemodynamic Measurements

On the day of the experiments, the rats were anesthetized and ventilated by a rodent ventilator as previously reported.16 A 2F micromanometer-tipped catheter (model SPR-307, Millar Instruments) was inserted through the right carotid artery and advanced into the LV for measurements of LVEDP and LV systolic pressure (LVSP). The hemodynamic measurements in the bosentan intervention study were performed 24 hours after the last dose of bosentan/vehicle.

Northern Blot Analysis

Isolation of RNA and Northern blot analysis were performed as previously described.17,18 The cDNA probes used were a fragment of the rat ADM cDNA (nucleotides 152 to 755, GenBank accession No. U15419) and a fragment of the rat ADM receptor cDNA (nucleotides 620 to 1068, GenBank accession No. S79811). The RAMP-2 cDNA probe was a fragment of the human RAMP-2 cDNA (nucleotides 315 to 572, GenBank accession No. AJ001015). The restriction fragments were radiolabeled by the random priming method in the presence of [α-<sup>32</sup>P]dCTP (specific activity ~6000 Ci/mmol). The filters were subjected to autoradiography on storage phosphor plates (48 hours’ exposure) and analyzed by a scanning phosphorimager (PhosphorImager 445 SI, Molecular Dynamics). Densitometric analysis of the bands was performed with the Image-Master software package (Pharmacia Biotech). To normalize the ADM, ADM receptor and RAMP-2 mRNA signals for variations in RNA loading and transfer efficiencies, the same filter membranes were rehybridized with a GAPDH cDNA probe (a fragment of rat GAPDH cDNA corresponding to nucleotides 458 to 994, GenBank accession No. M17701).

Immunohistochemistry

Rat hearts subjected to immunohistochemical analysis were perfused and fixed with Bouin’s solution and embedded in paraffin wax. Paraffin-embedded myocardial tissue was cut into 8-μm sections, dewaxed in xylene, and subsequently rehydrated in descending alcohol and buffer (20 mmol/L HEPES [pH 7.4], 5 mmol/L MgCl<sub>2</sub>, 10 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L EDTA, 1% BSA, 0.1 mg/mL benzamidine, 30 μg/mL aprotinin, and 0.2 mmol/L PMSF). Membranes were prepared by differential centrifugation. The final pellets were resuspended in 50 mmol/L HEPES, pH 7.4, containing 0.25 mol/L sucrose, 0.25 μg/mL leupeptin, 10 μg/mL pepstatin, 0.1 mg/mL benzamidine, 30 μg/mL aprotinin, and 0.2 mmol/L PMSF. Membranes were prepared by differential centrifugation. The final pellets were resuspended in 50 mmol/L HEPES, pH 7.4. Membrane aliquots (25 μg membrane protein) were incubated for 30 minutes at 4°C in binding buffer (20 mmol/L HEPES [pH 7.4], 5 mmol/L MgCl<sub>2</sub>, 10 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L EDTA, 1% BSA, 0.1 mg/mL benzamidine, 30 μg/mL aprotinin, and 0.2 mmol/L PMSF) containing 0.3 mmol/L (125)I-labeled ADM (specific activity ~2000 Ci/mmol). Bound and free radioligand were separated by rapid filtration through GF/C filters (Whatman International) pretreated with 0.3% polyethylenimine. Nonspecific binding was determined in the presence of 5 μmol/L of ADM.

Radioligand Binding Assay

ADM receptor binding was studied in myocardial tissue from the LV of sham-operated rats and HF rats. The tissue was homogenized in ice-cold 50 mmol/L HEPES, pH 7.4, containing 0.25 mol/L sucrose, 0.25 μg/mL leupeptin, 10 μg/mL pepstatin, 0.1 mg/mL benzamidine, 30 μg/mL aprotinin, and 0.2 mmol/L PMSF. The membranes were prepared by differential centrifugation. The final pellets were resuspended in 50 mmol/L HEPES, pH 7.4. Membrane aliquots (25 μg membrane protein) were incubated for 30 minutes at 4°C in binding buffer (20 mmol/L HEPES [pH 7.4], 5 mmol/L MgCl<sub>2</sub>, 10 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L EDTA, 1% BSA, 0.1 mg/mL benzamidine, 30 μg/mL aprotinin, and 0.2 mmol/L PMSF) containing 0.3 mmol/L (125)I-labeled ADM (specific activity ~2000 Ci/mmol). Bound and free radioligand were separated by rapid filtration through GF/C filters (Whatman International) pretreated with 0.3% polyethylenimine. Nonspecific binding was determined in the presence of 5 μmol/L of ADM.

### TABLE 1. Hemodynamic Measurements in Sham-Operated Rats and HF Rats

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=6)</th>
<th>HF-Vehicle (n=12)</th>
<th>HF-Bosentan (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVSP, mm Hg</td>
<td>125±6</td>
<td>131±5</td>
<td>134±5</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>2±2</td>
<td>2±2</td>
<td>2±2</td>
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</tbody>
</table>

Values are mean±SEM. *P<0.05 vs sham-operated rats; †P<0.05 vs HF-vehicle rats.

### TABLE 2. Hemodynamic Measurements in HF Rats After 15 Days of Treatment With Bosentan

<table>
<thead>
<tr>
<th></th>
<th>Sham (n=6)</th>
<th>HF-Vehicle (n=12)</th>
<th>HF-Bosentan (n=11)</th>
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</thead>
<tbody>
<tr>
<td>LVSP, mm Hg</td>
<td>131±5</td>
<td>107±3*</td>
<td>99±2*</td>
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<tr>
<td>LVEDP, mm Hg</td>
<td>2.0±0.9</td>
<td>23.2±0.7*</td>
<td>19.8±1.6*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 vs sham-operated rats; †P<0.05 vs HF-vehicle rats.
ence of 0.8 μmol/L unlabeled rat ADM. Specific binding, defined as total binding minus nonspecific binding, was determined by γ-spectrometry.

Statistical Analysis
All the data are presented as mean±SEM. Statistical analysis was assessed by the Mann-Whitney nonparametric test. Comparisons of the myocardial ADM mRNA levels between the 3 groups at different time points after MI were analyzed by ANOVA with the Kruskal-Wallis test. If the Kruskal-Wallis test revealed significant differences, subsequent pairwise analyses of individual group means were performed with the Mann-Whitney test and Bonferroni correction for multiple comparisons. Value of P<0.05 were considered to be statistically significant.

Results
Hemodynamic Measurements
Table 1 shows LVEDP and LVSP of sham-operated rats and HF rats at various time points after the surgical procedure. The hemodynamic measurements demonstrated progressive LV dysfunction in the HF groups, with LVSP significantly decreased and LVEDP significantly increased compared with sham-operated animals (P<0.05). Table 2 demonstrates LVEDP and LVSP of the rats in the intervention study. As shown, bosentan significantly decreased both LVEDP and LVSP compared with vehicle (P<0.05).

Myocardial ADM mRNA Expression
Left Ventricle
Northern blot analysis was performed to investigate the regulation of myocardial ADM mRNA levels at various time points after induction of ischemic HF. ADM mRNA expression could be identified with confidence in myocardial tissue from the LV of sham-operated rats (Figure 1). In the nonischemic region of the LV, maximal levels of ADM mRNA were observed 28 days after MI (1.5-fold increase compared with the sham group, P<0.05). In the ischemic area, the ADM mRNA levels were 2.3- and 4.9-fold, respectively, above the levels in the sham-operated groups at 7 and 28 days after the induction of MI (P<0.05).

Right Ventricle
ADM mRNA expression was also identified in myocardial tissue from the right ventricle. However, ADM mRNA expression was not significantly different in the HF rats compared with the sham-operated group during the period of observation (data not shown).

Atria
Low levels of ADM mRNA could be identified with confidence in myocardial tissue from the atria (Figure 2). There
were no statistically significant alterations of atrial ADM mRNA expression 7 days (Figure 2) or 42 days (data not shown) after induction of MI compared with the sham-operated rats.

**Myocardial ADM Receptor mRNA Expression**
Northern blot analysis using 4 µg of poly A⁺ RNA per lane and a single-stranded cDNA probe revealed low levels of ADM receptor mRNA in the LV of sham-operated rats (Figure 3). Increased ADM receptor mRNA levels were observed in the LV (ischemic and nonischemic regions analyzed together) 7 days (2.2-fold) and 42 days (2.3-fold) after ligation of the left coronary artery compared with the sham-operated groups (P<0.05).

**Myocardial RAMP-2 mRNA Expression**
RAMP-2 mRNA expression could be identified with confidence in myocardial tissue from the LV of sham-operated rats (Figure 4). Two, 7, and 42 days after MI, the RAMP-2 mRNA levels in the nonischemic region of the LV were 1.4-, 1.6-, and 1.5-fold, respectively, above the levels in the sham groups (P<0.05). RAMP-2 mRNA levels in the ischemic region 16 days after MI were 3 times the levels observed in the sham-operated rats (P<0.05; data not shown).

**Effects of ET Receptor Antagonism on LV ADM and RAMP-2 mRNA Expression**
Treatment with bosentan for 3 days did not affect the ADM or the RAMP-2 mRNA expression in the failing LV (data not shown). However, 15 days of intervention with bosentan prevented the increase of RAMP-2 mRNA levels in the nonischemic area of the failing LV (P<0.05) but did not cause significant alterations of the myocardial ADM mRNA levels (Figure 5). Intervention with bosentan did not affect the ADM or the RAMP-2 mRNA levels in the ischemic zone (data not shown).
ADM-Like Immunoreactivity in the Myocardium

Immunohistochemical analysis of the myocardium from both the left and the right ventricles of unoperated rats revealed the presence of ADM-like immunoreactivity (ADM-ir). The distribution of ADM-ir in the myocardium was not homogeneous. As shown in Figure 6B, the cardiomyocytes contain very low levels of ADM-ir. However, substantial ADM-ir was observed in the interstitium between the cardiomyocytes and in perivascular connective tissue. Furthermore, in the HF rats, increased myocardial anti-ADM immunostaining could be discerned compared with the control rats. The enhanced immunostaining was already evident 2 days after MI. In the myocardium distal to the ischemic zone, increased ADM-ir was observed predominantly in the microvascular endothelium, in the perimyocytic space (Figure 6D and 6E), as well as in the perivascular connective tissue (Figure 6D). However, the endothelial lining and circular smooth muscle cells of larger vessels in the failing myocardium did not display ADM-ir (Figure 6C and 6D). In the granulation tissue of the ischemic area and especially at the border zone between the ischemic and the nonischemic regions (Figure 6F), heavy anti-ADM immunostaining was observed. Microvascular endothelial cells in this region displayed strong ADM-ir. Conversely, the fully differentiated scar tissue displayed weak ADM-ir (Figure 6F). The numerous leukocytes observed in the ischemic area and in the tissue bordering this region, especially at 7 days after MI, did not display ADM-ir. Sections of hearts incubated with nonimmune rabbit serum did not demonstrate immunostaining of any of the cellular elements of the myocardial tissue, demonstrating specificity of the ADM antisera (Figure 6A).

Specific ADM Receptor Binding Sites in the Myocardium

Radioligand binding assay demonstrated specific binding of 125I-ADM in LV tissue. Four days after induction of MI, the density of ADM binding sites in the LV free wall contiguous to the ischemic region was increased 1.6-fold above the levels in sham-operated rats (194 ± 10 versus 121 ± 5 fmol/mg mem-
brane protein, P < 0.05), whereas the density of ADM binding sites in the interventricular septum and in the ischemic area were not significantly different from the density in sham-operated rats.

Discussion

The present study provides novel information on the regulation of the myocardial ADM signaling system in different regions of the heart during ischemic HF in rats. The major new findings are that both LV ADM and ADM receptor levels are significantly increased during postinfarction failure. In addition, the study demonstrates increased gene expression not only of ADM and its primary receptor during HF but also of the receptor-modifying protein RAMP-2. RAMP-2 has been shown to modify the posttranslational processing of the CRLR, leading to high-affinity ADM binding. Thus, this study demonstrates, for the first time, upregulation of RAMP mRNA during a pathological condition, indicating that these receptor-modifying proteins may participate in the dynamic control of ADM receptor binding activity and selectivity for agonist in disease.

It could be argued that the increase of myocardial ADM mRNA levels observed in the study was moderate and therefore of uncertain pathophysiological relevance. However, parallel increases of myocardial ADM-ir could be identified with confidence in microvascular endothelium and in perivascular tissue during ischemic HF. In addition to the induction of ADM mRNA, our results demonstrate that both myocardial ADM receptor and RAMP-2 mRNA levels are significantly upregulated during HF and not downregulated as a compensatory response to the increased levels of ADM. Furthermore, an increase of specific ADM binding sites in the failing myocardium was found. Thus, the concerted upregulation of ADM, ADM receptor, and RAMP-2 indicates that activation of the myocardial ADM signaling system in the failing myocardium could have an important pathophysiological role during postinfa

An intriguing issue is which mechanisms are involved in the induction of the ADM signaling system in the failing LV. Induction of LV ADM mRNA as well as ADM receptor and RAMP-2 mRNAs could be a general response to hemodynamic overload and increased wall tension of the LV. Elevated myocardial ADM mRNA levels during volume and
pressure overload in rats have recently been reported.\textsuperscript{19,20} In the present study, ET receptor antagonism for 15 days with subsequent reduction of cardiac load did not significantly affect the myocardial mRNA levels of ADM. However, treatment with bosentan prevented the induction of RAMP-2 mRNA expression in the nonischemic area of the LV, indicating that at least 1 component of the ADM signaling system may be subjected to regulation by hemodynamic load. However, the inhibitory effects of bosentan could also be due to blockade of putatively stimulatory actions of ET-1 on RAMP-2 mRNA expression. Although our results do not support a stimulatory action of ET-1 on myocardial ADM production, ET-1 has previously been shown to stimulate ADM secretion from cultured smooth muscle cells.\textsuperscript{21} It has

Figure 6. Photomicrographs of rat myocardial sections immunostained with an anti-ADM antiserum (B through F) or nonimmune serum as control (A). B through D, ADM-ir of myocardium of an unoperated rat (B) and of nonischemic myocardium of LV from HF rats 7 and 42 days after MI (C and D, respectively). E, ADM-ir in interstitium between cardiomyocytes and in microvascular endothelium (arrow) in nonischemic LV 42 days after MI. F, ADM-ir in microvascular endothelial cells (arrows) and in connective tissue surrounding these vessels in border zone between ischemic zone (top) and nonischemic LV (bottom) 42 days after MI. Magnification $\times$400.
also recently been demonstrated that stimulation of the ET3 receptors of cultured aortic endothelial cells increases the production and secretion of ADM. Activation of the sympathetic nervous system and release of other neurohumoral mediators may also stimulate ADM, ADM receptor, and RAMP-2 mRNA induction. The 5′ flanking region of the ADM gene has been reported to contain multiple binding sites for the transcription factor activator protein-2, suggesting that expression of ADM may be subjected to regulation by the protein kinase C or protein kinase A pathways. Other vasoactive substances activated in HF may also stimulate ADM, ADM receptor, and RAMP-2 mRNA expression. For instance, in rats infused with arginine-vasopressin and angiotensin II, 1.6- and 1.5-fold increases, respectively, of LV ADM mRNA levels have been reported. In addition, increased secretion of ADM from cultured vascular smooth muscle cells has also been reported after stimulation with angiotensin II.

Another mechanism potentially implicated in the induction of the LV ADM signaling system during postinfarction failure may be myocardial ischemia. In a rat model of cerebral ischemia, cerebral ADM mRNA levels were increased 20-fold above normal values. Furthermore, it has been demonstrated that ADM mRNA is induced by hypoxia in cultured adult rat ventricular cardiomyocytes and that this response is mediated by the hypoxia-inducible factor-1 consensus sites of the ADM promoter. This is consistent with our data demonstrating increased ADM and RAMP-2 mRNA levels in the ischemic area. The density of specific ADM binding sites was increased in LV tissue contiguous to the ischemic zone. Because the ADM signaling system mediates vasorelaxation, induction of the ADM system in the hypoxic regions may increase blood flow, resulting in reduced myocardial damage and improved myocardial function during postinfarction failure.

In the first weeks after MI, heavy myocardial leukocyte infiltration takes place in the ischemic region. These cells are known to secrete cytokines. Interestingly, it has been demonstrated in vitro that both interleukin-1 and tumor necrosis factor-α may stimulate synthesis and secretion of ADM in vascular smooth muscle cells, endothelial cells, and cardiac myocytes. Therefore, the increased levels of myocardial ADM mRNA in the ischemic area may be the result of such a stimulation.

The immunohistochemical analysis demonstrated the most substantial ADM-ir in microvascular endothelial cells and in connective tissue surrounding these vessels in the transition zone between the ischemic and nonischemic areas, ie, in areas with expected neovascularization. However, the endothelial cells exhibited various degrees of ADM immunoreactivity. Indeed, the endothelial lining of larger vessels did not display ADM-ir. The various degrees of anti-ADM immunostaining of the endothelial cells could be due to different degrees of exocytotic secretory activity. Thus, negative ADM immunostaining of endothelial cells of larger vessels could be explained by high secretory activity of these cells.

The functional role of increased myocardial ADM, ADM receptor, and RAMP-2 expression during postinfarction failure remains to be elucidated. ADM may enhance myocardial contractility via activation of adenyl cyclase and generation of cAMP. However, data from in vitro studies are conflicting, showing both positive and negative inotropic responses in isolated cardiomyocytes after stimulation with ADM. However, in an ovine model of pacing-induced HF, Rademaker et al. demonstrated reduced peripheral resistance and increased cardiac output after infusion of ADM. Evidence has recently been reported that the failing heart itself may secrete ADM into the circulation. Thus, it has been suggested that the vasorelaxing and diuretic actions of ADM could play a compensatory role, modulating the increased vascular tone and the increased intravascular volume associated with HF.

In conclusion, the present study demonstrates a concerted induction of the LV ADM signaling system in HF subsequent to MI in rats. Furthermore, immunohistochemical analysis demonstrated substantial ADM-ir in microvascular endothelial cells and in perivascular tissue. These observations suggest that the vessels are the main source of myocardial ADM production in normal and failing hearts and that ADM could act as an important regulator of vascular tone of these vessels. Therefore, it is suggested that ADM may play an important role in the pathophysiology of HF.

Acknowledgments

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References


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