Endothelin-1–Independent and Angiotensin II–Independent Induction of Adrenomedullin Gene Expression

Hannu Romppanen, Jutta Puhakka, Gábor Földes, István Szokodi, Olli Vuolteenaho, Heikki Tokola, Miklós Tóth, Heikki Ruskoaho

Abstract—Adrenomedullin (AM) may function as an autocrine and/or paracrine factor in the heart, but the exact mechanisms time course of induction of atrial and ventricular AM gene expression during pressure overload and to study whether endothelin-1 or angiotensin II plays a causal role in the activation of cardiac AM gene expression. The pressure overload was produced by arginine-vasopressin (AVP, 0.05 μg/kg per minute IV) infusion for 15 minutes, 30 minutes, 1 hour, 2 hours, or 4 hours in conscious rats. A significant increase in left ventricular AM mRNA levels was seen after 2 hours of pressure overload in the left ventricle and after 30 minutes in the left atrium. The left atrial immunoreactive AM (ir-AM) levels decreased significantly after 2 hours of pressure overload. Plasma ir-AM levels increased slightly in response to 4 hours of AVP infusion. Bolus injections of bosentan (mixed ETA/ETB receptor antagonist, 10 mg/kg IV), losartan (AT1 receptor antagonist, 10 mg/kg IV), and their combination had no effect on the increase of cardiac AM mRNA and ir-AM levels produced by 2 hours of pressure overload. In addition, losartan, bosentan, and their combination did not affect plasma ir-AM levels in the vehicle-infused and AVP-infused animals. The present study indicates that cardiac AM gene expression is rapidly upregulated in response to pressure. The induction of ventricular and atrial AM gene expression by pressure overload is angiotensin II–independent and endothelin-1–independent.

Key Words: adrenomedullin ■ gene expression ■ angiotensin II ■ endothelin

Cardiac overload is known to produce the hypertrophy of individual muscle cells and alter the expression of several cardiac-specific genes, including atrial natriuretic peptide and B-type natriuretic peptide (BNP), but it has not yet been established whether wall stretch acts directly or through local paracrine and autocrine factors liberated in response to hemodynamic load. In particular, local angiotensin II (Ang II) and endothelin-1 (ET-1) may play an important role in the adaptation of the heart to pressure and volume overload. Adrenomedullin (AM) is a novel hypotensive peptide, originally isolated from human pheochromocytoma. Infusion of AM causes vasodilation, diuresis, and natriuresis in normal animals (for reviews, see References 9 through 11). Subsequent studies have shown that AM peptide and mRNA are distributed in a variety of tissues, including the heart. Binding studies have demonstrated the presence of specific receptors for AM in the heart. AM increases cardiac output and left ventricular contractility in vivo and exerts a direct inotropic effect in vitro. The finding that AM attenuates Ang II–stimulated and serum-stimulated protein synthesis in cardiac myocytes further suggests a role for AM in paracrine and/or autocrine regulation of cardiac function. In addition, AM is a circulating hormone, and its plasma concentration is increased in various cardiorenal diseases such as hypertension, chronic kidney failure, and congestive heart failure. Previous studies have also revealed that ventricular AM levels are increased in several hypertensive models with cardiac hypertrophy. Left ventricular AM gene expression has been reported to increase in response to aortic banding in rats within 24 hours, whereas Kaiser et al did not find changes in AM mRNA levels during aortic banding from 30 minutes up to 28 days. We have recently reported that AM gene expression is increased in the left ventricle by pressure overload within 2 hours, whereas in a recent study, ventricular AM gene expression was upregulated only in advanced heart failure. Thus, the pathophysiological significance and time course of induction of AM gene expression in the heart as well as the mechanisms regulating cardiac AM production in hemodynamic overload remain unclear.

In the present study, to characterize the exact time course of induction of cardiac AM gene expression, we measured hemodynamics and tissue mRNA and peptide levels of AM and plasma-immunoreactive AM (ir-AM) levels at 15 min-

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utes, 30 minutes, 1 hour, 2 hours, and 4 hours after pressure overload produced by intravenous infusion of arginine-vasopressin (AVP) in conscious normotensive rats. We also assessed the effects of the mixed ET<sub>A</sub>/ET<sub>B</sub> receptor antagonist bosentan, the AT<sub>1</sub> receptor antagonist losartan, or their combination on the induction of cardiac AM gene expression to determine whether ET-1 or Ang II plays a causal role in the activation of AM gene expression by pressure overload in ventricles and atria. Furthermore, the actions of ET-1 and Ang II receptor antagonist on atrial and ventricular levels of AM mRNA and tissue and plasma peptide levels under basal conditions (without pressure overload) in conscious rats were also analyzed.

**Methods**

**Drugs**

AVP was obtained from Peninsular Laboratories Europe, bosentan from F. Hoffmann-La Roche Ltd and Actelion Ltd (Dr Martine Clozel), losartan from DuPont Merck Pharmaceutical Co (Dr Ronald D. Smith), and [32P]-deoxy-CTP and radioiodine from Amersham.

**Experimental Design in Conscious Rats**

The 2-month-old male Sprague-Dawley rats (n=56) were anesthe-
tized with 0.26 mg/kg fentanyl citrate, 8.25 mg/kg fluanisone, and 4.1 mg/kg midazolam IP and instrumented for vehicle and drug infusions as previously described. The experiments were started in conscious animals by measurement of mean arterial pressure (MAP) and heart rate for 25 minutes before 1.0 mL of blood was withdrawn for the measurement of plasma ir-AM. The volume was replaced with an equal volume of blood from a donor rat. Baseline hemodynamics were taken 5 minutes later, when MAP and heart rate had stabilized near the control values. AVP (0.05 μg/kg per minute IV) or vehicle (0.9% NaCl IV) was infused at 37.5 μL/min for 15 minutes, 30 minutes, 1 hour, 2 hours, and 4 hours. In a separate series of experiments, bosentan (10 mg/kg), losartan (10 mg/kg), their combination, or vehicle (0.9% NaCl) was injected as an intravenous bolus (injection volume, 0.1 mL/100 g body wt) followed by 2 hours of vehicle or AVP infusion. Arterial blood samples were taken at the end of infusions. Finally, Ang II (33 μg/kg per hour, n=9) or vehicle (0.9% NaCl, n=9) was infused for 12 hours through subcutaneously implanted osmotic minipumps (Alzet 2001). For telemetric monitoring of MAP and heart rate, the rats were instrumented with a catheter in the descending aorta coupled with a sensor and transmitter (PA-C40, Data Sciences International). Tissues were prepared as previously described<sup>22</sup> for the peptide and mRNA determinations at the end of drug and vehicle infusions. All cardiac tissue samples were blotted dry, weighed, immersed in the liquid nitrogen, and stored at −70°C until assayed. The experimental design was approved by the Animal Use and Care Committee of the University of Oulu.

**Isolation and Analysis of RNA**

RNA was isolated from ventricles and atria by the guanidine thiocyanate–CsCl method.<sup>5</sup> For the RNA Northern blot analysis, 20-μg samples of the RNA from the ventricles and 5-μg samples from atria were separated by electrophoresis on agarose gel and transferred to nylon membranes. A 390-bp fragment of rat BNP cDNA,<sup>25</sup> a cDNA probe (450 bp) for AM made by reverse transcription—polymerase chain reaction,<sup>22</sup> and a full-length cDNA probe complementary to rat glyceraldehyde 3-phosphate-dehydrogenase (GAPDH)<sup>26</sup> were labeled, and the membranes were hybridized and washed as described previously.<sup>22</sup> The hybridization signal of AM mRNA and BNP mRNA was normalized to that of GAPDH mRNA in each sample.

**Radioimmunoassays**

The AM and BNP radioimmunoassays were performed as previously described.<sup>5–22</sup> The sensitivities of the AM and BNP assays were 1

<table>
<thead>
<tr>
<th>Time</th>
<th>Mean Arterial Pressure, mm Hg</th>
<th>Heart Rate, bpm</th>
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<td></td>
<td>0 min</td>
<td>15 min</td>
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<tr>
<td>AVP (n=10)</td>
<td>120±2</td>
<td>159±3†</td>
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<td>107±5</td>
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<tr>
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<tr>
<td>L+B (n=6)</td>
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<td>98±5</td>
</tr>
<tr>
<td>L+B+AVP (n=6)</td>
<td>380±3</td>
<td>295±6†</td>
</tr>
</tbody>
</table>

L indicates losartan; B, bosentan.

Results are mean±SEM.

*P<0.05, †P<0.001 vs 0-minute time point (1-way ANOVA followed by Student-Newman-Keul’s post hoc test).
fmol/tube and 2 fmol/tube, respectively. The intra-assay and inter-assay variations were <10% and 15%, respectively. Serial dilutions of tissue and plasma extracts showed parallelism with the standards. Tissue AM and BNP are expressed as a concentration per milligram wet weight.

Statistics
The results are expressed as mean±SEM. For the comparison of statistical significance between 2 groups, the Student’s t test was used. The hemodynamic variables were analyzed with 1-way ANOVA followed by Student-Newman-Keuls post hoc test. A value of P<0.05 was considered statistically significant.

Results
Characterization of Pressure-Overload Model
Pressure overload was produced by infusion of AVP to study the regulatory mechanisms for induction of cardiac AM gene expression. To validate the model, we examined the activation of BNP synthesis in ventricles and atria because it is one of the earliest cardiac myocyte-specific markers of hemodynamic overload. AVP infusion rapidly raised MAP, which reached maximum value within 15 minutes. This increase in MAP was associated with a significant decrease in heart rate when compared with the vehicle group (Table). MAP and heart rate remained unchanged in the vehicle-treated animals (Table). AVP infusion caused a significant increase in BNP mRNA levels both in the endocardial and the epicardial layers of left ventricle from 1 hour onward (Figure 1 and Figure 2, A and B). The AVP infusion caused a significant increase of AM mRNA (vehicle, 1.0±0.7 versus Ang II, 1.4±0.13 arbitrary densitometric units, P<0.05) and 53% increase in ir-AM levels (vehicle, 0.17±0.01 versus Ang II, 0.26±0.02 fmol/mg, P<0.01) in the left ventricles.

Effects of Pressure Overload on Cardiac AM Gene Expression
AVP infusion caused rapid upregulation of ventricular AM gene expression. A significant increase in AM mRNA levels was observed at 2 and 4 hours both in the endocardial and epicardial layer of the left ventricle, the increase being 2-fold in both layers of the left ventricle in response to 4 hours of AVP infusion (Figure 1 and Figure 3, A and B). There were no differences in left ventricular ir-AM levels between vehicle-infused and AVP-infused animals (Figure 3, A and B). The AVP infusion caused a significant increase of AM mRNA levels also in the left atria. A 1.3-fold induction in AM mRNA levels was seen already after 30 minutes of AVP infusion, the greatest increase (3.5-fold) being observed after 4 hours of AVP infusion (Figure 3C). Like BNP, ir-AM levels decreased in the left atria by 46% (P<0.05) and by 40% (P<0.01) after 2 and 4 hours infusion of AVP, respectively (Figure 3C). In vehicle-treated animals, baseline left atrial concentrations of ir-AM (243±15 fmol/g) were higher than that in the left ventricle (endocardium: 62±3 fmol/g, epicardium: 80±2 fmol/g). AVP infusion had no effect on right atrial pressure and AM mRNA levels (data not shown) as well as on right BNP mRNA levels, supporting the hypothesis AVP has no direct effect on cardiac gene expression under these experimental conditions.

To strengthen the hypothesis that pressor overload stimulates AM gene expression, we infused Ang II in conscious rats by osmotic minipumps for 12 hours. Ang II infusion raised MAP (from 103±8 to 141±9 mm Hg, P<0.05) and decreased heart rate (from 373±29 to 320±16 bpm, P<0.05), whereas during the vehicle infusion, MAP and heart rate remained unchanged (MAP: 108±3 versus 106±2 mm Hg; heart rate: 366±18 versus 392±9 bpm). The pressor response to Ang II infusion was associated with 40% increase in AM mRNA (vehicle, 1.0±0.07 versus Ang II, 1.4±0.13 arbitrary densitometric units, P<0.05) and 53% increase in ir-AM levels (vehicle, 0.17±0.01 versus Ang II, 0.26±0.02 fmol/mg, P<0.01) in the left ventricles.
Effect of Losartan, Bosentan, and Their Combination on Hemodynamic Variables

To characterize the role of ET-1 and Ang II in the pressure-overload–induced AM gene activation, we studied the effects of mixed ETA/ETB receptor antagonist bosentan and AT1 receptor antagonist losartan on the increase of cardiac AM mRNA and ir-AM levels produced by 2 hours of AVP infusion. Both bosentan and losartan were administered at a concentration of 10 mg/kg IV as a bolus injection. Previously, we have shown that in conscious rats, bosentan at a dose of 10 mg/kg IV completely blocks any increase in MAP produced by big ET-1, and losartan at a concentration of 10 mg/kg completely blocks any increase in MAP produced by Ang II infusion.27 In agreement with the previous study in normotensive rats, 24 bolus injections of losartan and bosentan as well as their combination led to a significant decrease in MAP within 2 hours (Table). In contrast, infusion of AVP increased MAP similarly in vehicle- and drug-treated conscious rats (Table). In addition, heart rate decreased similarly in the vehicle-treated, bosentan-treated, losartan-treated, and bosentan plus losartan–pretreated animals (Table). These results show that drug injections did not alter the hemodynamic response evoked by AVP infusion, thus allowing us to examine the direct action of load versus a requirement for Ang II and ET-1 to mediate pressure overload–induced upregulation of cardiac AM gene expression.

Effects of Losartan, Bosentan, and Their Combination on Cardiac AM Gene Expression

Administration of losartan, bosentan, and their combination did not significantly influence baseline AM mRNA and ir-AM levels in the endocardial (Figure 4A) or epicardial (Figure 4B) layer of the left ventricle. The elevation of AM mRNA levels in response to pressure overload produced by 2 hours of AVP infusion was similar in both left ventricular endocardial and epicardial layers in vehicle- and drug-treated conscious rats, whereas no changes in left ventricular ir-AM concentrations were found (Figure 4, A and B). As shown in Figure 4C, injections of bosentan and losartan alone had no effect on AM mRNA and ir-AM levels in left atria, whereas a 37% decrease (P<0.05) in baseline left atrial AM mRNA levels was seen in conscious rats treated with both bosentan and losartan (Figure 4C). Losartan, bosentan, and their combination did not significantly affect the increase of left atrial AM mRNA levels in response to 2 hours of AVP infusion when compared with the vehicle group. Furthermore, left atrial ir-AM levels decreased in vehicle- and drug-treated animals, although this change was not statistically significant in losartan-treated animals (Figure 4C). Of note, the increase in left atrial AM mRNA levels in response to AVP was greater in losartan-pretreated than in bosentan-
Furthermore, hypertrophy, thus mimicking the rapid induction of proto-oncogenes in response to hemodynamic stress.1 Furthermore, a positive correlation between left ventricular mass index and left ventricular AM concentrations suggests that ventricular hypertrophy may activate AM gene expression in the progression of heart failure.28 In this study, the cardiac AM mRNA levels in the ventricles were significantly decreased by AVP infusion after 2 hours. However, the plasma AM levels were not elevated in pressure-overloaded rats compared with the control rats, except after 4 hours of AVP infusion. This increase in circulating AM may be due to the effect of pressure overload on the heart because left atrial AM mRNA levels decreased in AVP-infused animals after 2 hours. However, ventricles may also contribute to circulating AM levels because unchanged left ventricular AM peptide levels together with increased AM mRNA levels could be explained by an increased rate of release of AM from the ventricles promptly after its synthesis. The elevated plasma levels at 4 hours may also reflect mechanical, stress-stimulated AM production from systemic vascular walls.35 In the myocardium, immunoreactivity is located in cardiac myocytes, not nonmyocytes, and Ang II significantly activated AM gene expression within 2 hours in perfused rat heart preparation.33 Our present results do not support a role for ET-1 or Ang II in the induction of ventricular AM mRNA levels within 2 hours in perfused rat heart preparation.33 Ventricular AM gene expression is a very rapid and sensitive marker of increased pressure overload and suggest that AM plays an important role in cardiovascular regulation in concert with other neurohumoral mechanisms.

**Plasma AM Concentrations**

In contrast to the marked increase in cardiac AM gene expression, AVP infusion significantly raised plasma ir-AM concentrations only at 4 hours in conscious rats (49.7±7.7 versus 36.9±5.2 pmol/L, P<0.05) (Figure 5A). The administration of losartan, losartan, and their combination had no effect on the plasma ir-AM levels in the vehicle- and AVP-infused conscious rats (Figure 5B).

**Discussion**

Recent data show that in experimental congestive heart failure produced by rapid ventricular pacing in dogs, ventricular AM is activated in the progression of heart failure.28 Furthermore, a positive correlation between left ventricular mass index and left ventricular AM concentrations suggests that ventricular hypertrophy may activate AM gene expression.20 In the present study, we report that ventricular AM gene expression is activated at a very early stage of pressure overload, well before the development of left ventricular hypertrophy, thus mimicking the rapid induction of proto-oncogenes in response to hemodynamic stress.1 Furthermore, pressure overload significantly increased left atrial AM gene expression, even earlier than that in the left ventricle. These results indicate that cardiac AM gene expression is a very rapid and sensitive marker of increased pressure overload and suggest that AM plays an important role in cardiovascular regulation in concert with other neurohumoral mechanisms. The mechanisms by which pressure overload is transduced by the cardiac muscle cell and translated into myocyte hypertrophy are not completely understood. Candidates include neurohormonal factors such as Ang II, ET-1, and α-adrenergic agents.6,7,29,30 With the use of cultured neonatal rat heart cells, it has been reported that mechanical stretch is coupled with cellular release of Ang II and ET-1 and that they act as chemical mediators of stretch-induced myocyte hypertrophy.31,32 Thus, Ang II acting through the AT1 receptor and endogenous cardiac production of ET-1 may play a functional role in mechanical load–induced cardiac gene expression and thus also mediate the rapid induction of AM gene expression. In support of this, pressor overload produced by administration of Ang II for 12 hours (this study) and 2 weeks,22 respectively, in conscious rats increases left ventricular weight and AM mRNA levels, and administration of ET-1 can induce the increase of ventricular AM mRNA levels within 2 hours in perfused rat heart preparation.33 Our present studies show that the combination of losartan and bosentan significantly decreased AM mRNA levels in the left atrium, whereas losartan or bosentan alone had no significant effect. Because the drug treatments decreased MAP, and this decrease was greatest with the combination treatment of losartan and bosentan, it is likely that the decreased pressure load explains the decrease in left atrial AM mRNA levels. In addition, atrial AM gene expression appears to be more sensitive than ventricular AM gene expression to rapid alterations in cardiac load because drug treatments did not have any effect on left ventricular AM mRNA levels.

Although the concentration of plasma AM has been shown to be increased in patients with congestive heart failure and hypertension,24 the main source of circulating AM is unclear. A recent study with immohistochemical analysis showed that ventricular myocytes, not nonmyocytes, may be a major source of ventricular AM production in left ventricular hypertrophy.20 In this study, the cardiac AM mRNA levels gradually increased in a time-dependent manner, whereas the plasma AM levels were not elevated in pressure-overloaded rats compared with the control rats, except after 4 hours of AVP infusion. This increase in circulating AM may be due to the effect of pressure overload on the heart because left atrial AM mRNA levels decreased in AVP-infused animals after 2 hours. However, ventricles may also contribute to circulating AM levels because unchanged left ventricular AM peptide levels together with increased AM mRNA levels could be explained by an increased rate of release of AM from the ventricles promptly after its synthesis. The elevated plasma levels at 4 hours may also reflect mechanical, stress-stimulated AM production from systemic vascular walls.35 In the myocardium, immunoreactivity is located in...
the peripheral cytoplasm of cardiac myocytes, and so far, no AM granules have been reported in the myocardial cells. Therefore, cardiac AM secretion may be constitutive, and during the early phase of pressure load, atria rather than ventricles appear to contribute to the increase in circulating AM.

The present results are consistent with the hypothesis that AM may play a compensatory role in the maintenance of intravascular volume and cardiac filling pressures during increased cardiac workload, similar to atrial natriuretic peptide and BNP. AM appears to be regulated in a pattern similar to that of BNP, which is also synthesized both in atria and ventricles. Because BNP mRNA levels increased more prominently and earlier than AM mRNA levels, BNP appears to be a slightly more sensitive marker for acutely increased cardiac pressure load than AM. It is also noteworthy that left atrial AM and BNP mRNA levels responded to cardiac overload more sensitively than those of left ventricle and that the enhanced mRNA expression led to a significant increase in ventricular BNP but not AM peptide levels. This latter observation suggests that distinct pathways are involved in the regulation of ventricular BNP and AM peptide levels. Because plasma ir-AM levels increased only slightly during pressure overload, AM may function as a paracrine and/or autocrine factor in the heart rather than as a circulating hormone. Indeed, AM enhances cardiac contractility through cAMP-independent mechanisms and inhibits Ang II–stimulated hypertrophic response in cardiac myocytes. The use of specific AM receptor antagonists and transgenic approaches are necessary to determine the exact role of AM in the regulation of cardiac function.

In conclusion, our data show that cardiac wall stretch produced by pressure overload is a major stimulus for the early induction of AM gene expression both in the ventricle and atrium. We also found for the first time that the induction of cardiac AM gene expression is Ang II independent and ET-1 independent, suggesting that local ET-1 and Ang II production do not act as triggering factors to an early increase of cardiac AM gene expression. The increase in plasma ir-AM levels were small, suggesting a paracrine and/or autocrine factor in the heart rather than as a circulating hormone. Indeed, AM enhances cardiac contractility through cAMP-independent mechanisms and inhibits Ang II–stimulated hypertrophic response in cardiac myocytes. The use of specific AM receptor antagonists and transgenic approaches are necessary to determine the exact role of AM in the regulation of cardiac function.

Acknowledgments

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References

tricle is a major site of synthesis and secretion of brain natriuretic peptide. 


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