Increased gene expression of adrenomedullin and adrenomedullin-receptor complexes, receptor-activity modifying protein (RAMP)2 and calcitonin-receptor-like receptor (CRLR) in the hearts of rats with congestive heart failure

Kazuhito TOTSUNE*, Kazuhiro TAKAHASHI†, Harald S. MACKENZIE‡, Osamu MURAKAMI*, Zenei ARIHARA*, Masahiko SONE†, Toraichi MOURI§, Barry M. BRENNER‡ and Sadayoshi ITO*

*Second Department of Internal Medicine, Tohoku University School of Medicine, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan, †Department of Molecular Biology and Applied Physiology, Tohoku University School of Medicine, Sendai, Miyagi 980-8575, Japan, ‡Renal Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, U.S.A., and §Division of Neuroendocrinology, Mouri Clinic, Natori, Miyagi 981-1224, Japan

ABSTRACT

Adrenomedullin is a vasodilator peptide produced in various organs, including heart and kidney. A novel adrenomedullin receptor complex has recently been identified, namely the calcitonin receptor-like receptor (CRLR) and receptor-activity modifying protein (RAMP)2. In the present study, we have examined gene expression of RAMP2, CRLR and adrenomedullin in hearts and kidneys of rats with congestive heart failure caused by coronary artery ligation. Partial cloning was performed to determine the rat RAMP2 nucleotide sequence. Messenger RNA levels were then determined using competitive, quantitative reverse transcription-PCR techniques. Significantly increased expression levels (means ± S.E.) of RAMP2, CRLR and adrenomedullin mRNA were found in the atrium (1.8 ± 0.2-fold, 1.8 ± 0.2-fold and 2.1 ± 0.1-fold, respectively, compared with sham operated rats) and in the ventricle (1.4 ± 0.1-fold, 1.3 ± 0.03-fold and 3.0 ± 0.5-fold respectively). On the other hand, expression levels of RAMP2, CRLR and adrenomedullin mRNAs were not significantly changed in the kidney. These findings suggest potential roles of locally-produced and locally-acting adrenomedullin in the failing heart.

INTRODUCTION

Adrenomedullin is a novel vasodilator peptide with diuretic action [1,2]. Adrenomedullin is produced by various kinds of cells, including vascular endothelial cells [3], vascular smooth muscle cells [4], cardiomyocytes [5], fibroblasts [6], mesangial cells [7], macrophages [8], neurons [9,10], astrocytes [11] and some tumour cells [9,12–14]. Adrenomedullin is highly expressed in the heart and kidney [2,15]. Recently, several reports have suggested that adrenomedullin plays an important role in the pathophysiology of heart failure. Plasma adreno-
medullin levels were elevated in patients with heart failure [16,17], and levels of adrenomedullin expression were upregulated in the hearts of animals with experimental congestive heart failure [18–20].

McLatchie et al. [21] reported novel receptor complexes, consisting of calcitonin-receptor-like receptor (CRLR) and receptor-activity modifying proteins (RAMPs). Three isoforms of RAMPs have been identified. The two major forms, RAMP1 and RAMP2, together with CRLR, are important for the generation of receptors specific for calcitonin gene-related peptide and adrenomedullin respectively. The RAMP1–CRLR complex generates the calcitonin gene-related peptide specific receptor, and the RAMP2–CRLR complex generates the adrenomedullin-specific receptor. Another isoform of RAMP, RAMP3, also forms an adrenomedullin receptor in vitro in combination with CRLR [21], but RAMP3 expression is very low in rat tissues, including the heart [22]. Furthermore, adrenomedullin binding showed a tendency to vary with RAMP2 mRNA levels, but not with RAMP3 mRNA levels in various rat tissues [22]. Thus CRLR is a component common to the both receptors, whereas RAMP2 is a major component specific for the adrenomedullin receptor.

However, regulation of adrenomedullin receptor expression in pathophysiological states has not been studied in detail. Further, the main target organ of adrenomedullin in heart failure remains unclear. In the present study, we examined gene expression of RAMP2, CRLR, and adrenomedullin in the heart and kidney of rats with congestive heart failure induced by coronary ligation.

**METHODS**

**Animals**

Animal experiments were performed in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of Harvard Medical School. Studies were performed on representative rats from groups which had been used for a separate study [23]. Briefly, in the experimental animals, coronary ligation was performed on male Munich–Wistar rats weighing 250–270 g (n = 4) under methohexital anaesthesia (50 mg/kg). Sham operated (SO) rats (n = 4) underwent thoracotomy and closure of the thorax only. Rats received standard rat chow and water ad libitum for 3–4 weeks after surgery. All rats were then anaesthetized and the cardiac atria, ventricles and kidneys were harvested, snap-frozen in liquid nitrogen and maintained at −80 °C until RNA extraction. The profiles of SO rats and rats with congestive heart failure have been summarized previously [23], and increased plasma atrial natriuretic peptide levels (approx. 14-fold compared with SO rats) and left ventricular diastolic pressure (approx. 4-fold compared with SO rats) were reported for the rats with heart failure.

**Competitive and quantitative reverse transcription (RT)-PCR**

Total RNA was extracted by the guanidinium isothiocyanate/CsCl method and 4 μg of total RNA were reverse transcribed with 400 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT; Life Technologies, Rockville, MD, U.S.A.) using an oligo(dT) primer in a total volume of 20 μl, as described previously [24]. Expression of RAMP2, CRLR, adrenomedullin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs was determined using competitive, quantitative RT-PCR methods.

For rat RAMP2 PCR, a partial cloning of rat RAMP2 was performed because the nucleotide sequence of rat RAMP2 has not, to our knowledge, been previously reported. A sense primer, corresponding to nt 300–319, and an antisense primer, corresponding to nt 516–534 of human RAMP2 (accession number AJ001015), were used in a PCR to amplify rat RAMP2 at low stringency as follows: 35 cycles at 94 °C for 45 s, 38 °C for 45 s, 72 °C for 1.5 min and a final cycle at 72 °C for 5 min. PCR was performed in a total volume of 20 μl containing 1 μl of the first strand cDNA reverse transcribed from rat atrial total RNA, 10 mM Tris/HCl (pH 8.30), 50 mM KCl, 2 mM MgCl2, 0.001% (w/v) gelatin, 0.2 mM of each deoxynucleotide triphosphate, 0.25 μM of each primer and 0.4 units of Taq DNA polymerase (Pharmacia, Piscataway, NJ, U.S.A.). A 235-bp PCR product was obtained, ligated into pGEM-T vector (Promega, Madison, WI, U.S.A.) and sequenced using an autosequencer (Model 310, Applied Biosystems).

Primers used for RT-PCR analysis are summarized in Table 1 [2,25]. PCR protocols were as follows. For RAMP2: 20 cycles at 94 °C for 15 s, 61 °C for 1 min, 72 °C for 1 min; for CRLR: 24 cycles at 94 °C for 15 s, 61 °C for 30 s, 72 °C for 1 min; for adrenomedullin: 24 cycles at 94 °C for 15 s, 58 °C for 30 s, 72 °C for 1 min; each was then subjected to a final cycle at 72 °C for 5 min.

![Image](image-url)
543Adrenomedullin and adrenomedullin receptors in the failing heart

Figure 1 Polyacrylamide gels of competitive, quantitative RT-PCR for RAMP2 (top panel), CRLR (middle panel) and adrenomedullin (AM) (bottom panel) using increasing amounts of CRS DNA

Lane 1, standard DNA ladder; lanes 2–9, PCR products using 200 ng total RNA from normal rat kidney and increasing amounts of CRS-DNA/tube as templates; lane 10, normal kidney sample treated similarly but in the absence of reverse transcriptase, used as a negative-control template in the PCR.

Competitive, quantitative RT-PCR for GAPDH was performed as reported previously [24]. The competitive reference standard (CRS) DNA for RAMP2 was prepared using a PCR by deleting 40-bp (22–54 and 168–174) from a 194-bp RAMP2 cDNA fragment. The CRS DNA for the competitive, quantitative RT-PCR of CRLR was prepared with a PCR by deleting 138-bp (762–837 and 1195–1256) from the 533-bp CRLR cDNA fragment. The CRS DNA for adrenomedullin was obtained using a PCR MIMIC Construction Kit (Clontech, Palo Alto, CA, U.S.A.) according to manufacturer’s instructions. The CRS DNA measured 596-bp, 260-bp shorter than the objective PCR product. In the competitive, quantitative RT-PCR, a constant amount of wild-type cDNA and increasing amounts of CRS DNA (0.63 × 10^6 to 40 × 10^6 molecules for RAMP2, 0.13 × 10^6 to 8.0 × 10^6 molecules for CRLR and 0.06 × 10^6 to 4.0 × 10^6 molecules for adrenomedullin, each in a 1:2 dilution) were added to each tube, which was then subjected to PCR.

To determine the equivalent concentration point, the PCR products were separated by PAGE (5 % polyacrylamide gel), stained with 1.3 μmol/l ethidium bromide, revealed using a UV transilluminator and photographed. The photograph was digitized using a flat-bed scanner (ScanJet Iicx; Hewlett Packard), the image was inverted (Figure 1), and densitometric analysis was performed as reported previously [24].

RAMP2, CRLR and adrenomedullin mRNA concentrations were corrected for GAPDH mRNA expression and reported respectively as mmol/mol of GAPDH.

Statistical analyses
Data are given as mean ± S.E.M. mRNA concentration data were analysed by one-way ANOVA followed by Fisher’s protected least-squares difference test for comparisons of differences between SO and heart failure groups. Statistical significance was accepted at P < 0.05.

RESULTS
Sequence analysis of the rat RAMP2 homologue obtained by partial cloning showed 83 % similarity in nucleotide sequence and 77 % in amino acid sequence when compared with human RAMP2.

Amplification products of 194 bp of rat RAMP2, 533 bp of rat CRLR and 856 bp of adrenomedullin were detectable in all cardiac and kidney samples. RAMP2 mRNA levels in heart failure rats were significantly increased by 1.8 ± 0.2-fold (63.1 ± 7.9 mmol/mol GAPDH, P < 0.05) in the atrium and by 1.4 ± 0.1-fold (33.0 ± 2.3 mmol/mol GAPDH, P < 0.05) in the ventricle when compared with SO rats. Conversely, there was no significant change in kidney samples (1.1 ± 0.1-fold, 25.5 ± 1.9 mmol/mol GAPDH) when compared with SO rats (Figure 2).

CRLR mRNA levels in heart failure rats were significantly increased by 1.8 ± 0.2-fold (1.05 ± 0.12 mmol/mol GAPDH, P < 0.05) in the atrium and 1.33 ± 0.03-fold (0.84 ± 0.02 mmol/mol GAPDH, P < 0.05) in the ventricle when compared with SO rats. There was no significant elevation in CRLR mRNA levels in the kidney (1.2 ± 0.2-fold, 1.09 ± 0.18 mmol/mol GAPDH) when compared with SO rats (Figure 2).

Adrenomedullin mRNA levels were significantly increased by 2.1 ± 0.1-fold (0.59 ± 0.02 mmol/mol GAPDH, P < 0.01) in the atrium and 3.0 ± 0.5-fold (1.88 ± 0.30 mmol/mol GAPDH, P < 0.05) in the ventricle, when compared with SO rats (Figure 2). No significant upregulation of adrenomedullin mRNA expression was observed in the kidney of heart failure rats (Figure 2).

RAMP2 mRNA levels were markedly higher than CRLR mRNA levels; approx. 27- to 60-fold in heart and kidney of both SO and heart failure rats (Figure 2).
Figure 2  mRNA levels of RAMP2 (top panel), CRLR (middle panel) and adrenomedullin (AM) (bottom panel) in rat atrium (left), ventricle (centre) and kidney (right)

○ Indicates individual data; ○ shows means ± S.E.M. CHF, congestive heart failure. *P < 0.05 and **P < 0.01 versus SO rats.

DISCUSSION

The present study has shown that cardiac RAMP2 and CRLR gene expression is enhanced in rats with heart failure. In addition, we confirmed increased cardiac expression of adrenomedullin mRNA in heart failure rats, which is consistent with previous reports [18–20]. Kaiser et al. [20] reported only a modest (40%) increase in the left ventricle of rats with post-infarction heart failure by coronary artery ligation. The increase in adrenomedullin mRNA levels in their experiments was smaller than that in the present study (about 3-fold increase). This difference may be explained by the difference in the regions of the heart studied (the left ventricle in their study and the right and left ventricles in our study), or by differences in the strain of the rats used (Wistar rats and Munich-Wistar rats). The findings in the present study indicate that expression of both adrenomedullin receptors (RAMP2–CRLR) and adrenomedullin peptide is upregulated in failing hearts. Adrenomedullin is present in plasma, and plasma concentrations of adrenomedullin have been found to be elevated in patients with heart failure [16,17]. The levels, however, were relatively low when compared with the concentrations of adrenomedullin required to cause biological effects, e.g. vasodilatation [1]. It therefore seems likely that adrenomedullin acts as an autocrine or paracrine factor in various organs, including the heart. Our findings suggest a potential role for locally produced adrenomedullin in the failing heart.

Intravenous administration of adrenomedullin reduced ventricular preload and afterload, and improved cardiac output in sheep with heart failure [26]. Adrenomedullin has a potent vasodilatory action [1] and, therefore, adrenomedullin produced in the heart may dilate coronary arteries. Further, positive inotropic effects of adrenomedullin have been observed in vivo [27] and in vitro [28] in isolated perfused rat hearts. Moreover, it has been reported that adrenomedullin has an inhibitory effect on cardiac myocyte hypertrophy in vitro [29]. Thus increased expression of adrenomedullin receptors (RAMP2 and CRLR) and adrenomedullin mRNA in the failing heart may have beneficial effects on cardiac performance and may represent a defence mechanism against heart failure.

By contrast, there were no significant changes in the expression levels of RAMP2, CRLR or adrenomedullin mRNA in the kidney of heart failure rats. The direct
haemodynamic consequences of heart failure or the accompanying myocardial ischaemia might elevate the expression levels of RAMP2, CRLR and adrenomedullin mRNAs in the heart. It has been reported that hypoxia induced expression of adrenomedullin in cultured cardiac myocytes [30] and in cultured coronary artery endothelial cells [31]; however, the haemodynamics of the kidney in heart failure are quite different. Also, increased levels of cytokines in the cardiac tissues with infarction may affect the expression levels of adrenomedullin in the heart, because cytokines, such as tumour necrosis factor-α, are known to induce adrenomedullin expression in various kinds of cells [4, 6, 11].

Öie et al. [32] have recently shown increased mRNA expression of adrenomedullin and RAMP2 in the myocardium of heart failure rats by Northern-blot analysis, which is consistent with findings in the present study. In contrast, we used competitive, quantitative RT-PCR for the measurement of adrenomedullin, RAMP2 and CRLR mRNAs and were able to quantitate expression levels of CRLR mRNA, which were much lower than the levels of RAMP2 mRNA both in heart and kidney. Buhlmann et al. [33] reported that adrenomedullin binding was inhibited by co-expression of RAMP1 in CRLR-transfected cells, and suggested the presence of competitive interactions among the different RAMP–CRLR complexes. It seems likely that RAMPs compete with each other for CRLR, which is expressed in much smaller amounts in heart and kidney. Thus our findings may explain, in part, the competitive interactions of RAMP1 and RAMP2 with CRLR, although further studies are required to clarify this issue.

The present study has shown elevated cardiac expression of RAMP2, CRLR and adrenomedullin mRNAs in heart failure. Adrenomedullin produced in failing hearts may act as a paracrine or autocrine factor and have beneficial effects on cardiac performance in heart failure.

ACKNOWLEDGMENTS

The authors are grateful to Ms Kumi Kikuchi for her technical assistance. This study has been supported in part by a Research Grant from the Takeda Science Foundation and by a Research Grant from Renal Anaemia Research.

REFERENCES


Received 18 April 2000/17 July 2000; accepted 8 August 2000