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Decreased Gene Expression of Adrenomedullin Receptor in Mouse Lungs during Sepsis

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Plasma concentrations of adrenomedullin (AM) are markedly increased during sepsis, but the role of AM has not been clarified. Coexpression of calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein (RAMP) 2 or 3 have been reported to form the adrenomedullin (AM) specific receptor. We examined the expression of CRLR and RAMP1, 2, and 3 in several tissues from mice in a sepsis model induced by lipopolysaccharide (LPS). High expression of CRLR and RAMP2 mRNA was observed in lungs of normal mice, but it was markedly decreased in endotoxemic mice. It is suggested that the abundant binding sites of AM in lungs are formed by CRLR and RAMP2 in healthy subjects and that their reduction should contribute to the increase of plasma AM concentrations during sepsis. In contrast, LPS treatment markedly increased RAMP3 gene expression in lungs, spleen, and thymus. It is revealed that the distributions of receptor or binding sites of AM are changed in sepsis, and it is suggested that AM plays distinct roles in the clinical course of this syndrome.

Key Words: **adrenomedullin; receptor; CRLR; RAMPs; lung; sepsis; LPS.**

Adrenomedullin (AM) is a hypotensive peptide isolated from human pheochromocytoma tissue by monitoring the elevation of cyclic AMP (cAMP) in platelets (1). As several functions of AM has been reported, it is known as a multifunctional peptide (2). The amino acid sequences of AM show a slight homology with calcitonin gene-related peptide (CGRP), a neuropeptide which has a vasodilation activity. Previously, we reported that AM gene transcription was induced in almost all tissues from a dog septic model, and that its plasma concentration was also increased (3). Similar findings have been reported in a rat septic model and sepsis patients (4–6). It was reported that lipopolysaccharide (LPS) and inflammatory mediators, tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β), contributed to an increase in AM production (7).

In the clinical course of this syndrome, the AM functions appear to be emphasized. For example, AM, a potent vasodilator, is a possible factor which is responsible for severe hypotension in sepsis or septic shock (8, 9). Analysis of a sepsis model is useful to clarify the activities and mechanisms of AM. However, little is known about the physiological mechanisms and the pathological effects of AM. In addition, AM and CGRP have similar biological effects in *in vitro* experiments, but a comparative analysis of their physiological effects has not been discussed. One reason is that the AM receptor has not been revealed.

Recently, three isoforms of receptor activity modifying proteins (RAMPs), RAMP1, 2, and 3, were identified (10). These proteins interact with calcitonin receptor-like receptor (CRLR), which is an orphan receptor with seven-transmembrane-domains, and modify its ligand specificity, i.e., CRLR with RAMP1 is a CGRP specific receptor, and CRLR with RAMP2 or RAMP3 constitute AM specific receptors (10, 11). To collect further information on AM, we cloned and sequenced the murine CRLR, RAMP1, 2, and 3, and analyzed their gene transcriptions in a murine LPS-induced sepsis model.

Here we report the gene expressions of murine CRLR and RAMPs before and after treatment with LPS, which suggest a new function of AM.

MATERIALS AND METHODS

The present study was conducted according to the animal experimentation guidelines of Teikyo University School of Medicine, which

Abbreviations used: AM, adrenomedullin; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity modifying protein; CGRP, calcitonin gene-related peptide; LPS, lipopolysaccharide.

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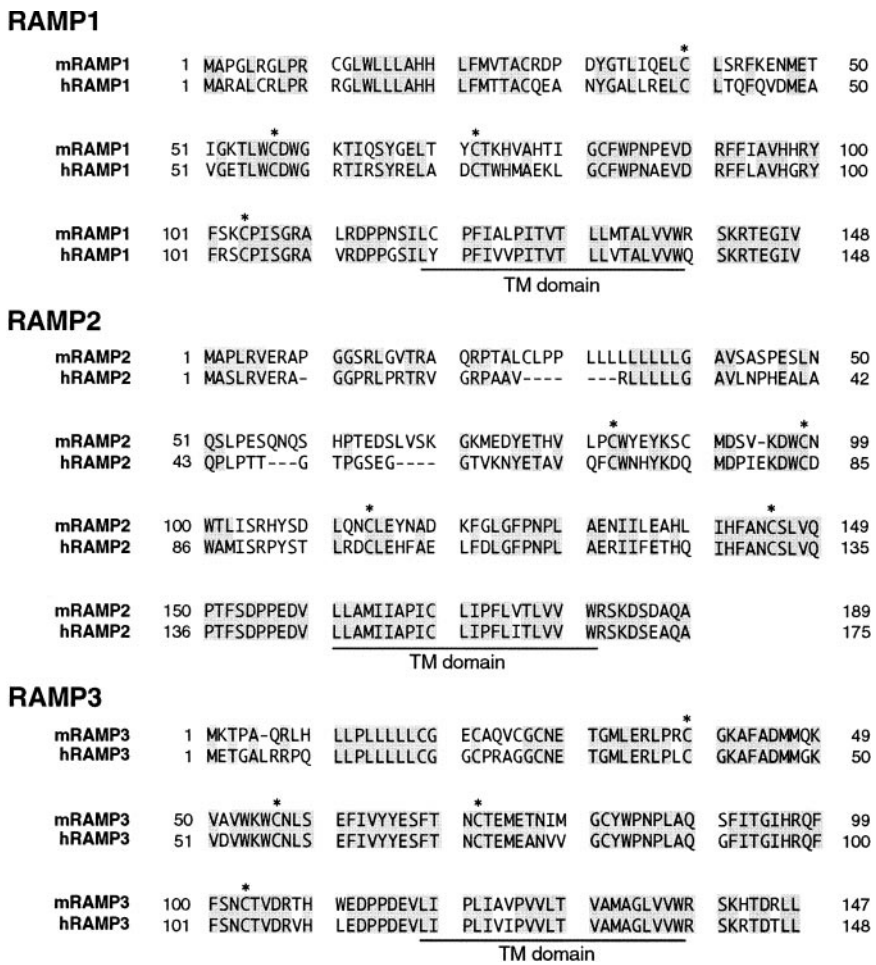

FIG. 1. Alignment of amino acid sequences of murine and human RAMPs. Deduced amino acid sequences begin with initiating methionine. Conserved cysteine residues among RAMPs are indicated by asterisks. The transmembrane domains are underlined.

adheres strictly to the National Institute of Health animal experimental guidelines, with the approval of the Teikyo University School of Medicine Animal Experimentation Committee. C57BL/6 mice (male, 4–8 weeks old) were used in all experiments.

cDNA cloning of mouse RAMP1, 2, 3, and CRLR. To examine the gene expression of CRLR and RAMPs in a septic mouse model, we cloned and sequenced the cDNAs of these murine proteins. Total RNA was extracted from murine brain and lung by TRIzol Reagent (Life Technologies). Poly(A)$^+$ RNA was isolated by Oligotex-dT30 "Super" (Takara Shuzo Co., Ltd., Kyoto, Japan). Double-stranded cDNAs were synthesized using a cDNA Synthesis Kit (Pharmacia LKB Biotechnology) according to the manufacturer's instructions. The cDNA was ligated to phage λZAPII arms and packaged *in vitro* using Gigapack III Gold (Stratagene).

The cDNA libraries were screened as previously described (3). To screen libraries, we cloned mouse EST clones encoding RAMP1, 2, and 3 from the GenBank database. We used the partial sequences of the EST clone 483161 (GenBank Accession No. AA063790), clone 808071 (Accession No. AA444361), and clone 761307 (Accession No. AA388255) for RAMP1, 2, and 3, respectively. To isolate murine CRLR, we used partial sequences of rat CRLR (Accession No. L27487). Both strands of the longest clones were sequenced using an automated DNA sequencer (373A, Applied Biosystems).

Sepsis model. Twenty-one mice were administered intraperitoneally with 2 mg/0.2 mL saline/head of lipopolysaccharide (LPS) as sepsis models, and six mice were injected with 0.2 mL of saline as controls. At various time points after the injection of LPS (0.5, 1, 2, 3, 4, 6, and 12 h), tissues (brain, lung, heart, liver, spleen, kidney, thymus, and testis) were obtained from three mice at each time point and RNA was extracted immediately. The controls were sacrificed at 0 and 12 h after injection of saline.

RNA blot analysis. The total RNA (5 μg/tissue) was electrophoresed on 1.0% agarose gel, then transferred to a nylon membrane (Zeta Probe, Bio-Rad). The probes were hybridized under high-stringency conditions (3). Band intensity of RNAs was estimated using an image analyzer (BAS 5000, Fuji Film), and the intensity of 18S ribosomal RNA was used as an internal standard for each tissue.

RESULTS AND DISCUSSION

Sequence Analysis of Murine CRLR and RAMPs

The deduced amino acid sequence of murine CRLR was 88.4% identical to human CRLR and 94.6% to rat CRLR (data not shown) (12, 13). Conserved cysteine residues and potential N-linked glycosylation sites

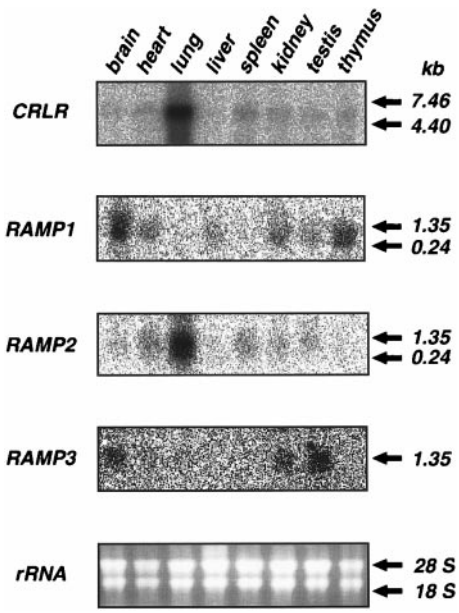

FIG. 2. Distribution of CRLR and RAMPs transcripts in mouse tissues. Five micrograms of total RNA from each mouse tissue was run in a Northern blot. Ribosomal RNA stained with ethidium bromide is shown for estimation of sample loading. Numbers on the right indicate migration of RNA marker fragments (size in kilobases (kb)).

were found in human, rat, and murine CRLR. The predicted amino acid sequences of murine RAMP 1, 2, and 3 shared 66.4, 48.8, and 79.0% identity with human RAMP1, 2, and 3, respectively (10). The deduced amino acid sequences of CRLR, RAMP1 and 3 are highly conserved between mammal species, whereas the sequences of RAMP2 show low sequence homologies, especially in the N-terminal extracellular region (Fig. 1). Four cysteine residues in the N-terminal region are conserved in the human RAMPs family (10). They are also conserved in the murine RAMPs family including RAMP2. Further analysis is required to clarify in detail the function of murine RAMP2 with CRLR. The EMBL/GenBank Data Library accession numbers for murine CRLR and RAMP1, 2, and 3 are AF209905, AF209904, AF209906, and AF209907, respectively.

Expression of CRLR and RAMPs in a Normal Mouse

To reveal the target tissues of AM, tissue distributions of CRLR, and RAMPs were investigated using Northern blot hybridization (Fig. 2). CRLR mRNA (6 kb) and RAMP2 mRNA (0.8 kb) were predominantly expressed in the lung. High levels of RAMP1 mRNA (0.8 kb) were observed in the brain and thymus. RAMP3 mRNA (1.3 kb) was high in testis, brain, and kidney.

Specific binding sites of radiolabeled AM were most abundant in the lung, and the plasma AM concentration of the pulmonary artery was higher than that of the aorta (14–16). Thus, it is expected that the lungs are the main clearance sites of plasma AM in the steady state. The present findings suggested that CRLR with RAMP2 contributed to the AM binding sites in the lungs of normal mice.

Although it was detected only at low levels in organs other than the lungs, CRLR mRNA was ubiquitously expressed among all mouse tissues studied. Considering that both CGRP and AM act in various tissues (2, 17), their site of action is probably determined by RAMPs distribution.

Expression of CRLR and RAMPs in a Sepsis Model

To examine the effects of LPS-induced sepsis on CRLR and RAMPs transcription, we analyzed mRNA levels in murine tissues at 12 h after LPS treatment, the latest stages of sepsis. This dose of LPS caused severe septic shock. As shown in Fig. 3, LPS treatment suppressed CRLR and RAMP2 mRNA levels in the lungs. In other tissues, CRLR expression change was minimal, and RAMP1 and 2 expressions were generally decreased in the sepsis model relative to normal mice.

Injection of LPS resulted in a time-dependent decrease in RAMP2 gene expression in the lungs (Fig. 4). Greater than 50% reduction was detected in RAMP2 mRNA at 0.5 h after treatment. A further reduction of RAMP2 mRNA was sustained for up to 3 h after LPS injection, and no additional decreases or rebounds were observed between 3 and 12 h. In lungs, LPS treatment produced similar large decreases in CRLR mRNA expression. CRLR mRNA levels showed increases of 2.5-fold at 0.5 h following LPS administration and the expression decreased gradually to the lowest level at 4 h after LPS treatment, and this level lasted to 12 h.

As previously reported, the plasma concentration of AM was obviously elevated during early stages of sepsis (3, 4). The present findings showed that the receptors conferred by CRLR and RAMP2 in the lung were diminished in a relatively early stage of sepsis. These findings indicate the marked reduction of AM binding sites in lungs, should contribute to elevation of plasma AM concentration in sepsis.

In contrast to RAMP1 and 2, RAMP3 message levels were markedly increased in the lungs, spleen, and thymus after 12 h of LPS injection (Fig. 3). In lungs, there appeared to be a new type of AM receptor presented by RAMP3 and the remaining CRLR. Although the CRLR mRNA was downregulated in lungs, the level was comparable with that of other organs in normal mice. Therefore, CRLR expression should be sufficient for constituting AM receptor with RAMP3.

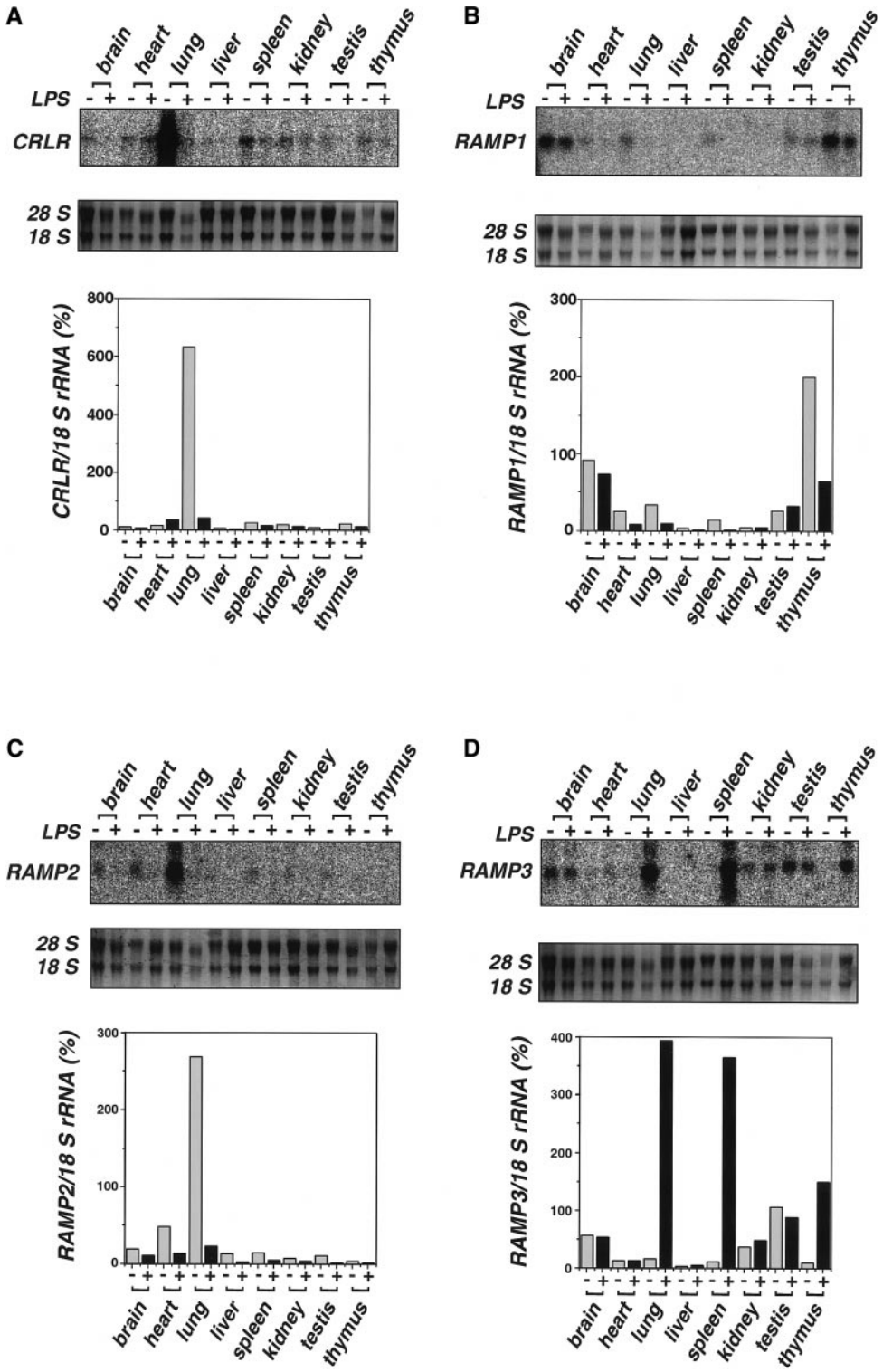

FIG. 3. Regulation of CRLR (A) and RAMPs (B, C, D) mRNA in various mouse tissues at 12 h after LPS treatment. (−) or (+) indicates control or LPS-injected mice. Top panels: Representative autoradiogram of Northern blot of murine CRLR and RAMPs. Each lane contained total RNA (5 μg) from normal and LPS-injected mice tissues. rRNAs stained with ethidium bromide are shown as internal controls. Bottom panels: Densitometric quantification of CRLR and RAMPs messages normalized to the 18S rRNA message. Each mRNA level of LPS treated mouse tissue (closed bars) is compared with the mRNA level of normal mouse tissue (open bars).

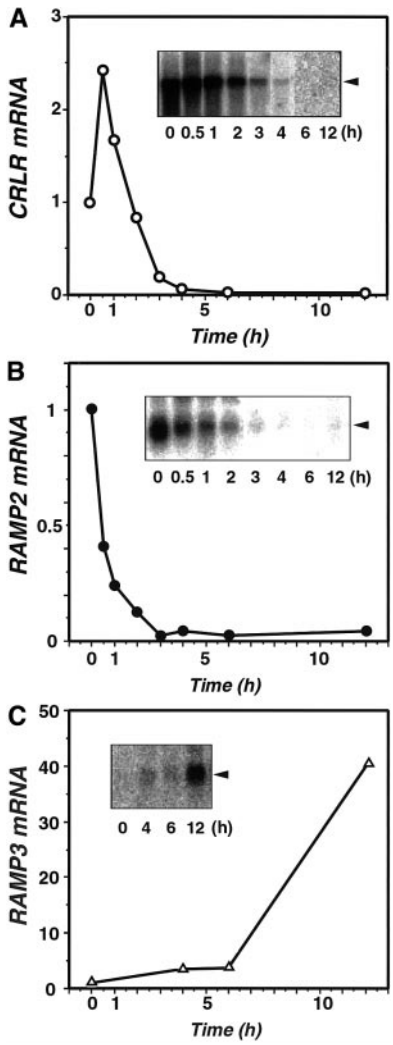

FIG. 4. Time course of stimulation of CRLR (A), RAMP2 (B), and RAMP3 (C) mRNA expressions in mouse lungs by LPS treatment. Total RNA was extracted from three individual animals per group, and mean levels of each group are shown. Densitometric quantification of CRLR and RAMPs 2 and 3 messages was normalized to 18S rRNA. Each mark indicates its level relative to the level at 0 h. (Inset) The autoradiography of Northern blot. The position of each mRNA is indicated with an arrowhead.

Little is known about the role of the "CRLR/RAMP3" receptor at present. The "CRLR/RAMP3" receptor shows similar functions to the "CRLR/RAMP2" receptor, which increases intracellular cAMP accumulation *in vitro* by AM treatment (11). However, the affinity of various types of human AM (AM1-52, AM13-52, and AM22-52) is different between these receptors (11). In general, human AM affinity to the "CRLR/RAMP2" receptor is higher than that to the "CRLR/RAMP3" receptor. The predominant expression of RAMP3 should be required to exert AM functions mediated by the "CRLR/RAMP3" receptor.

The tissue distribution of RAMP3 mRNA during the end stage of sepsis may provide a clue to its pathophysiological function, namely, the additional roles of AM in sepsis. The "CRLR/RAMP3" receptor appeared in spleen and thymus, in addition to the lungs. These tissues are principal organs of the immune system. In the thymus, the "CRLR/RAMP3" receptor appeared during sepsis, while the CGRP specific receptor "CRLR/RAMP1" was decreased by downregulation of RAMP1. It was reported that CGRP enhances apoptosis of thymocytes in normal mice (18).

RAMP3 expression levels in lungs increased threefold at 4 h after LPS injection. At 12 h after treatment, the RAMP3 mRNA level was approximately 40 times higher than that of controls (Fig. 4). There was no remarkable change in RAMP3 mRNA between 4 and 6 h. Similar findings were observed in the spleen and thymus (data not shown). It was suggested that the upregulation of RAMP3 mRNA was accelerated during the late stages of sepsis. The differences between the time course patterns of RAMPs may be due to various transcriptional factors such as the cytokines produced during the clinical course of sepsis. AM may function in the immune system via the "CRLR/RAMP3" receptor during end stage sepsis. Further studies are needed to reveal the function of AM mediated by the "CRLR/RAMP3" receptor.

In conclusion, the present study revealed dramatic changes in gene expressions of AM receptors formed by CRLR and RAMPs during sepsis. Decrease of the AM receptor "CRLR/RAMP2" in the lungs should be responsible for an increase of AM plasma concentration. On the other hand, increase of the AM receptor by RAMP3 in several tissues suggests that AM plays distinct roles under septic conditions.

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