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Saturation of adrenomedullin receptors plays an important role in reducing pulmonary clearance of adrenomedullin during the late stage of sepsis

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Abstract

Adrenomedullin (AM) is a potent vasodilator that plays a major role in the cardiovascular response during the progression of sepsis. Although pulmonary clearance of AM (i.e., the primary site of AM clearance) is reduced during the late, hypodynamic stage of sepsis, the role of AM receptors under such conditions remains unclear. This study was carried out to test the hypothesis that saturation of AM receptors is responsible for the decreased clearance of AM in the lungs during sepsis. Polymicrobial sepsis was induced in male adult rats by cecal ligation and puncture (CLP). At 20 h after CLP (i.e., the late phase), ^{125}I-labeled rat AM was administered through the jugular vein, both with (+) and without (−) pre-injection of the human AM fragment AM_{22-52} (an AM receptor antagonist). Pulmonary tissue samples were harvested after 30 min and the radioactivity was determined. In addition, lung levels of AM were determined at 5 and 20 h after CLP by radioimmunoassay. Alterations in gene expression of the recently identified AM receptor subunits calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein-2 and -3 (RAMP-2 and -3) were assessed in the lungs by reverse transcription–polymerase chain reaction (RT–PCR) at 5 and 20 h after CLP. The results indicate that there was a significant decrease in pulmonary [^{125}I]AM clearance at 20 h in $-AM_{22-52}$ CLP animals. Lung clearance in $+AM_{22-52}$ sham animals was significantly lower than in $-AM_{22-52}$ sham animals and was not statistically different from the $-AM_{22-52}$ CLP group. There was no statistical difference between $+AM_{22-52}$ and $-AM_{22-52}$ CLP groups. However, there was a significant increase in lung AM levels at 20 but not 5 h after CLP. In addition, RAMP-3 expression was significantly upregulated at 5 but not 20 h after CLP. There were no alterations in the expression of CRLR or RAMP-2 at either time point. These results suggest that pulmonary AM receptors become saturated as more AM enters the bloodstream, thereby reducing the ability of the lungs to clear this peptide during late sepsis. Early upregulation of RAMP-3 may be a compensatory mechanism to help clear the upregulated AM from the bloodstream. The lack of upregulation of RAMP-3 during late sepsis could also contribute to the decreased clearance observed during this phase. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Adrenomedullin receptor antagonist; Calcitonin receptor-like receptor; Receptor activity modifying protein; Cecal ligation and puncture; Vasoactive peptide

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1. Introduction

Adrenomedullin (AM), a recently reported potent vasodilatory peptide, has been shown to be upregulated under various adverse circulatory conditions, including heart failure [1], renal failure [2], ischemia and reperfusion [3], and endotoxic shock [4,5]. Previous studies from our laboratory using the cecal ligation and puncture (CLP) model of polymicrobial sepsis have shown that plasma levels of AM increase as early as 2 h after the onset of sepsis and remain elevated during the late, hypodynamic phase [6]. In addition, AM gene expression is upregulated in the small intestine, left ventricle, and aorta [6]. Moreover, the small intestine has been identified as the major source of AM release during sepsis [7]. With regard to the clearance of this peptide, it has been demonstrated that the lungs are the primary site of AM clearance [8–11]. We previously reported that pulmonary clearance of [^{125}I]AM is reduced during the late, hypodynamic stage of polymicrobial sepsis, which could contribute to the elevated plasma levels of AM observed during this stage [8]. However, it remains unknown whether the reduced pulmonary clearance of AM observed during the late stage of sepsis is due to saturation of AM receptors in the lungs. It could be possible that during the progression of sepsis, as more AM accumulates in the bloodstream, the clearance mechanism (i.e., AM receptors) in the lungs becomes saturated. In this study, we hypothesized that blockade of AM receptors with an AM antagonist, $AM_{22-52,}$ would significantly reduce the clearance capacity of the lungs in sham animals but would be ineffective in reducing AM clearance during late sepsis because of prior saturation by endogenous AM. The aim of this study, therefore, was to investigate the role of AM receptors in the clearance of this peptide during sepsis. We also tested the hypothesis that alterations in AM receptors could be responsible for the inability of the lungs to clear increasing amounts of AM from the bloodstream during the progression of sepsis. Reverse transcription–polymerase chain reaction (RT–PCR) was performed to determine alterations in gene expression of the newly identified AM receptor subunits: calcitonin receptor-like receptor (CRLR), and receptor activity modifying protein-2 and -3 (RAMP-2 and -3).

2. Materials and methods

2.1. Animal model of polymicrobial sepsis

Male Sprague–Dawley rats (275–325 g, Charles River Laboratories, Wilmington, MA) were subjected to polymicrobial sepsis by CLP as previously described [12]. Briefly, rats were fasted overnight, but allowed water ad libitum. After anesthetization with methoxyflurane inhalation, a 3-cm ventral midline incision was made, and the cecum was exposed and ligated with a 3-0 silk ligature just distal to the ileocecal valve. The cecum was then punctured twice with an 18-gauge needle, squeezed slightly to allow a small amount of fecal matter to flow from the holes, and then returned to the abdominal cavity. The incision was closed in layers and the animal received a subcutaneous injection of normal saline (3 ml/100 g body weight, i.e., fluid resuscitation). Sham-operated animals were subjected to the same procedure though the cecum was neither ligated nor punctured. The experiments described herein were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

2.2. Administration of AM receptor antagonist and radioactive AM

At 20 h after CLP (i.e., the late, hypodynamic phase of sepsis) [13] or sham operation, the animals were anesthetized again with methoxyflurane inhalation. A steady state of sedation was maintained with a subsequent intravenous injection of sodium pentobarbital ($\sim$50 mg/kg body weight). Polyethylene-50 catheters were placed in the right jugular vein and left femoral artery, and a bolus injection of the AM receptor antagonist human AM_{22-52} (Phoenix Pharmaceuticals, Belmont, CA; 18.67 μg/kg body weight) was administered through the jugular cannula. This dose produced a plasma concentration approximately 500-fold higher than circulating AM concentrations observed during the late stage of sepsis, presumably saturating all AM receptors. After 10 min, ^{125}I-labeled rat AM (Peninsula Labs, Belmont, CA; 0.67 ng/kg at a specific activity of 2995 Ci/mmol;

~300 000 cpm/rat) was then administered. The remaining radioactivity in the syringe was measured with a gamma counter (1480 Wizard, Wallac, Gaithersburg, MD), and this radioactivity count was subtracted from the initial, pre-injection count to determine the net injected radioactivity. Thirty minutes after the administration of [^{125}I]AM, a blood sample was drawn, the inferior vena cava was cut, and the animal was perfused with 100 ml of warm, oxygenated normal saline through the femoral arterial cannula to remove blood from tissues. Following the perfusion, the lungs were harvested and weighed, and the radioactivity (cpm) was measured with the gamma counter. The pulmonary distribution of [^{125}I]AM was calculated as the percentage of the total radioactivity administered per gram of wet tissue.

2.3. Determination of lung AM levels

Lung tissue samples were harvested at 5 and 20 h after CLP or sham operation, snap-frozen in liquid nitrogen, and stored at −80°C until use. The tissue samples were then homogenized in equal volumes of supplied Buffer A (1% trifluoroacetic acid in water) and saline solution, centrifuged at 3000 rpm for 30 min, and the supernatant was frozen at −80°C until further use. Lung AM levels were determined by radioimmunoassay according to the manufacturer's instructions and guidelines (Phoenix Pharmaceuticals, Mountain View, CA). Briefly, AM was extracted from the acidified tissue homogenates using SEP-columns packed with 200 mg C18 sorbent (Peninsula Laboratories, Belmont, CA) and eluted with supplied Buffer B (60% acetonitrile in 1% trifluoroacetic acid). The eluant was then evaporated overnight in a centrifugal concentrator (Savant Speed Vac Plus SC110A, Savant Instruments, Farmingdale, NY), reconstituted in assay buffer, and analyzed according to the manufacturer's protocol.

2.4. Determination of AM receptor subunit gene expression

Gene expression of AM receptor subunits (i.e., CRLR, RAMP-2, and RAMP-3) was assessed using RT–PCR analysis. Lung samples were harvested at 5 and 20 h after CLP or sham-operation, snap-frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted using Tri-reagent (Molecular Research Center, Cincinnati, OH) and 4 μg of RNA was reverse transcribed as previously described by us [6]. The resulting cDNAs were amplified by PCR using specific primer for rat CRLR (sense, 5′-CCAAACAGACTTGGGAGTCACTAGG-3′; antisense, 5′-GCTGTCTTCTCTTTCTCATGCGTGC-3′), RAMP-2 (sense, 5′-AGGTATTACAGCAACCTG-CGGT-3′; antisense, 5′-ACATCCTCTGGGGGAT-CGGAGA-3′), and RAMP-3 (sense, 5′-ACCTGT-CGGAGTTCATCGTG-3′; antisense, 5′-AC-TTC-ATCCGGGGGGTCTTC-3′) [14]. Rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH) served as a housekeeping gene (Clontech, Palo Alto, CA). PCR cycling proceeded as follows: 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. Following the RT–PCR procedure, the PCR amplification products were electrophoresed using a 1.6% agarose gel containing 0.22 mg/ml ethidium bromide. The gel was then photographed using Polaroid film. Optical density of target and housekeeping gene bands were determined by ChemiImager 5500 software. Target/G3PDH band density ratios were then calculated.

2.5. Statistical analysis

Results are presented as mean ± S.E.M. One-way analysis of variance (ANOVA) and Tukey's test were used for statistical analysis, and the differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Pulmonary [^{125}I]AM distribution after AM receptor blockade

Since we have previously demonstrated that [^{125}I]AM distribution was not altered at 5 h after CLP [7], we only determined effects of AM receptor antagonism on pulmonary [^{125}I]AM distribution at 20 h after CLP in the present study. As shown in Fig. 1, despite a 27% decrease in lung radioactivity at 20 h after CLP in animals with pre-injection of AM_{22-52} as compared to sham-operated animals, this difference was not significant. This lies in contrast to the results reported previously [8], which showed significantly reduced pulmonary clearance at 20 h after

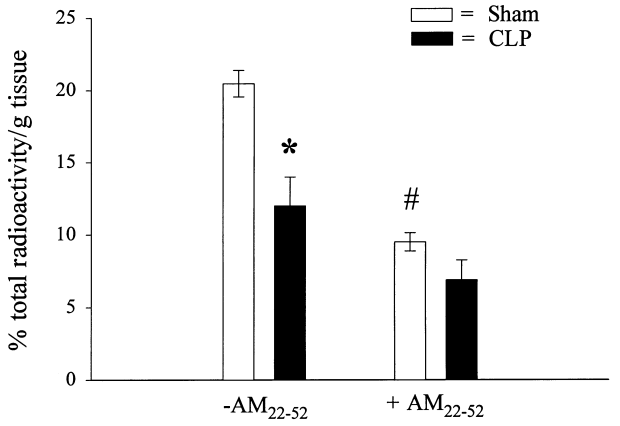

Fig. 1. Changes in pulmonary [^{125}I]AM clearance at 20 h after CLP, with (+) or without (−) pre-injection of the AM receptor antagonist AM_{22-52}. The data are presented as means ± S.E.M. (n = 6–7/group) and compared by one-way ANOVA and Tukey's test. *$P < 0.05$ versus the respective sham operated animals; $^{\#}P < 0.05$ versus the respective $-AM_{22-52}$ group. Lung clearance of [^{125}I]AM following pre-injection of AM_{22-52} was not statistically different between the sham and CLP groups, indicating that CLP does not further decrease AM uptake beyond the effect of AM_{22-52} injection alone. Please note that pulmonary distribution of [^{125}I]AM in animals without AM_{22-52} pre-injection has been previously published [8].

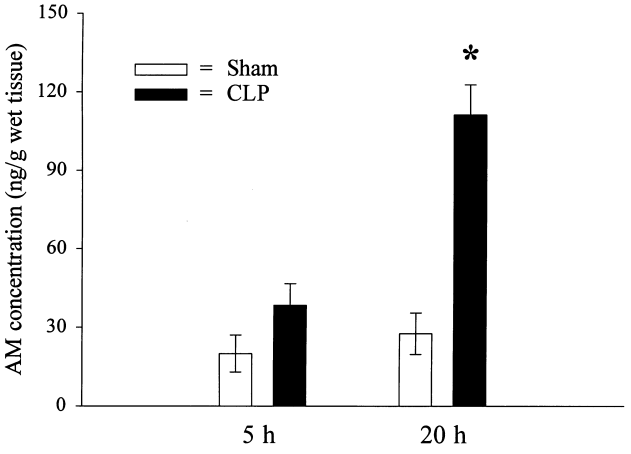

Fig. 2. Changes in pulmonary AM levels at 5 and 20 h after CLP or sham operation. The data are presented as means ± S.E.M. (n = 6–7/group) and compared by one-way ANOVA and Tukey's test. *$P < 0.05$ versus the respective sham-operated animals.

CLP without administration of AM_{22-52} (20.46 ± 0.75% total radioactivity/g tissue in sham group versus 11.99 ± 2.00% total radioactivity/g tissue in CLP group) (Fig. 1). It should be noted that the wet weight of lungs was 1.18 ± 0.07 g (SD) with body weight of 314 ± 12 g in randomly selected rats (n = 10). Thus, the distribution of [^{125}I]AM to the pulmonary tissue was approximately 24% of total radioactivity administered in sham-operated animals. In addition, there was a significant decrease in lung radioactivity in the sham group injected with AM_{22-52} as compared to the sham group without pre-injection of AM_{22-52}. In contrast, there was no statistical difference in lung levels of radioactivity at 20 h after CLP with and without AM_{22-52} pre-infusion ($P > 0.05$, Fig. 1). The distribution of [^{125}I]AM in blood was very low as compared to the pulmonary tissue. The level was 0.445 ± 0.019 and 0.462 ± 0.038% total radioactivity/ml in sham and CLP 5 h animals, respectively. At 20 h after CLP, it increased to 0.638 ± 0.011% total radioactivity/ml ($P < 0.05$ vs. sham). Please note that these results are comparable to our previously reported data without administration of AM_{22-52} [8].

3.2. Alterations in lung levels of AM

Alterations in lung AM levels are summarized in Fig. 2. At 5 h after CLP, there were no significant increases in lung AM levels. At 20 h after CLP, however, septic animals showed approximately a threefold increase as compared to sham-operated animals ($P < 0.05$, 111.3 ± 11.5 versus 27.7 ± 7.9 ng/g wet tissue in sham animals).

3.3. Alterations in AM receptor subunit gene expression

The results in Fig. 3 indicate that the RT–PCR products (181 bp) of the RAMP-3 gene increased

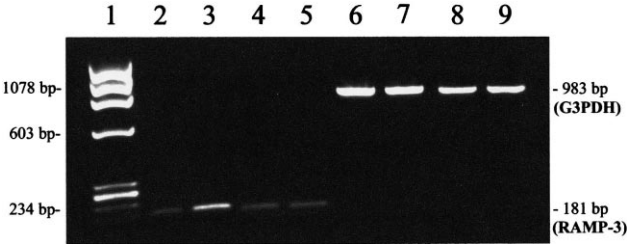

Fig. 3. Representative gel of gene expression of RAMP-3 (181 bp) in the lungs as well as the housekeeping gene G3PDH (983 bp). Lane 1, X174/*Hae* III size markers; lane 2, RAMP-3 at 5 h in sham animals; lane 3, RAMP-3 at 5 h in CLP animals; lane 4, RAMP-3 at 20 h in shams; lane 5, RAMP-3 at 20 h in CLPs. Lanes 6–9 are the corresponding G3PDH bands.

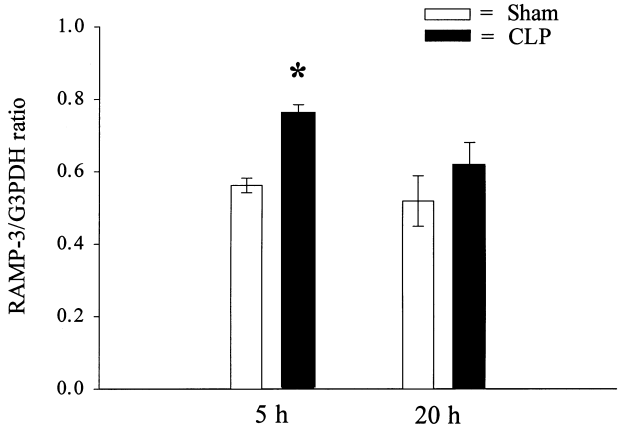

Fig. 4. Changes in pulmonary RAMP-3 gene expression at 5 and 20 h after (CLP) or sham operation. The data (expressed as ratio of target gene RAMP-3 and housekeeping gene G3PDH) are presented as means ± S.E.M. ($n = 4$–6/group) and compared by one-way ANOVA and Tukey's test. *$P < 0.05$ versus the respective sham-operated animals.

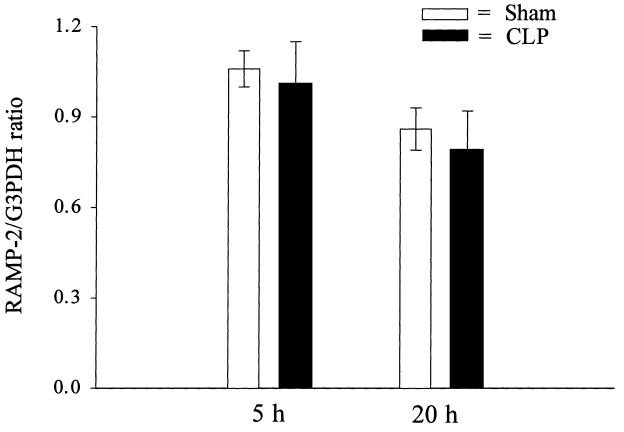

Fig. 6. Changes in pulmonary RAMP-2 gene expression at 5 and 20 h after (CLP) or sham operation. The data (expressed as ratio of target gene RAMP-3 and housekeeping gene G3PDH) are presented as means ± S.E.M. ($n = 3$–4/group) and compared by one-way ANOVA. There was no statistical difference between CLP and respective sham-operated animals.

in the lungs at 5 h after the onset of sepsis (lane 3) as compared to sham-operated animals (lane 2). In contrast, there were no alterations in RAMP-3 gene expression at 20 h after CLP (lanes 4 and 5). Fig. 3 indicates that expression of the housekeeping gene G3PDH (983 bp) was similar in both sham and CLP groups at both time points (lanes 6–9), indicating that similar levels of reverse transcription were achieved. The target/G3PDH band ratio results indicate that the RAMP-3 ratio increased significantly at 5 but not 20 h after the onset of sepsis as compared to the respective sham-operated group (Fig. 4). Unlike RAMP-3, there were no significant changes in CRLR or RAMP-2 gene expression at either time point (Figs. 5 and 6).

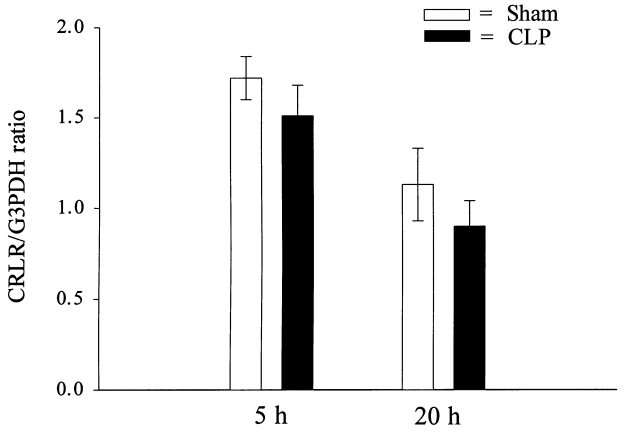

Fig. 5. Changes in pulmonary CRLR gene expression at 5 and 20 h after (CLP) or sham operation. The data (expressed as ratio of target gene RAMP-3 and housekeeping gene G3PDH) are presented as means ± S.E.M. ($n = 3$–4/group) and compared by one-way ANOVA. There was no statistical difference between CLP and respective sham-operated animals.

4. Discussion

After it was first isolated from human pheochromocytoma in 1993 by Kitamura et al. [15], AM was quickly established as an important mediator in several physiological as well as pathophysiological processes. In addition to its well-known vasodilatory effects, AM is known to be involved in the regulation of renal function [16], bronchodilation [17], inhibition of hormone secretion (including insulin, adrenocorticotropic hormone, aldosterone) [18–20], neurotransmission [21], and mitogenic activity [22,23]. Our recent studies have focused on the transition from the early, hyperdynamic stage of sepsis to the late, hypodynamic phase, and it has become increasingly clear that AM is an important mediator in this process [24]. We have shown that plasma levels of AM are elevated as early as 2 h after the onset of sepsis, with gene expression upregulated in the small intestine, heart, and aorta [6]. If AM is administered in normal rats, they exhibit a typical hyperdynamic re-

sponse, i.e., increased cardiac output, stroke volume and microvascular blood flow and decreased peripheral vascular resistance. Moreover, anti-AM antibodies administered shortly after CLP completely prevented the hyperdynamic response associated with early sepsis [25]. Although it has been suggested that AM is responsible for producing the severe drop in blood pressure associated with endotoxic shock [26], we have shown that the transition to the hypodynamic state appears to be the result of decreased vascular responsiveness to AM [27]. With regard to the clearance of this peptide, we have previously demonstrated that pulmonary clearance of AM is reduced during the late stage of sepsis [8]. Although saturating the clearance mechanism with large amounts of unlabeled AM produced similar results in normal rats, the role that receptors play in this phenomenon remains unknown. The present study was therefore conducted to elucidate their role by using the specific AM receptor antagonist AM_{22-52} to determine how much clearance is receptor mediated and how much is the result of non-specific metabolism. Although no synthetic AM receptor antagonist is currently available, the human AM fragment AM_{22-52} was first shown to act as a rat AM receptor antagonist by Eguchi et al., and it has been widely used to study ligand–receptor interactions of AM [28–30].

Our results indicate that blockade of AM receptors with AM_{22-52} significantly reduces the ability of the lungs to clear [^{125}I]AM in sham animals, as compared to the sham animals in our original study [8]. In that study, lung clearance in 20 h sham animals was 20.46% total radioactivity/g tissue, compared to 9.50% total radioactivity/g tissue in 20 h sham animals pre-injected with AM_{22-52}. Moreover, this value was not statistically different from the lung clearance of 20 h CLP animals in the original study (11.99% total radioactivity/g tissue). In contrast with the sham groups, there was no significant difference in lung clearance between CLP groups in the two studies. In addition, while there was no significant increase in lung levels of AM at 5 h after CLP, there was a three-fold increase at 20 h after CLP as compared to sham-operated animals, confirming the finding that lung tissue is the primary site of AM clearance. This would suggest that since the receptors are already saturated with the endogenous AM produced during the progression of sepsis, the antagonist has no effect on pulmonary AM clearance, and [^{125}I]AM is cleared non-specifically at levels similar to those seen in CLP rats without AM_{22-52} pretreatment. It appears that the lungs can clear AM from the bloodstream in two ways. The first is mediated by AM-specific receptors and accounts for about half of the clearance capacity (as evidenced by the $>50\%$ drop in radioactivity after administration of AM_{22-52} in sham animals) and the other is a non-specific-, non-receptor-mediated mechanism.

Nishikimi et al. have postulated that lung AM clearance is pronounced because this peptide preferentially regulates pulmonary vascular tone: AM more effectively dilates pulmonary vessels when compared to systemic vessels [31]. Little is known, however, about what cell populations in the lungs are responsible for the specific and non-specific clearance of AM. It is possible that endothelial and/or vascular smooth muscle cells mediate receptor-specific clearance, and macrophages or neutrophils mediate non-specific clearance. Further in vitro studies would be helpful in pinpointing the exact cell populations involved in AM clearance in the lungs.

Although there was some early controversy regarding the receptor through which AM acts, recent findings have elucidated the role that receptor activity modifying proteins (RAMPs) play in determining the phenotype of the calcitonin receptor-like receptor (CRLR). In 1998, McLatchie et al. were the first to show that the ligand specificity of CRLR could be changed based on the type of RAMP that was coexpressed [32]. When RAMP-1 was expressed with CRLR, a CGRP receptor was created, but when either RAMP-2 or RAMP-3 was expressed with CRLR, an AM receptor was created. RAMPs are thought to affect CRLR function in three ways: through receptor trafficking to the cell surface, receptor glycosylation, and receptor–ligand interaction through either direct or allosteric effects [32–34]. Another aim of our study was to determine whether alterations in gene expression could play a role in the decrease in [^{125}I]AM clearance during late sepsis. Our results indicate that RAMP-3 expression is increased in the lungs during the early, hyperdynamic stage of sepsis but not during the late, hypodynamic stage. There were no significant alterations in CRLR or RAMP-2 at either time point. The findings that

the increased RAMP-3 gene expression in early sepsis is not associated with significant changes in pulmonary AM clearance and that the absence of RAMP-3 expression changes in late sepsis is associated with decreased AM clearance may suggest that there is no direct relationship between AM clearance and AM receptor gene expression in sepsis. While further studies are required to determine whether changes in RAMP-3 gene expression in sepsis are associated with alterations in binding characteristics of AM receptors, we believe that upregulation of RAMP-3 may be part of a compensatory mechanism to help clear increasing amounts of AM from the bloodstream during the early stage of sepsis. Without the increase in AM receptor gene expression under such conditions, pulmonary AM clearance would have been reduced. Moreover, the lack of upregulation of RAMP-3 during the later stages of sepsis could be partly responsible for the decreased ability of the lungs to clear AM due to the diminished compensatory mechanism. Nonetheless, the findings that administration of AM receptor antagonist AM_{22-52} markedly decreases pulmonary AM clearance in sham-operated animals (Fig. 1) clearly point out the important role of AM receptors in AM clearance by the lungs. Various studies have indicated alterations in CRLR and RAMP expression under adverse circulatory conditions [35,36]. Ono et al. investigated the expression of CRLR and RAMP-1–3 in various organs using a mouse model of endotoxemia [37]. Although they also detected an increase in RAMP-3 expression in the lungs, they found that CRLR and RAMP-2 expression decreased, which lies in contrast to our expression data which showed no changes in these transcripts. It is possible that the endotoxemia model elicits a more severe response than the CLP model of polymicrobial sepsis, which could explain the differences between our data and the results of Ono et al. [37].

We have reported that AM levels in the heart and aorta increased by 93% and 51%, respectively, at 20 h after CLP [38]. Moreover, intestinal levels of AM increased by 72% at 20 h after the onset of sepsis [7]. In contrast to those moderate increases in AM levels, pulmonary AM increased by 302% at 20 h after CLP in the present study. The finding that the magnitude of AM increase is much higher in the pulmonary tissue at 20 h after the onset of sepsis than other tissues strongly suggests that AM is accumulated in the lungs during sepsis. Although we have not determined whether AM mRNA in the lungs is upregulated in sepsis, our previous studies have indicated that AM gene expression increases in the gut, heart and aorta in sepsis [6]. While pulmonary AM gene expression likely may be upregulated during sepsis, a 302% increase in pulmonary levels of AM versus increases of 93%, 51% and 72% increase in the heart, aorta, and gut, respectively, at 20 h after the onset of sepsis suggests that the increased pulmonary AM is due to uptake of AM from the blood in addition to increased local production. This is further supported by our previous study that left ventricular administration of [^{125}I]AM (i.e., lack of the first pulmonary passage) decreased pulmonary [^{125}I]AM distribution by 46.4% in sham-operated animals [8]. This result, along with our other findings, indicates that the lungs are indeed the major site for the clearance of AM in sepsis. Despite the fact that plasma and tissue levels of AM increase significantly at 20 h after the onset of sepsis, vascular AM responsiveness decreases markedly in the aorta and resistance blood vessels in the gut at the late stage of sepsis [27]. Although we did not determine pulmonary vascular responses to AM in our previous study [27], it is likely that the response of the pulmonary vascular bed to AM is reduced in late sepsis. In addition, hemoconcentration occurs during the late stage of sepsis, indicating that reduction in plasma volume occurs under such conditions. In view of this, it is possible that the altered pulmonary vascular tone and volume may also play a role in producing the reduction of pulmonary clearance of AM during the late stage of polymicrobial sepsis.

In summary, our results indicate that blockade of AM receptors with AM_{22-52} in sham animals produces similar levels of pulmonary [^{125}I]AM clearance as 20 h CLP animals without antagonist treatment. Moreover, lung AM levels are significantly increased at 20 but not 5 h after CLP. Gene expression of the AM receptor subunit RAMP-3 is significantly increased only during early sepsis, and there were no alterations in CRLR or RAMP-2 expression at either time point. These results, taken together, suggest that the decreased pulmonary clearance of [^{125}I]AM during late sepsis is partially due to the saturation of AM receptors in the lungs by endogenously pro-

duced AM, which contributes to the elevated plasma levels of AM observed under these conditions.

Acknowledgements

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