Adrenomedullin Binding Protein-1 Modulates Vascular Responsiveness to Adrenomedullin During the Late Stage of Sepsis

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Running Head: The role of adrenomedullin binding protein-1 in sepsis

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ABSTRACT

Adrenomedullin (AM), a potent vasodilatory peptide, plays an important role in initiating the hyperdynamic response during the early stage of sepsis. Moreover, the reduced vascular responsiveness to AM appears to be responsible for the transition from the early, hyperdynamic phase to the late, hypodynamic phase of sepsis. Although the novel specific AM binding protein-1 (AMBP-1) enhances AM-mediated action in a cultured cell line, it remains to be determined whether AMBP-1 plays any role in modulating vascular responsiveness to AM during sepsis. To study this, adult male rats were subjected to sepsis by cecal ligation and puncture (CLP). The thoracic aorta was harvested for determining AM-induced vascular relaxation. Aortic levels of AMBP-1 were determined by Western blot analysis and AM receptor gene expression in the aortic tissue was assessed by RT-PCR. The results indicate AMBP-1 significantly enhanced AM-induced vascular relaxation in aortic rings from sham-operated animals. Although vascular responsiveness to AM decreased at 20 h after CLP (i.e., the late, hypodynamic stage of sepsis), addition of AMBP-1 in vitro restored the vascular relaxation induced by AM. Moreover, the aortic level of AMBP-1 decreased significantly at 20 h after CLP. In contrast, AM receptor gene expression was not altered under such conditions. These results, taken together, suggest that AMBP-1 plays an important role in modulating vascular responsiveness to AM, and the reduced AMBP-1 appears to be responsible for the vascular AM hyporesponsiveness observed during the hypodynamic phase of sepsis.
INTRODUCTION

Despite advances in the management of sepsis with various novel therapeutic agents and aggressive fluid resuscitation, a large number of septic patients die of septic shock and multiple organ failure (1). The cardiovascular and hemodynamic response of experimental sepsis is characterized by a hyperdynamic circulation during the early stage, which is followed by a hypodynamic circulation at the late stage (31, 37). In order to prevent the occurrence of multiple organ failure and reduce the mortality rate of sepsis, it is important to identify the mediator/factor responsible for producing the transition from the hyperdynamic to the hypodynamic phase during the progression of sepsis. Adrenomedullin (AM) is a potent vasodilatory peptide and is expressed in a variety of tissues and cell populations (2, 16, 27, 39). Clinical studies have indicated that increased levels of plasma AM occurred with heart failure, renal failure, and particularly with sepsis, in which the highest levels of circulating AM were observed (11, 13, 14). In addition, a good correlation was observed between the changes in plasma levels of AM and various hemodynamic parameters (18). In this regard, our studies have demonstrated that the plasma level of AM and its gene expression in various tissues were significantly elevated during polymicrobial sepsis induced by cecal ligation and puncture (CLP) (34). The increased level of AM has been found to be responsible for initiating the hyperdynamic response observed during the early stage of sepsis (30) and the reduced vascular responsiveness to AM appears to be responsible for the transition from the hyperdynamic phase to the hypodynamic phase during the progression of polymicrobial sepsis (33). However, the mechanism responsible for the reduction of AM-induced vascular relaxation during the late, hypodynamic stage of sepsis remains unknown. A specific plasma AM binding protein from mammalian and avian blood has been identified recently (7). This binding protein (i.e., AM binding protein-1, AMBP-1) has been purified from human plasma and is identical to human
complement factor H, a 120- and/or 140-kDa protein complex (23). It has been demonstrated that AMBP-1 enhances AM-mediated cAMP accumulation in cultured fibroblasts and augments the AM-mediated growth of a cancer cell line (23). In line with the above reports, our recent results have shown that administration of AMBP-1 in combination with AM prevents the transition from the hyperdynamic to the hypodynamic phase of sepsis and reduces the mortality rate in the rat CLP model of sepsis (38). Based on these findings, we hypothesized that AMBP-1 plays a major role in modulating/enhancing vascular responsiveness to AM stimulation under normal as well as septic conditions. The present study was therefore carried out to test this hypothesis.
MATERIALS AND METHODS

Experimental Model of Sepsis. Polymicrobial sepsis was induced in male Sprague-Dawley rats (275-325g, Charles River Laboratory, Wilmington, MA) by CLP as described previously (31). Briefly, rats were fasted overnight prior to the induction of sepsis, but allowed water ad libitum. The animals were anesthetized with isoflurane inhalation and a 2-cm ventral midline abdominal incision was made. The cecum was then exposed, ligated just distal to the ileocecal valve to avoid intestinal obstruction, punctured twice with an 18-gauge needle, and returned to the abdominal cavity. The incision was closed in layers and the animals received 3 ml/100g body wt. normal saline subcutaneously immediately after CLP to provide fluid resuscitation. Sham-operated animals underwent the same surgical procedure except that the cecum was neither ligated nor punctured. Studies were then conducted at 20 h after the induction of sepsis or sham operation. It should be noted that 20 h after CLP represents the late, hypodynamic stage of sepsis (31, 37). This model of sepsis is associated with a mortality of less than 10% at 24h and 94% at 48h after CLP (35). The experiments described here were performed in adherence to the National Institutes of Health guidelines for the use of experimental animals and the work reported in this manuscript fully conformed with the “Guiding Principles for Research Involving Animals and Human Beings” by the American Physiological Society. This project was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Aortic Ring Preparation and Vascular Relaxation. Immediately following the death of the animals by an overdose of isoflurane inhalation, the thoracic cavity was rapidly opened and the thoracic aorta was removed. The blood vessels were immersed in ice-cold Krebs-Ringer bicarbonate solution (composition in mM: NaCl, 118.3; KCl, 4.7; CaCl₂, 2.5; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 25.0; Ca-EDTA, 0.026; glucose, 11.1, Na pyruvate, 1.0 ) that was aerated with 95% O₂:5%
CO₂ (pH 7.4; pO₂ 580 mm Hg) (29). The thoracic aorta was dissected with care to prevent any damage to the vessel and cut into approximately 1.2-mm rings (approximately 1.0 mg each). The aortic ring was then mounted on specimen holders and placed in a glass organ chamber containing 5-ml aerated Krebs-Ringer bicarbonate solution at 37°C. One holder was stationary and the other was connected to an isometric force-displacement transducer (Model FT03, Grass Instruments, Quincy, MA) coupled to a polygraph (Model 7D, Grass Instruments). The vascular rings were incubated for 30-60 min at a tension of 0.5 g during which time the organ chamber was rinsed every 15 min with the aerated Krebs-Ringer bicarbonate solution. When basal tension was stable, a contraction of approximately 0.6 g was induced by 2×10⁻⁹ M norepinephrine (Sigma, St. Louis, MO). Human AMBP-1 (Cortex Biochem, San Leandro, CA) at a concentration of 2×10⁻⁹ or 5×10⁻⁹ M was added to the organ bath and the percentage of vascular relaxation was then determined (29). Synthetic human AM (Phoenix Pharmaceuticals, Belmont, CA) at a concentration of 10⁻⁷ M was applied to the organ chamber alone or with AMBP-1 to determine the interaction between the two agents. At the end of the experiment, the viability of the vascular ring preparations was checked by an addition of norepinephrine, and there was no significant decrease in vascular contraction induced by this agent.

**Determination of Aortic Levels of AMBP-1.** At 20 h after CLP or sham operation, animals were killed by an overdose of isoflurane inhalation. The thoracic aorta (approximately 0.1 g) was rapidly removed and homogenized in a lysis buffer, which contains 10 mM Tris saline, 1% Triton-X, 1mM EDTA, 1 mM EGTA, 2 mM Na orthovanadate, 0.2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin. After centrifugation at 16,000 g for 10 min, the supernatant was collected and the protein concentration was determined by using a protein assay kit (Bio-Red, Hercules, CA). The sample was electrophoretically fractionated on a Bis-Tris gel in MOPS running buffer (Invitrogen, Carlsbad,
CA) under non-reducing conditions. Human complement factor H (5 ng, Cortex Biochem, San Leandro, CA) was used as a positive control. The protein on the gel was then transferred to a 0.45-µm nitrocellulose membrane, and blocked with 5% nonfat dry milk in Tris saline with 0.1% Tween 20 (TBS-T, pH 7.6). The membrane was incubated with 1:2000 rabbit anti-human complement factor H polyclonal antibodies (Accurate Chemical, Westbury, NY) followed by 1:5000 HRP-linked anti-rabbit IgG (Cell signaling Technology, Beverly, MA). ECL-Plus Western blot detection system (Amersham, Piscataway, NJ) was used to reveal the bands and a digital image system (Alpha Innotech, San Leandro, CA) was used to determine the band density. Our preliminary results indicate that the anti-human complement factor H antibodies recognize rat AMBP-1. We used such antibodies since anti-rat AMBP-1 antibodies are not commercially available.

Assessment of AM Receptor Gene Expression in the Aortic Tissue. The aortic tissue was harvested from septic and sham-operated rats at 20 h after surgery. Total RNA was extracted using Tri-reagent (Molecular Research Center, Cincinnati, OH) as previously described by us (34). RNA (4 µg) was then reverse transcribed as previously described (34). The resultant cDNAs were amplified by polymerase chain reaction (PCR) using specific primers for the rat AM receptor subunits calcitonin receptor-like receptor (CRLR), receptor-activity-modifying protein-2 (RAMP-2) and RAMP-3 (17). The primers for CRLR (L27487) are: 5'-CCAAACAGACTTTGGAGTCACTAGG-3' (forward) and 5'-GCTGTCTTCTCTTCTTCTC-ATGCGTGC-3' (reverse). The primers for RAMP-2 (AB028934) are: 5'-AGGTATTACAGCACTGCGGT-3' (forward) and 5'-ACATCCTCTGTGGGATCGGAGTA-3' (reverse). The primers for RAMP-3 (AB028935) are: 5'-ACCTGTGAGTGTCATCGT-G-3' (forward) and 5'-ACTTTCACTCGGGGGGATCTTC-3' (reverse). The PCR reaction was conducted at 30 cycles for CRLR and RAMP-3, and 25 cycles for RAMP-2. Each cycle consisted of 30 sec at 94 °C, 30 sec at 60 °C, 90 sec at 72 °C. Rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH, Clontech, Palo
Alto, CA) was used as a housekeeping gene and the PCR reaction was conducted at 30 cycles of 1 min at 94 °C, 2 min at 60 °C and 3 min at 72 °C. After the RT-PCR procedure, the PCR amplification products were electrophoresed by using a 1.6% agarose gel containing 0.22 µg/ml ethidium bromide. The gel was then photographed on Polaroid films and a digital image system (Alpha Innotech) was used to determine the band density. In addition, the L1 subtype of AM receptors (10) was determined by RT-PCR using specific rat L1 primer [forward primer, 5'-AGCGCCACCAGCACCGAATACG-3'; reverse primer, 5'-AGAGGATGGGGTTGGCGACACAGT-3' (19)] in aortic tissues at 2, 10, and 20 h after CLP in separate animals. The PCR reaction for L1 subtype of AM receptors was conducted at 25 cycles.

**Statistical Analysis.** All data are expressed as means ± SE and compared by Kruskal-Wallis one-way ANOVA on ranks and Tukey’s test, Mann-Whitney rank sum test, or unpaired Student’s t test. Differences in values were considered significant if $P < 0.05$. 
RESULTS

Alterations in AM-Induced Vascular Relaxation. To determine whether AMBP-1 synergistically enhances AM-induced vascular relaxation, experiments were first conducted in aortic rings isolated from sham-operated animals. The results in Figure 1 indicate that human AM at a concentration of $10^{-7}$ M induced an average of 27% vascular relaxation. Addition of $2 \times 10^{-9}$ M AMBP-1 in the presence of AM increased the vascular relaxation to an average of 43%. AMBP-1 at a higher concentration ($5 \times 10^{-9}$ M) further enhanced AM-induced vascular relaxation to approximately 70%, which was significantly higher than AM alone (P<0.05; Fig. 1). While AMBP-1 alone at a concentration of $2 \times 10^{-9}$ M did not induce significant relaxation of the aortic ring (by only 7%), AMBP-1 at $5 \times 10^{-9}$ M induced 24% vasorelaxation (Fig. 1). However, despite the increase in vascular relaxation by AMBP-1 at the higher concentration, it was not statistically significant (P=0.114). The finding that vasorelaxation induced by $10^{-7}$ M AM and $5 \times 10^{-9}$ M AMBP-1 (70%) is higher than the sum (51%) of $10^{-7}$ M AM-induced relaxation (27%) and $5 \times 10^{-9}$ M AMBP-1-induced relaxation (24%) suggests a synergistic potentiation of AMBP-1 on AM-induced vascular relaxation. As shown in Figure 2, AM-induced vascular relaxation decreased significantly at 20 h after the onset of sepsis, compared to sham-operated animals. However, addition of AMBP-1 at both of the concentrations tested restored the AM-induced vascular relaxation at 20 h after CLP (Fig. 2). Similar to sham-operated animals, AMBP-1 at $2 \times 10^{-9}$ M and $5 \times 10^{-9}$ M induced vascular relaxation by 9±2% and 23±5% (n=3), respectively, at 20 h after the onset of sepsis.

Alteration in Aortic Levels of AMBP-1. By using human anti-complement factor H polyclonal antibodies, we have observed a specific band of $M_r$ 140 kDa in rat aortic tissue (Fig. 3A), which is slightly above the commercially available human complement factor H ($M_r$ 120 kDa). This difference in apparent molecular weight could be explained by the utilization of tissues from different species. As shown in Figure 3A, AMBP-1 in the aortic tissue was reduced at 20 h after...
CLP as compared to sham-operated animals. Densitometry data show that the reduction of aortic levels of AMBP-1 was statistically significant (P < 0.05, Fig. 3B). It should be noted that a housekeeping protein was not determined due to the fact that the liver is the major site for AMBP-1 biosynthesis (8) and the vascular AMBP-1, as observed in this study, appears to be liver-derived. However, our previous unpublished data have shown that plasma levels of albumin were not significantly altered at 20 h after CLP. Although future studies are required to determine the alterations of AMBP-1 gene expression and the protein levels in the liver following the onset of sepsis, our present study has clearly indicated that the vascular levels of AMBP-1 are reduced at the later stage of sepsis.

Alteration in AM Receptor Gene Expression in the Aortic Tissue. The gene expression of AM receptor subunits CRLR, RAMP-2 and RAMP-3 was assessed by RT-PCR in aortic tissue. As demonstrated in Figure 4A, CRLR (323 bp), RAMP-2 (164 bp) and RAMP-3 (181 bp) gene expression did not appear to be altered at 20 h after CLP. Similarly, the housekeeping gene G3PDH bands in septic animals were similar to sham-operated animals (Fig. 4B). The ratios of the target genes to the housekeeping gene were not significantly altered at 20 h after the onset of sepsis (Fig. 4C). In contrast to CRLR, RAMP-2 and RAMP-3, the L1 subtype of AM receptors in aortic tissues appeared to be reduced at 20 h after CLP while it was not altered at 2 and 10 h after the onset of sepsis (Fig. 5A). The housekeeping gene G3PDH expression was not altered in sepsis in this portion of the experiment (Fig. 5B).
DISCUSSION

Studies have indicated that plasma levels of AM are elevated in patients with sepsis and septic shock (11, 21) as well as in the experimental animal model of polymicrobial sepsis (16). Our previous studies have demonstrated that circulating levels of AM are elevated as early as 2 h after CLP, progressively increase from 5-20 h, and remains at a high level at 30 h after the onset of sepsis (34). The upregulated AM plays an important role in producing the hyperdynamic circulation during the early stage of sepsis (30). Despite the persistent elevation of AM levels in the late stage of sepsis, the transition from the hyperdynamic to hypodynamic phase occurs during the progression of sepsis, and the reduced vascular responsiveness to AM appears to be responsible for the transition (33). Although it has been postulated that alterations in AM receptors may be associated with vascular hyporesponsiveness to AM at the late stage of sepsis (33), the precise mechanism responsible for the reduction of vascular responsiveness observed under such conditions remains unknown. Since the novel AM binding protein AMBP-1 synergistically enhances AM biological activity in cultured fibroblasts (23), it is possible that the interaction of AMBP-1 and AM is required for maintaining AM-induced vascular relaxation under normal as well as septic conditions. In this regard, our present study has clearly demonstrated that AMBP-1 significantly enhanced AM-induced vascular relaxation in the aortic ring isolated from sham-operated animals. This result further confirms the findings of Pio et al. (23) in which AMBP-1 enhanced AM-induced cAMP accumulation in cultured Rat-2 fibroblasts. Our results also indicate that the reduction of AM-induced vascular relaxation during the late stage of sepsis can be restored following the addition of AMBP-1 under in vitro conditions. Moreover, the aortic level of AMBP-1 decreased significantly at 20 h after CLP, suggesting that the reduced local level of AMBP-1 appears to play a critical role for vascular AM hyporesponsiveness observed during the late stage of sepsis. In contrast, the findings that gene expression of AM receptor subunits CRLR, RAMP-2, and RAMP-3 in the aortic...
tissue did not change significantly at 20 h after CLP suggest that alterations in the expression of above subtype of AM receptors may not be involved in vascular AM hyporesponsiveness in late sepsis. The finding that L1 subtype of AM receptors appears to be downregulated at 20 h after CLP may suggest its role in producing vascular AM hyporesponsiveness observed under such conditions. However, our findings that AMBP-1 significantly enhances AM-induced vascular relaxation suggests that the reduction of AMBP-1 binding capacity and the lack of adequate interaction between AM and AMBP-1 plays an important role in producing vascular hyporesponsiveness to AM in the late stage of sepsis.

It should be noted that the semi-quantitative RT-PCR technique was used to assess the gene expression of AM receptor subtypes. Our preliminary results have indicated that the amplification curve had not plateaued at 30 cycles. We therefore performed RT-PCR only at that number of cycles for AM receptor subtypes. In our recent study, we have demonstrated that, by using 25 cycles, the gene expression of RAMP-3 in the lungs increased at 5 h but returned to sham level at 20 h after CLP (22). In addition, gene expression of CRLR and RAMP-2 in the lungs did not change at 5 h and 20 h after CLP (22). Nonetheless, a determination of various subtypes of AM receptors by quantitative RT-PCR technique is required. While determination of AM-receptor gene expression is important, we plan to perform AM-receptor binding assays in the future, which should shed some light on the role of AM-receptors in producing vascular hyporesponsiveness to AM at the later stage of sepsis. Moreover, it could be argued that alteration in AM-induced relaxation in a conductance vessel (i.e., the aorta) may not reflect what happened in smaller, resistance blood vessels. Although AM-induced relaxation at the microcirculatory level was not examined in this study, we previously reported that AM-induced relaxation also decreased significantly at 20 h after the onset of sepsis in the intestinal resistance blood vessels by using isolated perfused gut preparation (33). Furthermore, pentoxifylline (a phosphodiesterase inhibitor) which downregulates
proinflammatory cytokines, prevents AM hyporesponsiveness in the aorta and gut resistance vessels in the late stage of sepsis (15). These results, taken together, would suggest that alterations of AM-induced vascular relaxation in sepsis occur at the macro- and microcirculatory level.

The presence of AMBP-1 in plasma was first reported by Elsasser et al. in 1999 (7). It has been demonstrated that AMBP-1 is a mixture of a 120-140 kDa protein complex in mammalian and avian blood. Human plasma AMBP-1 was identified in 2001 as being the same as complement factor H (23) which is a single chain glycoprotein containing 20 subunits called short consensus repeats (24). Factor H is present in plasma and has also been detected in extravascular compartments such as the synovial fluid (3). Although the liver is considered to be the main source of complement factor H, this protein can also be synthesized by extrahepatic cells such as mononuclear phagocytes, fibroblasts, endothelial cells, mesangial cells, neuroglia cells and neurons (8). Complement factor H inhibits the alternative complement pathway (9) and also binds to cell surfaces and modulates neutrophil and monocyte function (4, 12). Recent findings of the interaction between AM and complement factor H (i.e., AMBP-1) have opened a new avenue for further understanding of the AM biology. Two specific mechanisms are responsible for the vasodilatory effect of AM, i.e., a direct effect on vascular smooth muscle cells to increase intracellular cAMP levels by stimulating AM receptors and adenylate cyclase activity (6, 16, 19), and an indirect effect on vascular endothelial cells by stimulating Ca\textsuperscript{2+} mobilization to increase endothelium-derived nitric oxide (NO) release via the activation of constitutive NO synthase (cNOS) (16, 25). With regards to the mechanism responsible for AM-induced vascular relaxation [involving activation of endothelial cNOS and thereby activating NO-cGMP pathway (10, 20)], our preliminary results have indicated that the decreased acetylcholine-induced vascular relaxation (reflecting endothelial cNOS-derived NO) observed at 20 h after CLP was prevented following intravenous administration of AM/AMPB-1 (32). Moreover, the reduced gene expression of endothelial cNOS observed at 20 h
after CLP was prevented following administration of AM/AMBP-1 (32). This would suggest that the NO-cGMP pathway is indeed important in mediating the beneficial effects of AM/AMBP-1 in sepsis.

Although a full scale dose-response curve was not conducted for AMBP-1, we have tested two concentrations of this protein, i.e., $2 \times 10^{-9}$ and $5 \times 10^{-9}$ M (Fig. 1). The results indicate that AMBP-1 by itself has some effects on vascular reactivity. In this regard, our recent studies (36) have shown that AMBP-1 at a concentration of 50 nM significantly reduced LPS (10 ng/ml)-stimulated TNF-α production in Kupffer cells isolated from the rat. Co-incubation with anti-AM antibodies (1:1000 dilution) prevented the downregulatory effect of AMBP-1 on TNF-α. Since AM has anti-inflammatory properties and since co-incubation with AMBP-1 and anti-AM antibodies prevent the effect of AMBP-1 on TNF-α release in the isolated Kupffer cells, we believe that the effect of AMBP-1 on TNF-α production is primarily due to contamination of AMBP-1 preparation by AM and/or production of endogenous AM. It is therefore most likely that the effect of AMBP-1 on vascular reactivity is due to AM contamination in the AMBP-1 preparation used in this study. This is further confirmed by the fact that the binding capacity of AMBP-1 is extremely high (23). It should be pointed out that because a “housekeeping” protein is not determined, it could be argued that the data presented in Fig. 3B may reflect unequal loading. This may not be true since our preliminary data indicate that the binding capacity of AMBP-1 in plasma decreased and AMBP-1 gene expression in the liver is downregulated following the onset of sepsis. Nonetheless, future experiments determining AMBP-1 gene expression in vascular tissues are needed.

Recent studies have shown that chronic overexpression of AM in transgenic mice is protective against circulatory collapse, organ damage, and the mortality characteristic of endotoxic shock (26). AM gene delivery also attenuates hypertension, cardiac remodeling, and renal injury in deoxycorticosterone acetate-salt and Goldblatt hypertensive rats (5, 28). Since plasma levels of
AMBP-1 were not measured in the above studies, it is unclear whether the beneficial effect of upregulation of AM gene expression is due to the elevated AM by itself or due to accompanied upregulation of AMBP-1, which may occur following the chronic elevation of AM under the above conditions. Therefore, it would be very interesting to determine whether chronic overexpression of AM in normal animals is indeed associated with an elevation of AMBP-1 levels. Following infection, however, AMBP-1 binding capacity was found to be significantly reduced (7). Our present study also shows that vascular levels of AMBP-1 decreased significantly at the late stage of sepsis while it remains to be determined whether vascular AMBP-1 binding capacity is also altered during the progression of sepsis. Although our data have indicated that vascular levels of AMBP-1 decreased at 20 h after CLP, it remains unknown how AMBP-1 is delivered from the plasma to the binding site (i.e., AM receptors). Studies by Pio et al suggest that AMBP-1 enhances AM/AM-receptor binding capacity and/or increase the local concentration of AM, thereby improving the AM-induced vascular relaxation (23). However, it remains unknown whether or not specific receptors for AMBP-1 or AM/AMBP-1 complex exists. Since vascular AMBP-1 levels decreased during the late stage of sepsis and co-administration of AM and AMBP-1 attenuated the vascular AM hyporesponsiveness, we hypothesized that administration of AM and AMBP-1 could be a novel approach for preventing the transition from the hyperdynamic to the hypodynamic phase during the progression of sepsis. In this regard, our recent studies have demonstrated that administration of AMBP-1 and AM in combination maintained cardiac output, organ perfusion and oxygen delivery, attenuated hepatic injury, and reduced the mortality rate after the onset of sepsis (38). However, neither AM alone nor AMBP-1 alone produced a similar salutary effect in the CLP model of sepsis (38). Thus, the modulatory effect of AMBP-1 on vascular responsiveness to AM observed in the present study may be the underlying mechanism responsible for the beneficial effect of the administration of AM and AMBP-1 during the progression of polymicrobial sepsis.
It has been indicated that AM can stimulate its receptors on vascular smooth muscle cells by increasing intracellular cAMP to produce the vasodilatory effect (6). We have previously hypothesized that the reduced vascular hyporesponsiveness to AM in sepsis may be due to downregulation of AM receptors (33). In contrast to this hypothesis, our present study has clearly demonstrated that the gene expression of AM receptor subunits CRLR, RAMP-2, and RAMP-3 was not significantly altered at 20 h after the onset of sepsis. Similarly, our recent studies also show that pulmonary RAMP-3 increased at 5 h but returned to sham levels at 20 h after CLP while no significant alterations of CRLR and RAMP-2 occurred at either time point (22). Thus, the reduced vascular responsiveness observed in late sepsis appears to be due to the decrease of AMBP-1 modulatory function rather than downregulation of AM receptor gene expression. Although our data have shown that AM receptor expression in the vascular tissue was not changed at 20 h after the onset of sepsis, it remains to be determined whether the receptor binding capacity and/or affinity remain unaltered under such conditions. Moreover, studies are required to examine whether AM receptor signal transduction pathways are altered during the late stage of sepsis. As reported in our recent publication, $10^{-7}$ M synthetic rat AM produced an average of 69% vascular relaxation in sham-operated animals (33) while $10^{-7}$ M synthetic human AM produced an average of only 27% relaxation in the present study. These data indicate that rat and human AMs are of unequal potency. The reason for utilizing human AM in this study is due to the fact that rat AMBP-1 is not commercially available at present. Since rat AM is more potent than human AM, it remains unknown whether human AMBP-1 can potentiate $10^{-7}$ M rat AM-induced vascular relaxation. However, this can be tested using lower concentrations of rat AM.

In summary, our results have demonstrated that AMBP-1 enhances AM-induced vascular relaxation in the aortic tissues harvested from sham-operated as well as septic animals. Although AM receptor gene expression does not appear to be altered, vascular levels of AMBP-1 decrease
significantly during the late stage of sepsis. These results suggest that AMBP-1 plays an important role in modulating vascular responsiveness to AM during sepsis. Thus, maintenance of circulating AM-AMBP-1 complex should be considered as a novel approach in preventing vascular hyporesponsiveness and the transition from hyperdynamic to hypodynamic circulation during the progression of polymicrobial sepsis.
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FIGURE LEGENDS

**Figure 1.** Effects of adrenomedullin (AM), AM binding protein-1 (AMBP-1), or their combination on vascular relaxation in the aortic ring isolated from sham-operated animals. The data are expressed as percentage (%) of the vascular relaxation from the initial tension induced by 2 x 10^{-9} M norepinephrine. Values (n=5) are presented as means ± SE and compared by Kruskal-Wallis one-way ANOVA on ranks and Tukey’s test. *P < 0.05 versus the vascular relaxation induced by AM alone or AMBP-1 alone (both concentrations).

**Figure 2.** Effects of AMBP-1 on AM-induced vascular relaxation in the aortic ring at 20 h after cecal ligation and puncture (CLP). The data are expressed as percentage (%) of the vascular relaxation from the initial tension induced by 2 x 10^{-9} M norepinephrine. Values (n=5-6/group) are presented as means ± SE and compared by Mann-Whitney rank sum test (between sham and CLP groups), Kruskal-Wallis one-way ANOVA on ranks and Tukey’s test. *P < 0.05 versus sham-operated animals; #P < 0.05 versus the vascular relaxation induced by 10^{-7} M AM. Please note that the data of the sham group in this figure are the same data presented in Figure 1.

**Figure 3.** Alteration in AMBP-1 levels in the aortic tissue at 20 h after CLP. A. A representative blot from four different experiments in each group at 20 h after sham operation or CLP. B. Optical densities of aortic AMBP-1 blots. Values (n=4/group) are presented as means ± SE and compared by unpaired Student’s t test. *P<0.05 versus sham-operated animals.

**Figure 4.** Gene expression of AM receptor subunits and the housekeeping gene G3PDH in the aortic tissue at 20 h after sham operation or CLP. A. Representatives of RT-PCR products of AM receptor subunits CRLR (323 bp), RAMP-2 (164 bp) and RAMP-3 (181 bp). Lane “S” represents sham animals; Lane “C” represents animals at 20 h after CLP. B. Representative of RT-PCR products of the housekeeping gene G3PDH (983 bp) at 20 h after sham operation (S) or CLP (C). C. Effects of sepsis on the ratio of AM receptor subunits over housekeeping gene G3PDH at 20 h after CLP. The values (n=3-4/group) are presented as means ± SE and unpaired Student’s t test
indicates that there was no significant difference in the examined genes between septic and sham animals.

**Figure 5.** Gene expression of the L1 subtype of AM receptors and the housekeeping gene G3PDH in the aortic tissue at 2, 10, and 20 h after CLP. **A.** A representative of RT-PCR products of the L1 subtype of AM receptors (471 bp). Lane “S” represents animals at 20 h after sham operation; Lane “C2” represents animals at 2 h after CLP; Lane “C10” represents animals at 10 h after CLP; and Lane “C20” represents animals at 20 h after CLP. **B.** A representative of RT-PCR products of the housekeeping gene G3PDH (983 bp) after sham operation (S) or CLP (C). Please note that same tissue extracts were used for Gene expression of both L1 subtype of AM receptors and G3PDH.
**Figure 1**

Vascular Relaxation (%)

- 2x10^{-9}
- 5x10^{-9}
- 10^{-7}
- 2x10^{-9}
- 5x10^{-9} M [MPB-1]

- 10^{-7} M [AM]

Legend:
- **-** indicates absence of compound
- **10^{-7}** indicates 10^{-7} M concentration
- **5x10^{-9} M [AMBP-1]** indicates 5x10^{-9} M concentration with AMBP-1
Figure 2
Figure 3A

140 kDa (AMB-P-1)

Sham  CLP-20h
Figure 3B

AMBP-1 Density ($10^6$ pixels)

Sham             CLP-20h

*
**Figures 4A-B**
Figure 4C
Figures 5A-B