Adrenomedullin binding protein-1 modulates vascular responsiveness to adrenomedullin in late sepsis


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 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 determination of 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 that AMBP-1 significantly enhances 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 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.

Address for reprint requests and other correspondence: P. Wang, Center for Surgical Research, University of Alabama at Birmingham, 1670 University Blvd., Volker Hall, Rm. G094P, Birmingham, AL 35294 (E-mail: pwang@uab.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 (275–325 g, Charles River Laboratory, Wilmington, MA) by CLP as described previously (31). Briefly, rats were fasted overnight before 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 normal saline (3 ml/100 g body wt sc) immediately after CLP to provide fluid resuscitation. Sham-operated animals underwent the same surgical procedure, except 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 <10% at 24 h and 94% at 48 h 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 here fully conformed with the “Guiding Principles for Research Involving Animals and Human Beings” by the American Physiological Society. This project was approved by the Animal Care and Use Committee of the University of Alabama at Birmingham.

Aortic ring preparation and vascular relaxation. Immediately after 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: 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, 0.026 Ca-EDTA, 11.1 glucose, 1.0 sodium pyruvate) that was aerated with 95% O2-5% CO2 (pH 7.4, 580 mmHg PO2) (29). The thoracic aorta was dissected with care to prevent any damage to the vessel and cut into ~1.2-mm rings (~1.0 mg each). The aortic ring was then mounted on specimen holders and placed in a glass organ chamber containing 5 ml of aerated Krebs-Ringer bicarbonate solution at 37°C. One holder was stationary, and the other was connected to an isometric force-displacement transducer (model PT03, 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 ~0.6 g was induced by 2 × 10–9 M norepinephrine (Sigma, St. Louis, MO). Human AMBP-1 (Cortex Biochem, San Leandro, CA; 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; 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 (~0.1 g) was rapidly removed and homogenized in a lysis buffer, which contains 10 mM Tris saline, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 0.2 M phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotenin. 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-Rad, Hercules, CA). The sample was electrophoretically fractionated on a Bis-Tris gel in MOPS running buffer (Invitrogen, Carlsbad, CA) under nonreducing conditions. Human complement factor H (5 ng; Cortex Biochem) 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 (pH 7.6). The membrane was incubated first with 1:2,000 rabbit anti-human complement factor H polyclonal antibodies (Accurate Chemical, Westbury, NY) and then with 1:5,000 horseradish peroxidase-linked anti-rabbit IgG (Caltag Antibodies, 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 (RAMP)-2, and RAMP-3 (17). The primers are as follows: 5′-CCCAACAGACTTGAGTCTACTAGG-3′ (forward) and 5′-GCTGCTCTTTCTTCTTTCT-ATGCGTGC-3′ (reverse) for CRLR (L27487), 5′-AGGTATACAGCAGA-AGCTGCCGTG-3′ (forward) and 5′-ATCACCTCTGGGAGTCCAG-3′ (reverse) for RAMP-2 (AB028934), and 5′-ACCTTGCGGACTTCTCT-TCTG-3′ (forward) and 5′-ACCTCAGC8G88GGTGC8G8CTCTC-3′ (reverse) for RAMP-3 (AB028935). The PCR was conducted at 30 cycles for CRLR and RAMP-3 and 25 cycles for RAMP-2. Each cycle consisted of 30 s at 94°C, 30 s at 60°C, and 90 s at 72°C. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Clontech, Palo Alto, CA) was used as the housekeeping gene, and the PCR 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 using a 1.6% agarose gel containing 0.22 μg/ml ethidium bromide. The gel was then photographed with a Polaroid film 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 [5′-AGGCACCAACCGACCCGAAATACG-3′ (forward) and 5′-AGAGATGGGGGTGGCGACACATG-3′ (reverse)] (19) in aortic tissues at 2, 10, and 20 h after CLP in separate animals. The PCR for the 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.

Downloaded from http://ajpregu.physiology.org/ by 10.220.32.246 on October 30, 2017
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 Fig. 1 indicate that 10^{-7} M human AM 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 \sim 70%, which was significantly higher than vascular relaxation induced by AM alone (\*P < 0.05; Fig. 1). Although AMBP-1 alone at 2 \times 10^{-9} M did not induce significant relaxation of the aortic ring (by only 7%), 5 \times 10^{-9} M AMBP-1 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 Fig. 2, AM-induced vascular relaxation decreased significantly at 20 h after the onset of sepsis compared with 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, 2 \times 10^{-9} and 5 \times 10^{-9} M AMBP-1 induced vascular relaxation by 9 \pm 2 and 23 \pm 5% (n = 3), respectively, at 20 h after the onset of sepsis.

Alteration in aortic levels of AMBP-1. Using human anti-complement factor H polyclonal antibodies, we observed a specific 140-kDa band in rat aortic tissue (Fig. 3A), which is slightly above the molecular mass of the commercially available human complement factor H (120 kDa). This difference in apparent molecular mass...
mass could be explained by the utilization of tissues from different species. As shown in Fig. 3A, AMBP-1 in the aortic tissue was reduced at 20 h after sham operation or CLP. Similarly, the housekeeping gene GAPDH bands in septic animals were similar to those in sham-operated animals (Fig. 3B). The ratios of the target genes to the housekeeping gene were not significantly altered at 20 h after the onset of sepsis (Fig. 3C). 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, whereas it was not altered at 2 and 10 h after the onset of sepsis (Fig. 5A). The housekeeping gene GAPDH 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 between 5 and 20 h, and remain 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 the 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), RT-PCR in aortic tissue. As demonstrated in Fig. 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 GAPDH bands in septic animals were similar to those in 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, whereas it was not altered at 2 and 10 h after the onset of sepsis (Fig. 5A). The housekeeping gene GAPDH expression was not altered in sepsis in this portion of the experiment (Fig. 5B).
precise mechanism responsible for the reduction of vascular responsiveness observed under such conditions remains unknown. Because the novel 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 after 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 in 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 these AM receptor subunits may not be involved in vascular AM hyporesponsiveness under such conditions. However, our finding 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 play an important role in producing vascular hyporesponsiveness to AM in the late stage of sepsis.

The semiquantitative 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. Therefore, we 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 and 20 h after CLP (22). Nonetheless, a determination of various subtypes of AM receptors by quantitative RT-PCR is required. Although 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 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 an 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 in 1999 by Elsasser et al. (7). It has been demonstrated that AMBP-1 is a mixture of a 120- to 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, 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: 1) 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 2) an indirect effect on vascular endothelial cells by stimulating Ca²⁺ mobilization to increase endothelium-derived nitric oxide (NO) release via the activation of constitutive NO synthase (cNOS) (16, 25). With regard to the mechanism responsible for AM-induced vascular relaxation involving activation of endothelial cNOS and, thereby, activation of the 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 after intravenous administration of AM/AMBP-1 (32). Moreover, the reduced gene expression of endothelial cNOS observed at 20 h after CLP was prevented after 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.
with anti-AM antibodies (1:1,000 dilution) prevented the downregulatory effect of AMBP-1 on TNF-\(\alpha\). Because AM has anti-inflammatory properties and because coinubcation with AMBP-1 and anti-AM antibodies prevents the effect of AMBP-1 on TNF-\(\alpha\) release in the isolated Kupffer cells, we believe that the effect of AMBP-1 on TNF-\(\alpha\) 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 after 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). Because 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 accompanied upregulation of AMBP-1, which may occur after 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. After infection, however, AMBP-1 binding capacity was significantly reduced (7). Our present study also shows that vascular levels of AMBP-1 decreased significantly at the late stage of sepsis, whereas 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. (23) suggest that AMBP-1 enhances AM/AM receptor binding capacity and/or increases the local concentration of AM, thereby improving the AM-induced vascular relaxation. However, it remains unknown whether specific receptors for AMBP-1 or the AM-AMBP-1 complex exist. Because vascular AMBP-1 levels decreased during the late stage of sepsis and coadministration 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 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, whereas 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), whereas \(10^{-7}\) 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. Human AM was utilized in this study because rat AMBP-1 is not commercially available. Because rat AM is more potent than human AM, it remains unknown whether human AMBP-1 can potentiate vascular relaxation induced by \(10^{-7}\) M rat AM. 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.

This investigation was supported by National Institutes of Health Grants R01 GM-57468 and K02 AI-61461 (P. Wang).

REFERENCES


