Adrenomedullin binding protein-1 is downregulated during polymicrobial sepsis in the rat

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Abstract. We have previously shown that administration of adrenomedullin (AM) and AM binding protein-1 (AMBP-1) in combination maintains cardiovascular stability and reduces mortality in a rat model of sepsis. However, it is unknown whether AMBP-1 is reduced under the septic condition and, if so, whether lipopolysaccharide (LPS) plays a role in down-regulating AMBP-1. To determine this, male adult Sprague-Dawley rats were subjected to either polymicrobial sepsis by cecal ligation and puncture (CLP), or endotoxemia by intraperitoneal injection of LPS (15 mg/kg body weight). In an additional group of animals, LPS neutralizing agent polymyxin B (PMB) was given intramuscularly at 0.5 h before and 9 h after CLP. At 20 h after CLP (i.e. the late stage of sepsis) or endotoxemia, hepatic tissue and blood samples were collected. Hepatic AMBP-1 gene expression along with hepatic and plasma AMBP-1 protein levels were measured by RT-PCR and Western blot analysis, respectively. Our results showed that hepatic AMBP-1 gene expression decreased by 65%, hepatic AMBP-1 protein levels decreased by 72%, and plasma levels of AMBP-1 decreased by 59% at 20 h after CLP. Similar results were also seen in the animals receiving LPS injection. Administration of PMB, however, prevented the downregulation of AMBP-1 expression at 20 h after CLP. Thus, AMBP-1 is downregulated in the late phase of sepsis, and LPS plays a critical role in the reduction of AMBP-1.

Introduction

The recently reported adrenomedullin binding protein-1 (AMBP-1) was identified as complement factor H, which specifically binds adrenomedullin (AM) and regulates AM bioactivity (1-3). AMBP-1 does not change the affinity of AM receptors for AM, but has sequences which may bind to cell surface adhesion molecules. Therefore, AMBP-1 may bring AM near its receptors and raise the effective concentration of AM at the binding site (4,5). Our recent studies have shown that administration of AMBP-1 and AM in combination was able to prevent the transition from the hyperdynamic to hypodynamic phase of sepsis, increase organ perfusion, and attenuate multiple organ injury, eventually decreasing mortality in a rat model of sepsis (6,7). We also found that AM is upregulated during sepsis primarily due to elevated levels of lipopolysaccharide (LPS) (8). However, it remains unknown whether sepsis affects AMBP-1 expression and production. We hypothesized that AMBP-1 has a differential alteration in sepsis as compared to AM, and that AMBP-1 expression is downregulated under such conditions due to the elevated level of LPS. Since the liver is the major source of AMBP-1 (4), AMBP-1 gene expression and protein levels in hepatic tissues as well as its plasma protein levels were measured using reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis, respectively. The role of LPS in downregulating AMBP-1 expression was investigated following the administration of endotoxin (endotoxemia) or in septic animals receiving the endotoxin-neutralizing agent, polymyxin B (PMB).

Materials and methods

Experimental animals. Male Sprague-Dawley rats (average body weight 320 g; Charles River Laboratories, Wilmington, MA) were housed in a temperature-controlled room with a 12-h light/dark cycle, and fed on a standard Purina rat chow diet. The rats were fasted overnight but allowed water *ad libitum* before surgery, and anesthetized with isofluranne inhalation. The rats (n=5-6/group) were subjected to sham operation, polymicrobial sepsis, sepsis with LPS neutralization, or endotoxemia. The experiments 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 Feinstein Institute for Medical Research.

Polymicrobial sepsis. A 2-cm ventral midline abdominal incision was made. The cecum was exposed, ligated just distal to the ileocecal valve to avoid intestinal obstruction, and punctured twice with an 18 gauge needle. The punctured cecum was squeezed to expel a small amount of fecal material and

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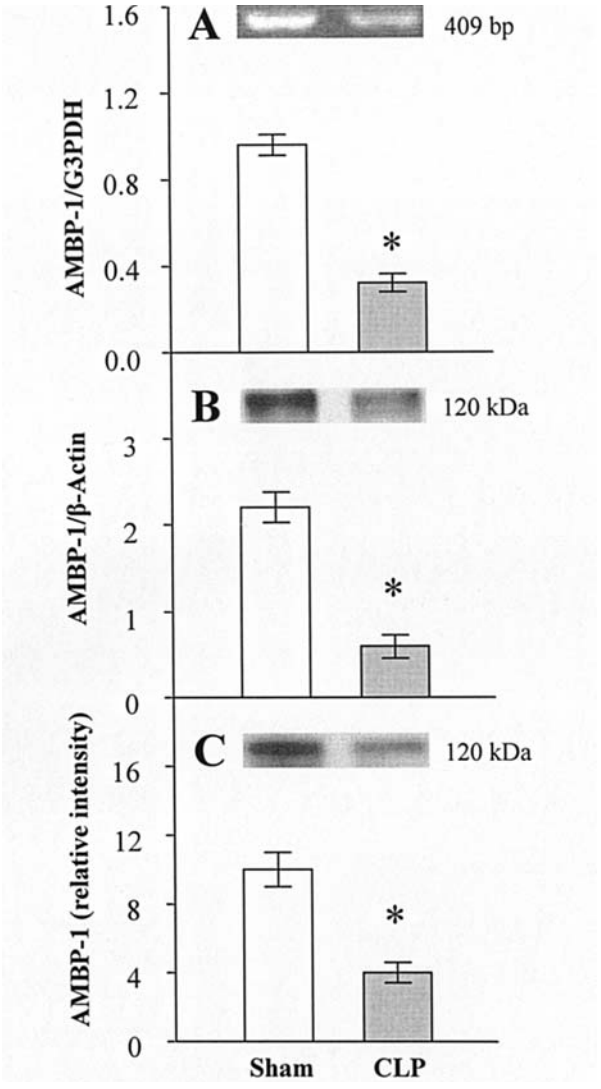

Figure 1. Alterations in hepatic AMBP-1 gene expression (A, the ratio of AMBP-1/G3PDH), hepatic AMBP-1 protein levels (B, the ratio of AMBP-1/ß-actin), and plasma AMBP-1 protein levels (C, the relative intensity) at 20 h after cecal ligation and puncture (CLP) or sham operation (Sham). Representative blots are also presented. Data are expressed as means ± SE (n=5-6/group) and compared by Student's t-test: *P<0.05 versus sham-operated animals.

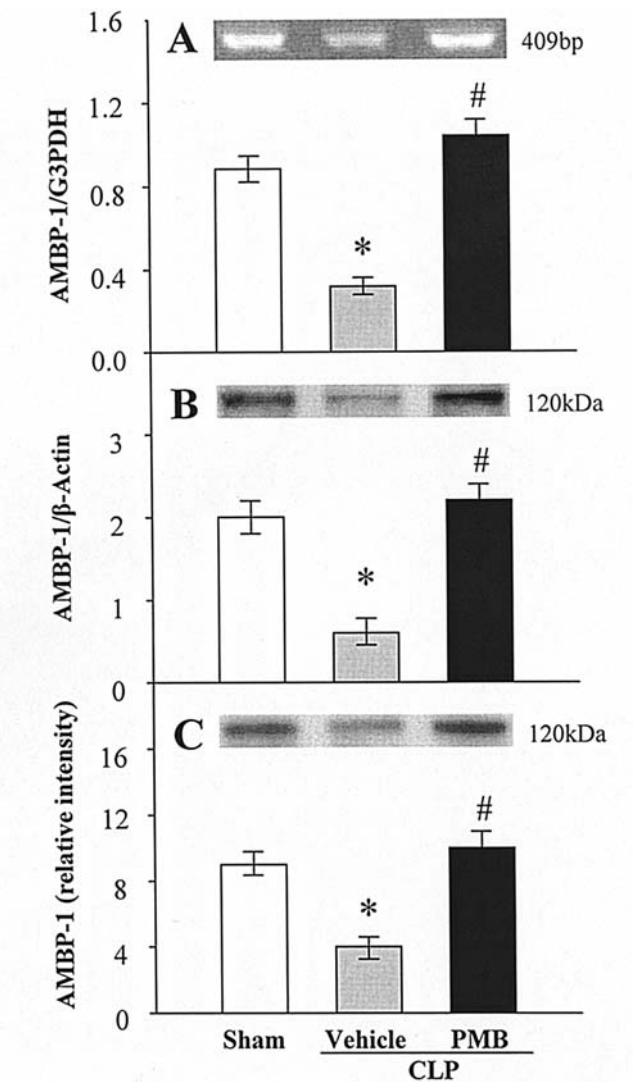

Figure 2. Alterations in hepatic AMBP-1 gene expression (A, the ratio of AMBP-1/G3PDH), hepatic AMBP-1 protein levels (B, the ratio of AMBP-1/ß-actin), and plasma AMBP-1 protein levels (C, the relative intensity) at 20 h after sham operation (Sham), cecal ligation and puncture (CLP) with vehicle or polymyxin B (PMB) administration. Representative blots are also presented. Data are expressed as means ± SE (n=5-6/group) and compared by one-way analysis of variance (ANOVA) and Student-Newman-Keuls test: *P<0.05 versus sham-operated animals; #P<0.05 versus CLP animals receiving vehicle treatment.

then returned to the abdominal cavity. The incision was closed in layers and the animals were resuscitated by 3 ml/100 g body weight saline subcutaneously immediately after CLP. Sham-operated animals underwent the same surgical procedure except cecum ligation and puncture. Blood and hepatic tissue were harvested at 20 h after induction of sepsis or sham operation. Please note that 20 h after CLP represents the late, hypodynamic phase of sepsis (9). Polymicrobial sepsis with LPS neutralization was achieved by administration of PMB (Sigma, St. Louis, MO), delivered intramuscularly at 0.5 h before CLP as well as 9 h after CLP at a dose of 2,000 U/kg body weight. PMB is a LPS binding antibiotic which binds and detoxifies lipid A. *In vitro* studies have confirmed that PMB neutralizes *E. coli* LPS activity (10,11). Our previous studies have shown that administration of PMB at the above dosage markedly decreased plasma levels of LPS after CLP in the rat (8).

Endotoxemia. Each rat received 15 mg/kg body weight *E. coli* LPS (Sigma). LPS was diluted to 1 ml normal saline followed by its intraperitoneal injection. Each rat in the vehicle group received 1 ml of normal saline (intraperitoneal injection).

Determination of hepatic AMBP-1 gene expression. Total RNA was extracted from the liver by Tri-Reagent (Molecular Research Center, Cincinnati, OH); 100 mg of tissue was homogenized in 1.5 ml Tri-Reagent and the homogenate was separated into aqueous and organic phases. The aqueous liquid was isolated and followed by chloroform addition and centrifugation. RNA was precipitated from the aqueous phase by addition of isopropanol, and washed with ethnol. The pellet was dissolved in 0.1% DEPC-treated, deionized, and distilled water. RNA concentration and purity were determined by measuring the absorbance at 260 and 280 nm, and RNA (4 μg)

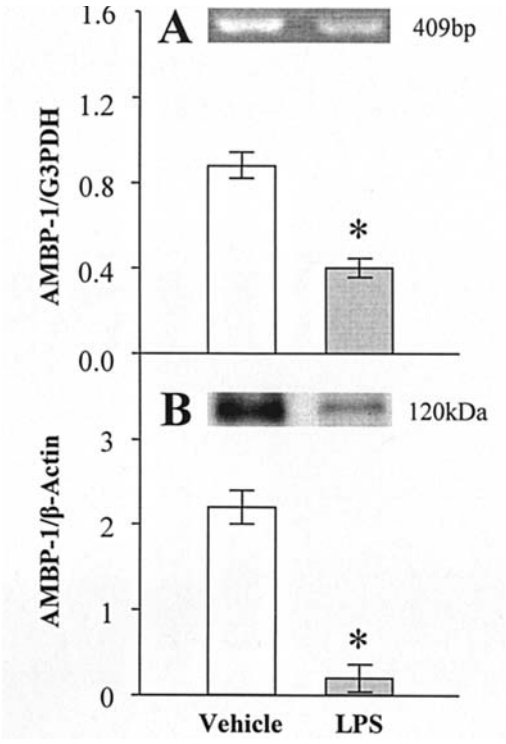

Figure 3. Alterations in hepatic AMBP-1 gene expression (A, the ratio of AMBP-1/G3PDH) and hepatic AMBP-1 protein levels (B, the ratio of AMBP-1/ß-actin) at 20 h after administration of lipopolysaccharide (LPS) or normal saline (Vehicle). Representative blots are also presented. Data are expressed as means ± SE (n=5-6/group) and compared by Student's t-test: *P<0.05 versus Vehicle.

was reverse-transcribed as previously described (12,13). The resulting cDNAs were amplified by polymerase chain reaction (PCR) using specific primers for rat AMBP-1 (forward 5'-CAC TTC CTT TTG CCT TGC TT-3', reverse 5'- TCA ATT ATC CCA CCT GCT CA-3', AA819055), and for rat housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (G3PDH) (forward 5'-TGA AGG TCG GTG TCA ACG GAT TTG GC-3', reverse 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3', M17701). The PCR was conducted at 25 cycles for both AMBP-1 and G3PDH. Each cycle consisted of 45 sec at 94˚C, 45 sec at 60˚C, and 2 min at 72˚C. The PCR products were electrophoresed on a 1.6% agarose gel containing 0.22 μg/ml ethidium bromide. AMBP-1 gel band intensities were normalized by G3PDH using the Bio-Rad image system (Hercules, CA).

Determination of AMBP-1 protein levels. Hepatic tissues (0.1 g) were lysed and homogenized in 1-ml lysis buffer (10 mmol/l TBS, 1 mmol/l EDTA, 1 mmol/l EGTA, 2 mmol/l Na ortho-vanadate, 0.2 mmol/l PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1% Triton-X100), cleared by centrifugation at 14,000 rpm for 15 min at 4˚C; 100 μg hepatic protein or 10 μg plasma protein sample was fractionated on 4-12% Trisacetate gel, and transferred to a 0.45-μm pore size nitrocellulose membrane. Nitrocellulose blots were blocked by 5% milk in TBST (10 mmol/l Tris-HCl, pH 7.5, 100 mmol/l NaCl, 0.1% Tween-20) for 1 h, incubated with rabbit anti-human complement factor H polyclonal antibodies (1:3,000, Accurate Chemocal, Westbury, NY) overnight at 4˚C, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:20,000, Cell Signaling Technology, Beverly, MA) for 1 h at room temperature. Protein blots were visualized using ECL (Amersham Biosciences, Piscataway, NJ), and band densities were determined using a Bio-Rad image system. Hepatic AMBP-1 protein bands were normalized by hepatic ß-actin. Our studies and others (6,14) showed that anti-human complement factor H antibodies are able to be cross-reactive with rat AMBP-1 (factor H).

Statistical analysis. The data were expressed as mean ± SE and compared by one-way analysis of variance (ANOVA) and Student-Newman-Keuls test or Student's t-test. Differences in values were considered significant at $P<0.05$.

Results

Alterations in AMBP-1 in sepsis. As shown in Fig. 1C, plasma levels of AMBP-1 decreased by 59% at 20 h after CLP in vehicle-treated animals as compared to sham-operated animals ($P<0.05$). The reduction of AMBP-1 levels in blood was due to the decreased production of AMBP-1 in the liver. This is confirmed by the results that mRNA expression in hepatic tissues decreased by 65% ($P<0.05$, Fig. 1A) and hepatic AMBP-1 protein levels decreased by 72% at 20 h after CLP ($P<0.05$, Fig. 1B).

Effects of endotoxin neutralization on AMBP-1 expression in sepsis. To explore whether LPS was responsible for AMBP-1 downregulation in septic animals, PMB was used to inhibit LPS activity. After intramuscular administration of PMB, downregulation of AMBP-1 gene expression in hepatic tissues was prevented (Fig. 2A). Similarly, both hepatic (Fig. 2B) and plasma levels of AMBP-1 protein (Fig. 2C) were reduced at 20 h after CLP, but such decrease was prevented after administration of PMB. This indicates that LPS is responsible for downregulation of AMBP-1 in the septic rat.

Alterations in AMBP-1 after endotoxin administration. To further confirm the downregulatory effect of LPS on AMBP-1 in septic animals, an animal model of endotoxemia was produced by LPS injection. The results showed that both mRNA expression and hepatic protein levels of AMBP-1 decreased significantly at 20 h after LPS administration ($P<0.05$). Hepatic AMBP-1 gene expression decreased by 54% (Fig. 3A), and protein levels in hepatic tissue decreased by 91% (Fig. 3B). The results further support that LPS plays a critical role in downregulation of AMBP-1 in the septic rats.

Discussion

A recent epidemiologic study shows that sepsis and its complications such as multiple organ failure are still prevalent causes of morbidity and mortality. It is estimated that there is a US hospital incidence of 751,000 cases per year with a mortality rate of 28.6%, despite rapid progress in critical care medicine (15). Thus, efforts continue in search for new therapies, and to understand sepsis and its related complications. The CLP model of sepsis in the rat has been widely used for the study of pathophysiology of sepsis. This

model of sepsis produces a similar pathological process compared to perforated appendicitis and peritonitis (16). The septic response in this animal model is biphasic with an early hyperdynamic phase generally occurring between 2 and 10 h after CLP, consisting of increased cardiac output, increased stroke volume, decreased systemic vascular resistance, and increased tissue perfusion. This hyperdynamic response is followed by a late hypodynamic phase starting approximately 16 h after CLP and characterized by decreased cardiac output, decreased stroke volume, increased systemic vascular resistance, and decreased tissue perfusion (9,17). Mortality rates in rats following CLP are 10% at 24 h and >50% at 48 h (18). Many studies have been conducted to investigate the mechanisms responsible for organ failure in sepsis using the CLP rat model. In this regard, we have shown that AM and its binding protein AMBP-1 play an important role in the cardiovascular stability during the progression of polymicrobial sepsis, and significantly decrease mortality in the rat CLP model of sepsis (4-7).

AM was first isolated in 1993 from human pheochromocytoma cells by its ability to raise cyclic adenosine 3'-5'-monophosphate (cAMP) levels in platelets (19). One of the major activities of AM is in the maintenance of cardiovascular homeostasis through vasodilation. The biological activity of AM is mediated by its receptors. AM receptors have recently been characterized as two associated proteins, which are calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein 2 or 3 (RAMP2 or RAMP3). CRLR is the seven-transmembrane domain, G-protein-coupled portion of the receptors, and the adjoined RAMP is a single-transmembrane-domain protein involved in transporting CRLR to the cell surface and determining the ligand specificity of the receptor. CRLR with RAMP2 or RAMP3 makes AM-specific receptors (20-22). The hemodynamic effects of AM are predominantly mediated by cAMP production resulting from activation of CRLR and RAMP.

Intravenous administration of AM increases blood flow predominantly in the tissues with the highest AM expression (23). AM production has been reported in a variety of tissues such as the lungs, gut, vascular tissues (both endothelial cells and smooth muscle cells), heart, kidneys, and adrenal glands (4). However, we found that the small intestine is a major source of AM production in sepsis and LPS may increase AM production (8,24). Elevated levels of AM play a major role in producing the early hyperdynamic phase of sepsis (25,26). On the other hand, in the late phase of sepsis, there is a decreased vascular response to AM, which is probably caused by the reduction of AMBP-1 (6). In addition, administration of AM and AMBP-1 in combination significantly decreases sepsis-induced mortality (7). AMBP-1 is considered to play an important role in the binding between AM and its receptors, although the exact mechanism by which the AMBP-1 and AM complex augments AM activity remains to be clarified. Theoretically, AMBP-1 acts as a carrier and reservoir of AM, which could provide high local levels of AM to stimulate its receptors. In this way, AMBP-1 would increase the AM effectiveness without modifying the affinity for its receptors (2).

Although our previous studies have indicated that administration of AM and AMBP-1 in combination produces a significant beneficial effect at the late stage of polymicrobial sepsis (4-7), it remains unknown whether AMBP-1 expression is downregulated during the progression of sepsis. In this regard, we hypothesized that AMBP-1 gene and protein expression in the liver and plasma level of AMBP-1 are reduced in the late stage of sepsis. Both RT-PCR technique and Western blot analysis were utilized to test this hypothesis. Our results indicated that AMBP-1, in contrast to AM, has a different response to sepsis. AMBP-1 gene expression and protein levels in the liver decreased significantly at 20 h after CLP. Similarly, plasma levels of AMBP-1 were markedly reduced at 20 h after the onset of sepsis. The reduced plasma levels of AMBP-1 at 20 h after CLP is most likely due to the downregulated AMBP-1 expression in the liver, since the liver has been identified as the major source of circulating AMBP-1 (4,27). To determine whether the increased endotoxin in sepsis plays any important role in downregulating AMBP-1, the LPS neutralizing agent PMB was administered prior to as well as after the onset of sepsis. The results clearly indicate that inhibition of LPS by PMB prevented the downregulation of hepatic AMBP-1 gene expression as well as the reduction of hepatic and plasma AMBP-1 protein at 20 h after CLP. This strongly suggests that endotoxin is a major mediator responsible for AMBP-1 downregulation observed at the later stage of sepsis. To further confirm this notion, endotoxemia was produced by intraperitoneal administration of *E. coli* LPS. The results indicate that endotoxemia by itself may significantly downregulate hepatic gene and protein expression. To explore the role of LPS in downregulating AMBP-1 expression in sepsis, more studies are required to determine whether this downregulatory effect on AMBP-1 is due to the direct effect of LPS or LPS-induced proinflammatory cytokines such as TNF-α and IL-1ß.

In summary, our results indicate that AMBP-1 gene expression in the liver and its protein levels in the liver and plasma decreased significantly at the late stage of polymicrobial sepsis. Neutralization of endotoxin, however, prevented downregulation of AMBP-1 in sepsis. Moreover, endotoxemia resulted in significant reduction in the AMBP-1 gene and protein expression in the liver. Therefore it is concluded that downregulation of AMBP-1 observed in sepsis is due to the increased level of endotoxin. Further studies are directed to investigate the precise mechanism responsible for the downregulatory effect of endotoxin on AMBP-1 expression in sepsis.

Acknowledgements

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