The small intestine plays an important role in upregulating CGRP during sepsis

MIAN ZHOU, ANGELÉ J. ARTHUR, ZHENG F. BA, IRSHAD H. CHAUDRY, AND PING WANG

Department of Surgery, University of Alabama at Birmingham
School of Medicine, Birmingham, Alabama 35294

Received 13 January 2000; accepted in final form 8 September 2000

Zhou, Mian, Angelé J. Arthur, Zheng F. Ba, Irshad H. Chaudry, and Ping Wang. The small intestine plays an important role in upregulating CGRP during sepsis. Am J Physiol Regulatory Integrative Comp Physiol 280: R382–R388, 2001.—Although studies have indicated that calcitonin gene-related peptide (CGRP), a potent vasodilatory peptide, is upregulated after endotoxic shock, it remains controversial whether this peptide increases during sepsis and, if so, whether the gut is a significant source of CGRP under such conditions. To study this, polymicrobial sepsis was induced by cecal ligation and puncture (CLP) followed by fluid resuscitation. Plasma levels of CGRP were measured at 2, 5, and 10 h after CLP (i.e., early, hyperdynamic sepsis) and at 20 h after CLP (late, hypodynamic sepsis). The results indicate that plasma CGRP did not increase at 2–5 h but increased by 177% at 10 h after CLP (P < 0.05). At 20 h after the onset of sepsis, however, the elevated plasma CGRP returned to the sham level. To determine the source of the increased plasma CGRP, the liver, spleen, small intestine, lungs, and heart were harvested, and tissue CGRP was assayed at 10 h after CLP in additional animals. Only the small intestine showed a significant increase in tissue levels of CGRP (by 129%, P < 0.05). Determination of portal vs. systemic levels of CGRP indicates that portal CGRP was 65.7 ± 22.7% higher than the systemic level at 10 h after CLP, whereas portal CGRP in sham-operated rats was only 4.9 ± 2.1% higher. Immunohistochemistry examination revealed that CGRP-positive stainings increased in the intestinal tissue but not in the liver at 10 h after the onset of sepsis. The distribution of CGRP stainings was associated with intestinal nerve fibers. These results, taken together, demonstrate that upregulation of CGRP occurs transiently during the progression of sepsis (at the late phase of the hyperdynamic sepsis), and the gut appears to be a major source of such an increase in circulating levels of this peptide.

vasoactive peptides; gut; liver; immunohistochemistry; cecal ligation and puncture; hyperdynamic sepsis; calcitonin gene-related peptide

CALCITONIN GENE-RELATED PEPTIDE (CGRP) is a single-chain polypeptide containing 37 amino acids (6). It is a potent endogenous vasodilatory neuropeptide and a vasoregulatory agent (18). Joyce et al. (17) reported that circulating levels of CGRP increased significantly in patients with sepsis. The increased plasma CGRP was found to be associated with the development of septic shock in humans (3). Similarly, administration of endotoxin in experimental animals increased CGRP production (14), and this was associated with the increased CGRP gene expression in sensory nerves (22). Thus it appears that there is a close association between the increased circulating levels of CGRP and the pathophysiological changes of sepsis. However, a recent study by Snider et al. (21) indicated that, although serum CGRP usually increased in neuroendocrine cancer, it was very low or undetectable in the systemic inflammatory response syndrome (SIRS) or sepsis. The above results may suggest that upregulation of CGRP could be related to a specific phase of sepsis. The objective of this study, therefore, was to determine whether CGRP plays any significant role in mediating the hyperdynamic response, observed during the early stage of sepsis (27), by examining the time course of this peptide in a rat model of polymicrobial sepsis. Moreover, the tissue source of this peptide was examined by determining CGRP levels in various target organs, including liver, spleen, small intestine, lungs, and heart, during sepsis.

MATERIALS AND METHODS

Animal model of sepsis. Polymicrobial sepsis was induced in male Sprague-Dawley rats (275–325 g; Charles River Laboratory, Wilmington, MA) by cecal ligation and puncture (CLP) as described previously (7). The sepsis model of CLP has two distinct phases characteristic in its progression: an early, hyperdynamic stage characterized by increased cardiac output, tissue perfusion, and decreased vascular resistance, and a late, hypodynamic stage characterized by decreased cardiac output and tissue perfusion (27, 30, 33). For the CLP procedure, rats were fasted overnight before the initiation of sepsis but were allowed water ad libitum. The animals were anesthetized with methoxyflurane inhalation, and a 2-cm midline incision was made. The cecum was then exposed, ligated just distally to the ileocecal valve to avoid any intestinal obstruction, punctured two times with an 18-gauge needle, and returned to the abdominal cavity. The abdominal incision was then closed in layers, and the animals received 3 ml/100 g body wt normal saline subcutaneously immediately after CLP to provide fluid resuscitation. In the sepsis model of CLP, 2–10 h after CLP represents the...
early, hyperdynamic stage of sepsis, and 20 h after CLP represents the late, hypodynamic stage of sepsis (27). Sham-operated animals underwent the same surgical procedure except that the cecum was neither ligated nor punctured. The animals were then divided into eight groups with five to seven animals in each group. Four groups of CLP animals and four groups of sham-operated animals were studied 2, 5, 10, and 20 h after CLP or sham operation, respectively. Blood samples were collected by the cardiac puncture at 2, 5, 10, and 20 h after CLP or sham operations. Tissue samples (i.e., liver, spleen, small intestine, lungs, and heart) were harvested immediately after blood sampling at 10 h after CLP or sham operation. The experiments described in this study were performed in adherence to the National Institutes of Health guidelines for use of experimental animals. This project was approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital (Providence, RI).

**Plasma CGRP determination.** At 2, 5, 10, or 20 h after CLP or sham operation, systemic blood samples (2–3 ml each) were collected in heparinized syringes by cardiac puncture and then transferred to polystyrene tubes containing EDTA (1 mg/ml) and aprotonin (0.56 trypsin inhibitor units/ml). In additional groups of animals, portal and systemic blood samples were collected simultaneously at 10 h after the onset of sepsis to determine whether the gut plays an important role in producing CGRP during sepsis. The plasma was separated immediately by centrifugation at 2,800 rpm for 15 min at 4°C and was stored at −70°C until assayed. Plasma levels of CGRP were quantified by using an RIA kit specific for rat CGRP according to the procedure provided by Peninsula Laboratories (Belmont, CA). Briefly, CGRP was extracted from 0.5 ml plasma on C18 columns eluted with 60% acetonitrile in 1% trifluoroacetic acid. The eluate was evaporated to dryness using a centrifugal concentrator. Samples were dissolved in RIA buffer (supplied with the assay kit) and then were incubated with the antibody against rat CGRP at 4°C for 20 h. 125I-labeled CGRP was then added and further incubated for 4 h at 4°C. Free and bound fractions of 125I-CGRP were separated by addition of a secondary antibody and centrifugation. Radioactivity of the pellet was then determined with a gamma counter. Assays were performed in duplicate. The sensitivity (IC50) of the RIA assay was 5 pg/tube (11 pM). Plasma levels of CGRP, even in sham-operated animals, were detectable by the RIA assay. The cross-reactivity of the RIA assay is as follows: 100% with rat CGRP, 14% with human CGRP, 23% with rat CGRP II, 13% with human CGRP II, and no cross-reactivity with human amylin and rat amylin amide and calcitonin. Because the primary aim of this study was to determine whether or not CGRP production increases during the course of polymicrobial sepsis, the values of plasma or tissue CGRP were not adjusted by CGRP’s recovery rate.

**Tissue CGRP determination.** Tissue samples (~0.5 g each) harvested at 10 h after CLP or sham operation were homogenized with 2 ml Tris·HCl (50 mM, pH 7.4) and centrifuged at 16,000 g for 10 min. The supernatant was collected and stored at −70°C until assayed. Tissue levels of CGRP were measured by using an RIA kit specific for rat CGRP from Peninsula Laboratories as described above. The levels of CGRP were expressed as picograms per gram wet tissue weight. To determine the recovery rate of CGRP, a spiked CGRP (128 pg/g sample) was added to hepatic samples (n = 4). The recovery rate of CGRP was found to be 58.3 ± 4.7% in hepatic tissues. Because the recovery rate of CGRP in other tissues and plasma samples was not measured routinely, the recovery rate was not used for the calculation of our data.

Therefore, the CGRP values reported in this study should not be considered as absolute values.

**Immunohistochemistry examination.** A portion of the small intestine (jejunum) and the liver was collected from additional groups of animals immediately after death by an overdose of pentobarbital sodium at 10 h after CLP or sham operation (3 rats/group). The tissue was fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and was washed with 0.1 M phosphate buffer (pH 7.4). The tissue was then cut on a Busan cryostat for 20 min at room temperature, followed by a reaction with an avidin-biotin-peroxidase complex (Vector Laboratories) for 1.5 h at room temperature. The reaction products were revealed by placing the tissue in 3,3′-diaminobenzidine solution (34). The sections were mounted on slides for light microscopy evaluation. For negative control, nonimmunized rabbit serum and TBS were substituted for the primary antibody.

**Statistical analysis.** Data are expressed as means ± SE. Plasma levels of CGRP at 2–20 h after CLP were compared by one-way ANOVA and Tukey’s test. Tissue levels of CGRP and the increase in portal levels of this peptide were compared by unpaired Student’s t-test. The differences were considered significant at a P value < 0.05.

**RESULTS**

**Alterations in plasma levels of CGRP.** As shown in Fig. 1, circulating levels of CGRP in septic animals were not different from the levels in sham-operated animals at 2 and 5 h after CLP. In contrast, plasma CGRP increased by 177% (P < 0.05) at 10 h after the onset of sepsis compared with the respective sham group. At 20 h after CLP, however, the plasma CGRP levels were similar to those of sham-operated animals.
Alterations in tissue levels of CGRP. Tissue levels of CGRP were determined at 10 h after CLP since plasma levels of the peptide did not increase at other time points studied. At 10 h after the onset of sepsis, hepatic and splenic levels of CGRP did not change compared with sham-operated animals (Fig. 1). In contrast, CGRP levels in the small intestine increased by 129% (P < 0.05). Although tissue levels of CGRP were elevated in the lungs and heart, the increase did not reach the statistically significant level (Fig. 2). Thus the gut appears to be a major CGRP-producing organ. To determine whether tissue levels of CGRP increase earlier than 10 h after the onset of sepsis, intestinal and cardiac samples were collected at 5 h after CLP or sham operation (n = 7/group). The results indicate that intestinal levels of CGRP were 863 ± 171 pg/g tissue in sham-operated animals and 634 ± 101 pg/g tissue in septic animals. There is no evidence indicating that intestinal CGRP levels increase at 5 h after CLP. Similarly, cardiac levels of CGRP were not altered at 5 h after the onset of sepsis (71 ± 35 pg/g in sham vs. 63 ± 22 pg/g in sepsis). Thus the upregulated CGRP in the small intestine at 10 h after CLP appears to be a transient phenomenon.

CGRP immunohistochemistry in the gut and liver. In sham-operated animals, CGRP immunostainings were observed in the mucosa and submucosa (Fig. 3A). The intestinal smooth muscle layer is also partially stained. The CGRP immunostainings were markedly increased at 10 h after the onset of sepsis (Fig. 3B). The mucosa and submucosa (primarily in nerve fibers and connective tissues) were densely stained (Fig. 3B). However, CGRP positive stainings were distributed in other locations such as the muscularis. In the negative control section (i.e., substitution of the primary antibody with a nonimmunized rabbit serum), CGRP immunoreaction was not observed (Fig. 3C). Although hepatic CGRP immunostainings could be observed around the portal triad, there was no apparent increase in CGRP stainings at 10 h after CLP (Fig. 3E) compared with sham-operated animals (Fig. 3D). It appears that CGRP immunostainings in the triad region were reduced at 10 h after CLP (Fig. 3E). However, the pathophysiological consequences of such apparent decrease in CGRP remains to be determined. The CGRP immunoreaction was also not observed in the hepatic tissue in the negative control section (Fig. 3F).

Differences in portal and systemic levels of CGRP. In additional groups of animals, the difference of CGRP levels in portal and systemic blood was determined. The results indicate that the percent increase in the portal level vs. the systemic level of CGRP was 4.9 ± 2.1 in sham-operated animals (n = 5). At 10 h after the onset of sepsis, however, portal levels of CGRP were 65.6 ± 22.7% higher than those in systemic blood (P < 0.05, n = 8; Table 1). Thus the percent increase in portal CGRP was significantly greater in septic than in sham-operated animals. It is most likely that the lower systemic levels of CGRP at 10 h after the onset of sepsis are due to the combination of the increased production by the gut and the enhanced clearance of this peptide by the liver.

DISCUSSION

CGRP was discovered in 1982 during alternative splicing of the primary calcitonin mRNA (4). Two components were generated from the gene CGRP (1, 20). CGRP is expressed predominantly in the nerve system (synthesized and released from small, capsaicin-sensitive sensory nerves), whereas calcitonin is expressed in the thyroid gland. The potent vasoactive peptide CGRP interacts with specific G protein-coupled receptors. The nerves responsible for CGRP production are found throughout the body (11, 19, 23). Several studies have indicated that circulating levels of CGRP increase during sepsis (3, 14, 17). Because of the close relationship between cardiovascular responses and upregulation of CGRP during the progression of sepsis, it has been postulated that CGRP may play a major role in the pathophysiology of sepsis. However, other studies have shown that plasma levels of CGRP did not increase or even were not detectable in patients with sepsis or SIRS (21). In view of these controversial findings, we hypothesized that, unlike endotoxemia or endotoxic shock, upregulation of CGRP during sepsis occurs only at a certain stage of sepsis. Moreover, organs rich in nerve fibers, such as the gut, may contribute to the increased levels of circulating CGRP after the onset of sepsis. Therefore, the present study was conducted to examine the temporal profile of CGRP using the CLP model of polymicrobial sepsis in the rat. In addition, tissue levels of CGRP were assayed to determine the major source of this peptide during sepsis.

Our results indicate that circulating levels of CGRP did not increase at 2 and 5 h after the onset of sepsis. However, this peptide increased by 177% at 10 h after CLP. Plasma CGRP returned to sham levels at 20 h after CLP. Our previous studies have demonstrated that the early, hyperdynamic stage of sepsis occurs...
between 2 and 10 h, and the late, hypodynamic stage occurs at 20 h after CLP (24, 27). Therefore, upregulation of CGRP is observed only at the late phase of the hyperdynamic stage of sepsis. Because the hypercardiovascular responses occur as early as 2 h after the onset of sepsis (25, 35), and upregulation of CGRP does not occur until 10 h after CLP, it suggests that this peptide does not appear to play a major role in initiating the hypercardiovascular response during sepsis. Moreover, because the increased levels of plasma CGRP returned to sham levels at 20 h after CLP, the role of CGRP during late sepsis and septic shock appears to be less important than previously reported.

Studies by Gardiner et al. (13) indicate that prolonged infusions of human α-CGRP (1.5 nmol·kg⁻¹·h⁻¹) in the rat produced sustained tachycardia and hypotension, a sustained reduction in renal flow, a transient reduction in mesenteric flow, and a relatively well-maintained hindquarter flow at the first day of infusion. However, by the second day of infusion and thereafter, cardiovascular parameters in the animals receiving vehicle and those receiving human α-CGRP were not different (13). In a separate report, the above authors show that human α-CGRP at a dose of 0.6 nmol/h caused substantial reduction in internal carotid vascular resistance in the rat (12). Similar to the ef-
fects of CGRP on normal animals, Huttemeier et al. (16) postulated that CGRP may mediate hypotension in endotoxic rats. In contrast, studies by Fox et al. (10) indicate that administration of a CGRP receptor antagonist, CGRP-(8-37), in a rat model of acute Pseudomonas pneumonia did not alter baseline hemodynamic variables and changes in pressor responses to hypoxia. Moreover, CGRP receptor blockade did not alter the distribution of blood flow in the lungs during normoxia or hypoxia (10). Their data suggest that, although the model of acute pneumonia is characterized by an attenuated hypoxic pressor response, the mechanism does not appear to be mediated by excessive release of the vasodilator CGRP (10). Our previous studies have indicated that mean arterial pressure was not altered at 10 h after CLP (24). The increased plasma levels of CGRP without hypotension under such conditions appear to be due to two reasons. First, the elevated levels of CGRP observed at 10 h after CLP are not high enough to induce hypotension. Second, the vascular responsiveness is reduced after the onset of sepsis.

The fact that plasma levels of CGRP increased only at 10 h after the onset of sepsis suggests that mediators other than this peptide may be responsible for producing the hyperdynamic response observed as early as 2 h after CLP (25, 27). In this regard, our recent studies have indicated that circulating levels of adrenomedullin (ADM, a potent vasodilatory peptide) increased significantly during sepsis (29). In addition, administration of synthetic rat ADM at a dose of 8.5 μg/kg increased cardiac output, stroke volume, and microvascular blood flow in various organs and decreased total peripheral resistance (26). Furthermore, administration of anti-ADM antibodies prevented the occurrence of the hyperdynamic response observed during the early stage of sepsis (26). These findings, taken together, suggest that ADM plays an important role in producing the hyperdynamic circulation after the onset of sepsis. It should also be pointed out that, despite the elevation of circulating ADM levels (29) during the late stage of sepsis, the hypodynamic response (i.e., reduced cardiac output and tissue perfusion) occurs under such conditions (27, 33). This appears to be due to the fact that the vascular responsiveness to ADM stimulation decreases significantly during the late stage of polymicrobial sepsis (28).

Tissue levels of CGRP were assayed at 10 h after the onset of sepsis. Our results indicate that, among the tissues tested (i.e., the liver, spleen, small intestine, lungs, and heart), only the small intestine showed a significant increase in CGRP levels under such conditions. Immunohistochemistry examination revealed that CGRP-positive immunostainings increased markedly at 10 h after CLP in the intestinal tissue but not in the hepatic tissue. Intestinal levels of CGRP were more than doubled, and portal levels of CGRP were 65% higher than that in systemic blood at 10 h after CLP. Thus the gut appears to be a major source of such an increase in circulating levels of this peptide. However, this does not exclude the possibility that tissues such as the kidneys, skeletal muscle, and blood vessels may also play a role in increasing plasma levels of CGRP at 10 h after the onset of sepsis. The possible cause for the increased CGRP in the small intestine is twofold: increased production of gut-derived CGRP and increased CGRP receptor-binding capacities. To differentiate these possibilities, blood samples were taken simultaneously from the portal vein and a systemic source for measuring plasma levels of CGRP. The results indicate that, unlike sham-operated animals (in which portal CGRP was similar to the systemic level), portal CGRP levels were 65% higher than those in systemic blood at 10 h after CLP, suggesting that the increased CGRP levels in the small intestine were primarily due to increased production of this peptide under such conditions. Although the nerve fibers (which were densely stained in the gut) are the proved source of CGRP, it is unlikely that connective tissues in the submucosa are the major source for CGRP synthesis. Because we used specific anti-rat CGRP polyclonal antibodies, it is possible that the positive stainings represent both CGRP synthesized locally and bound CGRP. Despite the fact that the gut is a major source of CGRP production during sepsis, it appears that CGRP-binding capacities also increase in intestinal tissues, resulting in increased immunostainings in connective tissues of the submucosa. Thus the gut appears to be responsible for the increased levels of circulating CGRP at 10 h after CLP. This notion is supported by the observations of Wang et al. (32), who reported that tissue levels of CGRP increased significantly in the duodenum, but not in the kidneys, heart, or adrenal gland, at 0.5 and 3 h after the administration of endotoxin in a rat model.

Because CGRP is synthesized and released from small, capsaicin-sensitive sensory nerves and because the extensive network of sensory nerves can be found in virtually all organs (9), it has been suggested that a variety of tissues produce and release this peptide. Although this may be true under normal conditions, our results indicate that the gut becomes an important CGRP-producing organ during sepsis. In this regard, studies have shown that CGRP is distributed extensively in neural tissue of the gut (5). It has been indicated that splanchnic organs may be the source of the elevated plasma CGRP levels after endotoxiaemia (14). In contrast, Arden et al. (2) reported that there is little evidence that the portal circulation is a major source of circulating CGRP levels during endotoxic shock in a pig model. Despite their conclusion that the contribution of the intestinal CGRP is insignificant during endotoxic shock, careful examination of the study revealed that portal levels of CGRP were indeed higher than those in carotid arterial blood at the 210 min after endotoxin administration (2). Thus it appears that intestinal CGRP increases also at a certain time point during endotoxic shock.

With regard to the involvement of prostaglandins in CGRP production, Wang et al. (31) reported that inhibition of cyclooxygenase activities, but not inhibition of thromboxane biosynthesis, significantly decreased en-
dotoxin-induced CGRP elevation. Because we have previously shown that circulating levels of PGE₂ increased at 5–10 h but not at 2 and 20 h after CLP (8), it is possible that the upregulated prostaglandins during sepsis may play a partial role in producing the elevation of circulating levels of CGRP observed in the present study.

In summary, our results indicate that plasma levels of CGRP did not increase at 2–5 h but increased significantly at 10 h after CLP. At 20 h after the onset of sepsis, however, the elevated plasma CGRP returned to the sham level. Thus upregulation of CGRP occurs transiently during the progression of sepsis. In addition, a significant increase in tissue levels of CGRP occurs at 10 h after CLP in the small intestine but not in the liver, spleen, small intestine, lungs, or heart. Similarly, immunohistochemistry examination revealed that CGRP-positive stainings increased in the intestinal tissue but not in the liver at 10 h after the onset of sepsis. Furthermore, portal levels of CGRP were significantly higher than systemic levels. These findings, taken together, suggest that upregulation of CGRP occurs transiently during the progression of sepsis (at the late phase of the hyperdynamic sepsis), and the gut may be a major source of the increased levels of circulating CGRP during sepsis.

**Perspectives**

The incidence of sepsis and septic shock has increased significantly over the past two decades despite advances in the resuscitation of trauma victims. Indeed, sepsis, septic shock, and multiple organ failure continue to be the most common causes of death in surgical intensive care units in the United States. Although the cardiovascular response to polymicrobial sepsis is typically characterized by an early, hyperdynamic phase followed by a late, hypodynamic phase, few studies have been conducted to examine the factors responsible for initiating the hyperdynamic response after the onset of sepsis. Moreover, the mechanisms responsible for the transition from the hyperdynamic to hypodynamic stage of sepsis are not fully understood. The lack of recognition and prevention of such a transition may lead to a progressive deterioration of cell and organ function. The present study was conducted to determine whether or not CGRP, a potent vasodilatory peptide, plays any role in initiating the hyperdynamic response during the early stage of sepsis. Our results indicate that upregulation of CGRP occurs only transiently at the late stage of hyperdynamic sepsis (i.e., 10 h after CLP) and that the small intestine appears to be a major source of such an increase in CGRP under such conditions. The finding that plasma levels of CGRP increased only at 10 h after the onset of sepsis suggests that mediators other than this peptide may be responsible for producing the hyperdynamic response observed as early as 2 h after CLP. In this regard, our recent studies have indicated that circulating levels of ADM (a newly reported vasodilatory peptide) increased very early after the onset of sepsis. In addition, administration of synthetic ADM produced hypercardiovascular responses, and anti-ADM antibodies prevented the occurrence of the hyperdynamic response observed during the early stage of sepsis. These findings, taken together, suggest that ADM plays an important role in producing the hyperdynamic circulation after the onset of sepsis. With advances in our understanding of the pathophysiology of sepsis, future studies and developments will lead to improved management of the septic patient and decreased morbidity and mortality.

We thank David Ornan for excellent assistance in preparing the revised version of this manuscript. This investigation was supported by National Institutes of Health (NIH) Grant RO1 GM-57468 and R29 GM-53008 (to P. Wang). P. Wang is the recipient of NIH Independent Scientist Award K02 AI-01461. A major portion of this work was performed while the authors were at Brown University School of Medicine and Rhode Island Hospital.


**REFERENCES**


13. Gardiner SM, Compton AM, Kemp PA, Bennett T, Foulkes R, and Hughes B. Regional haemodynamic effects of prolonged...