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CALCITONIN GENE-RELATED PEPTIDE INHIBITS LOCAL ACUTE INFLAMMATION AND PROTECTS MICE AGAINST LETHAL ENDOTOXEMIA

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ABSTRACT—Calcitonin gene-related peptide (CGRP), a potent vasodilatory peptide present in central and peripheral neurons, is released at inflammatory sites and inhibits several macrophage, dendritic cell, and lymphocyte functions. In the present study, we investigated the role of CGRP in models of local and systemic acute inflammation and on macrophage activation induced by lipopolysaccharide (LPS). Intraperitoneal pretreatment with synthetic CGRP reduces in approximately 50% the number of neutrophils in the blood and into the peritoneal cavity 4 h after LPS injection. CGRP failed to inhibit neutrophil recruitment induced by the direct chemoattractant platelet-activating factor, whereas it significantly inhibited LPS-induced KC generation, suggesting that the effect of CGRP on neutrophil recruitment is indirect, acting on chemokine production by resident cells. Pretreatment of mice with 1 μg of CGRP protects against a lethal dose of LPS. The CGRP-induced protection is receptor mediated because it is completely reverted by the CGRP receptor antagonist, CGRP 8-37. The protective effect of CGRP correlates with an inhibition of TNF-α and an induction of IL-6 and IL-10 in mice sera 90 min after LPS challenge. Finally, CGRP significantly inhibits LPS-induced TNF-α released from mouse peritoneal macrophages. These results suggest that activation of the CGRP receptor on macrophages during acute inflammation could be part of the negative feedback mechanism controlling the extension of acute inflammatory responses.

KEYWORDS—LPS, CGRP, cytokines, inflammation, endotoxemia

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide, localized in central and peripheral nerve system neurons (1, 2). CGRP, a potent microvascular vasodilator, is found in sensory unmyelinated C fibers and perivascular fibers terminations in virtually all vascular beds, suggesting a role for this peptide in local and systemic regulation of the blood flow (3). Receptors for CGRP are present in several cell types, including those from the immune system (4, 5). Negative regulatory effects of CGRP on the immune system were observed and include the inhibition of T cell proliferation by mitogen stimulation (6, 7), inhibition of IFN-γ-induced superoxide production by macrophages (8), inhibition of antigen presentation by macrophage and Langerhans cells (9, 10), and the increase of *Leishmania major* infection in mice (11).

Macrophages play a critical role in the response to a variety of pathogens and in inflammatory responses, and participates in several aspects of the body's homeostasis. The systemic inflammatory syndrome, triggered by several agents including gram-negative bacterial infections or by their cell wall component lipopolysaccharide (LPS), is characterized by an overproduction of proinflammatory cytokines by activated macrophages. TNF-α is able to reproduce the *in vivo* response to LPS, and its neutralization reduces lethality in experimental models (12). The events downstream of macrophage activation by LPS or TNF-α include neutrophil activation and extravasation, coagulopathy, increase of vascular permeability, shock, multiorgan failure, and eventually death. The role of vasoactive peptides, including neuropeptides, in the systemic inflammatory syndrome is still largely unknown. Some studies suggest a deleterious effect of vasoactive peptides in models of endotoxemia, including substance P (13), bradykinin (14), and endothelin (15). Furthermore, the disruption of the neutral endopeptidase gene by homologous recombination in mice induces an increase in the susceptibility to LPS (16). Neutral endopeptidase is a methaloprotease involved in the cleavage of bioactive peptides such as takikinins, enkefalins, bombesin-like peptides, endothelin, and atriopeptin. In contrast, some vasoactive peptides might have anti-inflammatory properties during endotoxemia. Indeed, we have previously shown that ligands of the pituitary adenylate cyclase-activating polypeptide type I receptor, such as pituitary adenylate cyclase-activating polypeptide 38 and the sand fly saliva peptide maxadilan, inhibit TNF-α production from macrophages and protect mice against lethal endotoxemia (17, 18).

Because CGRP has several inhibitory effects on macrophage activation, in the present study, we analyzed the effects of this neuropeptide on macrophage responses after LPS treatment *in vivo* and *in vitro*. Here, we show that CGRP inhibits local and systemic acute inflammation and reduces LPS-induced TNF-α production by mouse peritoneal macrophages.

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MATERIALS AND METHODS

Animals

Six to 8-week-old BALB-c mice were purchased from Charles River Laboratories (Boston, MA), and were housed at the Department of Public Health, Harvard School of Public Health animal facility. Eight-week-old CBA mice were raised and housed at the Fundação Oswaldo Cruz animal facility. Protocols were approved by the Harvard School of Public Health and the Oswaldo Cruz Institute's Animal Welfare Committee.

Reagents

LPS from *Escherichia coli* serotype 0111: B4, thioglycollate, platelet-activating factor (PAF), synthetic rat CGRP, and synthetic human receptor antagonist CGRP 8-37 were purchased from Sigma Chemical Co. (St. Louis, MO). All the reagents used contained <10 pg/mg endotoxin, measured by Limulus assay from BioWhittaker (Walkersville, MD).

Murine models of endotoxemia and treatments

Two *in vivo* models of endotoxemia were performed in mice, one using a low dose of LPS (0.25 μg) to analyze leukocyte recruitment, and the other using a high dose of LPS (500 μg) to study survival and plasma cytokine levels upon treatment with CGRP and/or CGRP receptor antagonist. Mice were injected i.p. with 200 μL of LPS*g* diluted in sterile saline. Control animals received equal volumes of saline. Groups of mice were pretreated with an i.p injection of indicated amounts of CGRP (1.0, 1×10^{-2}, 1×10^{-4}, and 1×10^{-6} μg/cavity) or CGRP 8-37 (2.0 μg/cavity) dissolved in 200 μL of phosphate-buffered saline (PBS) or PBS control. After 30 min, mice were injected with 0.25 μg of LPS for cell counting determination. The dose of *g*500 μg was consistently the LD 90-100 for the two batches of LPS used and the two mice strains (data not shown). All mice challenged with 500 μg of LPS appeared acutely ill and displayed lethargy, piloerection, shivering, and diarrhea. Survival was assessed every day for a total of 6 days. In endotoxic shock experiments, mice were pretreated with 1 μg of synthetic CGRP or CGRP 8-37 (2 μg/cavity) dissolved in 200 μL of PBS or with the same volume of PBS in the control groups, and after 2 hours, 500 μg of LPS was also injected i.p.

Cell counting

At 4 h of LPS (0.25 μg) or PAF (1 μg) injection, animals were sacrificed in a CO_2 gas chamber, blood was collected by cardiac puncture, and the peritoneal cavity washed with 1 mL of PBS. The peritoneal wash was recovered and the volume was measured in a graduated syringe. Peritoneal wash and blood aliquots were collected and diluted in Turk solution (2% acetic acid) for total leukocyte count in Neubauer chambers. Neutrophil numbers were determined by differential cell counts from cytospin slide preparations stained by May-Grunwald-Giemsa method.

Cytokine measurements

Plasma samples and peritoneal fluid from experimental animals were obtained at 90 min after LPS (500 μg/cavity) administration. Blood was collected by cardiac puncture after CO_2 euthanasia in tubes containing 50 μL of 3.2% sodium citrate. The plasma fraction was separated from the cellular components by centrifugation at 800*g* for 10 min and was stored at −70°C. Peritoneal fluid was obtained after washing the peritoneal cavity with 3 mL of PBS and was centrifuged at 2100 rpm for 10 min at 10°C before storage at −70°C. TNF-α, IL-6, macrophage inflammatory protein (MIP)-1α, KC, and IL-10 levels were measured by enzyme-linked immunoabsorbant assay (ELISA) using DuoSet kit from R&D Systems (Minneapolis, MN).

Macrophage cultures

To obtain peritoneal macrophages, mice were injected with 2 mL of sterile 3% thioglycollate in saline i.p., and after 4 days, peritoneal lavage was performed using 10 mL of cold Hanks' balanced salt solution. After two washes with Hanks' balanced salt solution, the cells were resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and plated in 24-well tissue culture plates at 2×10^6 per well in 1 mL. After 2 h of incubation at 37°C, nonadherent cells were removed by two washes with culture medium. This adherent population is 95% Mac-1^+ cells. Macrophages were then treated as described in the text or figure legends, and cell-free supernatants were frozen for subsequent cytokine measurement as described above.

Statistical analysis

Data were analyzed for significance using a nonpaired Student *t* test. Log-rank tests were used for comparisons of mortality rates. All data are expressed as mean ± SEM. Data with $P < 0.05$ were considered significant.

RESULTS

CGRP inhibits blood neutrophilia and neutrophil recruitment into the peritoneal cavity in a model of local acute inflammation induced by LPS

Injection of LPS into the peritoneal cavity of rodents induces an acute response characterized by blood neutrophilia and local neutrophil accumulation (19). In this model, resident macrophages release inflammatory mediators responsible for the mobilization of neutrophils from the bone marrow and their recruitment to the site of inflammation (20, 21). Intraperitoneal treatment with different doses of synthetic CGRP (1, 1×10^{-2}, 1×10^{-4}, and 1×10^{-6} μg/mL) 30 min before LPS injection caused a dose-dependent and statistically significant inhibition of neutrophil accumulation into the peritoneal cavity at 4 h (Fig. 1). As shown in Figure 2A, the receptor antagonist CGRP 8-37 (2 μg/cavity), completely prevented the CGRP-induced inhibition of LPS-induced neutrophil accumulation into the peritoneal cavity, although it had no effect on its own. This result indicates that CGRP inhibits neutrophil migration induced by LPS through activation of the CGRP receptor. Similarly, pretreatment with CGRP (1 μg/cavity) caused an inhibition of neutrophil accumulation to the peritoneal cavity after 4 h of thioglycollate instillation (data not shown). Interestingly, CGRP treatment significantly inhibited the increase in KC levels observed 90 min after LPS injection (Fig. 2B) and showed a trend toward inhibition of MIP-1α, although this result did not reach statistical significance (from 189.85 ± 93.02 pg/mL in LPS-injected animals to 120.40 ± 72.64 pg/mL in CGRP-treated animals). Treatment with CGRP (1 μg/cavity) also inhibited LPS-induced blood neutrophilia (Fig. 2C). Of note, at this dose, CGRP 8-37 fail to inhibit blood neutrophilia induced by LPS.

LPS induces PAF production *in vivo* and *in vitro*, and this lipid is an important trigger of inflammation and injury. Systemic administration of PAF to animals causes shock and bowel injury, and the effects of PAF and LPS are synergistic (22). Most of the adverse effects of PAF *in vivo* are dependent on the activation of polymorphonuclear neutrophils. Moreover, LPS-induced neutrophil recruitment depends on PAF (23). Treatment with CGRP does not inhibit PAF-induced peritoneal neutrophil accumulation

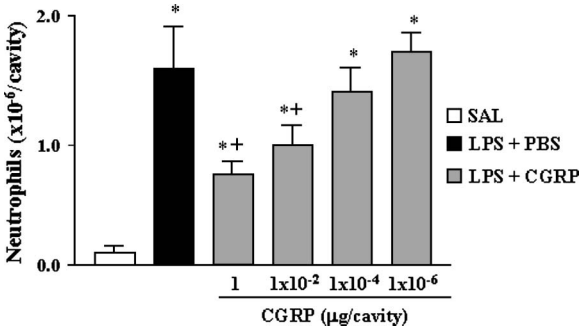

FIG. 1. **CGRP dose dependently inhibits the neutrophil accumulation induced by LPS.** Mice were pretreated with CGRP (1, 1×10^{-2}, 1×10^{-4}, 1×10^{-6} μg/cavity) and 30 min after were injected i.p. with 250 ng/cavity LPS. After 4 h, the peritoneal lavage fluid was obtained and used for total and differential cell count. A group of animals receiving an i.p. injection of saline (SAL) was used as control. Results are expressed as mean ± SEM from at least five animals. An asterisk denotes statistically significant differences when compared with control, and + indicates statistically different from LPS + vehicle group (LPS + PBS).

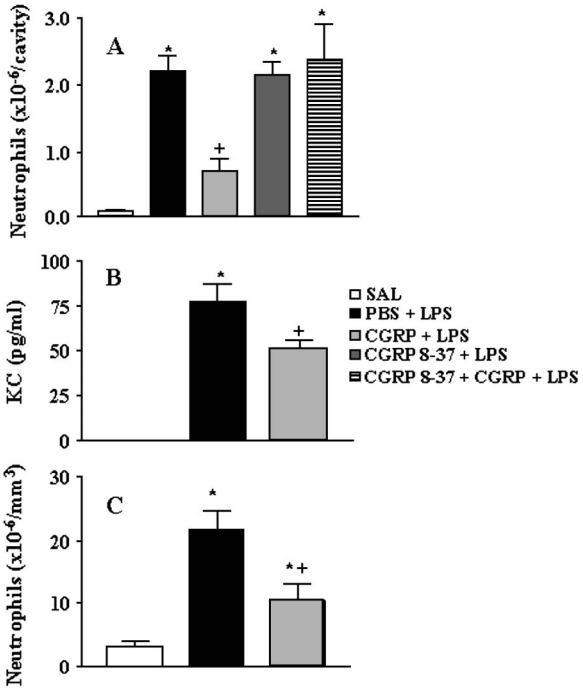

FIG. 2. **(A) CGRP receptor antagonist reverts the inhibitory effect of CGRP on LPS-induced neutrophil accumulation into the peritoneal cavity.** Mice were pretreated with CGRP (1 μg/cavity) or with CGRP and CGRP 8-37 (2 μg/cavity) and 30 min after were injected i.p. with 250 ng/cavity LPS. After 4 h, the peritoneal lavage fluid was obtained and used for total and differential cell count. A group of animals receiving an i.p. injection of saline (SAL) was used as control. Results are expressed as mean ± SEM from at least five animals. (B) CGRP treatment inhibited the increase in KC observed in the peritoneal fluid 90 min after LPS injection. Results are expressed as mean ± SEM from seven animals. (C) CGRP inhibits blood neutrophilia induced by LPS. A blood sample was obtained from animals receiving saline, LPS, or CGRP (1 μg/cavity) + LPS 4 h after challenge and total and differential leukocyte counts were performed as described in "Materials and Methods." Results are expressed as mean ± SEM from at least five animals. An asterisk denotes statistically significant differences when compared with control, and +) indicates statistically different from LPS + vehicle group (LPS + SAL).

(Fig. 3). This result indicated that CGRP inhibition of LPS-induced neutrophil accumulation is probably independent of any direct interference with the ability of PAF to induce neutrophil influx.

CGRP protects mice from lethal endotoxemia through activation of its receptor

Lethal endotoxemia was induced in groups of 10 mice by an i.p. injection of LPS (500 μg/mouse). Pretreatment with CGRP (1 μg/mouse) significantly blocked LPS-induced death (Fig. 4). CGRP was effective in protecting mice when the animals were treated between 6 h and 30 min before the lethal challenge, however, no protection was observed when mice received simultaneous treatment with CGRP and LPS, or when CGRP was used after LPS challenge (data not shown). CGRP-treated animals still exhibited signs of endotoxemia after LPS treatment, including piloerection, shivering, and lethargy, even though these signs were milder compared with the LPS-treated controls. To characterize whether the protective effect of CGRP was receptor mediated, animals were treated with CGRP in the presence or absence of the CGRP competing receptor antagonist, CGRP 8-37 (2 μg). Cotreatment with CGRP 8-37 and CGRP 2 h before LPS injection prevented the CGRP-induced protection to a lethal dose of LPS (Fig. 4).

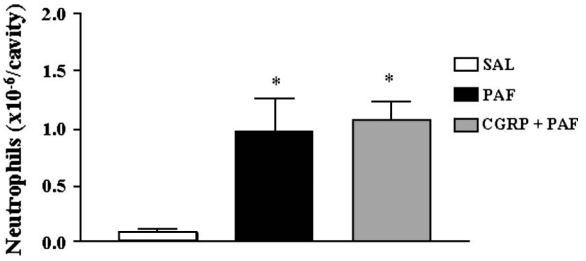

FIG. 3. **CGRP fails to inhibit PAF-induced neutrophil accumulation.** Mice were injected with PAF (1 μg/cavity) or CGRP (1 μg/cavity) + PAF and 4 h later, the peritoneal lavage fluid was obtained and used for total and differential cell count. A group of animals receiving an i.p. injection of saline (SAL) was used as control. Results are expressed as mean ± SEM from at least five animals. An asterisk denotes statistically significant differences when compared with control.

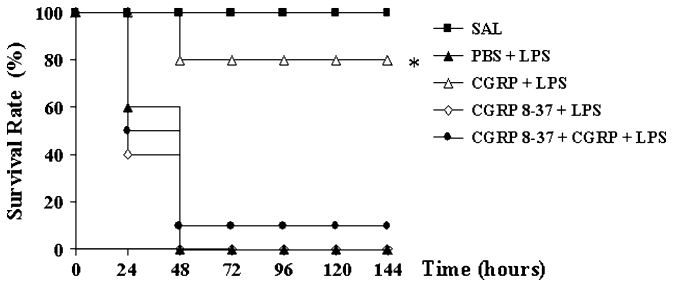

FIG. 4. **CGRP reduces mortality in endotoxic shock through interaction with its receptor.** Groups of 10 animals were injected intraperitoneally with LPS (500 μg/cavity), LPS + CGRP (1 μg/cavity), or CGRP 8-37 (2 μg/cavity) + CGRP + LPS. A group of animals receiving an i.p. injection of saline (SAL) was used as control. Mortality was recorded for 6 consecutive days. An asterisk denotes statistically significant differences (log-rank test) when compared with control (LPS + PBS).

CGRP inhibits TNF-α serum levels and enhances IL-6 and IL-10 levels

Previous studies (23, 24) suggest that the protective effect of CGRP could be related to an inhibition of proinflammatory cytokines or an induction of cytokines with anti-inflammatory properties. Because TNF-α is an essential mediator in the pathophysiology of the endotoxic shock, we wished to determine the effect of CGRP on serum TNF-α levels after LPS challenge. Pretreatment with CGRP reduced by 70% the LPS-induced increase in serum levels of TNF-α compared with the PBS-treated controls (Fig. 5A). IL-6 is considered to be an important marker for the inflammatory response, and a correlation between serum IL-6 levels and outcome in clinical as well as in experimental sepsis was observed (25). Interestingly, pretreatment with CGRP causes a 3-fold increase in IL-6 serum levels 90 min after LPS challenge compared with the control (Fig. 5B). IL-10 is a potent anti-inflammatory cytokine that inhibits LPS-induced TNF-α release *in vitro* and *in vivo*, protecting experimental animals against lethal endotoxemia (26). Analysis of IL-10 serum levels 90 min after LPS challenge revealed a marked increase in the CGRP-treated group compared with the PBS-treated control group (Fig. 5C).

CGRP inhibits LPS-induced TNF-αg production by macrophages

Because CGRP affects several macrophage functions and modulates the concentrations of TNF-α, IL-6 and IL-10 in the

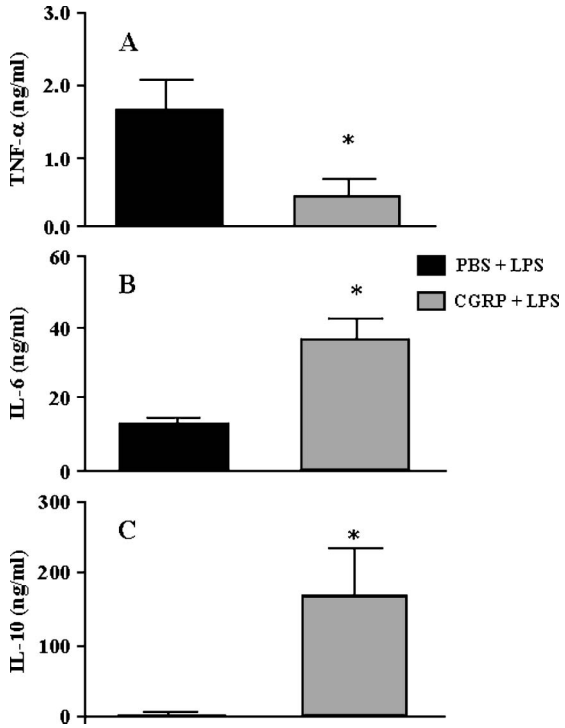

FIG. 5. **CGRP reduces serum levels of TNF-α, whereas it increases IL-6 and IL-10 after LPS challenge.** Mice (four per group) were pretreated with PBS or CGRP (1 μg/cavity) and challenged with LPS (500 μg/cavity). After 90 min, blood samples were collected by cardiac puncture and TNF-α (A), IL-6 (B), and IL-10 (C) serum concentrations were determined by ELISA. Each column is the mean from four animals with SEM indicated by a vertical line. An asterisk denotes statistically significant differences when compared with control.

sera of endotoxemic mice, we determined the effects of CGRP on LPS-induced cytokine release by macrophages in culture. Thioglycollate-elicited mouse peritoneal macrophages were pretreated with increasing concentrations of CGRP for 2 h, followed by LPS treatment (500 ng/mL) for an additional 4 h. As shown in Table 1, CGRP inhibited LPS-induced TNF-α secretion, while it enhanced IL-10 as measured by ELISA in cell-free culture supernatants. The effect of CGRP on macrophages was reverted by its receptor antagonist CGRP8-37.

DISCUSSION

Several vasoactive neuropeptides and humoral peptides are known to have potent proinflammatory properties during acute and chronic inflammation, including endotoxic shock. The results presented here demonstrate that the vasoactive neuropeptide CGRP has anti-inflammatory properties *in vivo*, inhibiting neutrophil influx into the peritoneal cavity and in the blood 4 h after LPS, and protecting mice against lethal endotoxemia. The protection induced by CGRP is receptor mediated because it was blocked by the specific CGRP receptor antagonist, CGRP 8-37. This protective effect of CGRP correlates with a potent inhibition of TNF-α and an induction of IL-6 and IL-10 in the sera 90 min after LPS challenge. The proinflammatory cytokine TNF-α is an essential mediator of endotoxic shock and its neutralization with antibodies or the reduction of secretion results in a protective effect in several models of endotoxemia (12). It is well documented that

TABLE 1. **CGRP inhibits TNF-α production and increases IL-10 levels induced by LPS-stimulated macrophages *in vitro*[1]**

Cytokines (ng/mL)	CGRP (ng/mL)	LPS	LPS + CGRP 8-37
TNF-α			
	0	7.29 ± 1.73	–
	0.1	10.84 ± 2.65	–
	1	5.92 ± 1.24	8.70 ± 1.71
IL-10			
	0	1.91 ± 0.21	–
	0.1	1.85 ± 0.25	–
	1	2.57 ± 0.47	1.93 ± 0.41

[1]Thioglycollate-elicited peritoneal macrophages were pre-incubated with different concentrations of CGRP (0.1 or 1 ng/mL) for 2 hours, in the present or not of CGRP 8-37 (20 ng/mL) LPS (500 ng/mL) was added for an additional 4 or 24 hours for TNF-α and IL-10 detection, respectively by ELISA. Values in PBS stimulated macrophages were (0.06 ± 0.01) in TNF-α levels and (0.79 ± 0.08) in IL-10 levels. Data are expressed as mean ± SEM from triplicates of one representative experiment out of tree experiments with similar results.

treatment with recombinant IL-10 protects mice against LPS-induced lethality, while it decreases the release of several proinflammatory cytokines including TNF-α *in vitro* and *in vivo* (26). Furthermore, endogenous IL-10 controls the magnitude of the inflammatory response during endotoxemia because its neutralization by specific antibodies or gene deletion by homologous recombination potentiates TNF-α production and increases LPS-induced lethality. The role of IL-6 is less clear because this cytokine displays pro- and anti-inflammatory activities. In this context, our results suggest that the induction of IL-6 and IL-10 by CGRP could be involved in the observed reduction of TNF-α induced by LPS even though the absolute levels of IL-6 were not extremely high, probably because of the time point chosen to analyze the samples.

It has been previously shown that CGRP inhibits several macrophage and Langerhans cell functions and increases the infection of the obligatory intracellular protozoan parasite *L. major* (6–11). These inhibitory effects of CGRP are in keeping with our results showing inhibition of LPS-induced TNF-α by CGRP on macrophages. The mechanism whereby CGRP affects macrophage functions, including the observed inhibition of TNF-α, was not fully investigated. However, it is well documented that CGRP is a potent inductor of cAMP in several cell types, including macrophages (27, 28). Because the increase of cAMP on macrophages is associated with inhibition of LPS-induced TNF-α (29), it is possible that the observed effect of CGRP on macrophages involves stimulation of adenylate cyclase and intracellular increase of cAMP. Interestingly, the vasoactive neuropeptide substance P that colocalizes with CGRP in several sensory neurons, stimulates secretion of TNF-α, IL-1, and IL-6 from human monocytes, and enhances TNF-α by LPS-stimulated neuroglial cells (30).

Leukocyte activation and migration to tissues is a critical step in the inflammatory process and in host defense. However, excessive accumulation of leukocytes, especially neutrophils, is considered important in the pathogenesis of endotoxemia (31, 32). In fact, depletion of neutrophils protects mice from endotoxic shock (33). In experimental inflammatory reactions in rabbit skin, CGRP acts synergistically with C5a, LTB4, PAF, FMLP, and IL-1, increasing edema and neutrophil

accumulation (34). Interestingly, in the present study, we observed anti-inflammatory effects of CGRP in models of acute peritonitis, reducing the neutrophil recruitment induced by treatment with LPS or thioglycollate, but not by PAF. These results suggest that the effect of CGRP on neutrophil recruitment is indirect, probably on cytokine and chemokine production by resident cells such as macrophages. In fact, we have measured the levels of two neutrophil chemotactic proteins, KC and MIP-1α, in the peritoneal fluid 90 min after LPS stimulation and observed that pretreatment with CGRP significantly reduced the levels of KC.

The synergistic effect of CGRP in combination with several inflammatory agents on edema formation and neutrophil recruitment in the skin suggest that in these models, the vasodilatory effect of CGRP is more important then its observed inhibitory effect on macrophage activation. It should also be mentioned that the strong potentiating effect of CGRP on neutrophil accumulation induced by IL-1 in the skin was obtained when the neuropeptide was injected 1 h after the inflammatory stimuli (35).

The protective effect of CGRP is possibly related to the ability of this peptide to reduce macrophage activation, including the inhibition of TNF-α secretion and of neutrophil chemotactic protein generation (KC) as well as to the reduction of neutrophil mobilization and migration. These effects could be related, at least in part, to its ability to induce IL-10 *in vivo*. The anti-inflammatory effects of CGRP compare with the proinflammatory effects of substance P, bradykinin, and endothelin, and suggest that different vasoactive peptides could participate in opposite ways on macrophage activation during local and systemic acute inflammation, and possibly bacterial sepsis.

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