British Journal of Pharmacology (1996) 118, 2164-2170

Rapid nitric oxide- and prostaglandin-dependent release of calcitonin gene-related peptide (CGRP) triggered by endotoxin in rat mesenteric arterial bed

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1 Our objective was to determine whether endotoxin (ETX) could directly trigger the release of calcitonin gene-related peptide (CGRP) from perivascular sensory nerves in the isolated mesenteric arterial bed (MAB) of the rat and to determine whether nitric oxide (NO) and prostaglandins (PGs) are involved.

2 ETX caused time- and concentration-dependent release of CGRP, and as much as a 17 fold increase in CGRP levels in the perfusate at 10-15 min after the administration of ETX (50 $\mu g\ ml^{-1}$).

3 CGRP-like immunoreactivity in the perfusate was shown to co-elute with synthetic rat CGRP by reverse-phase h.p.l.c.

4 Pretreatment of MAB with capsaicin or ruthenium red inhibited ETX-induced CGRP release by 90% and 71%, respectively. ETX-evoked CGRP release was decreased by 84% during Ca^{2+}-free perfusion.

5 The release of CGRP evoked by ETX was enhanced by L-arginine by 43% and inhibited by N^{ω}-nitro-L-arginine (L-NOARG) and methylene blue by 37% and 38%, respectively. L-Arginine reversed the effect of L-NOARG.

6 Indomethacin and ibuprofen also inhibited the ETX-induced CGRP release by 34% and 44%, respectively. No additive inhibition could be found when L-NOARG and indomethacin were concomitantly incubated.

7 The data suggest that ETX triggers the release of CGRP from capsaicin-sensitive sensory nerves innervating blood vessels. The ETX-induced CGRP release is dependent on extracellular Ca^{2+} influx and involves a ruthenium red-sensitive mechanism. Both NO and PGs appear to be involved in the ETX-induced release of CGRP in the rat mesenteric arterial bed.

Keywords: Calcitonin gene-related peptide; sensory nerve; endotoxin; nitric oxide; blood vessels; prostaglandin

Introduction

Calcitonin gene-related peptide (CGRP) is a very potent vasodilator neuropeptide in many isolated blood vessels from both man and rat (Brain *et al.*, 1985; Edvinsson *et al.*, 1985; Mulderry *et al.*, 1985). We have previously shown that plasma levels of CGRP are significantly elevated in rats and pigs following injection of endotoxin, and in a rat model of haemorrhagic shock (Wang *et al.*, 1991; 1992; Arden *et al.*, 1994); in addition, plasma CGRP levels were elevated in septic shock patients (Joyce *et al.*, 1990). We have also identified the portal circulation as a major source of circulating CGRP in the endotoxic rat (Wang *et al.*, 1991). Cyclo-oxygenase inhibitors decrease CGRP release during experimental endotoxemia (Wang *et al.*, 1995b). Therefore, CGRP may play an important role as a mediator of hypotension in both early and late stages of endotoxin shock, as well as in the late stages of haemorrhagic and septic shock.

In the vasculature, CGRP released appears to be the principal neurotransmitter from perivascular capsaicin-sensitive sensory nerves and may play a role in modulation of total peripheral resistance through an efferent function (Maggi, 1995). The levels of CGRP were the highest in rat mesenteric artery, suggesting that sensory nerves may be particularly important in modulating gastrointestinal vascular resistance (Mulderry *et al.*, 1985; Kawasaki *et al.*, 1988).

Endotoxin, a lipopolysaccharide, is the major antigen of the outer membrane of gram-negative bacteria, and is known to be a factor in many types of shock and further exacerbates systemic infection by promoting translocation of endogenous bacteria from the gut into the general circulation (Parrillo *et al.*, 1990). Endotoxin induces the profound physiological and behavioural changes that are associated with pain. The administration of endotoxin to animals *in vivo* produces a biphasic effect on blood pressure, i.e., an immediate transient fall in blood pressure after 1 to 5 min and a profound and unrelated hypotension several hours later (Parratt, 1983; Bhakdi, 1993). The possible mechanism of the endotoxin-induced hypotension has been proposed to involve the interaction of endotoxin with specific receptors on a number of cells, causing the release of several inflammatory mediators, such as bradykinin, nitric oxide (NO) and prostaglandins (PGs) (Fleming *et al.*, 1992; Smith *et al.*, 1994; Baydoun *et al.*, 1993). We have hypothesized that endotoxin might activate sensory nerve endings which transmit the inflammatory response message to the brain, elicit pain perception, and induce vasodilatation in blood vessels by releasing the sensory neuropeptide, CGRP. The release of CGRP may be triggered, at least in part, by local accumulation of various endogenous inflammatory mediators. In this study, we show that endotoxin can trigger the release of CGRP from perivascular nerves in a rat isolated mesenteric arterial bed, which is dependent on extracellular Ca^{2+} influx and involves a ruthenium red-sensitive mechanism. The release of CGRP is partially dependent on the enhanced production of NO and PGs. These data support our hypothesis. A preliminary account of this study has been published as an abstract (Wu *et al*, 1994).

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Methods

Tissue preparations and release experiments

The treatment of the laboratory animals and the experimental protocols of the present study adhered to the guidelines of Beijing Medical University and were approved by Institutional Authority for Laboratory Animal Care. All experiments were carried out in healthy, male Wistar rats (200–300 g) obtained from the animal laboratory of Beijing Medical University. Animals were housed in wire-mesh cages at 22°C ambient temperature and maintained on food and water *ad libitum* with a 12 h/12 h light/dark cycle for 1–2 weeks prior to all experiments.

The rat mesenteric arterial bed was removed and perfused according to the method described by Han *et al.* (1990a). The rats were anaesthetized with urethane (1 g kg^{-1}, i.p.) and heparinized by i.v. injection of heparin (1000 u ml^{-1}) into the caudal vein of rats. The mesenteric bed and the associated intestine were first excised from the abdomen by ligating the descending colon proximal to the rectum, the duodenum proximal to the stomach and the superior mesenteric artery proximal to the abdominal aorta. The superior mesenteric artery was cannulated with PE-50 tubing containing heparinized saline. The main branches of the superior mesenteric artery to the terminal ilium were then located and all other branches were ligated and severed. The remaining intestine was then separated from the vascular bed along the intestinal wall.

The dissected vascular bed was placed in a 10-ml organ bath maintained at 37°C and perfused intraluminally at constant flow rate of 3 ml min^{-1} and superfused at a rate of 0.5 ml min^{-1} by a Gilson minipump with modified Krebs solution (composition in mM: NaCl 118.5, KCl 4.74, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11 and EDTA 0.03), which was bubbled with 95% O_2/5% CO_2. After equilibration for 30 min, the preparations were perfused with a different concentration of endotoxin for 20 min. In another series of experiments, the preparations were preperfused with Krebs solution containing capsaicin, ruthenium red, N^{ω}-nitro-L-arginine (L-NOARG), L-arginine, indomethacin, ibuprofen or methylene blue for the first 20 min followed by another 20 min perfusion with endotoxin 50 μg ml^{-1}. Superfusate/perfusate samples were collected in 5 min fractions in ice chilled polystyrene tubes containing acetic acid (0.2 N final concentration). The samples at 10–15 min in response to various concentration of endotoxin were stored at −20°C and 3 ml samples were taken out to be evaporated to dryness using a Speed-Vac centrifugal vacuum evaporator-concentrator (Savant Instruments, Inc.) and stored at −20°C before CGRP measurement (see below). The data of CGRP release were divided by three and expressed as CGRP-LI pg per ml of superfusate/perfusate.

Reverse-phase high performance liquid chromatography (h.p.l.c.)

Superfusate/perfusate samples following the administration of 50 μg ml^{-1} endotoxin 10–15 min were centrifuged (2,800 *g*, 4°C, 20 min). The supernatant fractions were partially purified over SEP columns (Peninsula Laboratories, Inc.) containing 200 mg of C_{18}. After adding the samples to the columns and extensive washing with 0.1% trifluoroacetic acid (TFA, 4 × 5 ml), the sample CGRP was eluted from the columns with 3 ml of 60% acetonitrile (high performance liquid chromatography grade) in 0.1% TFA. The samples were evaporated to dryness with a Speed-Vac centrifugal vacuum evaporator-concentrator and reconstituted in a 0.1% TFA. Typically, 85–90% of synthetic CGRP (rat sequence), added (spiked) to some control (untreated) samples prior to purification and drying, was recovered in the RIA. The sample (100 ul) was injected onto the Beckman C_{18} ultraphere-ODS column (5 μM, 4.6 mm × 15 cm) or reverse-phase h.p.l.c. The column was developed at a flow rate 0.8 ml min^{-1} with a linear gradiem of 10–60% acetonitrile containing 0.1% TFA. The fractions (0.8 ml/fraction) were collected, evaporated, redissolved in H_2O and the CGRP immunoreactivity was determined by radioimmunoassay.

Radioimmunoassay (RIA) of CGRP

The RIA technique for the measurement of CGRP levels in the present study was similar to that in our previous studies investigating the release of CGRP in endotoxin and haemorrhagic shock in the rat (Wang *et al.*, 1991). Briefly, the samples were reconstituted in a RIA buffer (0.1 M phosphate buffer containing 0.1% bovine serum albumin, 0.01% NaN_3, 50 mM NaCl, and 0.1% Triton X-100, pH 7.4). Standards of synthetic CGRP (rat amino acid sequence) ranging from 2.5 to 1,000 pg/assay tube or the sample, dissolved in a volume of 200 μl RIA buffer, were incubated for 24 h at 4°C with 100 μl of anti-CGRP antibody (anti-human CGRP II antibody, Peninsula Laboratories, Inc.) diluted in RIA buffer. This antibody cross-reacts 100% with rat CGRP and shows <0.01% cross-reaction with human rat amylin and 0% cross-reaction with calcitonin, vasoactive intestinal polypeptide, substance P, and somatostatin (data from Peninsula Laboratories, Inc.). The mixture was then incubated for an additional 24 h at 4°C with 100 μl of ^{125}I-labelled CGRP (10,000 c.p.m./tube, Amersham Corp.) in RIA buffer. Free and bound fractions were separated by adding 100 μl of goat anti-rabbit IgG (second antibody) and 100 μl of normal rabbit serum for 2 h at room temperature. An additional 0.5 ml of RIA buffer was added, and the RIA test tubes were centrifuged (2,800 *g*, 4°C) for 20 min. After removal of the supernatant fractions, the RIA test tubes were counted for measurement of gamma radioactivity of ^{125}I remaining in the pellets.

Chemicals and drugs

Endotoxin (lipopolysaccharide B from *S. enteritides*) was purchased from Difco Laboratories, Detroit, MI, U.S.A. Synthetic CGRP (rat amino acid sequence), anti-CGRP antibody, goat anti-rabbit IgG and normal rabbit serum were purchased from Peninsula Laboratories, Inc, Belmont, CA, U.S.A. [^{125}I]-CGRP (human sequence) was purchased from Amersham Corporation, Arlington Heights, IL, U.S.A. Capsaicin, ruthenium red, L-NOARG, L-arginine, indomethacin, ibuprofen, methylene blue and other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.

Statistical analysis

The data are expressed as mean ± the standard error of the mean (s.e.mean). The data were analyzed by one-way ANOVA and further analyzed by the Student-Newman-Keuls (S-N-K) test for multiple comparisons between treatment groups. A P value of <0.05 was used to indicate significant differences. The n values given in the tables and the figure legends represent the number of individual rats used for providing the mesenteric arterial bed in each treatment group.

Results

Effects of endotoxin on the release of CGRP

Basal release of CGRP in the isolated rat mesenteric arterial bed was found to be 3.3 ± 0.7 pg ml^{-1} (Figure 1), and basal release remained stable for periods >40 min (data not shown). Endotoxin (2.5–50 μg ml^{-1}) caused a concentration-dependent release of CGRP (Figure 1). Endotoxin at concentration of 50 μg ml^{-1} increased CGRP level in perfusate by 17 fold (from 3.3 to 55 pg ml^{-1}). Figure 2 shows the time course of CGRP release induced by 50 μg ml^{-1} endotoxin in isolated mesenteric arterial bed of rat. The CGRP levels were elevated within 5 min, reaching their peak values at 10–15 min and declining after 15 min. Capsaicin (1 μM)-evoked CGRP release

was not significantly reduced after exposuring to endotoxin 50 $\mu g\ ml^{-1}$ (46 ± 4 vs 50.3 ± 6.3 pg ml^{-1}). The fractions of the immunoreactive peak were characterized by reverse-phase h.p.l.c. The retention time of the CGRP-like immunoreactive substance was 40 min, similar to that of the synthetic rat CGRP (Figure 3a and b).

Effects of capsaicin, ruthenium red and Ca^{2+}-free medium on endotoxin-induced CGRP release

Endotoxin (50 $\mu g\ ml^{-1}$)-evoked CGRP release was reduced by 90% after pretreatment of 10 μM capsaicin for 20 min and then wash out (Table 1). Ca^{2+}-free medium and pretreatment of 10 μM ruthenium red (an inhibitor of the accumulation of intracellular Ca^{2+} of sensory nerves) for 20 min inhibited endotoxin-induced release of CGRP by 84% and 71%, respectively (Table 1).

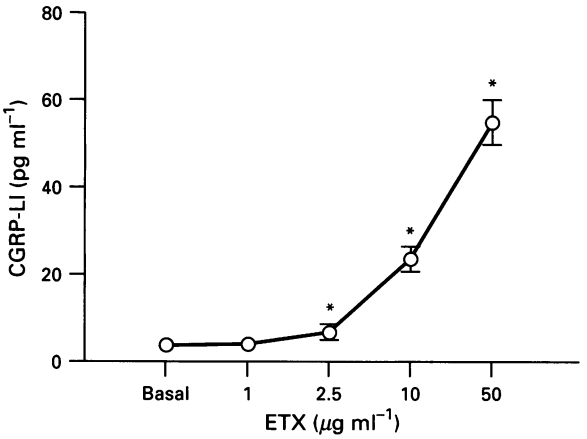

Figure 1 The effect of endotoxin (ETX) on the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from the isolated mesenteric arterial bed. The CGRP levels in successive 10–15 min fractions of perfusate in response to various concentration of endotoxin ($1-50\ \mu g\ ml^{-1}$) are shown. The results of CGRP release are expressed as CGRP-LI pg per ml of superfusate/perfusate. $n=6-8$ in each group. Endotoxin caused concentration-dependent elevation of CGRP level above basal (no endotoxin treatment). Values represent means $\pm$ s.e.mean. $^*P<0.05$ compared with control (no endotoxin treatment).

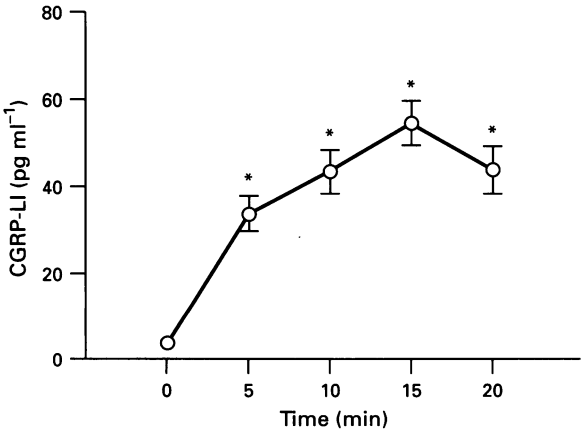

Figure 2 Time course of endotoxin (ETX)-evoked release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from the isolated mesenteric arterial bed. The CGRP levels in successive 5 min fractions of perfusate in response to $50\ \mu g\ ml^{-1}$ endotoxin are shown. The results of CGRP release are expressed as CGRP-LI pg per ml of superfusate/perfusate. $n=6$ in each group. The CGRP levels were elevated within 5 min, reaching their peak values at 10–15 min and declining after 15 min. $^*P<0.05$ compared with control (before endotoxin administration).

Effect of L-arginine, L-NOARG and methylene blue on endotoxin-induced CGRP release

Pretreatment with L-arginine (3 mM, a NO synthase substrate) for 20 min caused 43% enhancement of CGRP release induced by 50 $\mu g\ ml^{-1}$ endotoxin. Pretreatment with L-NOARG (0.3 mM a constitutive NO synthase inhibitor) or methylene blue (10 μM, an inhibitor of soluble guanylate cyclase) for 20 min caused inhibition of endotoxin-induced CGRP release by 37% and 38%, respectively. The inhibitory effect of L-

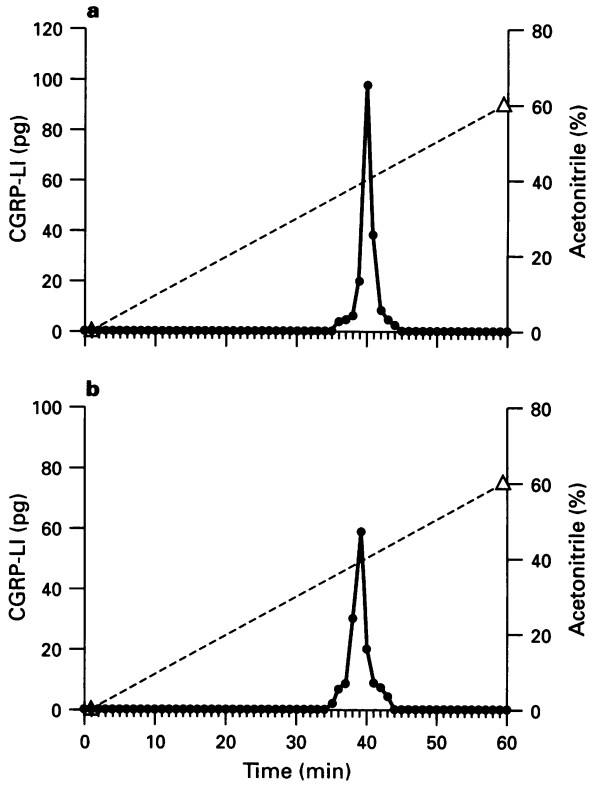

Figure 3 Endotoxin (ETX) triggers the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from the isolated mesenteric arterial bed proved by reverse-phase h.p.l.c. analysis. Reverse-phase h.p.l.c. analysis of the rat CGRP standard (a) and the perfusate of mesenteric arterial bed following the administration of endotoxin $50\ \mu g\ ml^{-1}$ (b). The extracted sample was subjected to h.p.l.c. and eluted as described in Methods. The retention time of the CGRP-LI substance was 40 min, similar to that of the synthetic rat CGRP. The dotted line indicates the linear gradient of percentage acetonitrile.

Table 1 Effects of capsaicin, ruthenium red and Ca^{2+}-free medium on endotoxin-induced CGRP release

	CGRP-LI ($pg\ ml^{-1}$)
Basal	2.9 ± 0.3
ETX	54.7 ± 4.9
ETX + capsaicin	8.3 ± 2.5*
ETX + Ca^{2+}-free	11.2 ± 2.1*
ETX + ruthenium red	18.0 ± 2.1*

Concentration of endotoxin (ETX) was $50\ \mu g\ ml^{-1}$. Capsaicin 10 μM was present for 20 min and then washed out. Pretreatment with ruthenium red (10 μM) was for 20 min. Endotoxin was administered in Ca^{2+}-free medium. The CGRP levels in successive 10–15 min fractions of perfusate in response to endotoxin are shown. The data of CGRP release are expressed as calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) pg per ml of superfusate/perfusate. Values are means $\pm$ s.e.mean. $n=6-8$ in each group. Endotoxin-evoked release of CGRP-LI was inhibited. $P<0.05$ compared with endotoxin alone.

NOARG could be completely reversed by L-arginine (Table 2). L-Arginine or L-NOARG or methylene blue alone had no effect on basal release of CGRP from the mesenteric arterial bed (data not shown).

Effects of cyclo-oxygenase inhibitors and/or L-NOARG on endotoxin-induced CGRP release

Pretreatment with indomethacin (5 μM) and ibuprofen (10 μM), inhibitors of cyclo-oxygenase, for 20 min reduced endotoxin-evoked CGRP release by 34% and 44%, respectively. No additive inhibition could be found when L-NOARG and indomethacin were concomitantly incubated (Table 3). Indomethacin or ibuprofen alone had no effect on basal release of CGRP from the mesenteric arterial bed (data not shown).

Discussion

Two important observations were made in this study. First, endotoxin causes a time- and concentration-dependent release of CGRP from capsaicin-sensitive sensory nerves innervating blood vessels in the isolated mesenteric arterial bed of rat. The release of CGRP is dependent on entry of extracellular Ca^{2+} and a ruthenium red-sensitive mechanism. Second, endotoxin-evoked rapid release of CGRP from perivascular nerves is partially via activation of constitutive NO synthase and activation of cyclo-oxygenase.

Table 2 Effects of L-arginine, L-NOARG and methylene blue on endotoxin-induced CGRP release

	CGRP-LI (pg mg^{-1})
Control	2.8±0.3
ETX	57.8±5.6
ETX + L-arginine	81.6±7.7*
ETX + L-NOARG	37.6±4.4*
ETX + L-arginine + L-NOARG	76.1±4.9*#
ETX + methylene blue	37.2±3.5*

Concentration of endotoxin (ETX) was 50 μg ml^{-1}. Isolated mesenteric arterial bed were pretreated with L-arginine (3 mM) or N^{w}-nitro-L-arginine (L-NOARG) (0.3 mM) or methylene blue (10 μM) for 20 min. The CGRP levels in successive 10–15 min fractions of perfusate in response to endotoxin are shown. The data of CRGP release are expressed as calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) pg per ml of superfusate/perfusate. Values are means±s.e.mean. $n=6$ in each group. Endotoxin-evoked release of CGRP-LI was changed. *$P<0.05$ compared with endotoxin plus L-NOARG treatment.

Table 3 Effects of cyclo-oxygenase inhibitors and/or L-NOARG on endotoxin-induced CGRP release

	CGRP-LI (pg ml^{-1})
Control	2.9±0.4
ETX	56.6±4.9
ETX + indomethacin	38.5±4.1*
ETX + ibuprofen	32.9±3.7*
ETX + L-NOARG	37.6±4.4*
ETX + L-NOARG + indomethcin	37.6±5.3*

Concentration of endotoxin (ETX) was 50 μg ml^{-1}. Isolated mesenteric arterial bed were pretreated with indomethacin (5 μM) or ibuprofen (10 μM) or N^{w}-nitro-L-arginine (L-NOARG) (0.3 mM) or L-NOARG plus indomethacin for 20 min. The CGRP levels in successive 10–15 in fractions of perfusate in response to endotoxin are shown. The data of CGRP release are expressed as calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) pg per ml of superfusate/perfusate. Values are means±s.e.mean. $n=6$ in each group. Endotoxin-evoked release of CGRP-LI was reduced. *$P<0.05$ compared with endotoxin alone.

In our previous studies with human patients, we have shown that CGRP levels were elevated more than 7 fold in the plasma of patients with severe, hyperdynamic septic shock (Joyce *et al.*, 1990). We have also shown that endotoxin administration to rats caused 4 fold elevations in plasma CGRP levels at 30 min, indicating that endotoxin triggers a relatively rapid release of CGRP and that CGRP may participate in early hypotension of endotoxin shock. After 3 h, plasma CGRP in endotoxin-treated rats had accumulated to levels more than 20 fold above control levels as well as a depletion of CGRP from nerves within blood vessels (Wang *et al.*, 1992). These data suggested that CGRP could be released from perivascular nerves into the circulation and accumulate within the blood plasma during the development of septic and endotoxaemic shock. CGRP is a very potent cardioaccelerator (Wang & Fiscus, 1989) and vasodilator (Brain *et al.*, 1985) and could therefore play a key role as one of the mediators of tachycardia and vasodilatation during circulatory shock. Huttemeier *et al.* (1993) have reported that a CGRP receptor antagonist, $hCGRP_{8-37}$ was used to block selectively the cardiovascular actions of endogenous CGRP during the development of endotoxin shock. Their data, coupled with previous reports from our and other laboratories, showing that the $hCGRP_{8-37}$ is an effective blocker of both vasodilatation and immunosuppression caused by CGRP (Han *et al.*, 1990b; Wang *et al.*, 1994; 1995a), have provided direct evidence that CGRP contributes significantly to the hypotension, tachycardia and immunosuppression during endotoxin shock. The question remaining is whether endotoxin can directly trigger the release of CGRP from perivascular nerves. The data in this study show that the release of CGRP from the mesenteric arterial bed is significantly increased in the first 5 min after endotoxin administration and is dependent on endotoxin concentration (Figures 1 and 2), indicating that endotoxin stimulates rapid release of CGRP from the isolated mesenteric arterial bed of rat. In the present study, CGRP tracer was purified by elution from reverse-phase h.p.l.c. with use of an acetonitrile gradient and it was demonstrated that the immunoreactivity released from the mesenteric arterial bed that was detected by antibody was eluted as a single peak in the same fraction as synthetic rat CGRP (Figure 3). This indicates that CGRP molecular form evoked by endotoxin was present in the perfusate of rat isolated mesenteric arterial bed and its biochemical characterization is similar to synthetic rat CGRP.

As regards the mechanisms of CGRP release, the endotoxin-evoked increase in CGRP release was prevented in the mesenteric arterial bed by pretreatment with a high dose of capsaicin (10 μM, depleting neurotransmitter from sensory nerve with toxic actions) for 20 min (Table 1). These results indicate that the CGRP found in the perfusate of endotoxin originates from mesenteric perivascular nerves of capsaicin-sensitive sensory nerves. Ca^{2+} is crucial as a mediator of effector function of neurones in excitation-secretion coupling (Simpson *et al.*, 1995). The observation that endotoxin-induced increase in CGRP release was largely dependent on extracellular Ca^{2+} fulfils a requisite for considering this event an active process of release (Table 1). Ruthenium red is known to interfere specifically with Ca^{2+} transport and blocks the accumulation of intracellular Ca^{2+} evoked by low dose capsaicin, which activates cation-selective channels on sensory nerves. However, the antidromic nerve stimulation-evoked CGRP release is not influenced by ruthenium red (Lundberg *et al.*, 1991; Maggi, 1995). The fact that endotoxin-induced CGRP release is blocked by ruthenium red further indicates that CGRP originates from capsaicin-sensitive sensory nerves, in which accumulation of intracellular Ca^{2+} is required (Table 1). The major mechanisms for CGRP release evoked by endotoxin from the mesenteric arterial bed have similar characteristics to some other stimuli, such as PGs, low pH, hypertonic media, lactic acid and low concentrations of cap-

saicin reported by our and other laboratories (Franco-Cereceda, 1989; Geppetti *et al.*, 1990; Franco-Cereceda & Lundberg, 1992; Wang *et al.*, 1993; Fiscus *et al.*, 1994).

When administered intravenously to the rat in lethal or near lethal doses, endotoxin can cause a biphasic effect on blood pressure, i.e an immediate (within 1 to 5 min) transient fall in blood pressure and unrelated hypotension several hours later (Parratt, 1983; Bhakdi, 1993). It has been proposed that this early phase of endotoxin-induced hypotension involves the immediate release of bradykinin, NO and PGs from blood vessels (Fleming *et al.*, 1992; Smith *et al.*, 1994). In cultured endothelial cells, endotoxin triggered the rapid release of both NO and PGI_2 within 1–5 min (Salvemini *et al.*, 1989). Some of these endogenous mediators involved in the immediate response to endotoxin have been reported to activate sensory afferent nerves (Barnes *et al.*, 1990) and probably trigger the release of neurotransmitter, such as CGRP, which may contribute significantly to the early and later hypotension of endotoxicosis. Our previous *in vivo* studies have shown that dexamethasone and the cyclo-oxygenase inhibitors, ibuprofen, indomethacin and high dose aspirin, given at doses known to block PG biosynthesis, improved the survival of rat during endotoxin shock, block the plasma accumulation of CGRP during endotoxicosis and suggest that PGs are involved in the endotoxin-induced release of CGRP (Wang *et al.*, 1991; 1995b). Local release of CGRP from sensory nerves has been shown to occur in response to PGs in guinea-pig isolated heart (Franco-Cereceda, 1989). The present study *in vitro* shows that an increase in cyclo-oxygenase products is partially responsible for endotoxin-induced CGRP release from the mesenteric arterial bed in the rat (Table 3). The results give further support to the idea that PGs are involved in the endotoxin-induced release of CGRP from perivascular nerves.

NO is produced by constitutive NO synthase (cNOS) not only in endothelial cells (ecNOS) but also in certain perivascular nerves containing the neural isoform of cNOS (ncNOS) (Iadecola, 1993). *In vivo* experiments using a rat model of endotoxicosis have shown that the early hypotension and the loss of vascular responsiveness to noradrenaline occurring within the first 1 h of shock was dependent on NO production and apparently involved the activation of cNOS in either endothelial or neural cells (Wright *et al.*, 1992; Szabo *et al.*, 1993). Some studies have shown that NO appears to increase CGRP release from perivascular nerves in cerebral arteries (Wei *et al.*, 1992), and microvessels in skin (Holzer & Jocic, 1994; Hughes & Brain, 1994; Kajekar *et al.*, 1995) by observation of blood flow in response to a CGRP receptor blocker. On the other hand, several studies have indicated that CGRP does not account for vasodilatation in response to NO (Ralevic *et al.*, 1992; Ayajiki *et al.*, 1994; Faraci & Breese, 1994; Brian *et al.*, 1995). Therefore, they suggest that release of CGRP is not important for responses to NO. By measuring CGRP directly, our present studies show for the first time that CGRP release is evoked by endotoxin. The endotoxin-induced release of CGRP is enhanced by L-arginine (a substrate of NOS) by 43%, and is inhibited by L-NOARG (a selective inhibitor of cNOS) and methylene blue (an inhibitor of guanylate cyclase) by 37% and 38%, respectively. The inhibitory effect of L-NOARG is reversed by L-arginine (Table 2). It is well established that NO activates guanylate cyclase, with a subsequent elevation of tissue levels of cyclic GMP (Fiscus, 1988). Our data with methylene blue suggest that sensory nerve activation and the CGRP release induced by endotoxin are, at least partially, mediated by the elevation of intracellular cyclic GMP induced by endogenous NO production in the mesenteric vasculature of rat. Therefore, the results of the present study are in agreement with the studies which show that NO appears to increase CGRP release from the perivasculature. The different results obtained by our group and others might be due to differences in stimuli, species and arterioles used.

The results of the present study cannot eliminate the possibility of involvement of inducible NO synthase (iNOS) in the endotoxin-evoked CGRP release. However, Brian *et al.* (1995) have recently shown that endotoxin-induced relaxation of rabbit cerebral arteriole was inhibited by both the CGRP antagonist $CGRP_{8-37}$ and the iNOS specific inhibitor, aminoguanidine. Since their effects were partially additive, they suggested that activation of iNOS probably does not contribute to the release of CGRP. Further studies are needed to obtain a conclusion.

NOS shares a number of similarities with cyclo-oxygenase. The pathway (s) leading to cyclo-oxygenase activation by NO are unknown but may involve an interaction at the iron-haeme centre of the enzyme. It is known that cyclo-oxygenase contains an iron-haeme centre (De Groot *et al.*, 1975; Kalyanaraman *et al.*, 1982). Both the constitutive and inducible form of cyclo-oxygenase are found in endothelial cells, fibroblasts and macrophages after treatment with proinflammatory agents, such as endotoxin (Salvemini *et al.*, 1993; Swierkosz *et al.*, 1995). Other studies have shown that NO activates cyclo-oxygenase in the hypothalamus, leading to corticotrophin-releasing factor release (Karanth *et al.*, 1993). NO increases arachidonic acid-stimulated PGE_2 production in fibroblasts (Salvemini *et al.*, 1993). A stimulatory role for NO in PGs release has been observed in endotoxin-stimulated chondrocytes (Stadler *et al.*, 1991) and the microcirculation of the rat (Warren *et al.*, 1992). However, there is another possibility that the interaction between cyclo-oxygenase products and NO is bidirectional. In the present study, coincubation of L-NOARG and indomethacin did not produce any additive inhibition on endotoxin-induced CGRP release from isolated mesenteric arterial bed (Table 3). It is possible that endotoxin may trigger CGRP release partially through the activation of NOS located in endothelial cells or in perivascular nerves (Iadecola, 1993). The increase of NO may lead to activation of cyclo-oxygenase by interaction with the haeme group, leading to the generation of PGs.

CGRP-containing nerves are located throughout the heart and in the adventitia of almost every artery and arteriole (Mulderry *et al.*, 1985; Uddman *et al.*, 1986). Most of the neurones that also contain substance P (SP) and neurokinin A (NKA) are classified as unmyelinated C-fibre afferent nerves and are thought to serve as sensory nerves, carrying information about pain or temperature perception toward the central nervous system. In addition, CGRP, SP and NKA within the peripheral ends of these nerves are released in the peripheral organs by various stimuli, e.g., bradykinin, histamine and PGs (Maggi, 1995). The release of CGRP and SP is now recognized as an important part of the inflammatory response, resulting in oedema, vasodilatation and release of histamine from mast cells (Lundberg *et al.*, 1991). CGRP can also inhibit the proliferation and killing function of T lymphocytes (Wang *et al.*, 1994; 1995a). CGRP released upon irritation of peripheral branches of primary afferent may evoke a variety of cardiovascular actions and immunoreactions during the development of endotoxicosis. Based on our present data, we hypothesize that first, endotoxin causes an immediate response, involving NO and PGs release, which enhances CGRP release from perivascular sensory nerves, leading to the increase of regional blood flow in some organs. Second, endotoxin causes a delayed response, involving the release of cytokines and potentially the induction of NO synthase, leading to long-term production of NO and CGRP release. The released CGRP may contribute to the oedema, immunosuppression, tachycardia and vasodilatation in endotoxicosis and septic shock.

In conclusion, we have found that endotoxin triggers the release of CGRP from perivascular nerves by a mechanism that is dependent on extracellular Ca^{2+} influx and blocked by ruthenium red. The endotoxin-induced release of CGRP was partially dependent on the biosynthesis of both NO and PGs. Further studies are needed to determine whether the NO enhanced release of CGRP may involve NO-mediated activation of cyclo-oxygenase and the local production of PGs, which could activate CGRP-containing perivascular nerves and cause enhanced release of CGRP.

This research project was supported by a Grant from the National Natural Science Foundation of China (No. 39425027) awarded to X.W., a Biotechnology Career Fellowship from the Rockefeller Foundation awarded to X.W. (Sponsored by R.R.F.), and the Vascular Medicine Grant from the China Medical Board of New York, Inc. No. 93-591.

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(Received January 2, 1996
Revised April 11, 1996
Accepted April 29, 1996)