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Effect of calpain inhibitor I, an inhibitor of the proteolysis of IκB, on the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat

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1 We compared the effects of calpain inhibitor I (inhibitor of the proteolysis of IκB and, hence, of the activation of nuclear factor κB (NFκB)) and dexamethasone on (i) the circulatory failure, (ii) multiple organ dysfunction and (iii) induction of the inducible isoforms of nitric oxide (NO) synthase (iNOS) and cyclo-oxygenase (COX-2) in anaesthetized rats with endotoxic shock.

2 Injection of lipopolysaccharide (LPS, *E. coli*, 10 mg kg^{-1}, i.v.) resulted in hypotension and a reduction of the pressor responses elicited by noradrenaline. This circulatory dysfunction was attenuated by pretreatment of LPS-rats with calpain inhibitor I (10 mg kg^{-1}, i.v., 2 h before LPS) or dexamethasone (1 mg kg^{-1}, i.v.).

3 Endotoxaemia also caused rises in the serum levels of (i) urea and creatinine (renal dysfunction), (ii) alanine aminotransferase (ALT), aspartate aminotransferase (AST) (hepatocellular injury), bilirubin and γ-glutamyl transferase (γGT) (liver dysfunction), (iii) lipase (pancreatic injury) and (iv) lactate. Calpain inhibitor I and dexamethasone attenuated the liver injury, the pancreatic injury, the lactic acidosis as well as the hypoglycaemia caused by LPS. Dexamethasone, but not calpain inhibitor I, reduced the renal dysfunction caused by LPS.

4 Endotoxaemia for 6 h resulted in a substantial increase in iNOS and COX-2 protein and activity in lung and liver, which was attenuated in LPS-rats pretreated with calpain inhibitor I or dexamethasone.

5 Thus, calpain inhibitor I and dexamethasone attenuate (i) the circulatory failure, (ii) the multiple organ dysfunction (liver and pancreatic dysfunction/injury, lactic acidosis, hypoglycaemia), as well as (iii) the induction of iNOS and COX-2 protein and activity in rats with endotoxic shock. We propose that prevention of the activation of NF-κB *in vivo* may be useful in the therapy of circulatory shock or of disorders associated with local or systemic inflammation.

Keywords: Inducible nitric oxide synthase; cyclo-oxygenase-2; circulatory shock; endotoxin; transcription factor

Introduction

The expression of inducible genes in eukaryocytes is largely controlled by proteins, such as nuclear factor-κB (NF-κB), which activate transcription (Nabel & Baltimore, 1987; Grimm & Baeuerle, 1993). NF-κB is itself activated by the exposure of cells to endotoxin (lipopolysaccharide, LPS), tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), IL-2 or phorbol 12-myristate 13-acetate (PMA) (Sen & Baltimore, 1986; Lowenthal *et al*., 1989; Arima *et al*., 1992; Henkel *et al*., 1993). NF-κB is a family of dimers, all of which are composed of members of the Rel/NF-κB family of polypeptides. The most frequent form of NF-κB is a dimer composed of two DNA-binding proteins, namely NF-κB (or p50) and RelA (or p65), although other dimeric combinations also exist (Siebenlist *et al*., 1994). Under physiological conditions, NF-κB is held (in an inactive form) in the cytoplasm by the inhibitory protein IκB-α, which avidly binds to most heterodimers including the NF-κB1/Rel A heterodimer. This inhibitory subunit can be considered as a cytoplasmatic anchor, as it prevents the nuclear uptake of NK-κB. Activation of NF-κB involves the release of the inhibitory subunit IκB-α from a cytoplasmic complex, which IκB forms together with the DNA-binding subunit RelA and NF-κB1 (Baeuerle & Baltimore, 1988a,b). Activation of NF-κB allows it to translocate to the nucleus and to induce the expression of specific genes. The cascade of events leading to the activation of NF-κB involves the signal-induced phosphorylation of IκB-α resulting in its proteolytic degradation and the release of NF-κB from its cytoplasmatic anchor. NF-κB then translocates into the nucleus, where it binds to different gene promotors and, hence, induces a large number of genes (Brown *et al*., 1993; Henkel *et al*., 1993; Sun *et al*., 1993; Miyamoto *et al*., 1994). The proteolytic degradation of IκB-α is inhibited by the cysteine protease inhibitor calpain inhibitor I, but not by other inhibitors of serine and cysteine proteases, such as chymostatin or leupeptin (Lin *et al*., 1995).

Local or systemic inflammatory responses are associated with the induction by cytokines (or lipopolysaccharides (LPS)) of the inducible isoforms of nitric oxide (NO) synthase (iNOS or NOS II) (see Moncada & Higgs, 1993; Thiemermann, 1994) and cyclo-oxygenase (COX-2) (see Xie *et al*., 1992; Vane *et al*., 1994). There is limited evidence that the expression of the genes for iNOS or COX-2 involves the activation of NF-κB, and the expression of iNOS caused by LPS or lipoteichoic acid is prevented by calpain inhibitor I (Xie 1994; Griscavage *et al*., 1995; 1996; Kengatharan *et al*., 1996). However, there is no evidence that interventions which specifically inhibit IκB protease and, hence, the activation of NF-κB exert beneficial effects in animal models of disease (e.g. local or systemic inflammation).

In the present study, we compared the effects of (i) agents known to attenuate the activation of NF-κB (e.g. calpain inhibitor I, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), N-carbobenzoxy-L-phenylalanine chloromethyl ketone (ZPCK), (ii) proteases which do not attenuate the activation of NF-κB (e.g. chymostatin, leupeptin; negative control) and (iii) dexamethasone (positive control) on the accumulation of nitrite in the supernatant of murine macrophages (cell line J774.1) activated with endotoxin. In a subsequent study, we investigated the effects of calpain in-

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hibitor I, chymostatin and dexamethasone on (i) the circulatory failure, (ii) the multiple organ dysfunction (MODS) and (iii) the induction of iNOS and COX-2 protein and activity in rats with endotoxic shock.

Methods

Cell culture

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum (Ruetten *et al*., 1996). Cells were cultured in 96-well plates with 200 μl culture medium until they reached confluence. To induce iNOS in macrophages, fresh culture medium containing *Escherichia coli* lipopolysaccharide (LPS, 1 μg ml^{-1}, serotype: 0127:B8) was added. Nitrite accumulation in the cell culture medium was measured after 24 h. To assess the effects of calpain inhibitor I (N-acetyl-leu-leu-norleucinal), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), N-carbobenzoxy-L-phenylalanine chloromethyl ketone (ZPCK), leupeptin, chymostatin (all 0.01 – 30 μM) or dexamethasone (0.01 – 1 μM) on the production of nitrite, agents were added to the cells 1 h before LPS.

Measurement of nitrite production

The amount of nitrite, an indicator of NO synthesis, in the supernatant of J774.2 was measured by the Griess reaction (Green *et al*., 1981) by adding 100 μl of Griess reagent to 100 μl samples of unfiltered serum or supernatant. The optical density at 550 nm (OD_{550}) was measured by a Molecular Devices microplate reader (Richmond, CA., U.S.A.). Nitrite concentrations were calculated by comparison with OD_{550} of standard solution of sodium nitrite prepared in control serum or culture medium.

Cell respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT (3 - (4,5 - dimethylthiazol - 2 - yl)- 2,5-diphenyltetrazolium bromide) to formazan (Mosman, 1983). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml^{-1} for 60 min). Culture medium was removed by aspiration and cells solubilized in dimethyl sulphoxide. Changes in absorbance at a wavelength of 550 nm (OD_{550}) were measured by a Molecular Devices microplate reader (Richmond, CA., U.S.A.). Formazan production was expressed as a percentage of the values obtained from untreated cells.

Measurement of haemodynamic changes

Male Wistar rats (240 – 320 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Intraval; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Statham, Oxnard, CA, U.S.A.) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR), which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA., U.S.A.). The femoral vein and jugular vein were cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 15 min. After recording baseline haemodynamic parameters, animals were given noradrenaline (1 μg kg^{-1}, i.v.), and 10 min later animals received vehicle for LPS (1 ml kg^{-1}, i.v., saline, $n=7$) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v., in 0.3 ml of saline) as a slow injection over 10 min. The pressor responses to noradrenaline were reassessed at 1 h, 3 h and 6 h after LPS injection. At 90 min after injection of LPS, blood was taken to evaluate the changes in the serum levels of TNFα (see below). At 6 h after LPS, blood was taken to measure the changes in the serum levels of various biochemical marker enzymes of MODS (see below). Animals were divided into 8 groups: *(1)* vehicle (50% ethanol/PBS, 1 ml kg^{-1}, i.p., 2 h before LPS) plus vehicle for LPS (saline), $n=4$; *(2)* vehicle (50% ethanol/PBS, 1 ml kg^{-1}, i.p., 2 h before LPS) plus LPS, $n=10$; *(3)* calpain inhibitor I (Cal-I, 10 mg kg^{-1}, i.p., 2 h before LPS) plus vehicle for LPS (saline), $n=3$; *(4)* calpain inhibitor I (Cal-I, 3 mg kg^{-1}, i.p., 2 h before LPS) plus LPS, $n=6$; *(5)* calpain inhibitor I (Cal-I, 10 mg kg^{-1}, i.p., 2 h before LPS) plus LPS, $n=7$; *(6)* calpain inhibitor I (Cal-I, 10 mg kg^{-1}, i.p., 2 h after LPS) plus LPS, $n=4$; *(7)* dexamethasone (Dex, 1 mg kg^{-1}, i.p., 2 h before LPS) plus LPS, $n=6$; *(8)* chymostatin (Chym, 10 mg kg^{-1}, i.p., 2 h before LPS) plus LPS, $n=5$).

Quantification of liver, renal, or pancreatic injury

At 6 h after the injection of LPS, 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from a catheter placed in the carotid artery. The blood sample was centrifugesd (6,000 r.p.m. for 3 min) to separate serum. All serum samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, U.K.). The following marker enzymes were measured in the serum as biochemical indicators of multiple organ dysfunction syndrome (MODS): (1) liver dysfunction and failure were assessed by measuring the rises in serum levels of alanine aminotransferase (ALT, a specific marker for hepatic parenchymal injury); aspartate aminotransferase (AST, a non-specific marker for hepatic injury), γ-glutamyl tranferase (γGT; an early indicator of hepatic injury) and bilirubin (an indicator of hepatic excretory function and predictor of the development of liver failure; Baue, 1993; Hewett *et al*., 1993). (2) Renal dysfunction and failure were assessed by measuring the rises in serum levels of creatinine (an indicator of reduced glomerular filtration rate, and hence, renal failure) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism). (3) Pancreatic injury was assessed by measuring the rises in the serum level of lipase (Thiemermann *et al*., 1995; Ruetten *et al*., 1996).

Measurement of glucose and lactate

Glucose and lactate concentrations were measured spectrophotometrically (Glucose and Lactate Kit, Sigma Chemical Co., Poole, Dorset, U.K.) according to the protocol provided by the manufacturer.

Nitric oxide synthase assay

NOS activity was measured as the ability of tissue homogenates to convert L-[^{3}H]-arginine to L-[^{3}H]-citrulline (Szabo *et al*., 1993). Lungs and livers were removed at 6 h after LPS and frozen in liquid nitrogen. Frozen organs were homogenized on ice with an Ultra-Turrax T25 homogenizer (Janke & Kunkel, IKA Labortechnick, Staufen i, Br., Germany) in a buffer composed of: Tris-HCl 50 mM, EDTA 0.1 mM, EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethysulphonyl fluoride 1 mM (pH 7.4). Briefly, tissue homogenates (30 μl, approx. 60 μg protein) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 25 min at 25°C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, U.S.A.). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-ctirulline formation independent of a

specific NOS activity. Experiments in the presence of NADPH, without calcium and with 5 mM EGTA, measured the calcium-independent iNOS activity, which was taken to represent iNOS activity. Protein concentration was measured spectrophotometrically in 96-well plates with Bradford reagent (Bradford, 1976), with bovine serum albumin as standard.

Western (immuno)blot analysis

Lungs and livers were homogenized on ice in an extraction buffer (pH 7.4) as previously described (De Kimpe *et al*., 1995; Bryant *et al*., 1995). The homogenates werre centrifuged (5000 *g*) for 15 min at 4°C and the supernatant was boiled for 10 min with gel-loading buffer in a ration of 1 : 1 (v/v). Protein concentrations of the supernatants were determined as above, and total protein equivalents for each sample were separated by one dimensional gel electophoresis (7.5% SDS gel for iNOS and 10% SDS gel for COX-2) together with molecular weight markers (SDS-7B; Sigma). After transfer to nitrocellulose by electrophoresis, nonspecific IgGs were blocked with 5% dried milk protein and incubated with specific antibodies to COX-2 (Cayman Chemicals, Ann Arbor, MI., U.S.A.) or iNOS (a generous gift from Dr C. Bryant) at a concentration of 1 : 5000. The blots were then incubated with a horseradish peroxidase-conjugated secondary antibody and developed by use of an enhanced horseradish peroxidase/luminol chemiluminescence reaction (ECL Western blotting detection reagents, Amersham International plc. Buckinghamshire, U.K.) and exposed to X-ray film for 30–60 s. Subsequently, the relative expression of iNOS or COX-2 protein in each tissue was quantified by densitometric scanning of the Western blots with a GS 700 Imaging Densitometer (Bio-Rad) and a computer programme (Molecular Analyst, Macintosh).

Measurement of the serum concentrations of tumour necrosis factor α

A blood sample was obtained at 90 min after injection to evaluate the effects of endotoxaemia and drugs on the serum levels of TNFα. This time point was chosen, as 90 min of endotoxaemia results in a maximal increase in the serum levels of TNFα in this species (De Kimpe *et al*., 1995). The content of TNFα in serum samples (50 μl) was determined by enzyme-linked immunosobent assay (ELISA) (Mouse TNF-α ELISA kit, Genzyme, Cambridge, MA., U.S.A.) in 96-well plates. Binding was detected by a peroxidase-conjugated polyclonal anti-mouse TNF-α antibody with tetramethylbenzidine as a substrate. Following acidification (sulphuric acid, 0.5 M final) the absorbance of each well was measured at 450 nm with a Molecular microplate reader.

Measurement of 6-keto $PGF_{1\alpha}$

At 6 h after the injection of LPS, 0.5 ml of blood was collected from the catheter placed in the carotid artery. The blood sample was centrifuged (10,000 r.p.m. for 3 min) to separate serum. The concentration of 6-keto $PGF_{1\alpha}$ present in the serum was measured by radioimmunoassay (Salmon, 1978).

Materials

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), NADPH, noradrenaline bitartrate, sulphanilamide, naphtyleneethylenediamide, phosphoric acid, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), N-carbobenzoxy-L-phenylalanine chloromethyl ketone (ZPCK), sulphuric acid,

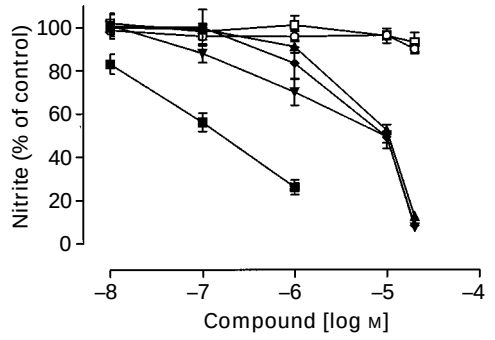

Figure 1 Effect of dexamethasone (■) calpain inhibitor (▲), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK, ▼), N-carbobenzoxy-L-phenylalanine chloromethyl ketone (ZPCK, ◆), chymostatin (○) or leupeptin (□) on the increase in the concentration of nitrite in the supernatant of cultured J774.2 macrophages activated with LPS for 24 h. Data are expressed as % control (mean ± s.e.mean (vertical lines) from triplicate determinations (well) from 4 separate experimental days ($n=12$)). Nitrite concentration in cultured medium of J774.2 cells activated with LPS at 24 h was 36 ± 1 μM.

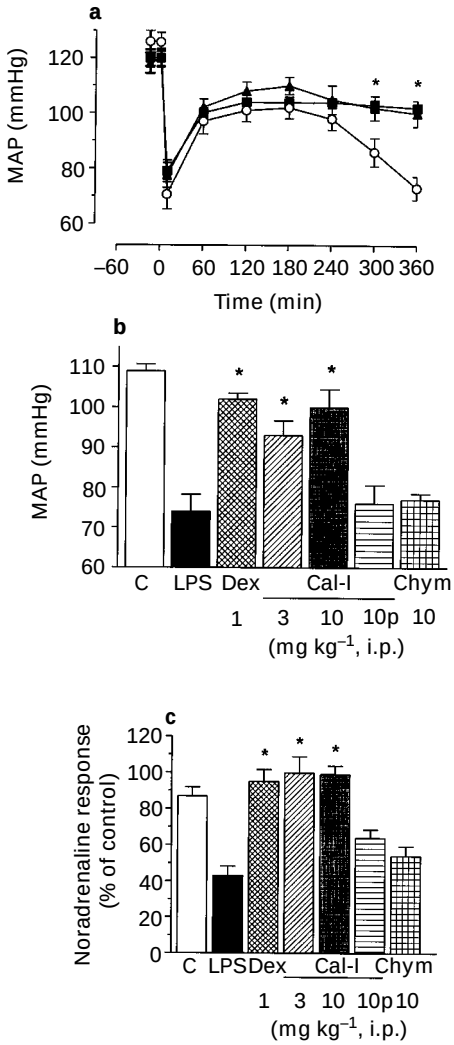

Figure 2 (a) Effects of calpain inhibitor I (10 mg kg^{-1}, i.p., ▲) or dexamethasone (1 mg kg^{-1}, i.p., $n=6$; ■) on mean arterial blood pressure (MAP) over time; effect of LPS alone (○) is also shown ($n=10$) (b) MAP 6 h after injection of LPS and (c) the pressor response elicited by noradrenaline in rats treated with LPS (10 mg kg^{-1}, i.v.). In (b) and (c), the effect of LPS alone (LPS-control or LPS) is also shown ($n=10$). One group of animals received vehicle rather than LPS (C, $n=4$). LPS rats were pretreated with dexamethasone (Dex, $n=6$) or calpain inhibitor I (Cal-I, $n=7$) or received calpain inhibitor I (10 mg kg^{-1}, i.p.) 2 h after LPS (Cal-I 10p, $n=4$). Another group of animals was pretreated with chymostatin (Chym, $n=5$; negative control). Data are expressed as mean ± s.e.mean (vertical lines) of n observations. $^*P<0.05$ represents significant difference when compared to LPS-controls.

leupeptin, L-glutamine, foetal calf serum, Bradford reagent, bovine serum albumin, Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Calpain inhibitor I (N-acetyl-leu-leu-morleucinal) and chymostatin were from Calbiochem (Nottingham, U.K.). L[2,3,4,5-^{3}H]-arginine hydrochloride and radiolabelled 6-keto $PGF_{1\alpha}$ were obtained from Amersham (Buckinghamshire, U.K.). Tetrahydrobiopterin (6**R**-L-erythro-5,6,7,8-tetra-hydrobiopterin) was obtained from Dr B. Schirks Laboratories (Jona, Switzerland) and sodium thiopentone (intraval Sodium) was from Rhone Merieux Ltd. (Harlow, Essex, U.K.).

Statistical analysis

A one-way analysis of variance (ANOVA) followed by, if appropriate, a Dunnett's *post hoc* test was used to compare means between groups (*in vivo* study). Student's unpaired *t* test was used to compare means between groups (*in vitro* study). A *P* value of less than 0.05 was considered as statistically significant.

Results

Effects of calpain inhibitor I, TPCK, ZBCK and dexamethasone on the formation of nitrite by macrophages activated with LPS

Activation of J774.2 macrophages resulted, within 24 h, in a significant increase in nitrite in the cell supernatant from 0.9 ± 0.2 μM (control) to 36 ± 1 μM ($n = 12$). Calpain inhibitor I, TPCK, ZPCK (IC_{50}: ~ 10 μM) and dexamethasone (IC_{50}: ~ 0.1 μM) caused concentration-dependent inhibitions of the formation of nitrite elicited by LPS (Figure 1). In contrast, neither chymostatin nor leupeptin, which are inhibitors of cysteine and serine protease activity, respectively; and which do not prevent the activation of NFκB (Lin *et al*., 1995), did not attenuate the increase in nitrite formation in the supernatant of macrophages activated with LPS (Figure 1).

Incubation of the cells with LPS alone caused a small ($\sim 20\%$) reduction in cell viability. Incubation of the cells with the drugs used (but without LPS) did not result in a significant reduction in cell viability. None of the drugs used attenuated the reduction in cell viability caused by LPS (data not shown).

The effect of calpain inhibitor I on the circulatory failure caused by LPS in the anaesthetized rat

Baseline values for MAP and HR of the animal groups studied ranged from 118 ± 4 mmHg to 125 ± 4 mmHg and from 368 ± 11 to 386 ± 8 beats min^{-1}, respectively, and were not significantly different between groups. Administration of LPS (10 mg kg^{-1}) caused a rapid (within 15 min), but transient fall in MAP which had partly recovered by 180 min. After 180 min, there was a second, further fall in MAP from 102 ± 4 mmHg to 73 ± 5 mmHg at 360 min (Figure 2a). This delayed, but not the early, fall in MAP, was abolished by both dexamethasone (1 mg kg^{-1}) and calpain inhibitor I (3 or 10 mg kg^{-1}) (Figure 2b). The mean baseline values for the pressor responses to noradrenaline (1 μg kg^{-1}, i.v.) ranged from 37 ± 4 to 43 ± 5 mmHg and were not significantly different between any of the experimental groups studied. Injection of LPS resulted, within 360 min, in a more than 50% reduction in the pressor response elicited by noradrenaline (Figure 2c). This vascular hyporeactivity to noradrenaline was attenuated by both dexamethasone and calpain inhibitor I (3 or 10 mg kg^{-1}, Figure 2c). However, the circulatory failure (hy-

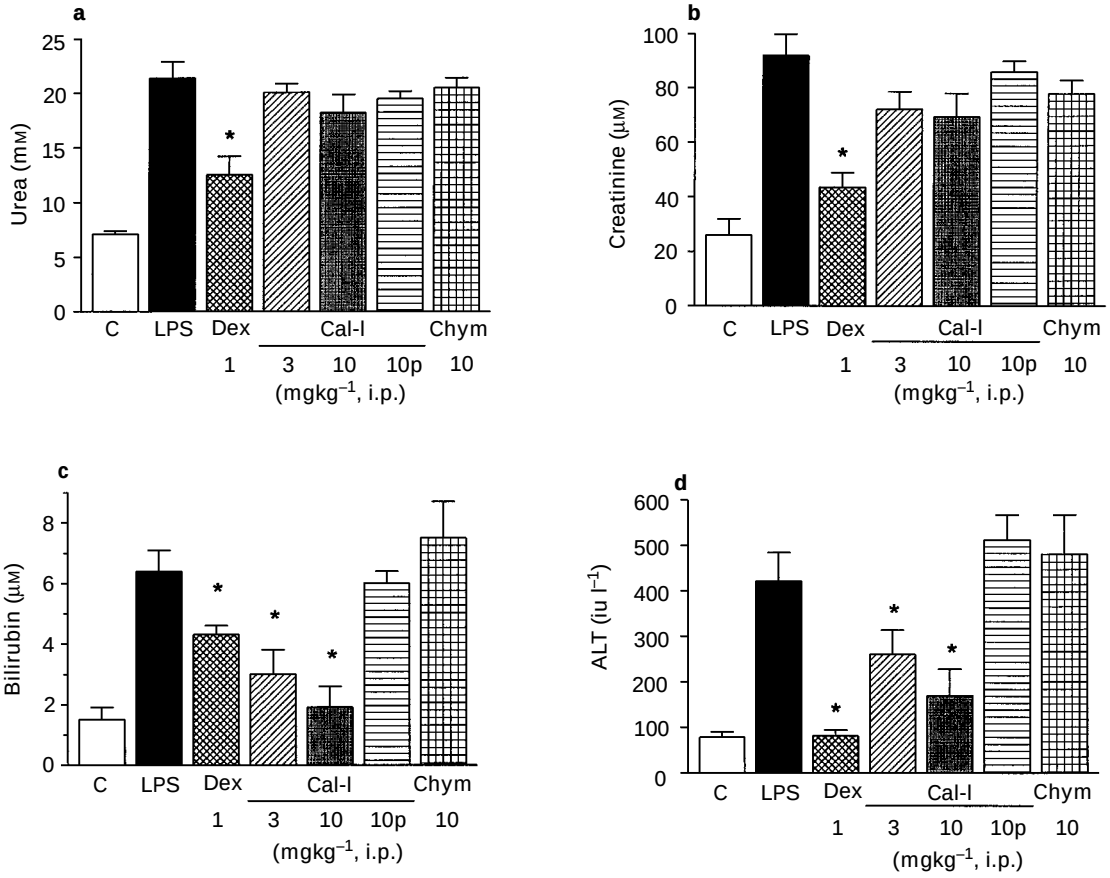

Figure 3 Effect of calpain inhibitor I on the LPS-induced increases in the serum concentrations of (a) urea, (b) creatinine, (c) bilirubin and (d) alanine aminotransferase (ALT) 6 h after injection of LPS (10 mg kg^{-1}). Different groups of animals received vehicle for LPS (C, $n = 4$), LPS alone (LPS, $n = 10$), LPS plus dexamethasone (Dex, $n = 6$), LPS plus 3 mg kg^{-1}, i.p., calpain inhibitor I (Cal-I, 3; $n = 6$), LPS plus 10 mg kg^{-1}, (i.p.) calpain inhibitor I (Cal-I, 10; $n = 7$), LPS plus late administration, 2 h after LPS, of 10 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 10p; $n = 4$) or LPS plus chymostatin (Chym; $n = 5$). Data are expressed as mean $\pm$ s.e.mean of n observations. *$P < 0.05$ represents significant difference when compared to LPS-controls.

Table 1 Effect of calpain inhibitor I on the rises in the serum concentrations of lipase, γGT, glucose and lactate elicited by LPS in the rat

Animal groups	*Lipase* (iu l^{-1})	*γGT* (iu l^{-1})	*Glucose* (mg dl^{-1})	*Lactate* (mg dl^{-1})
C (vehicle)	18±4	1.3±0.5	119±2	13±0.5
LPS	92±8	11.3±1.9	55±4	39±4
Dex (1 mg kg^{-1})	42±7*	2.2±0.4*	105±5*	18±3*
Cal-I (3 mg kg^{-1})	66±10*	9.6±1.5	71±6	34±3
Cal-I (10 mg kg^{-1})	54±9*	5.8±1.1*	96±7*	24±3*
Cal-I (10 mg kg^{-1}) post LPS	87±8	8.7±0.9	48±6	37±5
Chym (10 mg kg^{-1})	83±10	8.5±0.9	37±5	41±4

The serum concentrations of lipase, γGT, glucose and lactate were measured 6 h after injection of LPS (10 mg kg^{-1}, i.v.) or its vehicle. Animals received vehicle alone (50% ethanol/PBS, 1 ml kg^{-1}, i.p.; $n=4$), vehicle plus LPS ($n=10$), LPS plus dexamethasone (Dex,1 mg kg^{-1}, i.p.; $n=6$), LPS plus calpain inhibitor I (Cal-I, 3 mg kg^{-1}, i.p.; $n=6$), LPS plus high dose of calpain inhibitor I (Cal-I, 10 mg kg^{-1}, i.p.; $n=7$), LPS plus delayed injection of calpain inhibitor I (Cal-I, 10 mg kg^{-1}, i.p. 2 h after LPS; $n=4$) or LPS plus chymostatin (Chym, 10 mg kg^{-1}, i.p.; $n=5$). Data are expressed as mean ±s.e.mean of n observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

potension and vascular hyporeactivity to NA) was not affected by administration of calpain inhibitor I (10 mg kg^{-1}) given 2 h after LPS (late administration) (Figure 2b and c). Similarly, chymostatin (10 mg kg^{-1}) also did not affect the circulatory failure caused by LPS (Figure 2).

The administration of calpain inhibitor I (10 mg kg^{-1}, i.p., 2 h before LPS, $n=3$) without subsequent injection of LPS had no effects on any of the haemodynamic parameters studied (data not shown).

Effects of calpain inhibitor I on the multiple organ dysfunction syndrome caused by LPS in the rat

Endotoxaemia for 360 min was associated with a significant rise in the plasma levels of urea, creatinine (indicators of renal failure), bilirubin, the amino transferase ALT and AST, γGT (all indicators of liver injury or failure) and lipase (an indicator of pancreatic injury) ($P<0.05$; Figure 3, Table 1). In addition, endotoxaemia also resulted in substantial increases in the plasma levels of lactate and reductions in the plasma levels of glucose ($P<0.05$; Table 1). Dexamethasone, but not calpain inhibitor 1, reduced the rise in the plasma levels of urea and creatinine caused by endotoxin. In contrast, pretreatment of LPS-injected rats with calpain inhibitor I (3 or 10 mg kg^{-1}, i.v.) attenuated the rises in the plasma levels in bilirubin, ALT, AST and γGT, while the late administration of calpain inhibitor I (2 h after LPS) or chymostatin had no effect. Calpain inhibitor I (3 or 10 mg kg^{-1}) also reduced the increase in the plasma levels of lipase caused by endotoxin and (at 10 mg kg^{-1}) prevented the LPS-induced alterations in plasma glucose and lactate (Table 1).

The administration of calpain inhibitor I (10 mg kg^{-1}, i.p., $n=3$) without subsequent injection of LPS had no effect on any of the parameters of organ dysfunction/failure studied (data not shown).

The effect of calpain inhibitor I on the production of tumor necrosis factor α elicited by LPS in the rat

Injection of LPS resulted in an increase in the serum level of TNFα from below the detection limit (35 pg ml^{-1}) to 5757±442 (pg ml^{-1}) at 90 min (Figure 4). Dexamethasone, but not calpain inhibitor I or chymostatin, attenuated the rise in the serum level of TNFα (Figure 4).

The administration of calpain inhibitor I (10 mg kg^{-1}, i.p., $n=3$) without subsequent injection of LPS had no effect on the serum levels of TNFα (data not shown).

The effect of calpain inhibitor I on the expression of inducible nitric oxide synthase in the lung and liver of rats with endotoxaemia

Endotoxaemia for 6 h was associated with a substantial increase in iNOS activity in lung and liver homogenates

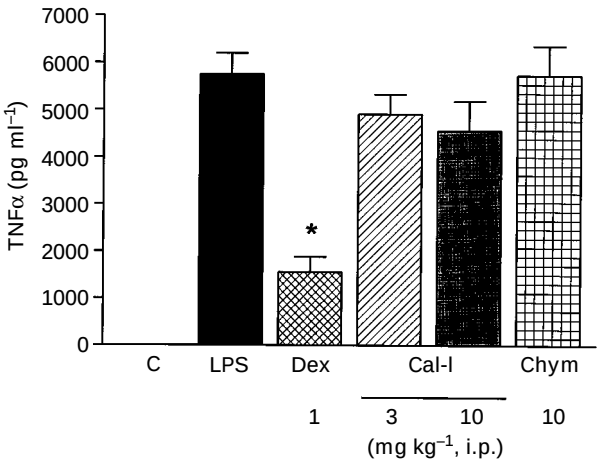

Figure 4 Effect of calpain inhibitor I on the increase of the plasma levels of tumour necrosis factor-α (TNFα) caused by LPS in the rat. Different groups of animals received vehicle for LPS (C, $n=4$), LPS alone (LPS, $n=10$), LPS plus dexamethasone (Dex, $n=6$), LPS plus 3 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 3; $n=6$) or 10 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 10; $n=7$) or LPS plus chymostatin (Chym; $n=5$). Please note that the plasma levels of TNFα in rats treated with vehicle rather than LPS (C) were below the detection limit. Data are expressed as mean±s.e.mean of n observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

($P<0.05$, Figure 5). Pretreatment of LPS-rats with dexamethasone or calpain inhibitor I (3 or 10 mg kg^{-1}) attenuated the increase in iNOS activity in lung and liver homogenates. In contrast, injection of calpain inhibitor I 2 h after LPS or chymostatin had no effect on the iNOS activity in these tissues (Figure 5). In addition, LPS also caused an increase in the expression of iNOS protein in lung and liver homogenates (Figure 6), which was significantly reduced by pretreatment with dexamethasone or calpain inhibitor I (10 mg kg^{-1}), but not by injection of calpain inhibitor I, 2 h after LPS, or chymostatin (Figure 6).

The administration of calpain inhibitor I (10 mg kg^{-1}, i.p., $n=3$) without subsequent injection of LPS had no effect on iNOS activity or protein expression (data not shown).

Calpain inhibitor I inhibits the increase in the serum levels of 6-keto-$PGF_{1\alpha}$ and the expression of COX-2 protein in the lung and liver of rats with endotoxaemia

Endotoxaemia for 6 h was associated with an 8 fold increase in the release into the serum of 6-keto-$PGF_{1\alpha}$ from 1.0±0.2 to 8.1±1.7 (pmol ml^{-1}) ($P<0.05$, Figure 7). Pretreatment of LPS-rats with dexamethasone or calpain inhibitor I abolished the increase in 6-keto-$PGF_{1\alpha}$ caused by LPS (Figure 7). In

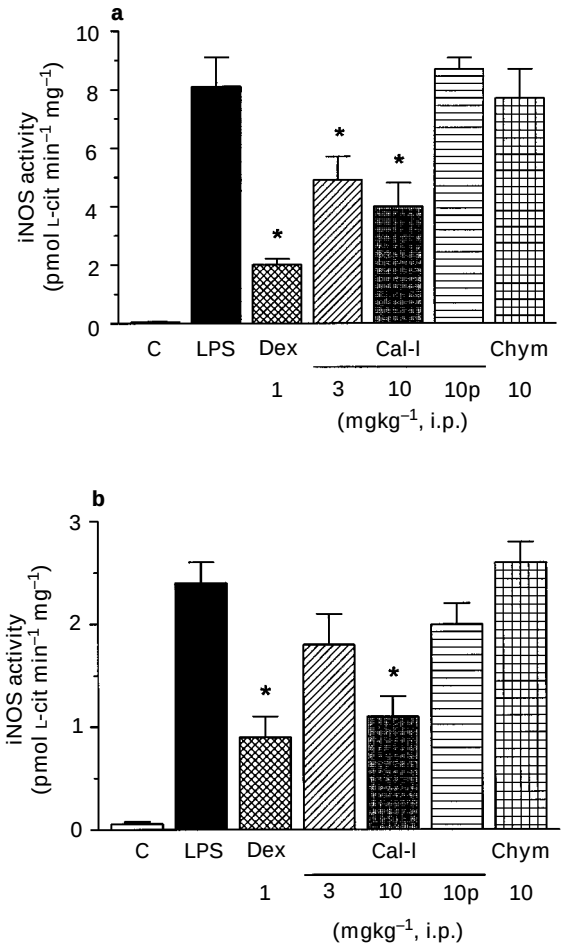

Figure 5 Effect of calpain inhibitor I on iNOS activity in (a) lung and (b) liver (measured 6 h after LPS). Different groups of animals received vehicle for LPS (C, $n=4$), LPS alone (LPS, $n=10$), LPS plus dexamethasone (Dex, $n=6$), LPS plus 3 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 3; $n=6$) or 10 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 10; $n=7$), LPS plus late administration, 2 h after LPS, of 10 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 10p; $n=4$) or LPS plus chymostatin (Chym; $n=5$). Data are expressed as mean ± s.e.mean of n observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

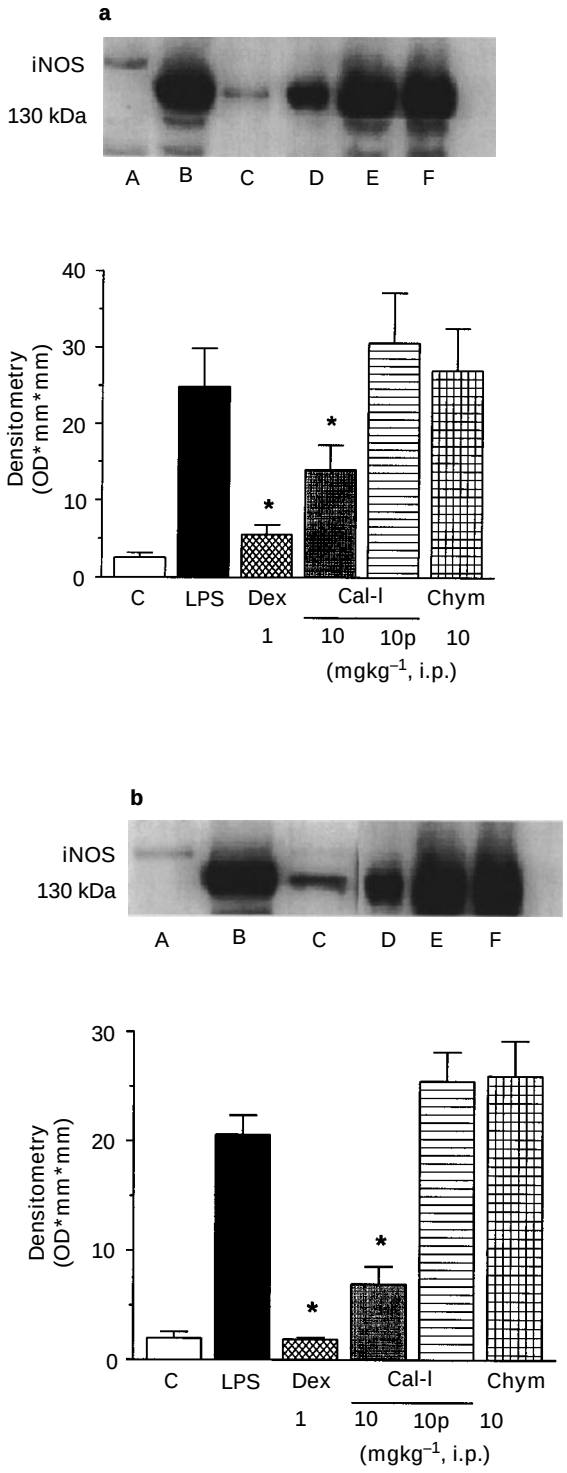

Figure 6 Effect of calpain inhibitor I on expression of iNOS protein in (a) lung and (b) liver (measured 6 h after LPS). (a) and (b) Representative Western blots (for each individual group of animals) of iNOS protein as well as the respective densitrometric analysis (of 3 Western blots per group) for lung and liver, respectively. Different groups of animals received vehicle for LPS (lane A of C, $n=4$), LPS alone (lane B or LPS, $n=10$), LPS plus dexamethasone (lane C or Dex, $n=6$), LPS plus 10 mg kg^{-1} (i.p.) calpain inhibitor I (lane D or Cal-I, 10; $n=7$), LPS plus late administration, 2 h after LPS, of 10 mg kg^{-1} (i.p.) calpain inhibitor I (lane E or Cal-I, 10p; $n=4$) or LPS plus chymostatin (lane F or Chym; $n=5$). *$P<0.05$ represents significant difference when compared to LPS-controls.

contrast, injection of calpain inhibitor I 2 h after LPS or pretreatment of rats with chymostatin had no effect on the production of 6-keto-PGF$_{1\alpha}$ (Figure 7). In addition, LPS also caused the expression of COX-2 protein in lung and liver homogenates (Figure 8); this effect significantly reduced by pretreatment with dexamethasone or calpain inhibitor I (10 mg kg^{-1}), but not by injection of calpain inhibitor I 2 h after LPS or pretreatment of rats with chymostatin (Figure 8).

The administration of calpain inhibitor I (10 mg kg^{-1}, i.p., $n=3$) without subsequent injection of LPS had no effect on COX-2 activity or protein expression (data not shown).

Discussion

Calpain inhibitor I, an inhibitor of cysteine proteases, blocks the degradation of IκB-α and, hence, prevents the activation of NF-κB but does not affect the upstream signal-induced phosphorylation of IκB-α (Lin *et al.*, 1995).

This paper demonstrates that calpain inhibitor I attenuates the formation of nitrite in cultured macrophages activated with LPS in a concentration-dependent fashion. The formation of nitrite is due to enhanced formation of NO secondary to the induction of iNOS, as it is attenuated by dexamethasone (an agent which prevents the induction of iNOS; Radomski *et al.*, 1990) and by selective inhibitors of iNOS activity (Southan *et al.*, 1995; Kengatharan *et al.*, 1996). The reduction by calpain inhibitor I of the formation of nitrite is likely to be due to

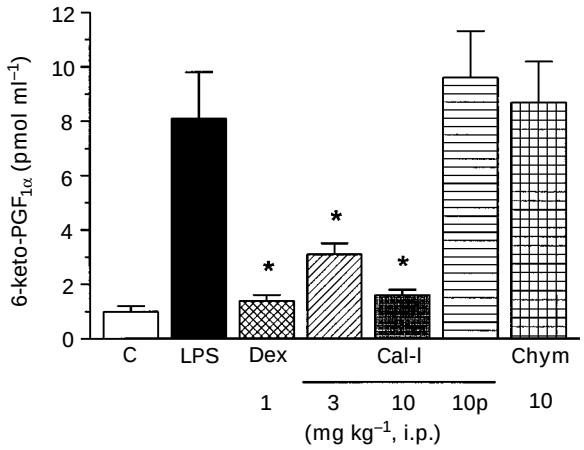

Figure 7 Calpain inhibitor I inhibits the increase in the serum levels of 6-keto-PGF$_{1\alpha}$ caused by endotoxin in the rat. Different groups of animals received vehicle for LPS (C, $n=4$), LPS alone (LPS, $n=10$), LPS plus dexamethasone (Dex, $n=6$), LPS plus 10 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 10; $n=7$), LPS plus late administration, 2 h after LPS, 10 mg kg^{-1} (i.p.) calpain inhibitor I (Cal-I, 10p; $n=4$) or LPS plus chymostatin (Chym; $n=5$). Data are expressed as mean$\pm$s.e.mean of n observations. *$P<0.05$ represents significant difference when compared to LPS-controls.

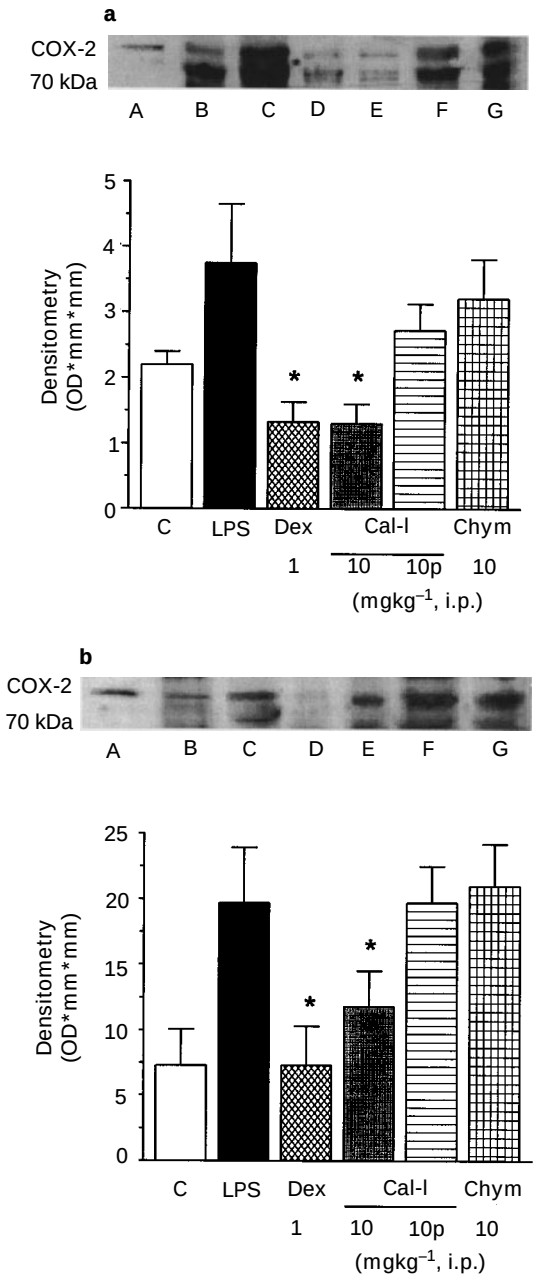

Figure 8 Effect of calpain inhibitor I on the expression of COX-2 protein in (a) lung and (b) liver of rats with endotoxaemia. (a) and (b) Representative Western blots (for each individual group of animals) of COX-2 protein as well as the respective densitrometric analysis (of 3 Western blots per group) for lung and liver, respectively. Different groups of animals received vehicle for LPS (lane A or C, $n=4$), LPS alone (lane B or LPS, $n=10$), LPS plus dexamethasone (lane C or Dex, $n=6$), LPS plus 10 mg kg^{-1} (i.p.) calpain inhibitor I (lane D or Cal-I, 10; $n=7$), LPS plus late administration, 2 h after LPS, of 10 mg kg^{-1} (i.p.) calpain inhibitor I (lane E or Cal-I, 10p; $n=4$) or LPS plus chymostatin (lane F or Chym; $n=5$). *$P<0.05$ represents significant difference when compared to LPS-controls.

inhibition of IκB proteolysis, as TPCK and ZPCK, two chemically distinct inhibitors of IκB proteolysis, also caused a concentration-dependent inhibition of nitrite formation in macrophages activated with LPS. In contrast, inhibition of the activity of serine or cysteine proteases is less likely to account for the effects of calpain inhibitor I on nitrite formation, as chymostatin and leupeptin – two chemically distinct inhibitors of serine and cysteine proteases (Lin *et al*., 1995) – did not attenuate the increase in nitrite formation by macrophages challenged with LPS. Our findings therefore support the hypothesis (Griscavage *et al*., 1996) that the expression of iNOS caused by LPS in macrophages involves the activation of NFκB.

This study provides the first evidence that calpain inhibitor I attenuates the circulatory failure (hypotension and vascular hyporeactivity to vasoconstrictor agents) caused by endotoxin in anaesthetized rats. The beneficial haemodynamic effects of calpain inhibitor I were dose-related and similar to those elicited by dexamethasone. Interestingly, calpain inhibitor I was only able to attenuate the circulatory failure caused by LPS when given 2 h before injection of LPS, but failed to exert any beneficial haemodynamic effects when given 2 h after the onset of endotoxaemia. Similarly, (other) agents which attenuate the induction of iNOS protein exert beneficial effects in rodent models of endotoxic shock when given before, but not 2 h after injection of LPS (Thiemermann, 1994).

The progression of shock to multiple organ dysfunction syndrome (MODS) is associated with an increase in the mortality such that with the number of organs failing (from 1–4), mortality progressively increases from 30% (in the absence of MODS) to 100% (Baue, 1993). In this rat model of endotoxic shock, six hours of endotoxaemia resulted in a substantial increase in the plasma levels of urea and creatinine and, hence, acute renal failure. Although the rises in the plasma levels of urea and creatinine were reduced by dexamethasone, calpain inhibitor I did not attenuate the acute renal failure caused by LPS. Prolonged periods of endotoxaemia were also associated with substantial rises in the plasma levels of ALT, AST, γGT and bilirubin indicating the development of acute liver injury (ALT, AST, γGT) and dysfunction (bilirubin) (Bau, 1993). Like dexamethasone, pretreatment of rats with calpain inhibitor I largely attenuated the acute liver dysfunction and injury associated with endotoxic shock. In contrast, the late administration (2 h after LPS) of calpain inhibitor I was without effect. Endotoxaemia also resulted in (i) an increase in the plasma activity of lipase, an indicator of pancreatic injury, (ii) a fall in glucose (hypoglycaemia) and, most importantly, (iii) an increase in the plasma levels of lactate demonstrating the development of tissue hypoxia, presumably secondary to an impairment in tissue oxygen extraction. Pretreatment of rats with calpain inhibitor I largely attenuated the pancreatic injury, the hypoglycaemia and the acidosis associated with endotoxaemia.

There are several explanations for the effects of calpain inhibitor I in modifying the circulatory failure and MODS

associated with endotoxaemia. one could argue that some of the effects of calpain inhibitor I are due to the ability of this agent to inhibit the activity of serine or cysteine proteases. However, this is unlikely as chymostatin, a potent inhibitor of such proteases (Lin *et al*., 1995), did not affect either the circulatory failure or the MODS caused by LPS. Thus, inhibition of protease activity is unlikely to account to the beneficial effects of calpain inhibitor I observed in this study. We therefore propose that the effects of calpain inhibitor I are due to the inhibition of the activation of the transcription factor NFκB. Although this conclusion appears likely, it should be noted that we have not investigated whether the doses of calpain inhibitor I used in this study do, indeed, attentuate the activation of NFκB *in vivo*.

Activation of the transcription factor NFκB plays an important role in the expression of iNOS (Xie *et al*., 1994; Griscavage *et al*., 1995; 1996; this study). An enhanced formation of NO by iNOS contributes to the circulatory failure, the liver injury and dysfunction as well as the pancreatic dysfunction caused by LPS in the anaesthetized rat (Thiemermann *et al*., 1995; Ruetten *et al*., 1996). We demonstrated here that inhibition of the activation of NFκB by calpain inhibitor I attenuates the increase in iNOS protein and activity in lungs and livers of rats with endotoxic shock. Thus, the reduction of the expression of iNOS by calpain inhibitor I may contribute to (or account for) the attenuation by this agent of the circulatory failure and the organ dysfunction and injury caused by LPS in the rat. This conclusion is supported by the finding that calpain inhibitor I, like several chemically distinct inhibitors of iNOS activity, attenuated the liver and pancreatic dysfunction, but not the renal failure caused by LPS in the rat.

TNFα causes the activation of NFκB (Scheinman *et al*., 1995) which, in certain cells, may result in the induction of iNOS (see above). Endogenous TNFα also mediates the induction of iNOS (Thiemermann *et al*., 1993) and, hence the circulatory collapse (Mozes *et al*., 1991; Thiemermann *et al*., 1993) as well as the liver injury (De La-Mata *et al*., 1990; Hewett *et al*., 1993) caused by endotoxin. We did not find a significant reduction in the plasma levels of TNFα in LPS-rats treated with calpain inhibitor I. Thus, a reduction of the formation of TNFα does not explain the inhibition by calpain inhibitor I of the expression of iNOS (and COX-2; see below) protein.

The promotor region of the murine and human COX-2 genes contain binding sites for NFκB (Sirois *et al*., 1993; Appleby *et al*., 1994). The expression of the COX-2 gene is activated by oxidant stress (Feng *et al*., 1995) and reactive oxygen intermediates cause the activation of NFκB (Schreck *et al*., 1991) suggesting that NFκB is one of the transcription factors involved. The increase in prostaglandin formation (COX activity) by murine osteoblasts (cell line MS3T3-E1) involves the activation of NFκB (Yamamoto *et al*., 1995). Here, we demonstrated that calpain inhibitor I attenuates the expression of COX-2 protein (in lung and liver) as well as the increase in the plasma levels of 6-keto-$PGF_{1\alpha}$ caused by endotoxin in the anaesthetized rat. The reduction of the expression of COX-2 protein and activity by calpain inhibitor I is associated with beneficial haemodynamic effects, but it is unclear whether the formation of arachidonic metabolites (by COX-2) contributes importantly to the pathophysiology of septic shock. Although there is some evidence that prostaglandins contribute to the haemodynamic alterations and the liver injury associated with endotoxic shock (Feuerstein & Hallenbeck, 1987), the effects of selective inhibitors of COX-2 activity (Griswold & Adams, 1996) on circulatory failure or MODS have not been investigated. Moreover, there are (to our knowledge) no studies evaluating the effects of antisense oligonucleotides against COX-2 mRNA in animal models of shock. Therefore, we cannot exclude the possibility that an enhanced formation of arachidonic acid metabolites by COX-2 contributes to the observed pathophysiology. It is impossible to predict if, and to what degree, reduction of the expression of COX-2 protein and activity contributes to the beneficial effects of calpain inhibitor I in rats with endotoxic shock. However, there is evidence that the formation of arachidonic acid metabolites by COX-2 contributes to the inflammatory response caused by injection of carageenan into a subcutaneous air pouch of the rat, as this response is blocked by NS-393 as well as dexamethasone (Masferrer *et al*., 1994). Thus, we propose that the reduction by calpain inhibitor I of the expression of COX-2 (caused e.g. by cytokines) results in a potent anti-inflammatory effect of this inhibitor of IκB-α proteolysis.

Like calpain inhibitor I, dexamethasone reduced the expression of iNOS and COX-2 and attenuated the circulatory failure, the hepatocellular injury and the pancreatic injury caused by LPS. Indeed, the effects of dexamethasone and calpain inhibitor I observed in this study were, with two exceptions ((1) prevention of renal failure by dexamethasone, but not calpain inhibitor I; (2) reduction of the rise in the serum levels of TNFα by dexamethasone, but not calpain inhibitor I), very similar suggesting a similar mechanism of action. Indeed, dexamethasone induces the transcription of the IκB α gene resulting in an increase in the synthesis of IκB α protein. Stimulation by TNFα causes the release of NFκB from IκB α (i.e. the activation of NFκB). In cell pretreated with dexamethasone, the NFκB released by TNFα rapidly reassociates with (the newly synthesized) IκB α. (Scheinman *et al*., 1995). Thus, both dexamethasone and calpain inhibitor I significantly reduce the amounts of NFκB which are able to translocate to the nucleus to initiate transcription of genes including those for COX-2 and iNOS. Indeed, there is some recent evidence that the prevention by dexamethasone of the expression of iNOS is also secondary to the prevention (by dexamethasone) of the activation of NFκB (Kleinert *et al*., 1996).

In conclusion, we demonstrated that calpain inhibitor I and dexamethasone, but not the serine and cysteine protease inhibitor, chymostatin, attenuate (i) the circulatory failure (hypotension and vascular hyporeactivity to noradrenaline), (ii) the multiple organ dysfunction (liver and pancreatic injury/dysfunction, increase in lactate, hypoglycemia) and (iii) the induction of iNOS and COX-2 protein and activity (in lung and liver) of rats with endotoxic shock. We propose that the reduction of the expression of iNOS and possibly COX-2 contributes to the beneficial effects of calpain inhibitor I observed. Our results support the view that attenuation or prevention of the activation of NFκB by calpain inhibitor I may be useful in the therapy of circulatory shock or of disorders associated with local or systemic inflammation.

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