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Role for intracellular platelet-activating factor in the circulatory failure in a model of Gram-positive shock

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1 This study investigates the effects of two structurally different antagonists of platelet-activating factor (PAF), BN52021 and WEB2086, on the circulatory and renal failure elicited by lipoteichoic acid (LTA) from *Staphylococcus aureus* (an organism without endotoxin) in anaesthetized rats.

2 Administration of LTA (10 mg kg^{-1}, i.v.) caused hypotension and vascular hyporeactivity to noradrenaline (1 $\mu g\ kg^{-1}$, i.v.). WEB2086 (5 mg kg^{-1}, i.v., 20 min before and 150 min after LTA) inhibited the delayed fall in mean arterial blood pressure (at 300 min: 99 ± 6 mmHg vs. 75 ± 6 mmHg, $P<0.01$) and prevented the decrease in pressor response to noradrenaline (at 300 min: 36 ± 5 mmHg min vs. 17 ± 5 mmHg min, $P<0.01$). Surprisingly, BN52021 (20 mg kg^{-1}, i.v., 20 min before and 150 min after LTA) neither prevented the hypotension (74 ± 6 mmHg) nor the vascular hyporeactivity (21 ± 5 mmHg min). However, BN52021 inhibited the hypotension to injections of PAF as well as the circulatory failure elicited by lipopolysaccharides (10 mg kg^{-1}, i.v.).

3 LTA caused an increase in plasma concentration of creatinine from 39 ± 5 μM (sham-operated) to 70 ± 8 μM and urea from 4.7 ± 0.1 to 13.1 ± 1.6 mM. The renal failure elicited by LTA was significantly inhibited by WEB2086 (creatinine: 45 ± 4 μM and urea: 5.7 ± 0.7 mM), but not by BN52021.

4 The induction of nitric oxide synthase activity in lungs by LTA was attenuated by WEB2086 from 98 ± 17 to 40 ± 15 pmol L-citrulline 30 min^{-1} mg^{-1} protein ($P<0.01$), but not by BN52021 (148 ± 21 pmol L-citrulline 30 min^{-1} mg^{-1} protein). Similarly, WEB2086, but not BN52021, inhibited the increase in plasma nitrite concentration associated with the delayed circulatory failure caused by LTA. The release of tumour necrosis factor-α (TNF-α) after injection of LTA was not attenuated by WEB2086.

5 The induction of nitrite release by cultured macrophages activated with LTA (10 $\mu g\ ml^{-1}$ for 24 h) was inhibited by $74 \pm 4\%$ by WEB2086 (3×10^{-4} M), but not by BN52021, indicating that only WEB2086 acts on intracellular PAF receptors.

6 Thus, the intracellular release of PAF contributes to the circulatory and renal failure and induction of nitric oxide synthase elicited by LTA in anaesthetized rats. The difference between the two structurally different PAF antagonists in our septic shock models using either LTA or lipopolysaccharide (LPS), shows the importance of models for Gram-positive sepsis in the elucidation of the pathophysiology of septic shock and for the evaluation of potential drugs.

Keywords: Septic shock; circulatory failure; platelet-activating factor; nitric oxide

Introduction

Platelet-activating factor (PAF) is a lipid mediator with a wide variety of pharmacological actions. When given exogenously to animals, PAF mimics many of the pathophysiological features of septic shock (for reviews, see Braquet *et al.*, 1987; Sánchez Crespo & Fernández-Gallardo, 1991; Koltai *et al.*, 1994). For instance, in various species, intravenous injection of PAF results in cardiovascular dysfunction, including hypotension, peripheral vasodilatation and plasma extravasation, and in multiple organ failure, such as a reduced kidney and lung function. In septic patients and animal models of endotoxaemia, plasma levels of PAF are elevated (Doebber *et al.*, 1985; Inarrea *et al.*, 1985; Chang *et al.*, 1987; Lopez-Diez *et al.*, 1989) and structurally different PAF receptor antagonists reduce mortality in various animal models of Gram-negative sepsis (Floch *et al.*, 1989; Chang *et al.*, 1990; Fletcher *et al.*, 1990; Qi & Jones, 1990). Other beneficial effects of PAF receptor antagonists in endotoxaemia or Gram-negative sepsis include the prevention of acute and delayed systemic hypotension, vascular hyporeactivity to catecholamines and lung injury (Doebber *et al.*, 1985; Casals-Stenzel, 1987; Chang *et al.*, 1987; Fletcher *et al.*, 1990; Qi & Jones, 1990; Rabinovici *et al.*, 1990; Torley *et al.*, 1992; Szabó *et al.*, 1993).

Production of the vasodilator and autacoid nitric oxide contributes importantly to the circulatory failure (hypotension and vascular hyporeactivity) in endotoxin shock (Julou-Schaeffer *et al.*, 1990; Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990). Nitric oxide is synthesized from L-arginine by constitutive (cNOS) or inducible (iNOS) nitric oxide synthases (for review, see Knowles & Moncada, 1994). The calcium-independent iNOS is induced in a variety of cells including macrophages and vascular smooth muscle cells by lipopolysaccharide (LPS), tumour necrosis factor (TNF)-α and interleukin (IL)-1. PAF is released in response to LPS, TNF-α, or IL-1, and, in turn, may mediate and/or enhance the release or action of these inflammatory cytokines (Pignol *et al.*, 1990; Poubelle *et al.*, 1991; Torley *et al.*, 1992; Ogata *et al.*, 1993). Interestingly, the PAF receptor antagonist WEB2086 (apafant; which blocks the effect of PAF intra- and extracellularly (Stewart *et al.*, 1990)) inhibits the induction of iNOS activity elicited by LPS in rats (Szabó *et al.*, 1993).

Most studies to elucidate the sequence of pathophysiological events in septic shock have employed LPS as a tool. Although, these studies help to gain a better understanding of the pathophysiology of Gram-negative shock, they may only provide a limited insight into the pathophysiology of circulatory shock caused by Gram-positive bacteria, for these organisms do not contain endotoxin. Interestingly, a cell wall component from *Staphylococcus aureus*, lipoteichoic acid (LTA), induces iNOS expression in anaesthetized rats and the

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subsequent enhanced formation of NO contributes importantly to the delayed hypotension and to the delayed hyporeactivity to noradrenaline elicited by LTA (De Kimpe *et al.*, 1995). The efficacy of BN52021 (ginkgolide B), an agent which selectively blocks extracellular PAF receptors (Marcheselli *et al.*, 1990), has been investigated in a randomised, placebo-controlled, double-blind, multicentre trial using 262 patients with severe Gram-positive and Gram-negative sepsis (Tenaillon *et al.*, 1993). In this study, no significant reduction in overall mortality was observed, but the mortality rate in the subset of 119 patients with Gram-negative sepsis receiving BN52021 was decreased by 42%. In the absence of documented Gram-negative infection no difference was observed.

The aim of the present study was to establish the role of PAF in a model of Gram-positive shock. Therefore, the effect of the PAF receptor antagonists WEB2086 and BN52021 on the cardiovascular dysfunction (hypotension and vascular hyporeactivity) and induction of iNOS elicited by LTA was investigated. In addition, we studied whether the renal failure elicited by LTA is prevented by treatment with these PAF antagonists.

Methods

Haemodynamic measurements

Animal experiments were performed in accordance with the Home Office regulations of the United Kingdom. Male Wistar rats (200–300 g, Glaxo Laboratories Ltd, Greenford, Middlesex, UK) were anaesthetized with thiopentone sodium (Trapanal, 3% solution, 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration, and rectal temperature was maintained at 37°C by using a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated with a catheter filled with heparinised saline (50 u ml^{-1} heparin in 0.9 M NaCl) and connected to a pressure transducer (model P23XL, Spectramed, Stratham, NH) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate, which were registered on a polygraph recorder (model 7D, Grass Instruments, Quincy, Mass). The right jugular vein was cannulated for the administration of drugs. After the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min.

Experimental procedure for LTA-induced shock

After recording baseline haemodynamic parameters, rats received the PAF antagonist WEB2086 (mg kg^{-1}, i.v., 20 min before LTA) or BN52021 (20 mg kg^{-1}, i.v., 20 min before LTA) or vehicle (5 mM HCl in saline for WEB2086 or BN52021-vehicle supplied by the manufacturer). After 15 min, the pressor response to noradrenaline (1 μg kg^{-1}, i.v.) was determined. Subsequently, animals received vehicle (0.2 ml 0.9% NaCl, i.v.) or LTA from *Staphylococcus aureus* (10 mg kg^{-1}, i.v.). Haemodynamic parameters were measured for 300 min. At 160 min after LTA, rats were injected for a second time with WEB2086 (5 mg kg^{-1}, i.v.) or BN52021 (20 mg kg^{-1}, i.v.) or vehicle. The pressor responses to noradrenaline were reassessed at 60 and 300 min after the administration of LTA.

Thus, the following groups were studied: (i) vehicle for WEB2086 ($n=4$) or vehicle for BN52021 ($n=4$) plus LTA. As none of the results obtained with vehicle for WEB2086 differed significantly from those obtained with vehicle for BN52021, these two groups were pooled to form one vehicle plus LTA-control group ($n=8$). (ii) WEB2086 plus LTA ($n=8$); (iii) BN52021 plus LTA ($n=5$); (iv) vehicle for LTA only ($n=5$); (v) WEB2086 plus vehicle for LTA ($n=6$); and (vi) BN52021 plus vehicle for LTA ($n=4$).

At the end of the experiment, plasma was collected for the measurement of nitrite (a primary break-down product of nitric oxide), serum was obtained for the measurement of urea and creatinine (indicative of kidney function), and lungs were removed from the animal for the measurement of iNOS activity. Plasma and lungs were snap frozen in liquid nitrogen and stored at −80°C until assayed (see below). Serum samples were analysed within 24 h by a contract laboratory for veterinary, clinical chemistry (Vetlab Services, Sussex, U.K.), for creatinine (an indicator of reduced glomerular filtration rate) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism).

Experimental procedure for LPS-induced shock

PAF contributes to the induction of iNOS-activity in anaesthetized rats injected with LPS (Szabó *et al.*, 1993). As a positive control, we investigated the effects of BN52021 on the circulatory failure and induction of iNOS elicited by LPS. After surgery and stabilization of haemodynamic parameters, rats received BN52021 (20 mg kg^{-1}, i.v., 20 min before LPS, $n=5$) or vehicle (supplied by the manufacturer, $n=5$). After 15 min, the pressor response to noradrenaline (1 μg kg^{-1}, i.v.) was determined. Subsequently, animals received LPS from *Escherichia coli* serotype 0127:B8 (10 mg kg^{-1}) as a slow injection over 10 min. Haemodynamic parameters were monitored for 180 min. The pressor responses to noradrenaline were reassessed at 60, 120 and 180 min after the administration of LPS. At the end of the experiment, plasma was collected for the measurement of nitrite, and lungs were removed for the measurement of iNOS activity. Plasma and lungs were snap frozen in liquid nitrogen and stored at −80°C until assayed (see below).

Experimental procedure for PAF-induced hypotension

To confirm that both PAF-antagonists were equipotent at the dose studied in this study, the ability of WEB2086 and BN52021 to inhibit the hypotension elicited by intravenous administration of PAF was investigated. Rats received either PAF alone (3 to 300 ng kg^{-1}) or PAF after pretreatment with WEB2086 (5 mg kg^{-1}, i.v.) or BN52021 (20 mg kg^{-1}, i.v.) 20 min before the first injection of PAF. Between subsequent injections of PAF, sufficient time was allowed for the blood pressure to return to baseline values (at least 10 min). The maximal decrease in blood pressure as well as the 50% recovery time from the hypotension caused by PAF (defined by the time period from injection to the time point where the decrease in blood pressure was recovered to 50% of the maximal response) was determined.

Measurement of plasma TNF-α levels

Rats were pretreated with WEB2086 (5 mg kg^{-1}, i.v., 20 min before LTA), or BN52021 (20 mg kg^{-1}, i.v., 20 min before LPS), or vehicle (see above). LTA (10 mg kg^{-1}, i.v., in experiments with WEB2086) or LPS (10 mg kg^{-1}, i.v., in experiments with BN52021) was administered and after 90 min plasma samples were taken, snap frozen in liquid nitrogen and stored at −80°C until assayed for TNF-α. Previous experiments by us and others (Rabinovici *et al.*, 1990; De Kimpe *et al.*, 1995), have demonstrated that TNF-α concentration in plasma is maximal at approximately 90 min after injection of LTA or LPS. TNF-α was measured in the plasma by a mouse TNF-α ELISA kit from Genzyme (Cambridge, MA, U.S.A.) which has also been used successfully to quantitate natural rat TNF-α (De Kimpe *et al.*, 1995). The samples were measured according to the instructions supplied with the ELISA kit by the supplier.

Plasma nitrite concentration

The nitrite concentrations in plasma were determined as an indicator of changes in NO production. Nitrite was assayed by adding 0.8 ml Griess reagent (0.5% sulphanilamide and 0.05% naphthylenediamine in 2.5% phosphoric acid) to 0.2 ml plas-

ma. After centrifugation, the difference in optical density between 540 nm and 600 nm was measured by a spectrophotometer. Nitrite concentrations (μM) were calculated by comparison with the optical density of standard solutions of sodium nitrite prepared in plasma.

iNOS activity assay

Frozen lung was homogenized on ice using an Ultra-Turrax T 25 in a Tris-buffer (pH = 7.4) composed of (mM): Tris-HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulphonylfluoride 1. The induction of iNOS activity was measured in the homogenates by the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline in the absence of calcium. Tissue homogenates (30 μl, approximately 100 μg protein) were incubated for 30 min in the presence of L-arginine/[^{3}H]-L-arginine (10 μM, 7.4 kBq per tube), NADPH (1 mM), calmodulin (300 u ml^{-1}), tetrahydrobiopterin (5 μM), L-valine (50 mM) and EGTA (1 mM) at room temperature in Tris-buffer (total reaction volume 100 μl). Reactions were stopped by addition of 1 ml ice-cold HEPES buffer (pH = 5.5) containing HEPES (20 mM), EDTA (2 mM) and EGTA (2 mM). Reaction mixtures were applied to DOWEX 50 W (sodium form) columns, and the eluted [^{3}H]-L-citrulline was measured by scintillation counting (model, Beckman Instruments Inc, Fullerton, California). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-citrulline formation independent of NOS activity. Protein concentrations were measured spectrophotometrically in 96-well plates by the method of Bradford using bovine serum albumin as standards (Bradford, 1976).

Induction of iNOS in cultured murine macrophages

Murine macrophages (J774.2 cell line) were cultured to confluency in 96-wells containing 200 μl DMEM (Dulbeco's Modified Eagle Medium) supplemented with 10% foetal calf serum. To induce NOS, macrophages were activated with LTA (10 μg ml^{-1}). After 24 h, nitrite accumulation was assayed by addition of 100 μl Griess reagent to 100 μl of cell culture supernatant. Using a microplate reader, the difference in optical density at 550 nm and 650 nm was measured and compared to the difference in optical density of standard concentrations of sodium nitrite to calculate the nitrite concentration in the supernatant. WEB2086 or BN52021 were added to the wells 20 min before LTA (final concentration $10^{-6}-3\times10^{-4}$ M).

Drugs

BN52021 (ginkgolide B) was a gift of Dr P. Braquet (Institute Beaufour, France) and apafant (3-[4-(-chlorophenyl)-9-methyl-6*H*-thieno[3,2-f]-[1,2,4]triazolo-[4,3-a][1,4]-diazepin-2-yl]-1-(4-morphonilyl)-1propanon), referred to as WEB2086, was provided by Boehringer Ingelheim (Bracknell, Berkshire, U.K.). Unless stated otherwise, all other compounds were purchased from Sigma (Dorset, U.K.). Heparin (Multiparin) was obtained from Evans Medical (Middlesex, U.K.) and thiopentone sodium (Intraval Sodium) from Rhône Mérieux Ltd. (Harlow, Essex, U.K.). L-(2,3,4,5-^{3}H)-arginine hydrochloride was obtained from Amersham (Buckinghamshire, U.K.). Tehtrahydrobiopterin (6R-L-erythro-5,6,7,8-tetrahydro-biopterin) was obtained from Dr B. Schircks Laboratories (Jona, Switzerland). Solutions for injection were prepared with nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.) and care was taken to prevent endotoxin contamination. PAF (Sigma) was dissolved in ethanol (1 mg ml^{-1}) and stored at −20°C. Dilutions in saline containing 0.25% bovine serum albumin were freshly prepared on the day of use.

Data analysis

All data are presented as mean ± s.e.mean of n observations. For macrophages, four independent experiments were performed in triplicate. The vasopressor response to noradrenaline was evaluated by determining the area under the curve and was expressed in mmHg min. Statistical analysis was performed by (one- or two-way) analysis of variance (ANOVA), when appropriate multiple comparison of single means was evaluated by use of Bonferroni's test for one-way ANOVA or Fisher's test for two-way ANOVA.

Results

Circulatory failure elicited by LTA

Injection of LTA (10 mg kg^{-1}, i.v.) resulted in an initial fall in MAP from 122 ± 5 mmHg (time 0, control) to 95 ± 7 mmHg at 60 min (n = 8, P < 0.01). This was followed by a second fall in MAP from 104 ± 8 mmHg at 120 min to 75 ± 6 mmHg at 300 min (Figure 1a). Treatment of rats with WEB2086 (5 mg kg^{-1}, i.v., 20 min before and 160 min after LTA, n = 8) did not significantly influence the early decrease in blood pressure at 60 min, but substantially reduced the delayed hypotension (Figure 1a). In contrast, treatment of rats with BN52021 (20 mg kg^{-1}, i.v., 20 min before and 160 min after LTA, n = 7) had no significant effect on the early and delayed hypotension elicited by LTA. Similarly, administration of LTA caused a significant attenuation of the pressor response

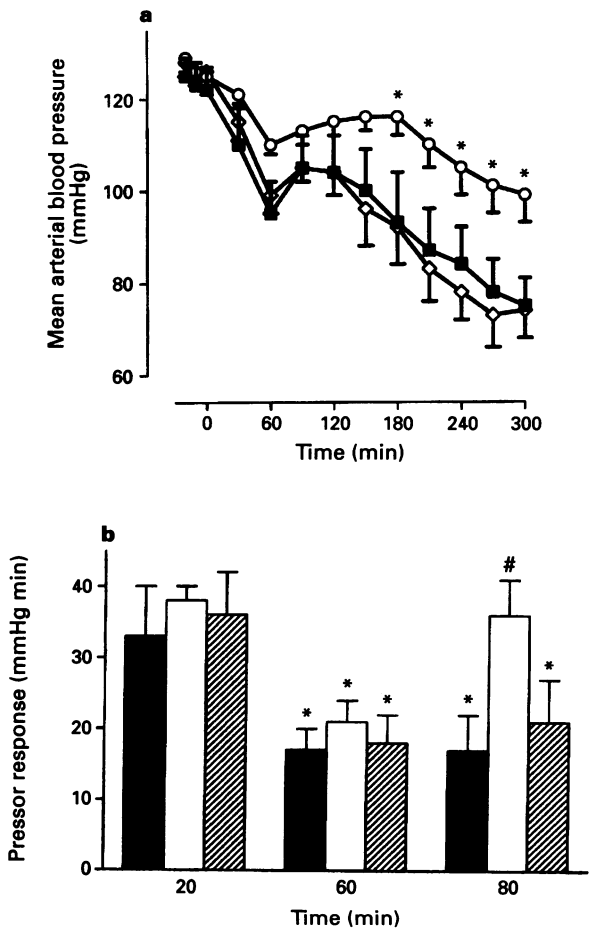

Figure 1 WEB2086, but not BN52021, inhibits hypotension (a) and hyporeactivity to noradrenaline (b; 1 μg kg^{-1}, i.v.) elicited by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.). Rats were treated with LTA (a; ■; b: solid columns; n = 8) or LTA and WEB2086 (5 mg kg^{-1}, i.v., 20 min before and 160 min after LTA) (a: ○; b: open columns; n = 8), or LTA and BN52021 (20 mg kg^{-1}, i.v., 20 min before and 160 min after LTA) (a: ◇; b: hatched columns; n = 7). Results are expressed as mean ± s.e.mean. (a): *P < 0.01 vs. LTA alone. (b), *P < 0.05 vs. time 0 and #P < 0.05 vs. LTA alone.

elicited by noradrenaline (1 $\mu g\ kg^{-1}$) at 60 and 300 min (Figure 1b). Neither WEB2086 nor BN52021 prevented the early vascular hyporeactivity to noradrenaline at 60 min after LTA. However, WEB2086, but not BN52021, prevented the decrease in pressor response to noradrenaline at 300 min after the administration of LTA (Figure 1b). There was no significant effect of LTA on heart rate (results not shown). Neither WEB2086 nor BN52021 (when given alone) had any effect on the haemodymic parameters when compared to sham-operated control rats (Table 1).

Induction of iNOS by LTA

At 300 min after LTA injection (10 mg kg^{-1}, i.v.), the calcium-independent iNOS activity in lung homogenates was strongly increased from 8 ± 2 pmol L-citrulline 30 min^{-1} mg^{-1} protein (sham-operated control rats) to 98 ± 17 pmol L-citrulline 30 min^{-1} mg^{-1} protein (LTA-treated rats; $n=8$; $P<0.01$; Figure 2). Additionally, plasma nitrite concentrations significantly increased from 1.7 ± 0.3 μM in sham-operated control rats ($n=5$) to 9.0 ± 1.1 μM in LTA-treated rats ($n=8$; $P<0.01$; Figure 2). Both the induction of iNOS activity and increase in plasma nitrite concentration elicited by LTA were significantly inhibited by treatment of the rats with WEB2086, but not with BN52021. Neither WEB2086 nor BN52021 (when given alone) had any effect on the iNOS activity and plasma concentration of nitrite when compared to sham-operated control rats (Table 1).

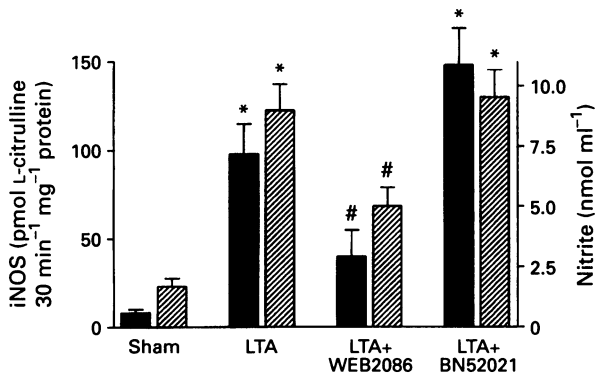

Figure 2 WEB2086, but not BN52021, inhibits the induction of nitric oxide synthase activity (iNOS, solid columns) and the increase in plasma nitrite concentration (hatched columns) by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.). The conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the absence of calcium and in the presence of EGTA (1 mM) in homogenates from lungs obtained from rats 300 min after the injection of LTA treated with WEB2086 (5 mg kg^{-1}, i.v., 20 min before and 160 min after LTA) or BN52021 (20 mg kg^{-1}, i.v., 20 min before and 160 min after LTA). Nitrite concentration in plasma was measured by the Griess method. Lungs and plasma were obtained from rats 300 min after injection of LTA or vehicle. Results are expressed as mean ± s.e.mean ($n=5-8$). *$P<0.01$ vs. sham-operated and $^{\#}P<0.01$ vs. LTA alone.

Renal failure elicited by LTA

Renal function was assessed by the measurement of serum creatinine and urea concentration. Injection of LTA (10 mg kg^{-1}, i.v.) resulted in a significant increase in serum levels of creatinine from 39 ± 5 μM ($n=5$) to 69 ± 8 μM ($n=5$; $P<0.01$) and urea from 4.7 ± 0.1 mM ($n=5$) to 13.1 ± 1.6 mM ($n=5$; $P<0.01$) at 300 min after vehicle or LTA, respectively (Figure 3). Both the increase in creatinine and urea levels elicited by LTA were prevented by WEB2086. In contrast, treatment with BN52021 had no significant effect on the increase in serum levels of creatinine or urea caused by LTA (Figure 3). Neither WEB2086 nor BN52021 (when given alone) had any effect on the plasma levels of creatinine or urea when compared to sham-operated control rats (Table 1).

Circulatory failure and induction of iNOS elicited by LPS

Intravenous injection of LPS (10 mg kg^{-1}) resulted in a rapid and sustained decrease in mean arterial blood pressure from 117 ± 6 mmHg (control, time 0) to 61 ± 7 mmHg at 60 min ($n=5$, $P<0.01$) and to 74 ± 11 mmHg at 180 min ($n=5$, $P<0.01$) (Figure 4a). Treatment of rats with BN52021 (20 mg kg^{-1}, i.v., 20 min before LPS, $n=5$) significantly inhibited the early hypotension at 60 min and the sustained hypotension at 180 min (Figure 4a). In addition, administration of LPS markedly attenuated the pressor response to noradrenaline at 60 and 180 min after injection (Figure 4b). Both, the early and the delayed hyporeactivity to noradrenaline were attenuated by treatment of the rats with BN52021.

Administration of LPS resulted in an induction of iNOS activity in lungs (213 ± 38 pmol L-citrulline 30 min^{-1} mg^{-1} protein, $n=5$), which was significantly inhibited by treatment

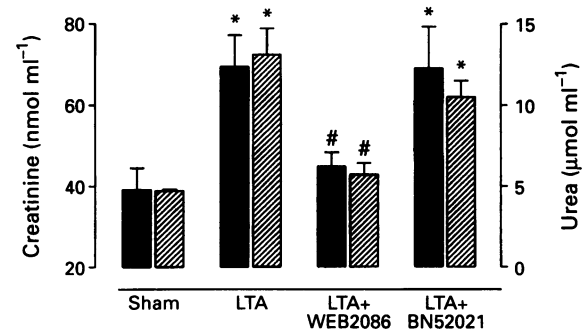

Figure 3 WEB2086 (5 mg kg^{-1}, i.v., 20 min before and 160 min after LTA), but not BN52021 (20 mg kg^{-1}, i.v., 20 min before and 160 min after LTA), inhibits the renal failure elicited by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.). Renal function was assessed by the measurement of concentrations of creatinine (solid columns) and urea (hatched columns) in plasma obtained from rats 300 min after the injection of LTA. Results are expressed as mean ± s.e.mean ($n=5-8$). *$P<0.01$ vs. sham-operated and $^{\#}P<0.01$ vs. LTA alone.

Table 1 Haemodynamic parameters in sham-operated rats at 300 min, after administration of WEB2086 or BN52021

Treatment	*MAP* (mmHg)	*Pressor response* (mmHg min)	*iNOS* (pmol 30 min^{-1} mg^{-1} protein)	*Nitrite* (μM)	*Urea* (mM)	*Creatinine* (μM)
Sham-operated	108 ± 5	38 ± 6	8 ± 2	1.7 ± 0.3	4.7 ± 0.1	39 ± 5
+ WEB2086	101 ± 6	44 ± 8	8 ± 2	1.9 ± 0.2	5.7 ± 0.7	38 ± 3
+ BN52021	100 ± 2	35 ± 9	12 ± 6	1.6 ± 0.3	6.2 ± 0.5	36 ± 4

Mean ± s.e.mean are given ($n=4-6$). Rats were treated with WEB2086 (5 mg kg^{-1}, i.v., 20 min before and 160 min after LTA) or BN52021 (20 mg kg^{-1}, i.v., 20 min before and 160 min after LTA). Mean arterial pressure (MAP), pressor response to noradrenaline, inducible nitric oxide synthase (iNOS) activity in lung, plasma concentrations of nitrite, urea and creatinine at 300 min are shown.

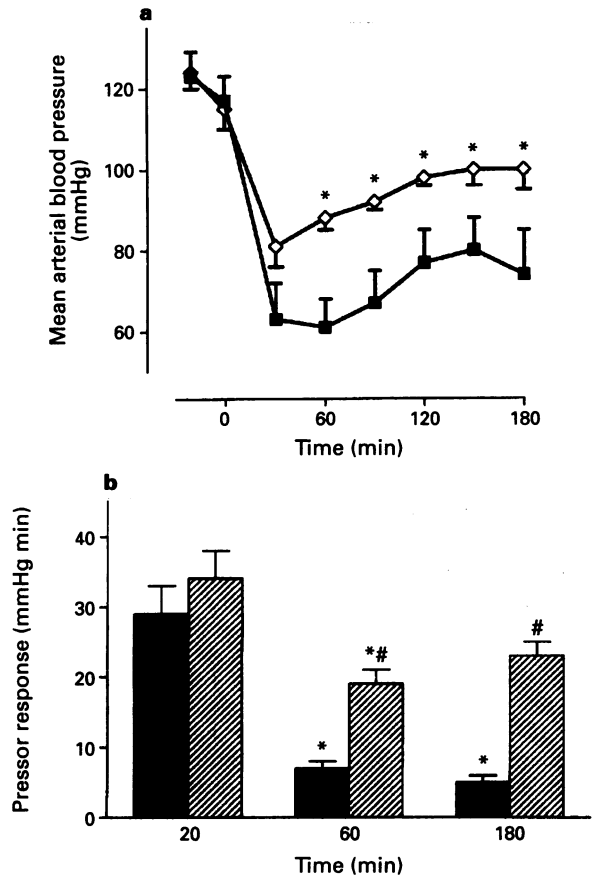

Figure 4 BN52021, inhibits hypotension (a) and hyporeactivity to noradrenaline (b; 1 $\mu g\,kg^{-1}$, i.v.) elicited by lipopolysaccharide (LPS, 10 $mg\,kg^{-1}$, i.v.). Rats were treated with LPS (a: ■; b: solid columns; $n=5$) or LPS and BN52021 (20 $mg\,kg^{-1}$, i.v., 20 min before LPS, $n=5$) (a: ◇; b: hatched columns). Results are expressed as mean ± s.e.mean. (a), $^{*}P<0.01$ vs. LPS alone. (b), $^{*}P<0.05$ vs. time 0 and $^{\#}P<0.05$ vs. LPS alone.

of rats with BN52021 (67 ± 14 pmol L-citrulline 30 min^{-1} protein, $n=5$, $P<0.01$). Similarly, BN52021 significantly inhibited the increase in plasma nitrite concentration elicited by LPS 180 min after administration (9.2 ± 0.6 μM for LPS, $n=5$, vs. 4.3 ± 0.2 μM for LPS + BN52021, $n=5$, $P<0.01$).

TNF-α measurements

As WEB2086 or BN52021 had beneficial effects on the circulatory failure elicited by LTA or LPS respectively, TNF-α levels were measured in the plasma from these rats to investigate the influence of PAF on TNF-α release. The basal level of TNF-α in plasma from sham-operated rats at 90 min after injection of vehicle (0.9% NaCl) was below the detection limit (0.07 ng ml^{-1}). Ninety min after the injection of LTA, the plasma TNF-α concentration was elevated to 5.0 ± 0.7 ng ml^{-1} ($n=8$). Treatment of the rats with WEB2086 (5 mg kg^{-1}, i.v., 20 min before LTA) did not significantly affect this increase in TNF-α release (6.0 ± 0.3 ng ml^{-1}, $n=4$). In rats receiving LPS, the TNF-α concentration increased to 3.8 ± 0.2 ng ml^{-1} ($n=5$) at 90 min after injection. This enhanced release of TNF-α by LPS was not significantly influenced by treatment of rats with BN52021 (20 mg kg^{-1}, i.v., 20 min before LPS) (3.7 ± 0.5 ng ml^{-1}, $n=3$).

Antagonism of hypotension elicited by PAF

Intravenous injections of PAF (3–300 ng kg^{-1}, $n=3$) resulted in a dose-dependent decrease in mean arterial blood pressure (Figure 5). Treatment of rats with WEB2086 (5 mg kg^{-1}, i.v., 20 min before PAF, $n=3$) or BN52021 (20 mg kg^{-1}, i.v., 20 min before PAF, $n=3$) inhibited the maximal decrease in mean arterial blood pressure elicited by PAF to a similar extent (Figure 5). In addition, the 50% recovery time after injection of PAF decreased markedly from 161 ± 26 s for PAF at 100 ng kg^{-1} to 3 ± 12 s after treatment with WEB2086 and 31 ± 9 s after treatment with BN52021 ($P<0.01$); with PAF at 300 ng kg^{-1} the 50% recovery time was decreased from 702 ± 71 s to 65 ± 25 s and 87 ± 17 s, respectively ($P<0.01$).

Induction of nitrite release in macrophages by LTA

Activation of the murine macrophage cell line J774.2 by LTA (10 $\mu g\,ml^{-1}$ for 24 h) resulted in an increase in nitrite accumulation from 1.9 ± 0.4 μM ($n=12$) to 30.0 ± 3.1 μM ($n=12$). Polymyxin B (0.5 $\mu g\,ml^{-1}$) only marginally attenuated the induction of nitrite release elicited by LTA (17 ± 4% inhibition, $n=12$), while a similar release of nitrite elicited by LPS (1 $\mu g\,ml^{-1}$) was nearly abolished (95 ± 1% inhibition, $n=12$). WEB2086, but not BN52021 (final concentrations 10^{-5}–3×10^{-4} M, added to the wells 20 min before LTA, $n=12$), inhibited concentration-dependently the induction of nitrite accumulation in the medium elicited by LTA (Figure 6).

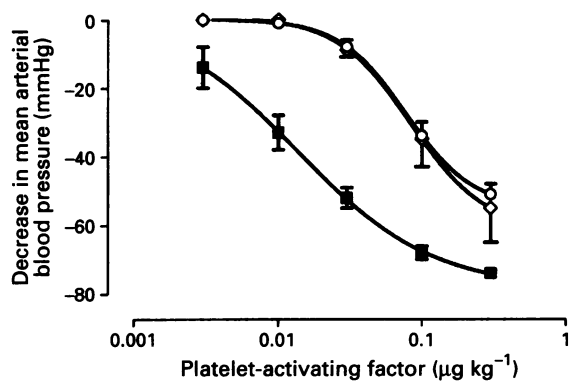

Figure 5 WEB2086 or BN52021 inhibits hypotension elicited by platelet-activating factor (PAF). PAF was administered in increasing concentrations (0.003–0.3 $\mu g\,kg^{-1}$, i.v.) allowing sufficient time for the blood pressure to recover to baseline values. Rats were treated with PAF (■), or PAF and WEB2086 (5 $mg\,kg^{-1}$, i.v., 20 min before PAF; ○), or PAF and BN52021 (20 $mg\,kg^{-1}$, i.v., 20 min before PAF; ◇). Results are expressed as mean ± s.e.mean ($n=3$).

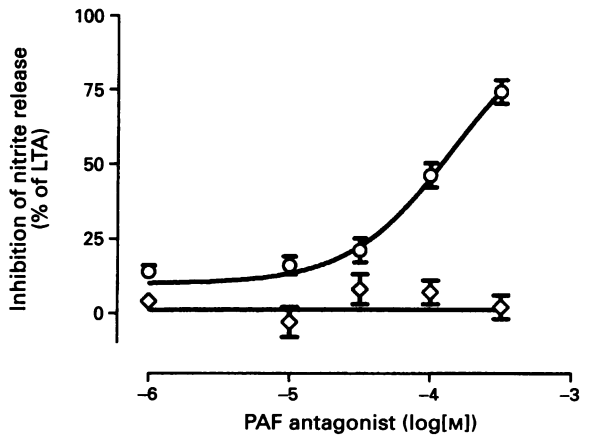

Figure 6 WEB2086 (○), but not BN52021 (◇), inhibits the formation of nitrite by cultured macrophages activated with lipoteichoic acid (LTA, 10 $\mu g\,ml^{-1}$ for 24 h). WEB2086 or BN52021 were added to wells containing confluent murine macrophages (J774.2 cell line) 20 min before LTA. The accumulation of nitrite elicited by LTA was measured by the Griess method. Results are expressed as mean ± s.e.mean of four independent experiments performed in triplicate.

Discussion

LTA from *Staphylococcus aureus* causes circulatory failure (hypotension and vascular hyporeactivity to noradrenaline) and renal failure (increase in plasma urea and creatinine) in the anaesthetized rat. Previously, we have shown that this model of septic shock is independent of endotoxin, confirming that products from Gram-positive organisms can elicit circulatory shock without causing endotoxaemia (Natanson *et al.*, 1989; Wakabayashi *et al.*, 1991; De Kimpe *et al.*, 1995). Treatment of rats with the PAF receptor antagonist WEB2086 prevents the delayed circulatory and renal failure elicited by LTA, suggesting that PAF is an important mediator in this model of Gram-positive shock. In contrast, the structurally different PAF antagonist BN52021 neither influences the circulatory nor the renal failure elicited by LTA. This discrepancy between WEB2086 and BN52021 is surprising, as both PAF receptor antagonists have well-described beneficial effects in animal models of endotoxaemia (Chang *et al.*, 1990; Fletcher *et al.*, 1990; Qi & Jones, 1990; Moore *et al.*, 1991; Mozes *et al.*, 1991; Szabó *et al.*, 1993). Therefore, as a positive control, the haemodynamic effects of BN52021 were also investigated in the circulatory failure elicited by LPS. Both the hypotension and vascular hyporeactivity to noradrenaline caused by injection of LPS were attenuated by BN52021. Thus, the beneficial effect of BN52021 on the cardiovascular dysfunction elicited by LPS (i) confirms that the release of PAF elicited by LPS contributes importantly to the pathophysiology of endotoxin shock (Floch *et al.*, 1989; Rabinovici *et al.*, 1990; Yue *et al.*, 1990; Sánchez Crespo & Fernández-Gallardo, 1991; Torley *et al.*, 1992; Ogata *et al.*, 1993; Koltai *et al.*, 1994), and (ii) demonstrates that the treatment protocol for BN52021 used in our studies should be sufficient to inhibit any potential effects of PAF released by LTA.

In the anaesthetized rat, an enhanced release of nitric oxide following the induction of iNOS contributes importantly to the delayed hypotension and vascular hyporeactivity to noradrenaline elicited by LPS or LTA (Julou-Schaeffer *et al.*, 1990; De Kimpe *et al.*, 1995). Here, injection of LTA or LPS results in the elevation of plasma nitrite and induction of iNOS activity associated with the delayed circulatory failure. Interestingly, WEB2086 prevents the increase in plasma nitrite concentration and induction of iNOS activity elicited by LTA. BN52021, however, does not influence the increase in plasma nitrite and iNOS activity in animals injected with LTA, but prevents the induction of iNOS activity and subsequent rise in plasma nitrite in our model of endotoxaemia. This again stresses the surprising discrepancy in the action of BN52021 in the circulatory failure elicited by LTA and LPS. Moreover, this shows that BN52021 does not interfere directly with iNOS to inhibit its activity, but attenuates the process leading to the expression of iNOS elicited by LPS. In rats, injection of PAF results in the induction of iNOS activity and WEB2086 attenuates the expression of iNOS elicited by LPS (Szabó *et al.*, 1993). Thus, our findings are consistent with the hypothesis that PAF contributes to the induction of iNOS activity and thereby the delayed circulatory failure elicited by LPS or LTA.

How, then, can we explain the different effects of the PAF antagonists WEB2086 and BN52021 on the circulatory and renal failure elicited by LTA? At the dosage used in this study, both WEB2086 or BN52021 inhibit the hypotension elicited by exogenous PAF to a similar extent. Interestingly, WEB2086, but not BN52021, prevents the induction of nitrite release in murine macrophages activated by LTA. These results demonstrate that BN52021 acts only extracellularly (PAF induced hypotension), while WEB2086 acts also intracellularly (PAF as a second messenger in the induction of nitrite release in macrophages activated by LTA). This is supported by findings that BN52021 preferentially binds to extracellular PAF receptors (Marcheselli *et al.*, 1990), and that WEB2086 inhibits the actions of PAF released intracellularly in leukocytes and endothelial cells (Stewart *et al.*, 1990). Interestingly, in many different cell types, including macrophages, neutrophils and endothelial cells, the majority of PAF is retained intracellularly upon stimulation (Lynch & Henson, 1986). The present results indicate that LTA causes the intracellular release of PAF, which, in turn, contributes importantly to the induction of iNOS activity, delayed circulatory failure and renal failure in anaesthetized rats.

In various animal models, injection of exogenous PAF results in systemic vasodilatation and hypotension (Braquet *et al.*, 1987; Sánchez Crespo & Fernández-Gallardo, 1991; Koltai *et al.*, 1994). Here, the early (60 min) hypotension and hyporeactivity to noradrenaline elicited by LPS are attenuated by BN52021, suggesting the extracellular release of PAF by LPS as a systemic vasodilator and mediator of the early decrease in pressor response to noradrenaline. In contrast, the early hypotension or hyporeactivity to noradrenaline elicited by LTA are not attenuated by either WEB2086 or BN52021. These results further confirm that a significant, bioactive amount of PAF is released extracellularly by LPS, but not by LTA, and also demonstrate that the early haemodynamic events elicited by LTA are not mediated by PAF.

Here, treatment of the rats with WEB2086, but not with BN52021, also attenuates the renal failure elicited by LTA, suggesting the intracellular involvement of PAF. An infusion of PAF reduces glomerular filtration and sodium excretion (Schlondorff & Neuwirth, 1986). A role for PAF in the pathophysiology of kidney injury has been demonstrated in several models of nephrotoxicity and post-ischaemic renal failure (Lopez-Farre *et al.*, 1990; Dos Santos *et al.*, 1991). The mechanisms by which renal failure develops in septic shock is still unclear and may involve cytokines and neutrophils (Karkar *et al.*, 1992; Kita *et al.*, 1993). Here, we demonstrate that intracellular PAF contributes to the pathophysiology of renal failure elicited by LTA.

It has been postulated that PAF may interact with the release and/or action of TNF-α (Koltai *et al.*, 1993). In septic patients and in various animal models of septic shock, inflammatory cytokines, such as TNF-α and IL-1, are released and these cytokines contribute to the induction of iNOS, circulatory failure, multiple organ (including the kidney) injury, and mortality (Dinarello, 1991; Freudenberg & Galanos, 1991; Karkar *et al.*, 1992; Aiura *et al.*, 1993; Kita *et al.*, 1993; Knowles & Moncada, 1994). Here, LTA or LPS increased plasma levels of TNF-α in the anaesthetized rat. However, the PAF receptor antagonists did not influence the rise in plasma TNF-α levels elicited by either LTA (WEB2086) or LPS (BN52021). Similarly, Rabinovici *et al.* (1990) demonstrated that TNF-α release in rats injected with LPS was unchanged by the PAF receptor antagonist BN50739 (like WEB 2086 a hetrazepine), while hypotension and mortality were attenuated. Other, structurally unrelated PAF antagonists, CL184,005 and TCV-309 (both pyridinium derivatives) attenuate the release of TNF-α in mice with endotoxaemia (Torley *et al.*, 1992; Ogata *et al.*, 1993), and L-659,989 (a tetrahydrofuran) reduces the levels of plasma TNF-α in rats subjected to splanchnic artery occlusion (Zingarelli *et al.*, 1992). Apart from differences in chemical structure and animal models, a possible explanation may be that PAF/cytokine interactions usually result in a bell-shaped concentration-effect curve, where lower concentrations of PAF enhance cytokine production, whereas high concentrations of PAF suppress their release (Pignol *et al.*, 1990; Poubelle *et al.*, 1991). Clearly, in the present study, the beneficial *in vivo* effects of (i) WEB2086 on the circulatory and renal failure and induction of iNOS activity elicited by LTA, and (ii) BN52021 on the circulatory failure and induction of iNOS activity elicited by LPS, are not due to the attenuation of the release of TNF-α by these compounds.

LTA generally exists as a hydrophilic poly(glycerophosphate) chain attached by phosphodiester bond to a glycolipid (for review, see Wicken & Know, 1980; Fischer, 1988). LTA is anchored in the cytoplasmic membrane by its lipid moiety, which consists of two to three fatty-acyl residues. The long polar poly(glycerophosphate) chain penetrates through the peptidoglycan cell wall structure and can be detected as a

surface antigen. Gram-positive bacteria excrete LTA into the external environment, which is enhanced by treatment of growing organisms with antibiotics. How LTA binds and activates cells is unclear, but appears to depend on lipophilic interaction. For instance, from two LTA fractions, the more lipophilic LTA fraction causes release of TNF-α from macrophages and regression of tumours in mice, whereas the more hydrophylic is inactive (Takada *et al.*, 1995). Also, only LTA, but not its deacylated form, causes injury to cultured human kidney cells (De Vuono & Panos, 1978). The hydrophobic component of LTA is simpler in structure and closer to eucaryotic membrane lipids than that of lipopolysaccharides and binding through intercalation into the membrane bilayer is probably easier for LTA than LPS. Further, studies are required to elucidate the molecular interaction between LTA and eucaryotic cells.

Thus, in the anaesthetized rat, the intracellular release of PAF elicited by LTA isolated from the cell wall of *Staphylococcus aureus* (a micro-organism without endotoxin) contributes importantly to the delayed circulatory and renal failure. The prevalence of sepsis resulting from Gram-positive organisms has risen markedly in the last decade, and it is possible that cases of Gram-positive sepsis may predominate in the years to come (Bone, 1994). Therefore, it is important to develop animal models without endotoxaemia in order to elucidate the pathophysiology of Gram-positive shock and to evaluate the effects of novel therapeutics. In a recent multicenter clinical trial, the PAF receptor antagonist BN52021 appeared to be beneficial in patients with severe Gram-negative, but not with Gram-positive sepsis (Tenaillon *et al.*, 1993). Strikingly, this surprising difference between Gram-negative and Gram-positive organisms, is also observed in our rat models of septic shock employing LPS or LTA.

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