Endotoxin-induced arterial endothelial barrier dysfunction assessed by an *in vitro* model

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Using an *in vitro* model in which albumin transfer across monolayers of bovine aortic endothelial cells (BAEC) was measured, we have shown that lipopolysaccharide (LPS) induces a concentration-dependent increase in endothelial permeability. This increase was biphasic, having an early peak at 2 h and rising again by 24 h. Both peaks were abolished by polymyxin B (PMB) but were unaffected by N^ω-monomethyl-L-arginine, N^ω-nitro-L-arginine methyl ester or dexamethasone. Furthermore, LPS did not stimulate nitric oxide production by BAEC following 24 h exposure. Thus, the LPS-induced increase in permeability may account for the vascular leakage of septic shock, but the L-arginine-nitric oxide system does not appear to be involved.

Keywords: Endothelial barrier function; endotoxin shock; lipopolysaccharide; nitric oxide; polymyxin B

Introduction Septic shock is characterized by severe hypotension, hyporeactivity to vasoconstrictors, profound vascular leakage, and ultimately multiple organ failure culminating in death (Pinsky *et al.*, 1993). Lipopolysaccharide (LPS) derived from gram-negative bacteria is a common cause of septic shock with various mediators having been implicated, including catecholamines, prostaglandins, free radicals, cytokines and nitric oxide (Wright *et al.*, 1992). Evidence suggests that nitric oxide is responsible for the hypotension associated with septic shock (Parker & Adams, 1993), however, the mediator(s) of the vascular leakage remains unknown.

Using an *in vitro* model, we wished to determine if LPS causes endothelial barrier dysfunction and so account for the vascular leakage of septic shock. If so, we wished to investigate the involvement of the L-arginine-nitric oxide system.

Methods *Albumin transfer experiments* Albumin transfer experiments were conducted as previously described (Berman & Martin, 1993). Briefly, bovine aortic endothelial cells (BAEC) were isolated, grown in 80 cm² culture flasks until confluent and seeded onto Transwell (Costar) polycarbonate membranes (6.5 mm diameter, 3 µm pore size). Following incubation with drugs as indicated in Results, membranes were washed twice in Krebs solution comprising (mM): NaCl 118, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, NaHCO₃ 2.4, glucose 11 and HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 5 at 37°C and pH 7.4, and transferred to 24-well plates. Krebs solution (600 µl) was placed in the lower chamber of each well, with 100 µl of Krebs containing 4% trypan blue-labelled albumin being placed in the upper chamber, above the monolayer. For experiments involving polymyxin B (PMB), N^ω-monomethyl-L-arginine (L-NMMA) and N^ω-nitro-L-arginine methyl ester (L-NAME), these were then re-added to both chambers. The plates were transferred to an orbital shaker and incubated for 90 min at 37°C after which the extent of equilibration of albumin across the monolayers was determined spectrophotometrically at 590 nm.

Drugs Dexamethasone, lipopolysaccharide from *Salmonella typhosa*, N^ω-nitro-L-arginine methyl ester and polymyxin B sulphate were obtained from Sigma (Poole, Dorset) whilst N^ω-monomethyl-L-arginine acetate was a gift from Dr D.D. Rees, Wellcome Laboratories (Beckenham). All drugs were dissolved in distilled water except for dexamethasone which

Figure 1 (a) Effects of 24 h exposure to 0–1000 ng ml⁻¹ lipopolysaccharide (LPS) on albumin transfer across monolayers of BAEC. Each column is the mean with s.e.mean of 6–23 observations. ***P < 0.001, indicates a significant difference from control (no LPS). (b) Time-course of the development of the increases in albumin transfer across monolayers of BAEC induced by 30 ng ml⁻¹ LPS. Each point is the mean with s.e.mean of 12–24 observations. ***P < 0.001, indicates a significant difference from control (0 h).

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Table 1 Effects of polymyxin B (PMB), N^ω-nitro-L-arginine methyl ester (L-NAME), N^ω-monomethyl-L-arginine (L-NMMA) and dexamethasone on the increases in albumin transfer stimulated by 2 h and 24 h exposure to 30 ng ml⁻¹ lipopolysaccharide (LPS)

LPS 2 h Treatment	Equilibration of albumin (%)			
	Control	LPS	Treatment alone	LPS + treatment
PMB, 10 µg ml ⁻¹	9.6 ± 0.5	15.9 ± 2.0**	9.2 ± 0.4	11.1 ± 1.1††
L-NAME, 500 µM	7.8 ± 0.7	26.4 ± 3.6***	8.6 ± 2.0	24.8 ± 1.6***
L-NMMA, 2 mM	4.5 ± 1.1	19.1 ± 1.5***	8.1 ± 1.9	28.3 ± 3.1***
Dexamethasone, 1 µM	9.2 ± 1.4	18.0 ± 1.6***	7.3 ± 1.0	15.6 ± 0.6**
LPS 24 h Treatment	Equilibration of albumin (%)			
	Control	LPS	Treatment alone	LPS + treatment
PMB, 10 µg ml ⁻¹	11.0 ± 0.7	24.5 ± 0.7***	10.4 ± 0.4	12.4 ± 1.3†††
L-NAME, 500 µM	8.2 ± 0.9	43.1 ± 2.6***	6.2 ± 0.6	45.8 ± 1.1***
L-NMMA, 2 mM	5.1 ± 0.4	16.1 ± 0.8***	5.3 ± 0.5	19.9 ± 2.3***
Dexamethasone, 1 µM	9.2 ± 0.8	32.1 ± 1.8***	6.6 ± 0.9	26.4 ± 2.6***

Levels of albumin transfer across BAEC monolayers are shown following incubation both in the absence of drugs (control) and in the presence of LPS. Effects of concomitant addition of PMB, L-NAME and L-NMMA, and of 20 h pretreatment with dexamethasone, are also shown. Results are expressed as the mean ± s.e.mean of 6 observations.

** $P < 0.01$; *** $P < 0.001$, indicates a significant difference from control.

†† $P < 0.01$; ††† $P < 0.001$, indicates a significant difference from LPS.

was dissolved in ethanol. The final concentration of ethanol in experiments did not exceed 0.2%.

Statistical analysis Results are expressed as means ± s.e.mean and were compared by one-way analysis of variance followed by Fisher's test. $P \leq 0.05$ was taken as significant.

Results Following 24 h exposure, LPS (3–1000 ng ml⁻¹) produced a concentration-dependent increase in the equilibration of albumin across BAEC monolayers over 90 min (Figure 1a).

The time-course of the increase in albumin transfer stimulated by 30 ng ml⁻¹ LPS is shown in Figure 1b. An early, transient peak was observed after 2 h which then declined towards basal levels before rising again by 24 h.

The increase in albumin transfer stimulated by exposure to LPS for both 2 h and 24 h was abolished by PMB (10 µg ml⁻¹) which binds and inhibits LPS, but was unaffected by the nitric oxide synthase inhibitors, L-NMMA (2 mM) and L-NAME (500 µM), or by 20 h pretreatment with the glucocorticoid, dexamethasone (1 µM) (Table 1).

Using a chemiluminescence assay (Cox, 1980), we found no significant increase over basal levels (29.2 ± 4.0 nmol/10⁶ cells) in nitrite accumulation in the medium above BAEC exposed to up to 1000 ng ml⁻¹ LPS for 24 h (21.8 ± 4.5 nmol/10⁶ cells).

Discussion The principal new finding is that LPS induced a concentration-dependent increase in albumin transfer across BAEC monolayers and this may account for the profound vascular leakage observed in septic shock (Pinsky *et al.*, 1993).

The LPS-induced permeability increase developed with a biphasic time-course, exhibiting a transient peak at 2 h which then declined, before rising again, nearing maximum by 24 h. At both time points PMB, which inhibits LPS activity by binding it with high affinity (Lasfargues *et al.*, 1989), abolished the increases in albumin transfer.

Interestingly, the nature of this biphasic time-course parallels that of the hypotension induced by LPS *in vivo* (Wright *et al.*, 1992). Evidence shows that the early fall in blood pressure results from production of nitric oxide by the vascular endothelium (Salvemini *et al.*, 1990) and that the subsequent delayed fall results from induction of nitric oxide synthase in the vascular smooth muscle (Wright *et al.*, 1992). We found, however, that L-NMMA and L-NAME, inhibitors of nitric oxide synthase, had no effect on LPS-stimulated increases in BAEC permeability, suggesting that nitric oxide is not involved. Two further findings led us to conclude that LPS had not stimulated an increase in permeability through nitric oxide production. Firstly, dexamethasone, a glucocorticoid which prevents induction of the Ca²⁺-independent, inducible form of nitric oxide synthase (Moncada, 1992), exhibited no inhibitory effect when added 20 h prior to LPS. Secondly, on determining nitrite levels as an indicator of nitric oxide production, we found no stimulated increase by LPS over 24 h.

Our findings differ from those of Palmer *et al.* (1992) who showed that LPS-stimulated cytotoxicity in pig aortic endothelium is inhibited by L-NMMA, dexamethasone and hydrocortisone. However, in their study cell death was taken as a marker of LPS activity, using a standard stimulus 300 times greater than the concentration we used to induce an increase in albumin transfer. To what extent cell viability is impaired by LPS in our study is unclear, but, whilst nitric oxide might be required to cause cell death in response to much higher concentrations of LPS, it does not appear to mediate the observed increases in endothelial permeability.

In conclusion, we have shown that LPS clearly induces endothelial barrier dysfunction and this may account for the profound vascular leakage associated with septic shock. This dysfunction does not appear, however, to be mediated by the L-arginine-nitric oxide system.

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