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Endotoxin-induced vasodilatation in anaesthetized rat skin involves nitric oxide and prostaglandin synthesis

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1 The effect of intradermally injected endotoxin on skin blood flow was investigated in anaesthetized male Wistar rats *in vivo*.

2 Local skin blood flow changes were measured hourly for 6 h in the shaved dorsal skin with a laser-Doppler flow probe and compared to changes in control sites which had been injected with 100 μl of phosphate-buffered saline. By 3 h, skin blood flow increased above basal by 129 ± 27% and 186 ± 29% with 1 and 10 μg of endotoxin respectively. Blood flow remained significantly elevated at 6 h, the corresponding figures being 129 ± 24% and 154 ± 31% ($P<0.05$, $n=6$ rats, mean ± s.e.mean).

3 In further experiments, the response to 3 μg of endotoxin was measured at 4 h and treatment with a cyclo-oxygenase inhibitor, nitric oxide synthase inhibitors or a topical steroid all significantly inhibited this response ($P<0.05$ in each case, $n=6$ rats in each group with duplicate sites in each animal).

4 Indomethacin 3×10^{-9} mol per site injected 3.5 h after injection of endotoxin suppressed the mean 4 h response to endotoxin by 78%; N^G-nitro-L-arginine methyl ester (L-NAME) 10^{-7} mol per site suppressed the response by 95%; N^G-monomethyl-L-arginine (L-NMMA) 10^{-7} mol per site suppressed the response by 50%; whereas the D-isomer of N^G-monomethyl-arginine 10^{-7} mol per site had no significant effect.

5 Topical application of the corticosteroid, betamethasone 17-valerate (1% solution) 18 h before injection of endotoxin inhibited the mean 4 h response to endotoxin by 66% and the 6 h response by 48%.

6 In the same model, the vasodilator response to arachidonic acid was inhibited by both indomethacin and nitric oxide synthase inhibitors ($P<0.05$ in each case).

7 These data suggest that the microcirculatory vasodilator response to endotoxin and arachidonic acid injected locally involves both nitric oxide synthase and cyclo-oxygenase in this *in vivo* model.

Keywords: Nitric oxide; nitric oxide synthase; endotoxin; vascular smooth muscle; endothelium; vasodilatation; septic shock; prostaglandins; arachidonic acid

Introduction

Bacterial endotoxins are lipopolysaccharides (LPS) found in the outer membrane of Gram negative bacteria. The LPS molecule consists of an oligosaccharide core with a chain of repeating sugar units at one end and lipid A at the other. The structure of lipid A is conserved among Gram negative bacteria and when released from bacteria can bind to the plasma membrane of most cell types. Endotoxin is a powerful stimulus to the immune response and can promote adhesion between leucocytes and the endothelium. Endotoxin can also interact with immune complexes, affect endothelial function to increase permeability and when given systemically can cause endotoxic shock with associated multiple organ failure (Ginsberg & Henson, 1978; Dinarello, 1983; Meyrick *et al.*, 1986).

Endotoxin stimulates the production of many mediators from leucocytes and endothelial cells such as tumour necrosis factor, platelet activating factor, prostacyclin and fragments of complement. Some of these mediators have been implicated in the pathogenesis of endotoxin-induced vasodilatation and pharmacological antagonists to some have protected against septic shock (Beutler *et al.*, 1985; Wise *et al.*, 1985; Casals-Stenzel, 1987).

One likely mediator of the vasodilator response to endotoxin is nitric oxide, synthesized from the amino acid L-arginine by many cell types (Moncada *et al.*, 1991). Two nitric oxide synthase enzymes have been described. The first is a Ca^{2+}-dependent constitutive enzyme which is present in endothelial cells and is involved in the control of vascular tone (Moncada *et al.*, 1991). The second is an inducible Ca^{2+}-independent enzyme which predominates in vascular smooth muscle cells, although it is present to a lesser extent in endothelial cells as well (Wood *et al.*, 1990; Moncada *et al.*, 1991). When precontracted vascular tissue is incubated with endotoxin *in vitro*, relaxation is induced progressively over several hours. This effect is mediated mainly by the inducible enzyme in vascular smooth muscle, requires new protein synthesis and is inhibited by pretreatment with corticosteroids (Julou-Schaeffer *et al.*, 1990; Di-Rosa *et al.*, 1990; Radomski *et al.*, 1990; Kilbourn *et al.*, 1990; Fleming *et al.*, 1991).

Endotoxaemia is characterized by vasodilatation (Wakabayashi *et al.*, 1987) and the subsequent hypotension is often refractory to vasoconstrictors. In both animal models and in man the hypotension can be reversed by inhibitors of nitric oxide synthase (Thiemermann & Vane, 1990; Petros *et al.*, 1991). Pretreatment of animal models with corticosteroids can prevent the hypotension (Rees *et al.*, 1990) suggesting that the vasodilatation is mediated by the inducible form of nitric oxide synthase.

The aim of the present study was to measure the vasodilator effect of endotoxin in the microcirculation and to determine the mechanisms involved. The microcirculatory effects of endotoxin are likely to mediate in part the tissue response to Gram negative bacterial infection.

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Methods

Animals

Male Wistar rats weighing 230–350 g were sedated by intramuscular injection of Hypnorm (fentanyl citrate/fluanisone) 0.3 ml kg^{-1}. The dorsal skin was shaved with electric clippers and depilated with a commercial depilating cream (Immac) followed by gentle close shaving with a glass microscope slide. The dorsal skin was then rinsed thoroughly with warm water and the rats left for 1 h before measurements were made.

Blood flow measurements (laser Doppler)

Before injection of test compounds, baseline blood flow was measured in the dorsal skin with a laser-Doppler flow probe (Perimed II, Sweden). This was repeated at either 6 or 8 marked sites, the number depending upon the protocol. During the experiment, the rats were sedated with Hypnorm in an air-conditioned room at 23–25°C. Each reading took 15 s with a 10 s interval between readings and the mean of three readings per site was taken. Results were recorded as red blood cell flux (the number of moving red cells detected by the laser beam × mean cell velocity) and expressed as a percentage of a standardized signal. The laser Doppler was set at 4 Hz, gain 10 and a time constant of 3 s. The output was recorded on a Maclab and Macintosh Apple Computer (Apple Computer Inc, Cupertino, Ca, U.S.A.) set at an input of 10 V and chart speed of 2 mm s^{-1}. Test compounds were injected in a randomized, balanced site pattern.

Experimental protocols

In the first experiment the vasodilator responses to two doses of endotoxin (*E. coli* serotype 055:B5) were determined in six rats. Basal blood flow was measured in six marked sites as described above and then phosphate-buffered saline, endotoxin 1 μg and endotoxin 10 μg were injected in duplicate sites in 100 μl volumes. Red cell flux was measured hourly for 6 h.

To assess the effects of inhibitors on the response to endotoxin, a single dose of 3 μg of endotoxin, assessed at 4 h, was chosen. N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) were used as nitric oxide synthase inhibitors and N^G-monomethyl-D-arginine (D-NMMA) was used as a control. Endotoxin or control phosphate-buffered saline were injected after taking basal readings. The inhibitors or vehicle control were injected in 100 μl volumes 3.5 h later and the blood flow response measured at 4 h. Batches of six rats were used to test for the effect of L-NAME 10^{-7} mol per site, L-NMMA 10^{-7} mol per site and indomethacin 3×10^{-9} mol per site.

In a further experiment, six rats were pretreated with a corticosteroid 18 h before the injection of endotoxin. Betamethasone 17-valerate was dissolved in 20% diethylacetamide, 40% acetone, 40% ethanol (DAE 2:4:4) and this vehicle was used as the control. The skin was prepared as above and then 0.5 ml of steroid at a 1% w/v concentration was painted evenly on the sites of the right flank and the left flank was treated with vehicle control. Eighteen hours later basal blood flow was measured and then two sites on each flank were injected with phosphate-buffered saline and two with endotoxin as before and the response measured 4 h and 6 h later.

To confirm that the dose of indomethacin used caused effective inhibition of cyclo-oxygenase, six rats were injected in duplicate sites with phosphate-buffered saline, arachidonic acid 3×10^{-9} mol per site, arachidonic acid + indomethacin 3×10^{-9} mol per site and arachidonic acid + L-NAME 10^{-7} mol per site. The change in blood flow from the basal reading was assessed at 30 min. Two other batches of six rats were used to assess the effect of the active L-isomer and inactive D-isomer of NMMA on the response to arachidonic acid and the effect of L-NAME on the response to the stable prostacyclin analogue, iloprost.

Drugs and chemicals

Indomethacin was made as a stock solution of 10^{-2} M in ethanol and then diluted in phosphate-buffered saline. Endotoxin and all other drugs were dissolved and diluted in phosphate-buffered saline. Iloprost was the generous gift of Schering AG Pharmaceutical Research, Berlin, Germany. Pentobarbitone sodium was from May & Baker. All other drugs and chemicals were from the Sigma Chemical Co., Poole, Dorset.

Statistical analysis

Data are presented as mean ± s.e.mean. The suppression of the mean response by inhibitors was calculated as:

$$\frac{(\text{endotoxin and inhibitor} - \text{control})}{(\text{endotoxin} - \text{control})} \times 100$$

Statistical analysis was performed by analysis of variance (ANOVA) and results termed significant if $P < 0.05$.

Results

Figure 1 shows that endotoxin caused slow onset vasodilatation when injected intradermally in rat skin *in vivo*. The response was dose-related and increased progressively over 3 h. A comparison of the mean of the data from 1–6 h showed that both doses of endotoxin were significantly different from control and that the 10 μg dose had a significantly greater effect than the 1 μg dose ($P < 0.05$ in each case, ANOVA). Subsequent experiments were carried out with a 3 μg dose and the response measured at 4 h.

Figure 2 shows that the co-injection of indomethacin at 3.5 h inhibited the vasodilator response to endotoxin when measured at 4 h ($P < 0.05$). The suppression of the mean response by indomethacin was 78% of the response caused by endotoxin alone. Both nitric oxide synthase inhibitors, L-NAME and L-NMMA, inhibited the response to endotoxin by 94% and 50% respectively ($P < 0.05$ in each case, Figure 3). Injection of the inactive isomer D-NMMA had no significant effect on endotoxin-induced vasodilatation (in a further experiment the mean L-NMMA response was 46% of the

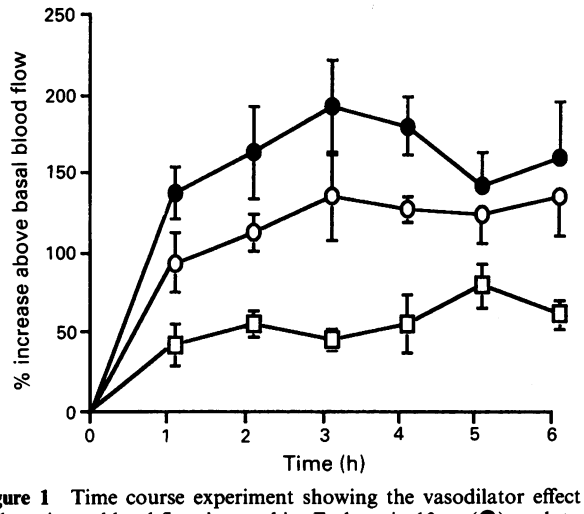

Figure 1 Time course experiment showing the vasodilator effect of endotoxin on blood flow in rat skin. Endotoxin 10 μg (●), endotoxin 1 μg (○) and phosphate-buffered saline (□) were injected in duplicate sites in each animal following the measurement of basal blood flow. Results, expressed as the percentage increase in basal blood flow, are the mean (± s.e.mean, vertical bars) for 6 rats.

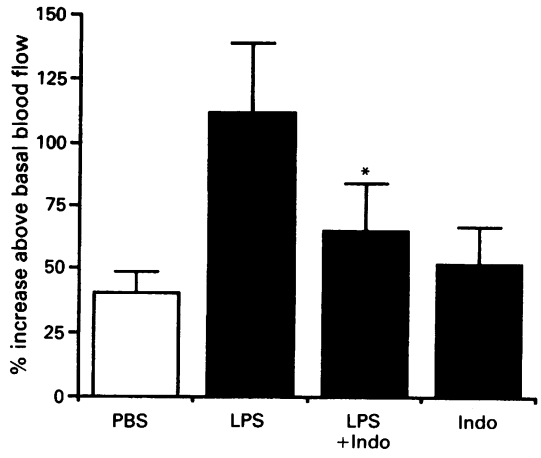

Figure 2 Inhibition of the vasodilator response to endotoxin 3 μg/ site (LPS) by indomethacin in rat skin. Indomethacin 3×10^{-9} mol per site (Indo) or control were injected 3.5 h after the injection of endotoxin or control and the vasodilator response measured at 4 h. The vasodilatation induced by endotoxin was inhibited significantly by indomethacin ($*P<0.05$). Experiments were performed in duplicate in each rat and the results are the mean (± s.e.mean, vertical bars) of 6 rats.

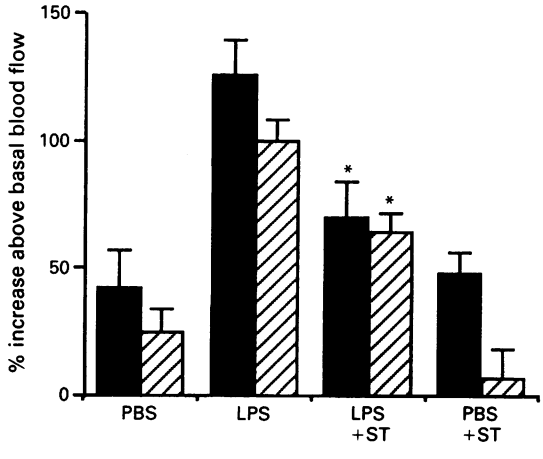

Figure 4 The inhibition of the vasodilator response to endotoxin 3 μg/ site by topical betamethasone-17-valerate in rat skin. The steroid was applied as a 1% solution to one flank and control solution to the other flank 18 h before the injection of endotoxin. The vasodilator response, measured at 4 h (solid columns) and 6 h (hatched columns) after the injection of endotoxin, was inhibited significantly by pretreatment with topical steroid ($*P<0.05$). Experiments were performed in duplicate in each rat and the results are the mean (± s.e.mean, vertical bars) of 6 rats.

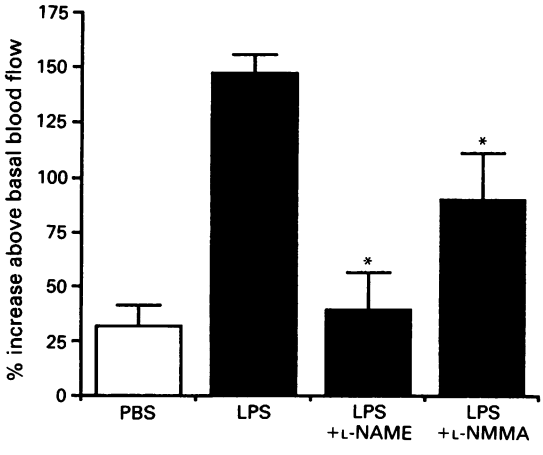

Figure 3 The inhibition of the vasodilator response to endotoxin 3 μg/ site (LPS) by the nitric oxide synthase inhibitors N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) in rat skin. L-NAME (100 nmol per site), L-NMMA (100 nmol per site) or control were injected 3.5 h after the injection of endotoxin or control and the vasodilator response measured at 4 h. The vasodilatation induced by endotoxin was inhibited significantly by both nitric oxide synthases inhibitors ($*P<0.05$ in each case). Experiments were performed in duplicate in each rat and the results are the mean (± s.e.mean, vertical bars) of 6 rats.

mean LPS response, $P<0.05$, and the D-NMMA response 128%, NS, $n = 6$ rats each in duplicate). It can be seen from Figures 2–5 that the injection of phosphate-buffered saline alone caused a slight increase in blood flow 30 min later, presumably a non-specific response to the 100 μl volume of the injection. In a separate experiment the injection of L-NAME in control sites had no significant effect compared to the injection of buffer when assessed at 30 min: blood flow increased by 24 ± 26% with buffer and 11 ± 7% with L-NAME 10^{-7} mol per site ($n = 4$ rats, experiment performed in triplicate in each rat).

Figure 4 shows the effect of pretreatment with the topical steroid, betamethasone-17-valerate, 18 h before injection of endotoxin or control. The responses were recorded at both 4 h and 6 h after the injection of endotoxin. The steroid significantly inhibited the response to endotoxin at both time points ($P<0.05$ in each case, $n = 6$ rats). Pretreatment with the steroid had no significant effect on blood flow in control sites at 18 h (data not shown in figure), the mean ± s.e.mean change in blood flow at 18 h being −4 ± 11% in sites painted with vehicle solution and 18 ± 13% in sites painted with steroid solution (NS, $n = 6$ rats, each in duplicate).

The vasodilator response to arachidonic acid, assessed at 30 min, was used to test if the chosen dose of indomethacin was sufficient to inhibit cyclo-oxygenase. Figure 5a shows that indomethacin significantly inhibited the response to arachidonic acid with the additional unexpected result that this response was blocked by L-NAME ($P<0.05$ in each case, $n = 6$ rats). Figure 5b shows that L-NAME did not suppress increased blood flow caused by the vasodilator prostaglandin analogue, iloprost ($n = 6$ rats). Figure 5c shows further, that the response to arachidonic acid was blocked by the nitric oxide synthase inhibitor L-NMMA ($P<0.05$, $n = 6$ rats), but not significantly by its inactive D-isomer.

Discussion

The present study shows that endotoxin injected intradermally causes a dose-dependent increase in blood flow in the microcirculation of rat skin *in vivo*. The effect is delayed in onset and can be inhibited by nitric oxide synthase inhibitors. These data suggest that endotoxin-induced arteriolar dilatation is mediated by nitric oxide synthesized by the inducible form of nitric oxide synthase. It is likely that the enzyme is present in vascular smooth muscle as the response to endotoxin in large arteries *in vivo* is endothelium-independent (Fleming *et al.*, 1991), although the endothelium may play a part also, as this tissue can release nitric oxide rapidly in response to endotoxin (Salvemini *et al.*, 1990). The inducible form of nitric oxide synthase has been shown to be steroid-sensitive (Radomski *et al.*, 1990; Knowles *et al.*, 1990) and indeed the response to endotoxin was inhibited in the present experiment by local pretreatment with the steroid betamethasone.

Other cell types may have been involved in the endotoxin-mediated vasodilatation observed in the present experiment. Endotoxin can release intermediates such as tumour necrosis factor or interleukin-1, both of which have been shown to

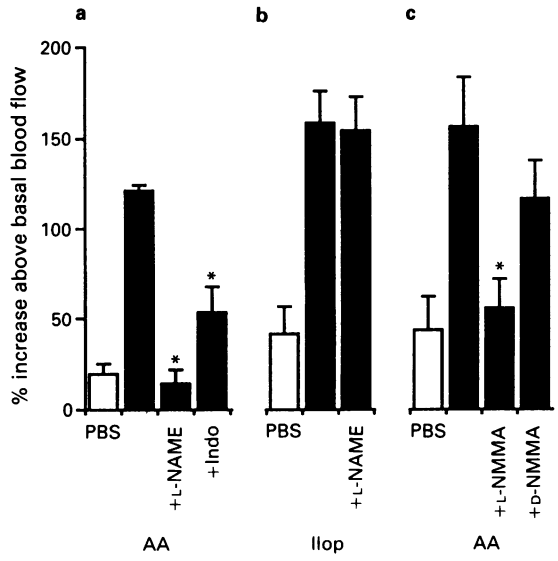

Figure 5 The effect of nitric oxide synthase inhibitors and indomethacin on the vasodilator response to arachidonic acid and iloprost. Responses were measured 30 min after the injection of test compounds. Three groups of 6 rats were studied and experiments carried out in duplicate in each rat. The response to arachidonic acid 3×10^{-9} mol per site (AA) was inhibited significantly by the co-injection of indomethacin 3×10^{-9} mol per site (Indo) and abolished by the co-injection of N^G-nitro-L-arginine methyl ester (L-NAME) 10^{-7} mol per site (a), ($^*P<0.05$ in each case). The response to arachidonic acid was inhibited by the active isomer of methyl arginine, N^G-monomethyl-L-arginine (L-NMMA) ($^*P<0.05$), but not significantly by the inactive isomer, D-NMMA (b). The response to iloprost 10^{-9} mol per site (Ilop) was unaffected by the co-injection of L-NAME 10^{-7} mol per site (c).

induce nitric oxide synthase (Busse & Mulsch, 1990; Robert *et al.*, 1992). When tested at the same dose, the nitric oxide synthase inhibitor L-NAME had a greater effect than L-NMMA in the present experiment, possibly because L-NMMA can be metabolized to L-arginine (Hecker *et al.*, 1990). Interestingly, L-NMMA is a more potent inhibitor than L-NAME in macrophages, where the inducible form of nitric oxide synthase predominates (Gross *et al.*, 1990).

Our results suggest that the endotoxin-induced vasodilatation was mediated, at least in part, by the release of nitric oxide and that this is an important vasodilator in the microcirculation *in vivo*. The nitric oxide-mediated vasodilatation may contribute to the second component of inflammation, oedema formation. Several vasodilators have been shown to potentiate oedema formation (Williams & Peck, 1977; Williams, 1982; Warren *et al.*, 1992) and the oedema response to substance P in rat skin is attenuated by nitric oxide synthase inhibition (Hughes *et al.*, 1990). The injection of *E. coli* intradermally had been used by others to study the response of the rabbit microcirculation where bacteria, probably by the release of endotoxin, can elicit vasodilatation, oedema formation and cell accumulation (Kopaniak *et al.*, 1980).

The inhibition of endotoxin-induced vasodilatation by indomethacin suggests that prostaglandins contribute to the hyperaemia, although we did not measure prostaglandin production directly. The response to endotoxin is slow in onset, suggesting possible induction of an enzyme involved in prostaglandin synthesis. The inhibitory effect of corticosteroids could involve the inhibition of phospholipase A_2 and the suppression of endogenous free arachidonate, and hence prostaglandin and leukotriene synthesis. Vasodilatation induced by arachidonic acid was inhibited by nitric oxide synthase inhibition in the present experiment suggesting that a prostanoid or leukotriene might mediate the release of nitric oxide. One candidate could be prostaglandin D_2 (PGD_2) a vasodilator in rat skin (Flower *et al.*, 1976), which is capable of stimulating nitric oxide synthesis (Braun *et al.*, 1991). Another pathway could be the metabolism of arachidonate to intermediates via the lipoxygenase enzyme, possibly involving leucocytes (Borgeat & Samuelsson, 1979).

There are other possibilities for an interaction between prostaglandins and nitric oxide. One could be that their vasodilator effect on the microcirculation is synergistic, analogous to the synergistic inhibition of platelet aggregation by nitric oxide and prostacyclin (Moncada *et al.*, 1991) or their synergistic action in the gastric mucosa (Whittle *et al.*, 1990). Prostaglandins are vasodilators in the microcirculation, in contrast to their lack of relaxant effect in some large vessels (Gryglewski *et al.*, 1989).

In conclusion, endotoxin induced a delayed vasodilatation in the microcirculation of rat skin *in vivo*. The response was inhibited by pretreatment with a corticosteroid suggesting the inducible form of nitric oxide synthase is involved. That the vasodilator response was inhibited by both indomethacin and nitric oxide synthase inhibition suggests either a synergy between prostaglandins and nitric oxide or that their release is linked in the microcirculation.

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