Involvement of nitric oxide synthase in the delayed vasodilator response to ultraviolet light irradiation of rat skin *in vivo*

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1 The role of nitric oxide synthase and cyclo-oxygenase in the skin blood flow response to ultraviolet light B (u.v.B) irradiation was investigated in the rat *in vivo*.

2 Local skin blood flow changes were measured in the shaved dorsal skin of anaesthetized male Sprague-Dawley rats with a laser Doppler flow probe.

3 u.v.B irradiation caused delayed onset vasodilatation and by 18 h basal blood flow increased by $125 \pm 25\%$ ($P < 0.05$, $n = 12$ rats, mean $\pm$ s.e.mean).

4 Indomethacin, 3 nmol per site, N^G-nitro-L-arginine methyl ester (L-NAME) 100 nmol per site, but not D-NAME 100 nmol per site, injected locally 17.5 h after u.v.B irradiation abolished the 18 h increase in blood flow.

5 The nitric oxide synthase inhibitor L-NAME, N^G-monomethyl-L-arginine (L-NMMA) and canavanine, 10 and 100 nmol per site injected at 17.5 h, suppressed significantly the u.v.B 18 h response in a dose-dependent manner. The order of potency was L-NAME > canavanine = L-NMMA. The effect of L-NAME was reversed partially by the co-injection of an excess of L-arginine.

6 Topical application of the corticosteroid, clobetasol 17-propionate, immediately after irradiation inhibited the 18 h u.v.B response in a dose-dependent manner.

7 The delayed onset microcirculatory vasodilatation induced by u.v.B involves both nitric oxide synthase and cyclo-oxygenase in this *in vivo* model. Topical corticosteroids may attenuate the response by inhibiting both prostaglandin and nitric oxide synthesis pathways.

Keywords: Nitric oxide; nitric oxide synthase; ultraviolet light; sunburn; inflammation; corticosteroids; vascular smooth muscle; endothelium; vasodilatation; prostaglandins

Introduction

Vasodilatation of the microcirculation is a key component of the inflammatory reaction and influences both oedema formation and leucocyte accumulation. The skin response to u.v.B irradiation was chosen as an inflammatory model for the present study as changes in blood flow can be assessed readily by a non-invasive laser Doppler flow probe. The sunburn reaction, caused primarily by u.v.B irradiation, causes increased local blood flow. The mechanisms controlling this reaction are not understood, although in man the reaction is attenuated by both non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids (Hughes *et al.*, 1992). Exposing multiple small areas of skin to u.v.B allows the comparison, within the same animal, of several test agents injected locally into irradiated sites.

Both cyclo-oxygenase and nitric oxide synthase (NOS) influence microvascular tone and both enzymes contribute to the inflammatory response. Prostaglandins are implicated in several models of inflammation and NSAIDs diminish the vasodilator response to inflammatory stimuli (Williams & Peck, 1977; Warren *et al.*, 1992). Corticosteroids also inhibit this vasodilator response by a prostaglandin dependent mechanism via the suppression of phospholipase A₂ (Flower & Blackwell, 1979). Corticosteroids differ from NSAIDs in their additional ability to inhibit the inducible form of nitric oxide synthase, iNOS (Di-Rosa *et al.*, 1990; Radomski *et al.*, 1990; Smith *et al.*, 1991; McCall *et al.*, 1991; Nathan, 1992). This enzyme is induced to release NO by several inflammatory stimuli such as interleukin-1, tumour necrosis factor and endotoxin (Moncada & Higgs, 1991; Nathan, 1992). In rat skin, both nitric oxide and prostaglandins are involved in the response to endotoxin injected locally (Warren *et al.*,

1992). The aim of the present study was to determine if similar mechanisms were involved in the vasodilator response of rat skin to u.v.B.

Two main subtypes of the NOS enzyme have been described. The Ca²⁺-dependent, constitutive enzyme (cNOS) is present in endothelial cells and is involved in the control of vascular tone (Moncada & Higgs, 1991; Nathan, 1992). The second, iNOS, is Ca²⁺-independent and predominates in vascular smooth muscle cells, although present to a lesser extent in endothelial cells (Moncada & Higgs, 1991; Nathan, 1992). It has been proposed that these two subtypes differ in their sensitivity to inhibition by arginine analogues. Thus, L-canavanine, a basic amino acid which resembles L-arginine, inhibits iNOS but not cNOS (McCall *et al.*, 1989; Iyengar *et al.*, 1987). It has been suggested also that N^G-nitro-L-arginine methyl ester (L-NAME) is 100 fold more potent than N^G-monomethyl-L-arginine (L-NMMA) at inhibiting cNOS in endothelial cells whereas L-NMMA is more potent than L-NAME at inhibiting iNOS in leucocytes (McCall *et al.*, 1989; Gross *et al.*, 1990). Three inhibitors of NOS, L-NAME, canavanine and L-NMMA, were chosen for the present study to determine if their relative potency in inhibiting iNOS was similar to that of previous studies. The potency of a topical corticosteroid, clobetasol 17-propionate, at preventing the vasodilator response to u.v.B irradiation, was also assessed.

Methods

Animals

Male Sprague-Dawley rats were anaesthetized with Hypnorm (fentanyl citrate/fluanisone) 0.3 ml kg⁻¹, i.m. The dorsal skin was shaved with electric clippers and depilated with a commercial depilating cream (Immac) followed by close shaving

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with a glass microscope slide. The dorsal skin was then rinsed thoroughly with warm water and the rats left for 1 h before use. Six to eight sites, depending on the protocol, were marked out on the dorsal skin with a black paper stencil. Each of the holes in the stencil was 6 mm diameter. Baseline skin red cell flux, as an index of blood flow, was measured in the marked sites using a laser Doppler flow meter. With the paper stencil in position to shield the remaining skin, the sites were exposed to u.v.B irradiation. Blood flow responses were measured 4 or 18 h later, depending on the protocol, with the rats sedated with Hypnorm. All experiments were performed in a temperature controlled laboratory at 23–25°C. All injections were given as 100 µl volumes via a 27 wire gauge needle. The lesions induced by u.v.B caused no signs of distress and similar, or more marked, lesions in the skin of human volunteers were not painful (preliminary observations).

Blood flow measurements (laser Doppler)

Blood flow was measured in the dorsal skin with a laser-Doppler flow probe (Perimed II, Stockholm, Sweden). Each reading took 15 s with a 10 s interval between readings and the mean of three readings per site were taken. Results were recorded as red blood cell flux (the number of moving red cells detected by the laser beam $\times$ mean cell velocity) and expressed as a percentage of a standardized signal obtained in a particulate control medium. The laser Doppler was set at 4 Hz, gain 10 and a time constant of 1.5 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 4 mm s⁻¹. Test compounds were injected in a randomized site pattern.

Ultraviolet B irradiation

A high dose u.v.B lamp was used (StaFL 400 W, Starma Ltd., Romford, Essex). A filter was used to prevent heat transmission. The u.v.B intensity at the skin surface was measured with a UVX radiometer (UVX-31, UVP Ltd, Cambridge, GB) with spectral sensitivity in the wavelength range of 280–310 nm. The intensity was 20 mW cm⁻² with the lamp held 30 cm from the skin. All sites were exposed for 9 min. This dose caused approximately a 100% increase in skin blood flow at 18 h.

Experimental protocols

In a preliminary time course experiment, baseline blood flow was measured in marked sites in 6 rats and this was repeated 4 h and 18 h after irradiation.

A further 12 rats were irradiated in 5 sites after baseline blood flow was measured in each site. At 17.5 h the sites were injected with either phosphate buffered saline as control, indomethacin 3 nmol per site, L-NAME 100 nmol per site, L-arginine 10 µmol per site or L-NAME 100 nmol + L-arginine 10 µmol per site and blood flow re-measured at 18 h. In an additional 3 sites per rat, which were not irradiated, baseline blood flow measurements were followed by the injection of either control, indomethacin 3 nmol per site or L-NAME 100 nmol per site and blood flow re-measured 30 min after injection. To determine if the small change in blood flow in non-exposed skin induced by indomethacin or L-NAME was significant, an additional 16 rats were studied to examine effects on basal blood flow of control, indomethacin and L-NAME as above.

A separate batch of 6 rats received either D-NAME 100 nmol per site or control to duplicate sites 17.5 h after u.v.B irradiation and blood flow was measured at 18 h.

To determine if L-NAME or indomethacin act as non-specific vasoconstrictors, their effect on iloprost-induced vasodilatation was determined. Blood flow response were measured 30 min after the injection of control, iloprost

1 nmol per site, iloprost 1 nmol + L-NAME 100 nmol per site, and iloprost 1 nmol and indomethacin 3 nmol per site in 6 rats.

Three inhibitors of nitric oxide synthase were compared in a further experiment. Baseline blood flow was measured in 8 sites in 6 rats. One site was not irradiated as control and the other 7 irradiated and then injected 17.5 h later with the inhibitors L-NAME, canavanine, L-NMMA (each at 10 and 100 nmol per site), or phosphate buffered saline as control, and blood flow measured again at 18 h.

To test the effect of a topical corticosteroid on the u.v.B response, baseline blood flow readings were measured in 8 marked sites per rat. Two sites on each flank were covered whilst the other two sites on each flank were exposed to u.v.B. After exposure, the 4 sites on the left flank were painted with vehicle solution 0.5 ml and the 4 sites on the right flank with steroid solution 0.5 ml. Blood flow was re-measured in all 8 sites 18 h after irradiation and the animals then overdosed with anaesthetic. Three groups of 9 rats each received either 0.1, 0.01 or 0.001% concentrations of clobetasol 17-propionate.

Drugs and chemicals

Indomethacin was made as a stock solution of 10⁻² M in ethanol and then diluted in phosphate-buffered saline. Clobetasol 17-propionate was dissolved in 20% diethylacetamide, 40% acetone and 40% ethanol and this vehicle was used as the control. Iloprost was the generous gift of Schering AG Pharmaceutical Research, Berlin, Germany. All other drugs were dissolved and diluted in phosphate-buffered saline. Pentobarbitone sodium was from May & Baker. Other drugs and chemicals were from the Sigma Chemical Co., Poole, Dorset.

Statistical analysis

Data are presented as mean $\pm$ s.e.mean. Statistical analysis was performed by analysis of variance (ANOVA) and results termed significant if $P < 0.05$.

Results

Ultraviolet B irradiation caused a delayed onset increase in skin blood flow when injected intradermally in rat skin *in vivo*. This was readily detected by laser Doppler flow probe, but was only visible as a slight reddening of the skin between 15 and 24 h after exposure. In a preliminary experiment in six rats the % increase in basal blood flow in irradiated sites was 66 $\pm$ 23% at 4 h and 124 $\pm$ 20% at 18 h (18 h response significantly greater than the 4 h response, $P < 0.05$ ANOVA). In three animals the inhibition of the 18 h u.v.B response was greater if L-NAME was injected at 17.5 h than if injected at 17 h; injection immediately after irradiation had no effect on the 18 h response, D-NAME, 100 nmol per site, injected 17.5 h after u.v.B exposure had no significant effect on the 18 h response; the % change in blood flow was $-8 \pm 6\%$, $146 \pm 25\%$ and $113 \pm 15\%$ in non-exposed sites injected with phosphate-buffered saline, exposed sites injected with phosphate-buffered saline, and exposed sites injected with D-NAME, respectively ($n = 6$ rats).

Figure 1 shows that L-NAME had a small vasoconstrictor effect in control skin sites when blood flow was re-measured 30 min after injection ($P < 0.05$, ANOVA). This could indicate basal release of the endogenous vasodilator nitric oxide, although this response may depend upon the mild irritation of shaving. Exposure of sites to u.v.B irradiation increased basal blood flow significantly at 18 h in control sites which had been injected with phosphate buffered saline at 17.5 h and the effect of u.v.B was abolished by the local injection of either indomethacin or L-NAME ($P < 0.05$ in each case, ANOVA).

Figure 1 The inhibition of the vasodilator response to u.v.B irradiation by the nitric oxide synthase inhibitor, N^G -nitro-L-arginine methyl ester (L-NAME) and the cyclo-oxygenase inhibitor, indomethacin, in rat skin. Control (solid columns); indomethacin 3 nmol per site (open columns); L-NAME 100 nmol per site (hatched column); L-NAME + L-arginine 10 μ mol per site (cross-hatched column); or L-arginine 10 μ mol per site (horizontally lined column); were injected 17.5 h after exposure of the shaved dorsal skin to u.v.B. The vasodilator response was measured in both irradiated and non-irradiated skin sites at 18 h. L-NAME significantly reduced blood flow in non-irradiated sites and both L-NAME and indomethacin significantly reversed the effect of u.v.B irradiation (* $P < 0.05$ in each case). The effect of L-NAME on u.v.B irradiation was partially reversed by the co-injection of L-arginine (* $P < 0.05$). Each column represents the mean $\pm$ s.e.mean percentage change in basal blood flow for 12–28 rats.

Figure 2 Comparison of the attenuation of the blood flow response to u.v.B irradiation in rat skin by 3 inhibitors of nitric oxide synthase N^G -nitro-L-arginine methyl ester (L-NAME), canavanine and N^G -monomethyl-L-arginine (L-NMMA) (hatched columns) were injected at 10 and 100 nmol per site 17.5 h after exposure of the shaved dorsal skin to u.v.B irradiation and the vasodilator response measured at 18 h. The change in blood flow in control injected, non-irradiated sites is shown by the first solid column, and in control injected and irradiated sites by the second solid column. All three agents significantly inhibited the response and this was dose-dependent (* $P < 0.05$ in each case). Results are the mean $\pm$ s.e.mean of 6 rats.

The possibility that L-arginine could reverse the effects of L-NMMA was tested. L-NAME at 100 nmol per site was not reversed completely by an excess of L-arginine; in the experiment depicted in Figure 1, irradiated sites injected with L-arginine 10 μ mol per site showed a similar response to those irradiated and injected with control buffer. There was partial reversal of the effects of L-NAME by the co-injection of L-NAME with an excess of L-arginine, $P < 0.05$, ANOVA, $n = 12$. In a further four rats, a lower dose of L-NAME was reversed to a greater extent, the increase in blood flow at 18 h in irradiated sites was $70 \pm 1\%$, -55 ± 4 and $45 \pm 35\%$ when injected with control, L-NAME 10 nmol per site, and L-NAME 10 nmol + L-arginine 10 μ mol per site respectively (all mean $\pm$ s.e.mean).

L-NAME and indomethacin did not significantly affect the response to iloprost. Iloprost 1 nmol per site caused a $158 \pm 18\%$ increase in blood flow at 30 min compared to $42 \pm 15\%$ change in control sites. Co-injection of L-NAME or indomethacin with iloprost caused a $154 \pm 19\%$ and $106 \pm 20\%$ change in blood flow respectively ($n = 6$).

All three inhibitors of nitric oxide synthase suppressed the 18 h blood flow response to u.v.B in a dose-dependent manner when injected locally into sites which were irradiated 17.5 h previously (Figure 2, $P < 0.05$ in each case, ANOVA). Canavanine and L-NMMA were equipotent whereas L-NAME was at least ten fold more potent than the other two. In a separate experiment, canavanine 100 nmol per site had no significant effect on basal skin blood flow at 30 min the effect in canavanine injected sites being $38 \pm 18\%$ change and $5 \pm 12\%$ in control sites ($n = 6$ rats).

Figure 3 shows that increased blood flow in u.v.B irradiated skin was attenuated by topical steroid treatment. Clobetasol 17-propionate applied topically immediately after exposure suppressed the response in a dose-dependent manner. It was effective at 0.1, 0.01 and 0.001% concentrations ($P < 0.05$ in each case compared with control). With the 0.1% concentration there is the suggestion that the control response is diminished, consistent with some systemic absorption of clobetasol from the treated side acting on the control side. In non-irradiated sites the blood flow changes at 18 h in

Figure 3 The inhibition of the vasodilator response to u.v.B in rat skin by the corticosteroid, clobetasol, applied topically immediately after irradiation. The graph shows the increase in blood flow at 18 h in the irradiated sites for those sites treated with vehicle control (solid columns) and those treated with clobetasol (open columns). The results at each concentration are the mean $\pm$ s.e.mean of 9 rats, * $P < 0.05$ in each case.

vehicle- and steroid-treated sites were respectively $-6 \pm 6\%$ and $-13 \pm 8\%$ in the 0.1% steroid group; $4 \pm 2\%$ and $2 \pm 4\%$ in the 0.01% steroid group; and $13 \pm 7\%$ and $16 \pm 5\%$ in the 0.001% group.

Discussion

The present study shows that exposure to u.v.B irradiation causes a delayed increase in blood flow in shaved rat skin *in vivo*. Three different inhibitors of NOS antagonized the increase in blood flow indicating that endogenous nitric oxide is involved. The inhibition by canavanine implicates the inducible form of NOS, as iNOS from leucocytes is inhibited

by canavanine whereas cNOS is not (Iyengar *et al.*, 1987; McCall *et al.*, 1989). It has been suggested that L-NMMA is at least as effective as L-NAME at inhibiting leucocyte iNOS (McCall *et al.*, 1989; Gross *et al.*, 1990), however, L-NAME was at least ten fold more potent in the present study at inhibiting the u.v.B response. Discrepancies in the relative potency of the arginine analogues may arise from the minor differences in the subclasses of the NOS group of enzymes (Nathan, 1992). The delayed onset of the blood flow response and its inhibition by a corticosteroid makes it most likely that it is the inducible form of the enzyme that is involved.

The effect of L-NAME was reversed by an excess of L-arginine which suggests that NO was formed from L-arginine. The reversal by L-arginine was not complete, particularly when a high dose of L-NAME was used, consistent with L-NAME having some irreversible inhibitory action on NOS (Dwyer *et al.*, 1991). We have shown previously that the D-isomer of NMMA is inactive in rat skin (Warren *et al.*, 1992) and the D-isomer of NAME had no effect on the u.v.B response in the present experiment.

An intermediate in the reaction to u.v.B that can induce iNOS is interleukin-1 (IL-1). Epidermal cells irradiated *in vivo* or *in vitro* increase their synthesis of IL-1 (Schwarz *et al.*, 1987) and this may contribute to the sunburn reaction in man (Murphy *et al.*, 1989). IL-1 is a potent stimulus to iNOS, an action prevented by corticosteroids (Busse & Mulsch, 1990; Schini *et al.*, 1991; Moncada & Higgs, 1991; Nathan, 1992). Future experiments with a specific IL-1 antagonist may elucidate the role of IL-1 further.

The cellular origin of the nitric oxide in the present model is not clear. Vascular smooth muscle is the most likely source as iNOS predominates in this tissue, in contrast to the endothelium where cNOS predominates. Neurones also release nitric oxide, although it is possible to induce an erythema response to u.v.B in denervated skin in man (Lewis *et al.*, 1927), suggesting normal nerve function is not a pre-requisite. Leucocytes are yet another source that has not

been excluded. There is evidence to suggest that nitric oxide-mediated vasodilatation contributes to the oedema component of inflammation as several vasodilators potentiate oedema (Williams & Peck, 1977a; Williams, 1982; Warren *et al.*, 1992) and nitric oxide is involved in the oedema response to substance P in rat skin (Hughes *et al.*, 1990).

The inhibition of the u.v.B-induced hyperaemia by indomethacin implicates prostaglandins in the pathogenesis of this response, although prostaglandin production was not measured directly. The response to u.v.B is slow in onset suggesting enzyme induction, possibly of the inducible form of cyclo-oxygenase (Masferrer *et al.*, 1992). Corticosteroids may suppress the u.v.B response by inhibiting the inducible form of cyclo-oxygenase (Masferrer *et al.*, 1992), by inhibiting phospholipase A₂ (Axelrod, 1983), as well as by preventing the induction of iNOS. Prostaglandins are potent vasodilators of the microcirculation (Messina *et al.*, 1975; Williams, 1979; Messina & Kaley, 1980), in contrast to their low activity in large blood vessels (Gordon & Martin, 1983; Gryglewski *et al.*, 1986). Prostaglandins and nitric oxide might be synergistic in the microcirculation, analogous to their combined inhibition of platelet aggregation (Moncada & Higgs, 1991), although there is no evidence this is the case in the vasculature.

In conclusion, the pathogenesis of u.v.B-induced vasodilatation of the microcirculation of rat skin *in vivo* involves endogenous nitric oxide. The response was abolished by NOS inhibitors and the effectiveness of canavanine implicates the inducible form of NOS as the enzyme involved. Indomethacin was as effective as the NOS inhibitors suggesting a link between vasodilator prostaglandins and nitric oxide within the microcirculation. The suppression of the reaction by corticosteroids may be explained by the dual inhibition of both phospholipase A₂, and hence prostaglandin production, and the inhibition of iNOS.

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