Br. J. Pharmacol. (1993), **110**, 1515–1521 © Macmillan Press Ltd, 1993

Role of prostaglandins and nitric oxide in acute inflammatory reactions in guinea-pig skin

[1]M.M. Teixeira, T.J. Williams & P.G. Hellewell

Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 Oedema formation in skin is dependent on a synergism between mediators that increase vascular permeability and mediators that enhance local blood flow. Leukocyte accumulation is also enhanced by mediators that increase local blood flow. In this study, we have investigated whether nitric oxide (NO), an important endogenous vasodilator, could modulate oedema formation and leukocyte accumulation in guinea-pig skin.

2 Local administration of the NO synthesis inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), dose-dependently inhibited the oedema formation induced in response to intradermal injection of bradykinin or histamine. L-NAME, but not N^G-nitro-D-arginine methyl ester (D-NAME), also inhibited oedema formation in response to i.d. injection of platelet-activating factor (PAF), zymosan-activated plasma (ZAP) and in a passive cutaneous anaphylactic (PCA) reaction.

3 N-iminoethyl-L-ornithine (L-NIO) was less effective and about 100 times less potent than L-NAME in inhibiting bradykinin-induced oedema formation. The cyclo-oxygenase inhibitor, ibuprofen, had little effect on oedema responses induced by bradykinin, PAF and in a PCA reaction. On the other hand, histamine-induced oedema formation was significantly suppressed by ibuprofen.

4 The inhibition by L-NAME of bradykinin-induced oedema formation was reversed by co-injection of sodium nitroprusside (SNP) or prostaglandin E_1 (PGE_1).

5 L-NAME inhibited ^{111}In-eosinophil and ^{111}In-neutrophil accumulation induced by i.d. injection of ZAP. ^{111}In-eosinophil accumulation induced by PAF and in the PCA reaction was also inhibited by L-NAME but not by D-NAME.

6 Co-injection of SNP or PGE_1, reversed the inhibition by L-NAME of ZAP-induced oedema formation and ^{111}In-neutrophil accumulation. SNP, but not PGE_1, also reversed the effects of L-NAME on ZAP-induced ^{111}In-eosinophil accumulation.

7 L-NAME caused a significant decrease in basal cutaneous blood flow when injected alone or with bradykinin. Again, SNP or PGE_1 reversed the effects of L-NAME suggesting that the inhibitory action of L-NAME on oedema formation and cell accumulation was due to an inhibition of vasodilator tone in the microcirculation.

8 Thus, it appears that in guinea-pig skin the inhibition of the production of endogenous NO inhibits both leukocyte accumulation and oedema formation induced by different mediators of inflammation. Since administration of L-NAME also causes a local decrease in basal blood flow, we suggest that this is the mechanism by which it exerts anti-inflammatory effects in this model.

Keywords: Nitric oxide; prostaglandins; eosinophils; neutrophils; inflammation; oedema; vasodilatation; N^G-nitro-L-arginine methyl ester

Introduction

Oedema formation, one of the features of the cutaneous inflammatory response, is dependent on a synergism between mediators that increase vascular permeability and mediators that increase blood flow (Williams & Morley, 1973; Williams & Peck, 1977). Thus, drugs such as indomethacin may inhibit inflammatory responses by virtue of their ability to inhibit production of vasodilator prostaglandins which are thought to act on arterioles to increase local blood flow at the site of injury or insult. Indeed, in rabbit skin, indomethacin inhibits oedema formation in various inflammatory reactions (Williams & Peck, 1977; Williams *et al.*, 1986; Needham *et al.*, 1988; Hellewell *et al.*, 1992). This synergism between mediators also implies that mediators which decrease local blood flow may attenuate oedema formation. In support of this, it has been shown that vasoconstrictor agents such as endothelin (Brain *et al.*, 1988) may decrease oedema formation induced by various mediators of inflammation (Brain *et al.*, 1989). The accumulation of leukocytes into sites of inflammation in the skin of various animals is also modulated by an increase in local blood flow (Issekutz & Movat, 1979; Issekutz, 1981; Buckley *et al.*, 1991). Thus, the regulation of blood flow at the site of an inflammatory insult appears to be an important mechanism in determining the outcome of the response.

Since first described by Furchgott and Zawadzki (1980), endothelium-derived relaxing factor has received great attention as an important endogenous dilator. The major vasodilator activity accounting for the effects of endothelium-derived relaxing factor has been attributed to nitric oxide (NO) (Palmer *et al.*, 1987). NO is a short-lived molecule which has been shown to have a role in the control of microvascular tone under basal conditions and under stress conditions (Ignarro, 1989; Moncada *et al.*, 1991). After appropriate stimulation, many different cells, including leukocytes, can express an inducible form of NO synthase which generates large amounts of NO (Moncada *et al.*, 1991). Both the endothelial (constitutive) and leukocyte (inducible) forms of NO synthase can be inhibited by L-arginine analogues and this has made it possible to investigate the role of NO in various pathophysiological conditions (for review see Moncada *et al.*, 1991).

In guinea-pig skin, local oedema formation and neutrophil accumulation are enhanced by intradermally-injected prostaglandins although the synergism is not as marked as that observed in rabbit skin (Williams & Morley, 1973; Williams

[1] Author for correspondence.

& Peck, 1977; Teixeira et al., 1993). This may be a reflection of higher basal flow in guinea-pig skin than in the rabbit. In this study we have investigated the role of endogenous prostaglandins and NO as modulators of acute inflammatory responses in guinea-pig skin.

Methods

Preparation of zymosan-activated plasma

Guinea-pig heparinized ($10\ u\ ml^{-1}$) plasma was incubated with zymosan ($5\ mg\ ml^{-1}$) at 37°C. After 30 min, zymosan was removed by centrifugation (2×10 min at 3000 g). The activated plasma obtained was then desalted using a PD-10 Sephadex G-25M column and stored in aliquots at − 20°C. Zymosan-activated plasma (ZAP) was used as a 10% solution in saline unless stated otherwise.

Preparation of passive cutaneous anaphylaxis sera and reactions

Details of the preparation of IgG_1-rich anti-sera are described elsewhere (Weg et al., 1991). Briefly, male guinea-pigs (Harlan Porcellus, Oxon, 350–400 g) were immunized with BGG in Freund's complete adjuvant (0.2 mg BGG $0.2\ ml^{-1}$ of adjuvant s.c.). On day 21, these animals received a boost of antigen in Freund's incomplete adjuvant and the serum was collected and prepared on day 30. Skin sites were sensitized for passive cutaneous anaphylatic (PCA) reactions by giving an i.d. injection of a 1/50 dilution of anti-BGG anti-serum. After allowing antibody to fix to tissue cells for 16–20 h, PCA reactions were induced by an i.d. injection of antigen (BGG, 1 μg of antigen per site).

Induction, purification and radiolabelling of guinea-pig-eosinophils

The method is described in detail by Faccioli et al. (1991). Briefly, ex-breeder female guinea-pigs (Harlan Porcellus, Oxon; 700–800 g) were treated with horse serum (1 ml) i.p. every other day for two weeks. A boost was given on the day prior to collection of the cells. The animals were killed by exposure to CO_2 and their peritoneal cavities washed out with heparinized saline ($10\ u\ ml^{-1}$). The cells obtained were layered onto a discontinuous Percoll-HBSS (calcium- and magnesium-free) gradient prepared on the day of the procedure and the eosinophils collected from the 1.085/1.090 and 1.090/1.095 interfaces. Cells were used only if purity was greater than 95%. The main contaminating cells were mononuclear cells and the presence of neutrophils ($>1\%$) was a major exclusion criteria for the use of the preparation. Viability was assessed in some experiments by trypan blue exclusion and was found to be greater than 98%. The purified eosinophils (1.5 to 3.0×10^7 cells) were radiolabelled by incubation with ^{111}Indium ($100\ \mu Ci$ in $10\ \mu l$) chelated to 2-mercaptopyridine-N-oxide ($40\ \mu g$ in 0.1 ml of 50 mM PBS, pH 7.4) for 15 min at room temperature. The cells were then washed twice in HBSS (calcium and magnesium-free) containing 10% guinea-pig platelet-poor plasma and resuspended at a final concentration of 10^7 cells ml^{-1} prior to i.v. injection.

Induction, purification and radiolabelling of guinea-pig neutrophils

Neutrophils were elicited in the peritoneal cavity of naive ex-breeder guinea-pigs by the i.p. injection of 15 ml of a 5% (w/v) solution of casein. After 12–16 h, the animals were killed and the peritoneal cavity washed with heparinized saline ($10\ u\ ml^{-1}$). The rest of the procedure was followed as described for the eosinophils. The cells were also collected from the 1.085/1.090 and 1.090/1.095 interfaces. The purity of the preparation was always greater than 99% and the rare contaminants were eosinophils and occasional mononuclear cells. Viability, tested by trypan blue exclusion, was greater than 98%. Labelling of the cells was carried out as described above for the eosinophils.

Measurement of local oedema formation and ^{111}In-leukocyte accumulation in guinea-pig skin

Radiolabelled leukocyte infiltration and oedema formation were measured simultaneously in the skin. Guinea-pigs (Harlan Porcellus, Oxon, 350–400 g) were anaesthetized with Hypnorm (0.2 ml, i.m.) and received an i.v. injection of ^{111}In-labelled eosinophils or neutrophils (5×10^6 cells per animal) together with ^{125}I-human serum albumin ($5\ \mu Ci$). After 5 min, inflammatory mediators or antigen were injected i.d. into the dorsal skin of shaved animals with or without the NO synthase (NOS) or cyclo-oxygenase inhibitors. In some experiments, PGE_1 (3×10^{-10} mol per site) or SNP (10^{-7} mol per site) were also added. All drugs were mixed before the i.d. injection. Each animal received a duplicate of each treatment following a randomized injection plan and the inflammatory response (^{111}In-labelled cell accumulation and oedema formation) was assessed after 2 h. A maximum of 15 different treatments was given per animal and all experiments assessing the effects of an inhibitor were done in the same animals. Experiments were carried out over 2 h since previous time course studies indicated that the majority of plasma leakage or leukocyte accumulation was complete at this time (Faccioli et al., 1991; Weg et al., 1992; Collins et al., 1993). At 2 h, a blood sample was obtained by cardiac puncture and plasma prepared; the animals were killed by an overdose of sodium pentobarbitone and the dorsal skin was removed. Skin sites were punched out with a 17 mm punch and, together with plasma samples, counted in an automatic 5-head gamma-counter (Canberra Packard Ltd, Pangbourne, Berks.) with cross-channel correction for the two isotopes.

The number of leukocytes accumulating at the site of inflammation was expressed as ^{111}In-labelled cells per skin site based on the specific activity of the leukocytes (i.e. counts per cell) in each experiment. Oedema formation was expressed as μl of plasma, obtained by dividing ^{125}I counts of the skin sample by the ^{125}I counts in 1 μl of plasma.

Measurement of skin blood flow

Guinea-pigs (350–400 g) were shaved and depilated the day before the experiment to avoid any non-specific effect of the depilating agent (Immac, Reckitt & Colman Products, Hull). The animals were allowed to acclimatize to the laboratory temperature for at least 1 h and then anaesthetized (Hypnorm. 0.15 ml, i.m.). Temporal alterations of red blood cell flux, taken as an index of skin blood flow, were determined with a Perimed II laser-Doppler flow meter (Perimed, Stockholm, Sweden) and recorded on a MacLab device (ADI Instruments, Reading, Berks.) coupled to an Apple Macintosh Classic (Apple Computer Inc., CA, U.S.A.). Triplicate basal readings of the site to be injected were obtained and the drugs (mixed prior to injection when necessary) were injected i.d. Ten min later, triplicate readings of the same sites were obtained again and averaged. Each animal received up to 6 injections. Results were expressed as % change of basal flow (i.e. flow in the site prior to the i.d. injection).

Reagents

The following compounds were purchased from Sigma Chemical Company (Poole, Dorset): bradykinin, casein, bovine gamma globulin (BGG), L-arginine, D-arginine, N^G-nitro-L-arginine-methyl ester (L-NAME), sodium nitroprusside (SNP) and zymosan. N^G-nitro-D-arginine-methyl ester (D-NAME) and PAF (C16) were purchased from Bachem (Saffron Walden, Essex). N(5)-(1-iminoethyl)-L-ornithine (L-

NIO, batch CZ92) was purchased from Cookson Chemicals Ltd, Southampton). Hanks balanced salt solution (HBSS), HEPES and horse serum were purchased from Gibco Limited (Paisley, Renfrewshire). Percoll was from Pharmacia (Milton Keynes, Bucks.) and prostaglandin E_1 (PGE_1) from Janssen Pharmaceuticals (Belgium). Ibuprofen was a gift from Boots Chemical Company plc (Nottingham, Notts.). $^{111}InCl_3$ (>10 mCi per μg in 0.04 M HCl) and ^{125}I-human serum albumin (2.5 μCi per mg in sterile isotonic saline) were purchased from Amersham International (Amersham, Herts.).

Statistics

Data are presented as means ± s.e.mean for the number of experiments indicated. Data were analyzed by two-way analysis of variance on normally distributed data. For blood flow experiments, Student's paired t test was used comparing the values before and after the local treatment. Values of $P<0.05$ were considered statistically significant.

Results

Effect of ibuprofen on oedema formation induced by bradykinin, histamine, PAF and in a PCA reaction

The potentiation of bradykinin-induced oedema formation by low doses of arachidonic acid (AA, 1.5×10^{-9} mol per site) has been shown previously to be dependent on the production of vasodilator prostaglandins (Williams & Peck, 1977). Ibuprofen, at a dose (10^{-7} mol per site) which inhibited this potentiation by >95% had little effect on oedema formation induced by bradykinin alone, PAF or in a PCA reaction (Table 1). In contrast, ibuprofen significantly suppressed histamine-induced oedema formation by 30% (Table 1).

Effect of L-NAME on oedema formation induced by bradykinin, histamine, PAF and in a PCA reaction

The concomitant injection of increasing doses of L-NAME with bradykinin (10^{-10} mol per site) caused a dose-dependent inhibition of bradykinin-induced oedema formation (Figure 1). At a dose of 10^{-6} mol per site, L-NAME inhibited bradykinin-induced oedema formation by 89% (Figure 1). Interestingly, oedema formation induce by histamine, at a dose (2.5×10^{-8} mol per site) selected to induce a similar response to bradykinin (10^{-10} mol per site), was not inhibited to the same extent with a maximal inhibition of 62% (Figure 1). The dose of L-NAME used in all subsequent experiments was 10^{-6} mol per site. The inhibition was specific for L-NAME since D-NAME, at a dose of 10^{-6} mol per site, had no effect on bradykinin-induced oedema formation (bradykinin 10^{-10} mol per site, 33.8 ± 4.5 μl; bradykinin + D-NAME 10^{-6} mol per site, 29.6 ± 2.0 μl; bradykinin + L-NAME 10^{-6} mol per site, 12.6 ± 1.4 μl; saline, 8.6 ± 0.6 μl, $n = 4$). L-NAME also effectively suppressed the oedema formation induced by PAF (10^{-9} mol per site) and a PCA reaction by up to 68% and 81%, respectively (Table 2). However, these responses were not altered by D-NAME (Table 2). There was no additional inhibition of histamine- and bradykinin-induced oedema formation if ibuprofen was added to L-NAME (data not shown).

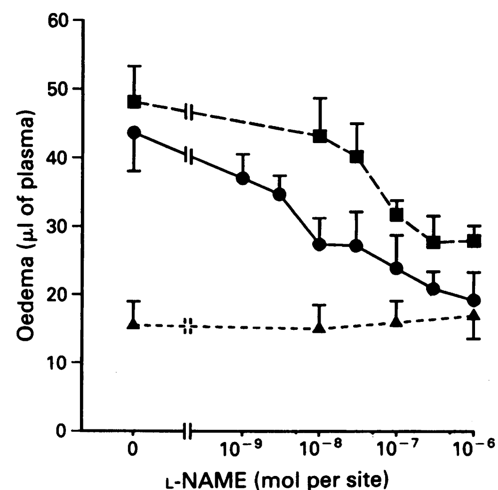

Figure 1 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on oedema formation induced by i.d. injection of bradykinin (10^{-10} mol per site, ●) or histamine (2.5×10^{-8} mol per site, ■) in guinea-pig skin. The effect of L-NAME alone (injected in saline) is shown by (▲). Oedema formation was assessed 2 h after the i.d. injections. Values are mean ± s.e.mean from 4–6 guinea-pigs.

Table 1 Effect of ibuprofen on oedema formation in guinea-pig skin

	Control (μl of plasma)	*Ibuprofen* (μl of plasma)
BK	32.2 ± 2.6	31.0 ± 2.8
BK + AA	44.8 ± 2.4	31.6 ± 1.7*
PAF	50.4 ± 2.8	48.6 ± 5.9
PCA	29.3 ± 7.0	25.6 ± 3.6
Hist	54.4 ± 5.2	38.4 ± 7.0*
Saline	7.4 ± 1.8	5.6 ± 0.7
AA	9.4 ± 2.1	ND

The mediators or antigen were mixed with ibuprofen (10^{-7} mol per site) prior to their i.d. injection and oedema formation assessed 2 h later. The following stimuli were used: bradykinin (BK, 10^{-10} mol per site), arachidonic acid (AA, 1.5×10^{-9} mol per site), platelet-activating factor (PAF, 10^{-9} mol per site), histamine (Hist, 2.5×10^{-8} mol per site) and a passive cutaneous anaphylaxis (PCA) reaction (1 μg of BGG per site). Results are mean ± s.e. mean of at least 4 animals in each group. *$P<0.05$ when compared to control values. ND, not determined.

L-NIO has been shown previously to be a potent inhibitor of NO synthase in leukocytes and rat skin (McCall *et al.*, 1991; Mulligan *et al.*, 1992). Therefore we decided to compare the activity of L-NIO with that of L-NAME on bradykinin-induced oedema formation in the guinea-pig skin. Figure 2 shows the effects of L-NIO and L-NAME on oedema formation induced by bradykinin in guinea-pig skin. In these experiments, bradykinin-induced oedema responses were suppressed by up to 80% and 30% by L-NAME and L-NIO, respectively. Oedema formation induced by histamine was also less effectively inhibited by L-NIO than L-NAME (data not shown). The L-NIO preparation used was highly effective as assessed by its ability to block the relaxation induced by acetylcholine (10^{-6} M) of rat aortic rings pre-contracted with phenylephrine (5×10^{-7} M). In this preparation, L-NAME (10^{-6} M) and L-NIO (10^{-6} M) inhibited acetylcholine-induced relaxation by 89% and 80%, respectively ($n = 2$).

Effect of PGE_1 and SNP on the inhibition by L-NAME of bradykinin-induced oedema formation

The addition of PGE_1 or SNP to the mixture of bradykinin and L-NAME prior to their injection reversed the inhibitory effects of L-NAME on bradykinin-induced oedema formation (Figure 3). L-Arginine, in doses up to 50 times greater than L-NAME, only partially reversed the inhibitory effects of L-NAME (data not shown). The D-enantiomer of arginine (5×10^{-6} mol per site) displayed inflammatory effects when injected alone, so it was not possible to draw any conclusions from its use (data not shown).

Table 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) and D-NAME on ^{111}In-eosinophil accumulation and oedema formation in guinea-pig skin

		Oedema (µl of plasma)			*^{111}In-eosinophils*		
		Control	*L-NAME*	*D-NAME*	*Control*	*L-NAME*	*D-NAME*
ZAP	10%	31.4 ± 6.8	12.6 ± 2.2*	29.1 ± 5.1	6172 ± 781	738 ± 143*	5898 ± 914
	30%	41.5 ± 2.8	23.0 ± 2.6*	ND	9618 ± 1325	4552 ± 918*	ND
PCA		29.1 ± 5.1	14.2 ± 2.5*	28.2 ± 7.5	6391 ± 844	3605 ± 876*	7141 ± 1920
PAF		48.3 ± 6.3	22.9 ± 2.6*	43.2 ± 12.9	9186 ± 1698	1267 ± 242*	9087 ± 1136
BK		33.8 ± 4.5	12.6 ± 1.4*	29.6 ± 2.0	252 ± 51	198 ± 33	555 ± 114
Sal		10.4 ± 1.5	10.6 ± 1.4	11.0 ± 2.5	454 ± 41	368 ± 42	362 ± 73

The mediators or antigen were mixed with L-NAME (10^{-6} mol per site) or D-NAME (10^{-6} mol per site) prior to i.d. injection and oedema formation and ^{111}In-eosinophil accumulation assessed after 2 h. The following stimuli were used: zymosan-activated plasma (ZAP, 10% and 30% in saline), a passive cutaneous anaphylaxis (PCA) reaction (1 µg of BGG per site), platelet-activating factor (PAF, 10^{-9} mol per site), bradykinin (BK, 10^{-10} mol per site) and in saline injected sites (Sal). Results are expressed as the mean ± s.e.mean for 4–6 animals. *$P<0.05$ when compared to control values. ND, not determined.

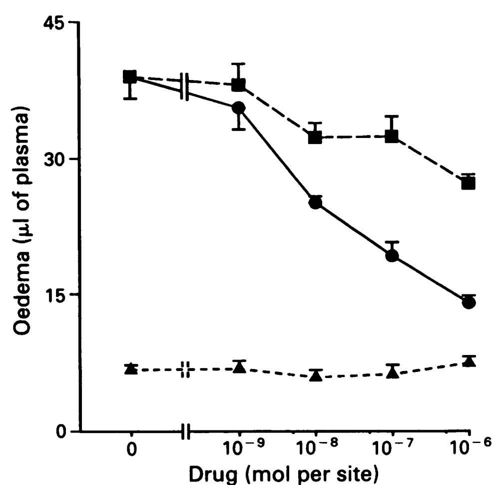

Figure 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME, ●) and N(5)-(1-iminoethyl)-L-ornithine (L-NIO, ■) on oedema formation induced by i.d. injection of bradykinin (10^{-10} mol per site) in guinea-pig skin. The effect of L-NIO alone (injected in saline) is shown by (▲). Oedema formation was assessed 2 h after the i.d. injections. Values are mean ± s.e.mean from 4 guinea-pigs.

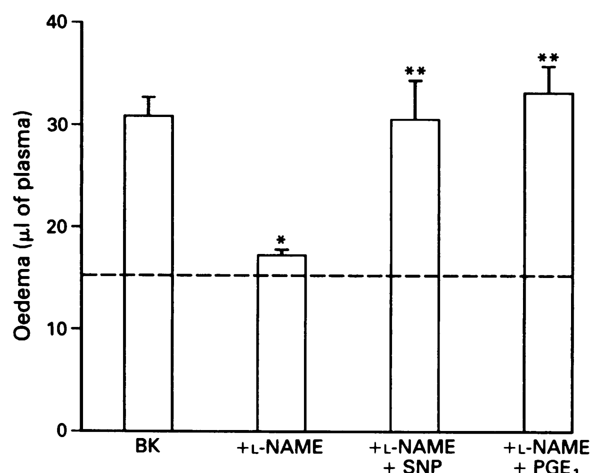

Figure 3 Reversal of the inhibitory effect of N^G-nitro-L-arginine methyl ester (L-NAME) on bradykinin-induced oedema formation in guinea-pig skin by sodium nitroprusside (SNP) or prostaglandin E_1 (PGE_1). Bradykinin (BK, 10^{-10} mol per site) was injected alone, with L-NAME (10^{-6} mol per site) and with L-NAME plus either SNP (10^{-7} mol per site) or PGE_1 (3×10^{-10} mol per site) and oedema formation was assessed after 2 h. The dashed line across the graph represents the background value obtained after i.d. injection of saline. Values are means ± s.e.mean from 7 guinea-pigs. *$P<0.05$ when compared to bradykinin; **$P<0.05$ when compared to bradykinin + L-NAME.

Effect of L-NAME on ZAP-induced oedema formation and leukocyte accumulation

The local injection of ZAP in guinea-pig skin has been shown previously to induce an inflammatory response in which the accumulation of leukocytes is a prominent feature (Faccioli *et al.*, 1991). When ZAP was injected with L-NAME, a significant inhibition of both oedema formation and cell accumulation was observed (Table 2, Figure 4), but D-NAME had no effect on ZAP-induced responses (Table 2). The inhibitory effects of L-NAME on ZAP-induced oedema formation (Figure 4a) and neutrophil accumulation (Figure 4b) were reversed by co-injection of PGE_1 (3×10^{-10} mol per site) or SNP (10^{-7} mol per site). However, the inhibition of ZAP-induced eosinophil accumulation by L-NAME was reversed by SNP, but not by PGE_1 (Figure 4c).

Eosinophil accumulation induced by PAF (10^{-9} mol per site) and in a PCA reaction were also suppressed by co-injection of L-NAME (Table 2). In addition, L-NAME suppressed PAF-induced neutrophil accumulation by 72%. Neither eosinophil accumulation nor oedema formation induced by these mediators was affected by D-NAME (Table 2).

Effect of L-NAME on blood flow in guinea-pig skin

In order to determine whether the inhibitory effects of L-NAME on oedema formation and cell accumulation could be due to a local decrease in blood flow, we measured cutaneous blood flow using a laser-Doppler flow system. Induction of anaesthesia with Hypnorm did not alter blood flow in uninjected sites (7.6 ± 5.1% increase in flow, $n = 3$, $P>0.05$) so all subsequent experiments were carried out in anaesthetized animals. As shown in Figure 5, L-NAME caused a significant decrease in basal flow, measured 10 min after its i.d. injection, compared with saline which did not change blood flow from the basal level. The co-injection of L-NAME with bradykinin also led to a significant decrease of local basal flow which was reversed by both PGE_1 and SNP (Figure 5). Basal flow in conscious animals was also inhibited by L-NAME (10^{-6} mol per site; 48.7 ± 7.1% decrease in flow, $n = 3$, $P<0.05$).

Discussion

Basal release of NO from endothelial cells has been previously shown and it is thought to contribute to the control of microvascular tone (Ignarro, 1989). By inhibiting NO production, an increase in vascular tone is observed and this may lead to systemic hypertension (Rees *et al.*, 1989). NO inhibition can also affect the inflammatory response induced by different mediators or reactions under different conditions (Garside *et al.*, 1992; Ialenti *et al.*, 1992; Lippe *et al.*, 1993; Miller *et al.*, 1993).

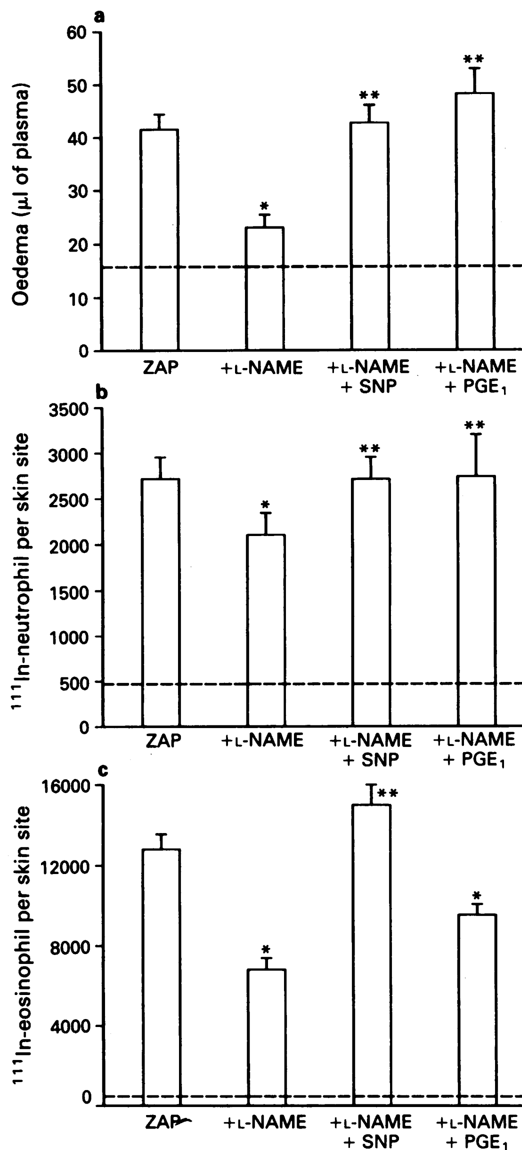

Figure 4 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on zymosan-activated plasma (ZAP)-induced (a) oedema formation, (b) neutrophil accumulation and (c) eosinophil accumulation and reversal by sodium nitroprusside (SNP) and prostaglandin E_1 (PGE_1). ZAP (10% in saline) was injected alone, with L-NAME (10^{-6} mol per site) and with L-NAME plus either SNP (10^{-7} mol per site) or PGE_1 (3×10^{-10} mol per site) and oedema formation or radiolabelled cell accumulation assessed after 2 h. The dashed line across each graph represents the background value obtained after i.d. injection of saline. Values are means ± s.e.mean from 4–8 guinea-pigs. $^*P<0.05$ when compared to ZAP alone; $^{**}P<0.05$ when compared to ZAP + L-NAME.

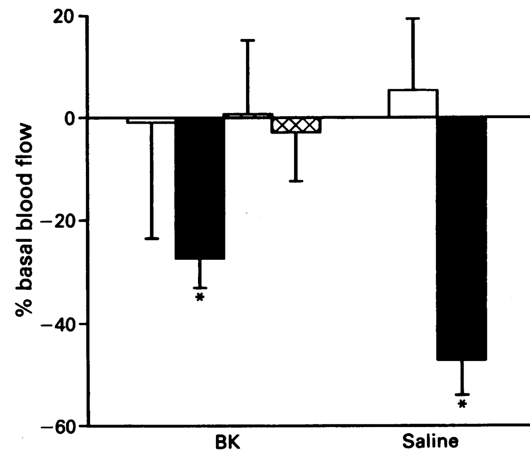

Figure 5 Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on blood flow in guinea-pig skin and reversal by sodium nitroprusside (SNP) or prostaglandin E_1 (PGE_1). Triplicate measurements were obtained in each site before and 10 min after the i.d. injections. Bradykinin (BK 10^{-10} mol per site) or saline were injected alone (open columns), with L-NAME (10^{-6} mol per site, solid columns) and with L-NAME plus SNP (10^{-7} mol per site, hatched column) or PGE_1 (3×10^{-10} mol per site, cross-hatched column). Values are means ± s.e.mean of 4–5 guinea-pigs. $^*P<0.05$ when compared to basal flow before the i.d. injection.

When the NO inhibitor, L-NAME, was co-injected with bradykinin, we observed dose-dependent inhibition of bradykinin-induced oedema formation in guinea-pig skin. This inhibitory effect of L-NAME, but not D-NAME, on oedema formation was also observed when histamine, PAF, a PCA reaction or ZAP were used as inflammatory stimuli. Nevertheless, the degree of inhibition of PAF- and histamine-induced oedema formation was less marked than that towards bradykinin. This observation has been previously noted, at least for histamine (Paul *et al.*, 1992), and it is thought to be due to differences in mechanisms by which these mediators produce oedema formation in guinea-pig skin. For example, it is possible that L-arginine liberated on breakdown of bradykinin in skin may be converted to NO, contributing to further vasodilatation. However, injection of L-arginine (10^{-5} mol per site) with bradykinin (10^{-10} mol per site) or ZAP (10%) had no effect on plasma leakage, arguing against such a possibility. It is also possible that histamine (and also PAF) may induce local release of prostaglandins which may contribute to the local maintenance of blood flow. In fact, histamine-induced oedema was significantly inhibited (by 30%) by ibuprofen at a dose that blocked cyclo-oxygenase in skin by >95% (as assessed by inhibition of responses to bradykinin potentiated by arachidonic acid). However, oedema responses induced by bradykinin, PAF and in a PCA reaction were unaltered by cyclo-oxygenase inhibition. This is in contrast to inflammatory responses in rabbit skin where local oedema formation induced by polycations, a PCA reaction and a reversed passive Arthus reaction are reduced by approximately 50% by administration of indomethacin (Williams *et al.*, 1986; Needham *et al.*, 1988; Hellewell *et al.*, 1992).

L-NIO has been described as a potent inhibitor of both the inducible and the constitutive form of NO synthase (Moncada *et al.*, 1991). In an immune complex-induced vasculitis model in rat skin, L-NIO was much more potent in inhibiting oedema formation than other inhibitors of NO synthase (Mulligan *et al.*; 1992). L-NIO has also been reported to be more potent than other inhibitors in suppressing NO production by activated leukocytes (McCall *et al.*, 1991) which might explain its potent effects on plasma leakage induced by deposition of immune complexes in rat skin, a response known to be neutrophil-dependent (Mulligan *et al.*, 1992). In our model, L-NIO was about 100 times less potent than L-NAME in inhibiting oedema formation induced by bradykinin. However, the same batch of the compound effectively inhibited relaxation of rat aorta induced by acetylcholine. The relative refractoriness of guinea-pig skin to the inhibitory action of L-NIO has not been described previously.

The inhibitory effect of L-NAME on bradykinin-induced oedema was reversed by SNP, a nitrovasodilator which releases NO as its active moiety (Ignarro, 1989). PGE_1 also effectively reversed L-NAME inhibition of bradykinin-induced oedema formation. Prostaglandins act on EP prostanoid receptors which are linked to G-proteins and may lead to an increase in adenosine 3':5'-cyclic monophosphate (cyclic) AMP levels (via EP_2 receptors) or activate phospholipase C (via EP_1 receptors) (Coleman & Humphrey,

1993). We are not aware of any study showing that prostaglandins may alter cyclic GMP levels within any type of cell. The most likely explanation for reversal of the effects of L-NAME on bradykinin-induced oedema formation by PGE_1 is local vasodilation. In rabbit skin, PGE_1 has been shown to have direct vasodilator activities as demonstrated using a xenon clearance technique (Williams, 1979). However, in guinea-pig skin using the laser Doppler method, we were able to detect only minor and inconsistent increases in local blood flow above basal levels when PGE_1 was injected alone ($19.6 \pm 14.8\%$ increase, $n = 4$). This could mean that the microcirculation was almost maximally dilated (under the control of endogenous NO) or that the laser Doppler technique is not as sensitive as xenon clearance. A more likely explanation is that the laser-Doppler method measures flow in superficial tissues of the skin, whereas the xenon clearance method measures flow in the deeper dermis in the region of direct exposure to PGE_1.

L-Arginine was weak in reversing the effects of L-NAME on bradykinin-induced oedema formation. This also seems to be the case in rabbit and human skin (S. Larkin, personal communication). It is possible that L-arginine does not achieve a sufficiently high concentration at skin sites to reverse the effect of L-NAME. However, the fact that D-NAME was ineffective in inhibiting oedema formation by any of the mediators tested provided good evidence that the inhibition of inflammation by L-NAME was due to inhibition of NO synthase rather than a non-specific action (Moncada *et al.*, 1991).

ZAP is a potent leukocyte chemoattractant in guinea-pig skin (Faccioli *et al.*, 1991). When ZAP was co-injected with L-NAME, not only was an inhibition of ZAP-induced oedema formation noticed but also a significant inhibition of ZAP-induced eosinophil and neutrophil accumulation (Figure 4). PAF- and the PCA reaction-induced eosinophil accumulation were also inhibited by L-NAME, but not by D-NAME (Table 2), again suggesting the stereospecific nature of the inhibition. Interestingly, the inhibition of ZAP-induced neutrophil accumulation was also reversed by SNP and PGE_1. Since neutrophil accumulation has been previously shown to depend on the local flow at the inflammatory site (Issekutz & Movat, 1979; Buckley *et al.*, 1991), it is also possible that both SNP and PGE_1 are reversing the inhibition of ZAP-induced neutrophil accumulation by reversing the decrease in local basal flow induced by L-NAME. The inhibition of ZAP-induced eosinophil accumulation by L-NAME was reversed by SNP, whereas PGE_1 was much less effective. Since both PGE_1 and SNP appeared to reverse the L-NAME-induced decrease in local flow to the same extent (Figure 5), it is unlikely that a difference in flow could explain the different effects of these two vasodilators on eosinophil accumulation. We have recently described the observation that i.d. administration of PGE_1 dose-dependently inhibits eosinophil accumulation induced by various stimuli in guinea-pig skin (Teixeira *et al.*, 1993). This effect, which occurred at the same time that PGE_1 enhanced oedema formation and neutrophil accumulation, is thought to be due to the capacity of PGE_1 to increase cyclic AMP levels in the target tissue (which could be the eosinophil itself or the venular endothelial cell) since the inhibitory effect was mimicked by isoprenaline (Teixeira *et al.*, 1993). Thus, in the case of leukocyte accumulation, PGE_1 can have two opposing effects.

There has been considerable interest in the study of the involvement of NO in inflammatory responses since the discovery that, with appropriate stimuli, inflammatory cells may produce large amounts of NO and that NO can be toxic to tissues, tumour cells and parasites (see Moncada *et al.*, 1991). The participation of NO in endotoxic shock has also been recently highlighted (Moncada *et al.*, 1991) as has its participation in different acute inflammatory models (Ialenti *et al.*, 1992; Mulligan *et al.*, 1992; Lippe *et al.*, 1993). The precise cellular target for the anti-inflammatory effects of NO inhibitors *in vivo* are nevertheless unknown. Recent work suggests that the inhibition of the production of NO in a model of adjuvant arthritis in rats may be due to inhibitory effect on T lymphocyte proliferation (Ialenti *et al.*, 1993).

In our model, the capacity of an NO inhibitor (L-NAME) to reduce inflammation appears to be linked to its ability to reduce skin blood flow. Others have found no suppression of leukocyte accumulation on a model of acute inflammation in the skin of rats even though oedema formation and red blood cell extravasation were potently inhibited (Mulligan *et al.*, 1991). In that series of experiments, the authors used rats rather than guinea-pigs and it is possible that the dependence of skin blood flow on NO is greater in the latter.

Kubes *et al.* (1991) and Kubes & Granger (1992) found that inhibitors of NO led to an increased oedema formation and leukocyte accumulation in a cat mesenteric preparation. They suggested that, by inhibiting NO production, there would be a loss of a protective (anti-adherence) effect of NO on neutrophils which would lead to their increased adhesiveness and injury to endothelial cells (Kubes *et al.*, 1991) and thence to increased vascular permeability. An antibody against CD11/CD18 partially blocked the increase in plasma exudation induced by inhibition of NO (Kubes & Granger, 1992). Vascular permeability was also enhanced in the rat coronary circulation after pre-treatment with L-NAME (Filep *et al.*, 1993). The neutrophil-dependence of hypoxic injury (Romson *et al.*, 1983) and the capacity of NO inhibitors to increase expression of CD11/CD18 may explain the increase in vascular permeability observed in these models. It is also possible that NO does not play a major role in modulating basal vascular flow in the cat mesentery or rat heart, though in the latter there is some evidence for NO involvement in control-ling perfusion pressure *in vitro* (Baydoun & Woodward, 1991).

We have shown that an NO inhibitor, L-NAME, significantly inhibits oedema formation and leukocyte accumulation in guinea-pig skin. These effects were reversed by SNP and by PGE_1, except in the case of eosinophil accumulation where PGE_1 was not as effective as SNP. Since both SNP and PGE_1 were also capable of inhibiting the L-NAME-induced reduction of basal flow, we suggest that the inhibitory effects of L-NAME on guinea-pig skin are related to its ability to reduce basal blood flow. This may also explain part of the inhibitory effects of NO inhibitors in other inflammatory models. The observation that cyclooxygenase inhibitors may inhibit inflammation through the inhibition of vasodilator prostaglandins (Williams & Peck, 1977) suggests that inhibitors of NO may also have a role as anti-inflammatory drugs.

We thank the National Asthma Campaign and Sandoz, Switzerland for support. We thank Miss A. Wilson for helpful technical support with rat aorta experiments.

References

BAYDOUN, A.R. & WOODWARD, B. (1991). Effects of bradykinin in the rat isolated perfused heart: role of kinin receptors and endothelium-derived relaxing factor. *Br. J. Pharmacol.*, **103**, 1829–1833.

BRAIN, D.S., CROSSMAN, D.C., BUCKLEY, T.L. & WILLIAMS, T.J. (1989). Endothelin-1: demonstration of potent effects on the microcirculation of humans and other species. *J. Cardiovasc. Pharmacol.*, **13** (Suppl 5), S147–S149.

BRAIN, S.D., TIPPINS, J.R. & WILLIAMS, T.J. (1988). Endothelin induces potent microvascular constriction. *Br. J. Pharmacol.*, **95**, 1005–1007.

BUCKLEY, T.L., BRAIN, S.D., COLLINS, P.D. & WILLIAMS, T.J. (1991). Inflammatory edema induced by interactions between interleukin-1 and the neuropeptide calcitonin gene-related peptide. *J. Immunol.*, **146**, 3424–3430.

COLEMAN, R.A. & HUMPHREY, P.P.A. (1993). Prostanoid receptors: their function and classification. In *Therapeutic Applications of Prostaglandins*. ed. Vane J. & O'Grady J. London: Edward Arnold, (in press).

COLLINS, P.D., WEG, V.B., FACCIOLI, L.H., WATSON, M.L., MOQBEL, R. & WILLIAMS, T.J. (1993). Eosinophil accumulation induced by human interleukin-8 in the guinea pig in vivo. *Immunology*, **79**, 312–318.

FACCIOLI, L.H., NOURSCHARGH, S., MOQBEL, R., WILLIAMS, F.M., SEHMI, R., KAY, A.B. & WILLIAMS, T.J. (1991). The accumulation of ^{111}In-eosinophils induced by inflammatory mediators in vivo. *Immunology*, **73**, 222–227.

FILEP, J.G., FOLDES-FILEP, E. & SIROIS, P. (1993). Nitric oxide modulates vascular permeability in the rat coronary circulation. *Br. J. Pharmacol.*, **108**, 323–326.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARSIDE, P., HUTTON, A.K., SEVERN, A., LIEW, F.Y. & MOWATT, A.M. (1992). Nitric oxide mediates intestinal pathology in graft-versus-host disease. *Eur. J. Immunol.*, **22**, 2141–2145.

HELLEWELL, P.G., JOSE, P.J. & WILLIAMS, T.J. (1992). Inflammatory mechanisms in the passive cutaneous anaphylactic reaction in the rabbit: evidence that novel mediators are involved. *Br. J. Pharmacol.*, **107**, 1163–1172.

IALENTI, A., IANARO, A., MONCADA, S. & DI ROSA, M. (1992). Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.*, **211**, 177–182.

IALENTI, A., MONCADA, S. & DI ROSA, M. (1993). Modulation of adjuvant arthritis by the L-arginine: NO pathway. *Br. J. Pharmacol.*, **108**, 10P.

IGNARRO, L.J. (1989). Biological actions and properties of endothelium-derived nitric oxide formed and released form artery and vein. *Circ. Res.*, **65**, 1–21.

ISSEKUTZ, A.C. (1981). Effect of vasoactive agents on polymorphonuclear leukocyte emigration in vivo. *Lab. Invest.*, **45**, 234.

ISSEKUTZ, A.C. & MOVAT, H.Z. (1979). The effect of vasodilator prostaglandins on polymorphonuclear leukocyte infiltration and vascular injury. *Am. J. Pathol.*, **107**, 300–309.

KUBES, P. & GRANGER, D.N. (1992). Nitric oxide modulates microvascular permeability. *Am. J. Physiol.*, **262**, H611–H615.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 4651–4655.

LIPPE, I.T., STABENTHEINER, A. & HOLZER, P. (1993). Participation of nitric oxide in the mustard oil-induced neurogenic inflammation of the rat paw skin. *Eur. J. Pharmacol.*, **232**, 113–120.

MCCALL, T.B., FEELISCH, M., PALMER, R.M.J. & MONCADA, S. (1991). Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase in phagocytic cells. *Br. J. Pharmacol.*, **102**, 234–238.

MILLER, M.J.S., SADOWASKA-KROWICKA, H., CHOTINARUEMOL, S., KAKKIS, J.L. & CLARK, D.A. (1993). Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J. Pharmacol. Exp. Ther.*, **264**, 11–16.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.*, **43**, 109–142.

MULLIGAN, M.S., HEVEL, J.M., MARLETTA, M.A. & WARD, P.A. (1991). Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6338–6342.

MULLIGAN, M.S., MONCADA, S. & WARD, P.A. (1992). Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis. *Br. J. Pharmacol.*, **107**, 1159–1162.

NEEDHAM, L., HELLEWELL, P.G., WILLIAMS, T.J. & GORDON, J.L. (1988). Endothelial functional responses and increased vascular permeability induced by polycations. *Lab. Invest.*, **59**, 538–548.

PALMER, R.M., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.

PAUL, W., LAWRENCE, L., DOUGLAS, G.J., SCHACHTER, M. & PAGE, C.P. (1992). Modulators of the L-arginine-nitric oxide pathway: differential effects on cutaneous permeability to bradykinin and histamine in the guinea pig. *Br. J. Pharmacol.*, **107**, 287P.

REES, D.D., PALMER, R.M.J. & MONCADA, S. (1989). Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3375–3379.

ROMSON, J.L., HOOK, B.G., KUNKEL, S.L., ABRAMS, G.D., SCHORK, M.A. & LUCCHESI, B.R. (1983). Reduction of the extent of ischemic myocardial injury by neutrophil in the dog. *Circulation*, **67**, 1016–1023.

TEIXEIRA, M.M., WILLIAMS, T.J. & HELLEWELL, P.G. (1993). E-type prostaglandins enhance local oedema formation and neutrophil accumulation but suppress eosinophil accumulation in guinea pig skin. *Br. J. Pharmacol.*, **110**, 416–422.

WEG, V.B., WATSON, M.L., CORDEIRO, R.S.B. & WILLIAMS, T.J. (1991). Histamine, leukotriene D_4 and platelet activating factor in guinea pig passive cutaneous anaphylaxis. *Eur. J. Pharmacol.*, **204**, 157–163.

WEG, V.B., WATSON, M.L., FACCIOLI, L.H. & WILLIAMS, T.J. (1992). [^{111}In]-eosinophil accumulation during passive cutaneous anaphylaxis in the guinea pig. *Br. J. Pharmacol.*, **105**, 127P.

WILLIAMS, T.J. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes of inflammation. *Br. J. Pharmacol.*, **65**, 517–524.

WILLIAMS, T.J., HELLEWELL, P.G. & JOSE, P.J. (1986). Inflammatory mechanisms in the Arthus reaction. *Agents Actions*, **19**, 66–72.

WILLIAMS, T.J. & MORLEY, J. (1973). Prostaglandin as potentiators of increased vascular permeability in inflammation. *Nature*, **246**, 215–217.

WILLIAMS, T.J. & PECK, M.J. (1977). Role of prostaglandin-mediated vasodilatation in inflammation. *Nature*, **270**, 530–532.

(Received June 14, 1993
Revised July 15, 1993
Accepted August 2, 1993)