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Effect of peroxynitrite on plasma extravasation, microvascular blood flow and nociception in the rat

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1 Peroxynitrite ($ONOO^-$) is a cytotoxic species, formed by the reaction between nitric oxide and superoxide free radicals, that may be involved in inflammation. In this study we have investigated the effect of peroxynitrite on plasma extravasation and microvascular blood flow in the dorsal skin and on nociceptive responses in the hind paw of the rat.

2 Male Wistar rats were anaesthetized and their dorsal skin shaved. Plasma extravasation was measured by the extravascular accumulation of ^{125}I-labelled albumin over 0–45 min and 0–240 min. Blood flow was measured by laser-Doppler flowmetry over 0–240 min. Studies in the hind paw were carried out in the conscious rat. Hind paw weight changes were determined by volume displacement and nociception by a mechanical hyperalgesia technique.

3 Intradermal (i.d.) peroxynitrite (100–200 nmol site^{-1}) produced a significant ($P<0.01$) dose-dependent increase in plasma extravasation in dorsal skin over 0–45 min which was not increased over 45–240 min. Plasma extravasation was significantly ($P<0.001$) decreased in rats pretreated with the anti-inflammatory steroid dexamethasone (1 mg kg^{-1}, i.v.; −180 min), but not modulated by treatment with the hydrogen peroxide deactivator catalase (2200 u site^{-1}), or the superoxide scavenger superoxide dismutase (500 u site^{-1}), effective doses of the tachykinin NK_1 antagonist SR140333 (1 nmol site^{-1}), the cyclo-oxygenase inhibitor indomethacin (358 μmol site^{-1}), or combined pretreatment with mepyramine (histamine H_1-receptor antagonist; 2.8 nmol site^{-1}) and methysergide (5-HT antagonist; 1.9 nmol site^{-1}).

4 Microvascular blood flow was significantly ($P<0.05$) increased 30 and 120 min after i.d. peroxynitrite (100 nmol site^{-1}) in dorsal skin and remained raised until the end of the recording period (240 min). The increase in blood flow was unaffected by dexamethasone (1 mg kg^{-1}, i.v.; −180 min) or indomethacin (10 mg kg^{-1}, s.c.; −30 min).

5 Hind paw volume was significantly ($P<0.001$) increased 30 min after intraplantar peroxynitrite (87.5 and 175 nmol paw^{-1}) and remained raised for the duration of the experiment (360 min). By comparison, nociception was not altered by intraplantar peroxynitrite.

6 These data indicate that peroxynitrite can cause an increase in both plasma extravasation and blood flow, suggesting that peroxynitrite could be of biological relevance to microvascular responses. These findings may be of importance in the pathology of inflammatory diseases in which peroxynitrite formation occurs.

Keywords: Peroxynitrite; microvascular; plasma extravasation; oedema formation; algesia

Introduction

The formation of nitric oxide *in vitro* is catalysed by the enzyme nitric oxide synthase (NOS, for a review, see Moncada *et al*., 1991). Nitric oxide synthase has at least three isoforms, two of which are constantly expressed and termed constitutive NOS (cNOS). The third isoform can be induced in many cell types including endothelial cells, macrophages, neutrophils and synoviocytes and is known as inducible NOS (iNOS; Nussler & Billiar, 1993). iNOS can be induced by several stimuli including inflammatory mediators such as IL-1 and IFNγ (Busse & Mulsch, 1990) and bacterial endotoxin (Boughton-Smith *et al*., 1993). iNOS has been linked to pathophysiological conditions associated with inflammation such as rheumatoid arthritis (Connor *et al*., 1995) and psoriasis (Sirsjo *et al*., 1996) as it synthesizes large amounts of nitric oxide for long periods.

Nitric oxide is a free radical and can react rapidly with other free radicals such as superoxide (O_2^-). This reaction results in the formation of peroxynitrite ($ONOO^-$). At physiological pH, peroxynitrite protonates and decomposes to generate powerful oxidants capable of interacting with lipids, thiols, proteins and DNA (for reviews, see Beckman & Crow, 1993; Beckman *et al*., 1994; Darley-Usmar & Halliwell, 1996). Superoxide production can be stimulated during the early phase of an inflammatory reaction and may be involved in the recruitment of inflammatory cells to the site of injury (Miller & Britigan, 1995). Superoxide dismutase can attenuate endothelial injury indicating the involvement of the superoxide anion (Kondo *et al*., 1996). Furthermore, peroxynitrite is generated in cells, such as endothelial cells, containing iNOS, suggesting that peroxynitrite and other nitric oxide-dependent nitrating species can be formed as a consequence of iNOS induction which leads to increased NO synthesis (Miller *et al*., 1995). Therefore, production of superoxide and large amounts of nitric oxide has the potential to yield peroxynitrite at sites of inflammation and it is possible that peroxynitrite contributes to the tissue injury observed in pathological conditions. Although the formation of peroxynitrite cannot be determined directly due to its short half-life at physiological pH, the detection of nitrotyrosine, a product of the nitration of tyrosine residues by peroxynitrate provides evidence for its formation (Beckman *et al*., 1994). Elevated levels of nitrotyrosine have been detected in a number of inflammatory diseases, for example levels are increased in serum and synovial fluid from rheumatoid arthritis patients compared to levels in healthy controls (Kaur & Halliwell, 1994) and Miller *et al*. (1995) have demonstrated colocalisation of nitrotyrosine with immunoreactivity of iNOS in a guinea pig model of ileitis.

The aim of this study was to investigate the effects of peroxynitrite on microvascular responses and nociception in the rat. Salvemini *et al*. (1996) have provided indirect evidence that

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peroxynitrite is involved in the oedema formation observed after subplantar injection of carrageenan. However, the effect of peroxynitrite on microvascular events which contribute to the inflammatory process has only been investigated in preliminary experiments (Ridger & Brain 1996; Greenacre *et al*., in press).

Methods

Animals

Male Wistar rats (220–250 g; from Tucks Ltd., Essex, U.K.) were used. They were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p., plus maintenance doses: 6 mg kg^{-1}, i.v.) for dorsal skin assays. The dorsal skin was shaved and, for blood flow experiments, depilated using a commercial depilatory cream (Immac; Reckitt & Colman, Hull, U.K.). Animals were left for 30 min before administration of test agents. Experiments were carried out in the unanaesthetized rat for hind paw experiments. The rats were allowed to habituate in the laboratory for 60 min before the initial measurement of nociceptive threshold and paw volume.

Plasma extravasation measurement in dorsal skin

Plasma extravasation, in response to i.d. agents (0.1 ml $site^{-1}$, in a modified Tyrod's solution) was measured by the extravascular accumulation of i.v. ^{125}I-human serum albumin (^{125}I-HSA; 0.2 MBq, i.v., −5 min). Evans blue dye (1 ml of a 2.5% solution, i.v., −5 min) was administered to allow observation of oedema formation. Test agents were administered intradermally (0.1 ml in Tyrode's solution) according to a balanced site pattern. Plasma extravasation was measured over 0–45 min or 0–240 mm. At the end of the accumulation period a cardiac puncture was taken to obtain a blood sample. The animals were killed by anaesthetic overdose followed by cervical dislocation. The dorsal skin was then removed and injection sites punched out with a 16 mm gasket punch. Blood was centrifuged (8000 *g*, 4 min, room temperature) to obtain plasma samples and these and the skin sites were then analysed for radioactivity in a gamma counter (Wallac, U.K). Results are expressed as the volume of plasma extravasated (μl) in each site compared to that in the plasma and statistical significance evaluated by ANOVA followed by Bonferroni's test for multiple comparisons.

Blood flow measurements

Blood flow was measured in the dorsal skin of anaesthetized rats using a FloLab flowmeter (Moor Instruments, Devon, U.K.). Basal flow was recorded via laser-Doppler probes at selected sites. Test agents were then administered i.d. and the probes replaced on each site. The mean flux over a 5 min period at each time point was calculated and measurements were taken every 30 min for 240 min. Results were recorded as blood cell flux (arbitrary units; number of cells detected by the beam × velocity) and expressed as mean change in flux compared to flux at 0 min. Results are expressed as mean ± s.e.mean and statistical differences evaluated by ANOVA followed by Dunnett's test for multiple comparisons with readings at 0 min for results shown in Figure 4 and by Bonferroni's test for multiple comparisons for results shown in Figure 5.

Nociception measurements

The weight of the paw, used to assess inflammatory oedema formation, was evaluated by volume displacement and the nociceptive threshold to mechanical stimulus was measured with a Ugo-Basile algesimeter (Laird *et al*., 1996; Handy *et al*., 1997). Peroxynitrite (30–175 nmol paw^{-1}) or control solutions (see below) were injected intraplantarly into one hind paw of the rat and responses determined in both hindpaws 30 min and thereafter until 360 min. Results are expressed as the difference between readings before and after treatment and significance was evaluated by Bonferroni's test for multiple comparisons.

Chemicals

^{125}I-HSA (Amersham International, U.K.), Sagatal (May and Baker, U.K.) carrageenan, catalase (from bovine liver; 48700 U mg^{-1}), dexamethasone, Evans blue, hydrogen peroxide, indomethacin, mepyramine, sodium nitrite and superoxide dismutase (from bovine erythrocytes; 5800 U mg^{-1},) all from Sigma Chemicals, Poole, U.K.); methysergide (gift, Sandoz, Feltham, U.K.), GR73632 (a gift from Dr D. Beattie, Glaxo-Wellcome, Stevenage, U.K.) and SR14033 (a gift from Dr Emonds-Alt, Sanofi, Nice, France). Peroxynitrite (Alexis Corporation, San Diego, U.S.A.) was used in addition to peroxynitrite, synthesised in house, as described below.

Peroxynitrite synthesis and preparation

In preliminary experiments we have shown that purchased peroxynitrite (Alexis Corporation) acts to increase microvascular permeability (Ridger & Brain, 1996). In this study we have used peroxynitrite from the Alexis Corporation for experiments shown in Table 1 and Figure 6 where a volume (3–20 μl) of stock peroxynitrite in 1.2 M sodium hydroxide was added to Tyrode solution, to give the required initial concentration and then injected into skin as soon as possible and always by 30 s after dilution. The Alexis Corporation also provided a negative control which was corrected for pH and administered as a control for each dose of peroxynitrite. The concentration of stock peroxynitrite was calculated before the start of each experiment by using the absorption coefficient (1670 M^{-1} cm^{-1}) and measuring the increase in absorbance at 302 nm of the stock solution in sodium hydroxide 1.2 M as previously discussed in detail (Greenacre *et al*., in press).

For all other studies peroxynitrite was synthesised in our laboratory by mixing 50 ml acidified (0.6 M HCl), hydrogen peroxide (0.7 M) and 50 ml $NaNO_2$ (0.6 M), followed by immediate quenching in 50 ml sodium hydroxide (1.2 M). Excess hydrogen peroxide was removed by passing the peroxynitrite through a column containing manganese (IV) oxide. The peroxynitrite was stored in 1.2 M sodium hydroxide at −20°C overnight and peroxynitrite was removed from the liquid upper layer formed by freeze fractionation. It was then further stored in aliquots until quantification (see above) and use. A degraded form of peroxynitrite, which had no absorbance at 302 nm under alkaline conditions, was prepared from the same stock as the active form by allowing it to remain at room temperature for 72 h. Peroxynitrite was added to buffer immediately before i.d. injections and all samples prepared such that identical amounts of sodium hydroxide (final pH 10–10.5) and other constituents of the peroxynitrite solution (Beckman *et al*., 1994) were present in the test and vehicle control solutions.

Results

Effect of i.d. administration of peroxynitrite

The effect of peroxynitrite on plasma extravasation is shown in Figure 1 and Table 1. Peroxynitrite, as previously reported, induces significant ($P<0.01$) plasma extravasation in the rat dorsal skin when compared to the alkaline vehicle controls (Ridger & Brain, 1996; S.A.B. Greenacre *et al*., unpublished results). Results in Table 1 indicate that the plasma extravasation induced by peroxynitrite is not inhibited by mast cell amine antagonists, mepyramine and methysergide, at doses which substantially inhibited plasma extravasation induced by the mast cell degranulating agent compound 48/80. The sub-

stance *P* antagonist, SR140333, was also unable to inhibit peroxynitrite-induced plasma extravasation at a dose which inhibited plasma exudation induced by the tachykinin NK_1 agonist, GR73632, in the presence of the vasodilator neuropeptide calcitonin gene-related peptide, CGRP (See Brain & Williams, 1985). Systemic pretreatment with the cyclo-oxgenase inhibitor indomethacin did not result in a significant reduction in peroxynitrite-induced extravasation. However, the oedema formation is inhibited by the anti-inflammatory steroid dexamethasone. Figure 1 shows the effect of peroxynitrite (50–200 nmol site^{-1}) on plasma extravasation in rat dorsal skin over 0–45 min. The peroxynitrite used for this series of experiments was synthesized from hydrogen peroxide and sodium nitrite and then treated in a manner which led to the removal of hydrogen peroxide. This is important as hydrogen peroxide itself increases extravasation (see Figure 2). The lack of response to a degraded sample from the same batch of peroxynitrite is shown for comparison. It should be noted that sodium nitrate ($NaNO_2$, 10 μmol site^{-1}), a concentration in excess of that used in the synthesis, did not induce plasma extravasation.

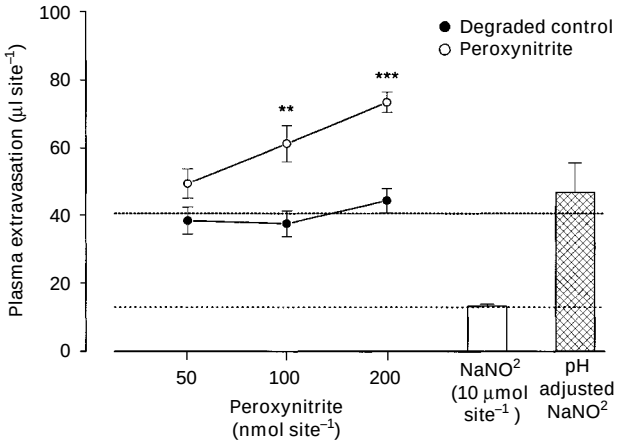

Figure 1 Dose–response relation for peroxynitrite (50–200 nmol site^{-1}) on plasma extravasation in rat dorsal skin. The responses to Tyrode's solution is shown as a dotted line and when adjusted to the same pH as peroxynitrite with sodium hydroxide is shown as a dashed line. The degraded control was prepared exactly as the equivalent dose of peroxynitrite and is shown for comparison. The response to sodium nitrite ($NaNO_2$, 10 μmol site^{-1};) in Tyrode's (▬) and in pH adjusted medium (▨) is also shown. Results are expressed as mean±s.e.mean, $n=6$. Statistical significance was evaluated using ANOVA followed by Bonferroni's modified *t* test for multiple comparisons. $^{**}P<0.01$; $^{**}P<0.01$ compared to vehicle control.

Effect of superoxide dismutase (SOD) and catalase on peroxynitrite-induced plasma extravasation

The effects of the superoxide scavenger, SOD, and the hydrogen peroxide scavenger catalase on peroxynitrite and hydrogen peroxide-induced oedema is shown in Figure 2. Both SOD and catalase had no effect on either vehicle control or peroxynitrite responses, but co-administration of catalase with hydrogen peroxide caused significant ($P<0.001$) attenuation of hydrogen peroxide-induced plasma extravasation, as expected. This indicates that all hydrogen peroxide had been removed from the peroxynitrite stock and further that peroxynitrite is not acting via a synergistic mechanism which involves the presence of low concentrations of hydrogen peroxide, as has been recently shown in platelets (Naseem *et al*., 1996).

Effect of time on peroxynitrite-induced plasma extravasation

A comparison of the plasma extravasation induced by peroxynitrite over 0–45 min and 0–240 min is shown in Figure 3. Neither of the doses of peroxynitrite caused increased plasma extravasation over 0–240 min when compared to that seen

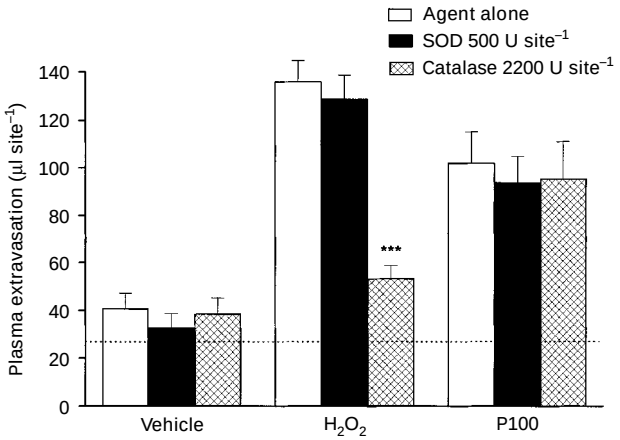

Figure 2 Effect of SOD (500 U site^{-1}) and catalase (2200 u site^{-1}) on peroxynitrite (100 nmol site^{-1}; P100), hydrogen peroxide (10 μmol site^{-1}) and vehicle control (0.1 ml site^{-1}) is shown. The dotted line refers to the response to Tyrode's alone. Results are expressed as mean±s.e.mean, $n=6$. Statistical significance was evaluated using ANOVA followed by Bonferroni's modified *t* test for multiple comparisons. $^{***}P<0.001$ compared to hydrogen peroxide control.

Table 1 Effect of peroxynitrite on plasma extravasation in untreated sites (Alone) and the modulating effect of mast cell amine inhibitors (mepyramine, MEP 2.8 nmol site^{-1} and methysergide, METH 1.9 nmol site^{-1} coinjected), a tachykinin NK_1 antagonist (SR140333 1 nmol site^{-1} co-injected), a cyclo-oxygenase inhibitor (indomethacin, INDO, 358 μmol site^{-1}) and the anti-inflammatory steriod dexamethasone (DEX 1 mg kg^{-1}; −3 h pretreatment)

Injected agents (dose site^{-1})	*Alone*	*MEP and METH*	*SR140333*	*INDO*	*DEX*
Tyrode (0.1 ml)	11.0±1.3	ND	9.3±1.4	10.0±1.9	5.1±0.5
GR73632 (30 pmol)+CGRP (10 pmol)	68.7±5.4	ND	22.0±5.6**	80.3±14.5	20.8±3.1***
Compound 48/80 (100 ng)	90.1±13.4	20.9±4.4**	ND	ND	ND
Peroxynitrite (40 nmol)	30.8±4.7	34.1±4.6	ND	41.3±5.3	12.1±1.6
Vehicle control for peroxynitrite (40 nmol)	25.0±3.0	ND	ND	ND	ND
Peroxynitrite (100 nmol)	56.8±5.1†	42.1±5.4	41.2±4.8	62.0±6.1	19.4±1.4**
Vehicle control for peroxynitrite (100 nmol)	30.0±5.1	ND	ND	ND	ND
Peroxynitrite (200 nmol)	89.2±7.1†††	120.0±13.0	73.4±10.9	76.3±11.7	28.7±3.0***
Vehicle control for peroxynitrite (200 nmol)	30.0±3.0	ND	ND	ND	ND

Pooled data from a number of experiments (mean±s.e.mean μl plasma extravasation site^{-1}, $n=5–11$) ND=not determined. Statistics are calculated by determining differences with control sites in the same rat when co-treatments are given, or at control sites in paired rats which receive vehicle for systemic pretreatments. $^{\dagger}P<0.05$; $^{\dagger\dagger\dagger}P<0.001$ compared to vehicle control. $^{**}P<0.01$; $^{***}P<0.001$ compared to control site.

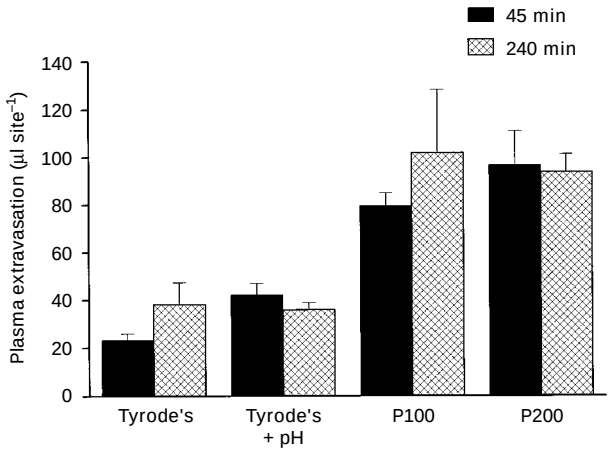

Figure 3 Comparison of the plasma extravasation observed over 0–45 min and 0–240 min after i.d. injection of peroxynitrite (P100, 100 nmol site^{-1} and P200, 200 nmol site^{-1}). Results for the response to Tyrode's (0.1 ml) and also to the vehicle control are also shown. Results are expressed as mean ± s.e.mean, $n=4$. Statistics were evaluated by ANOVA followed by Bonferroni's modified t test and no significant difference between the plasma extravasation at 0–45 min and 0–240 min was observed.

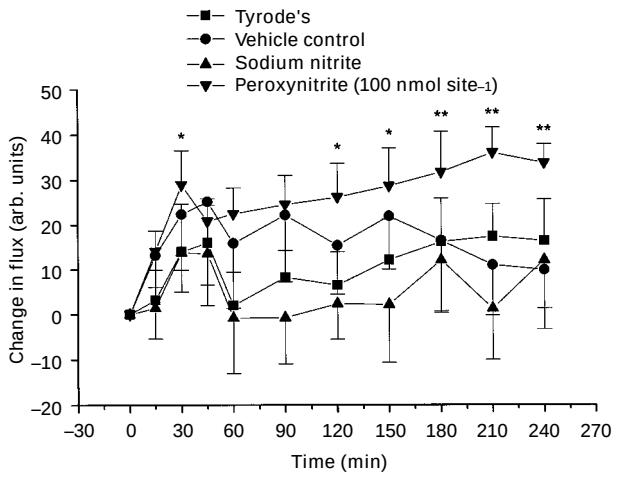

Figure 4 Effect of i.d. peroxynitrite (100 nmol site^{-1}) compared to Tyrode's (0.1 ml site^{-1}), vehicle control (0.1 ml site^{-1}) and sodium nitrite ($NaNO_2$, 10 µmol site^{-1}) on microvascular blood flow over 0–4 h. Results are expressed as mean change in flux (arbitrary units) ± s.e.mean, $n=6$. Statistics were evaluated using ANOVA followed by Dunnetts t test. *$P<0.05$, **$P<0.01$ compared to blood flow in that site at time of injection.

after 45 min, suggesting that peroxynitrite plasma extravasation is not time dependent and that extravasation is complete within 45 min after administration.

Effect of peroxynitrite on blood flow over 0–240 min

Figure 4 shows the effect of peroxynitrite on dorsal skin blood flow as measured by laser-Doppler flowmetry over 0–240 min. The effects of intradermal Tyrode's, vehicle control and sodium nitrite (10 µmol site^{-1}) are also shown. Peroxynitrite induced a significant ($P<0.05$) increase in blood flow, at 30 min and flow was consistently raised after 120 min. Figure 5 shows the effect of indomethacin (10 mg kg^{-1}, s.c.; −30 min) and dexamethasone (1 mg kg^{-1}, s.c.; −180 min) pretreatment on the increased blood flow observed after i.d. injection of peroxynitrite. Blood flow was measured at 240 min at sites injected with Tyrode's, vehicle control and peroxynitrite (100 nmol site^{-1}; 200 nmol site^{-1},) in animals pretreted with either vehicle (0.25 ml of 0.05% Na_2CO_3), indomethacin or dexamethasone. Neither indomethacin or dexamethasone had any effect on peroxynitrite induced blood flow.

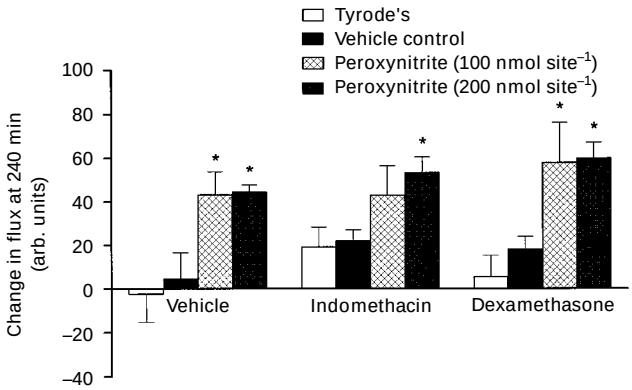

Figure 5 Effect of vehicle (0.25 ml of 0.05% Na_2CO_3; −30 min), indomethacin (10 mg kg^{-1}, s.c.; −30 min) and dexamethasone (1 mg kg^{-1}, s.c.; −180 min) pretreatment on peroxynitrite-induced increase in blood flow at 240 min. Two doses of peroxynitrite were administered (100 nmol site^{-1}; 200 nmol site^{-1}). Responses to Tyrode's (0.1 ml site^{-1}) and vehicle control for peroxynitrite (0.1 ml site^{-1}) are shown for comparison. Results are expressed as mean change in flux (arbitrary units) ± s.e.mean, $n=4–5$. Statistics were evaluated using ANOVA followed by Bonferroni's modified t test. *$P<0.05$ compared to Tyrode's control.

Effect of peroxynitrite on nociception and oedema formation in the rat hind paw

Intraplantar peroxynitrite (30–175 nmol) induced a dose-dependent increase in oedema formation, measured as an increase in paw volume as shown in Figure 6a, but had no effect on nociception (Figure 6b). The oedema formation induced by peroxynitrite in the rat paw was observed at an early stage after injection (by 30 min) and for the remainder of the experiment. By compariso, in parallel experiments, carrageenan (2%) induced a significant increase in oedema (0.4 ± 0.1 g and 1.2 ± 0.1 g increase in weight at 30 min, mean ± s.e.mean, $n=6$, $P<0.05$) and nociception (−9.7 ± 1.3 arbitrary units at 180 min, post-minus pre-nociceptive threshold, $P<0.05$) as expected (see Handy & Moore, 1996; Handy *et al*., 1997).

Discussion

These studies have shown that peroxynitrite, or species derived from its complex decomposition reactions, are capable of inducing plasma extravasation and increased blood flow but are devoid of algesic activity. Peroxynitrite has a short half-life at neutral pH and is rapidly decomposed, thus break down products (e.g. hydroxyl radicals), (see Darley-Usmar & Halliwell 1996), could be involved in mediating the responses. All peroxynitrite solutions are contaminated with other products (hydrogen peroxide, sodium nitrite, etc) but our results show that these substances cannot account for the effects observed (lack of effect of catalase, sodium nitrite or decomposed peroxynitrite control). The study provides evidence to show that peroxynitrite has the ability to increase microvascular permeability in a dose-dependent manner, leading to plasma extravasation and oedema formation, when injected intradermally into rat dorsal skin, or intraplantar injection into the rat paw.

The plasma extravasation observed in response to peroxynitrite appears to be a discreet microvascular response which is observed within 45 min and significantly higher than that seen in the presence of pH adjusted vehicle (necessary to prevent breakdown of peroxynitrite before injection (S.A.B. Greenacre *et al*., unpublished results). It would appear that substance P, NK_1-mediated responses, prostanoids and mast cells amines do not contribute to the plasma extravasation as antagonists

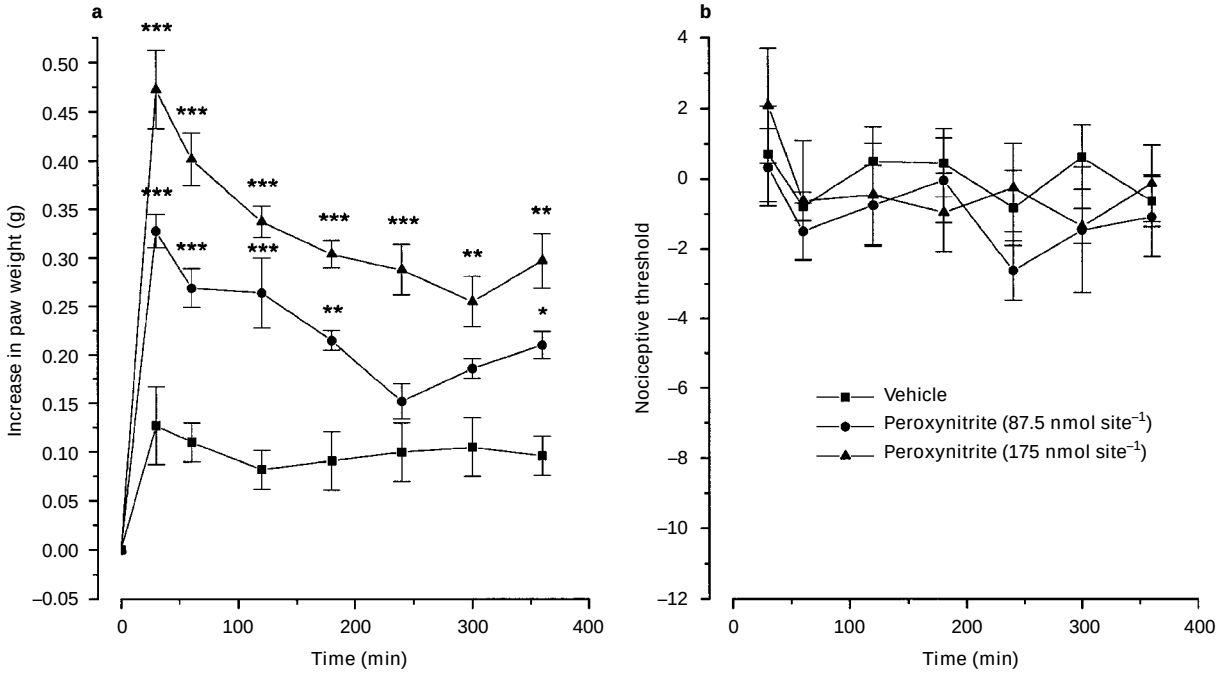

Figure 6 Effect of intraplantar peroxynitrite on oedema formation and nociception in the rat hind paw. (a) The time dependent effect of peroxynitrite (87.5 nmol paw^{-1} and 175 nmol paw^{-1}) on oedema formation, as assessed by increase in paw volume and (b) on nociceptive threshold, as assessed by a mechanical hyperalgesia assay. The response to vehicle control (matched for pH to the highest dose of peroxynitrite) is also shown. Results are expressed at mean$\pm$s.e.mean, $n=6$. ***$P<0.001$.

and/or inhibitors of these agents have no effect on plasma extravasation. Furthermore, the plasma extravasation is of relatively short duration, in that extravasation measured over 240 min was similar to that measured over 0–45 min in dorsal skin and maximal oedema formation was observed at 30 min in the rat hind paw. This would suggest that the effect of peroxynitrite is not ongoing and is reversible. Anti-inflammatory steroids act in a protein synthesis-dependent manner to inhibit mediator-induced plasma protein extravasation (Yarwood *et al*., 1993). The inhibition of peroxynitrite-induced plasma extravasation by steroids indicates that peroxynitrite is not merely acting to destroy the postcapillary venule endothelial barrier. Interestingly, the increased blood flow observed in these experiments was delayed, compared to the plasma extravasation and resistant to indomethacin, suggesting that the vasodilator mechanism is prostanoid-independent. Furthermore, it was not attenuated by dexamethasone suggesting that the increased blood flow is not a consequence of the earlier plasma extravasation process. Recently the acute vasodilator properties of peroxynitrite on a large blood vessel, the rat pulmonary artery, have been reported where is was suggested that peroxynitrite acts via a poly (adenosine 5′-diphosphoribose) synthase mechanism to vasodilate (Chabot *et al*., 1997); it will be of interest to determine whether this pathway is involved in the present findings.

Peroxynitrite is considered to be involved in the inflammatory process (Beckman & Crow, 1993; Darley-Usmer & Halliwell, 1996). However, the elucidation of specific proinflammatory actions of peroxynitrite has been difficult, in part due to the problems relating to its stability and administration. Rachmilewitz *et al*. (1993) showed that intrarectal peroxynitrite induced pronounced chronic colonic inflammation, which included tissue swelling when measured 1–21 days after administration. This study, together with our findings would suggest that, if peroxynitrite is produced at sites close to the postcapillary endothelial cell barrier, it could act to increase microvascular permeability and affect vascular tone. We have injected bolus amounts of peroxynitrite, while it might be expected that peroxynitrite may be produced *in vivo* for sustained periods, with perhaps, lower active concentrations present at any one time at vasoactive sites. Thus discussion of the present results is complicated. The doses of peroxynitrite used in this study are comparable to those used in an *in vitro* study of bactericidal activity (Beckman & Crow, 1993) and peroxynitrite is equipotent with histamine but about 100 fold less potent that substance P in inducing oedema formation in skin (see Brian & Williams, 1985). Perhaps it may be more important to point out that peroxynitrite is produced at time points when the inflammatory response is ongoing, thus it may have an important proinflammatory interactive role with other mediators at the inflamed site.

It has recently been shown by Boota *et al*. (1996) that microvascular smooth muscle cells *in vitro* are stimulated by IL-1β, leading to an upregulation of inducible nitric oxide synthase and an increase in superoxide production, that results in the formation of peroxynitrite. The cell viability was not affected. Thus microvascular smooth muscle cells could be a source of damaging peroxynitrite in inflamed tissues (Boota *et al*., 1996). Alternatively endothelial cells themselves, as well as ultraviolet B irradiated keratinocytes (relevant to this study in skin), are stimulated to produce significantly raised levels of nitric oxide, superoxide and peroxynitrite (Deliconstantinos *et al*., 1996a,b); although peroxynitrite is not cytotoxic to keratinocytes (Spencer *et al*., 1996). Furthermore, it has been shown that peroxynitrite modulates calcium signalling in endothelial cells (Elliott, 1996). Thus, a range of cells could contribute to the generation of peroxynitrite in inflamed skin.

It has been suggested that peroxynitrite does not mediate increased microvascular permeability in a rat lung model of ischaemia reperfusion injury over 90 min (Moore *et al*., 1996), a relatively short time period with respect to iNOS induction and related peroxynitrite generation. However, in another study it has been concluded that peroxynitrite is involved in pulmonary oedema formation (Johnson & Ferro, 1996). It has also been suggested that peroxynitrite is involved in the mucosal damage observed in the rat gastric mucosa (Lamarque & Whittle, 1995) and in carrageenan-induced oedema formation observed in the rat paw (Salvemini *et al*., 1996). Interestingly, our results suggest that the algesia observed in the carrageenan model is not primarily mediated by peroxynitrite, although we cannot rule out that peroxynitrite may play an interactive role in mediating algesia when present with other mediators.

In conclusion, our results suggest that peroxynitrite, if generated at an inflamed site, could contribute to the oedema and ongoing increased blood flow observed in inflammatory disease. These results are in keeping with recent studies of vascular and other cells in culture, as well as *in vivo* studies, which are now providing evidence to indicate that peroxynitrite is produced in a range of inflammatory situations, relevant to the microvasculature (Darley-Usmar & Halliwell, 1996). Evidence is also growing to suggest a relation between peroxynitrite production and inflammatory responses. The present findings could have clinical implications, because inflammatory oedema formation and increased blood flow are characteristic features in patients suffering from a range of inflammatory diseases. However, the results do not support the concept that peroxynitrite, at least by itself, is involved in the inflammatory hyperalgesia component.

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