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Investigation of 6-hydroxydopamine-induced plasma extravasation in rat skin

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Abstract

Perfusion of 6-hydroxydopamine into the rat knee and trachea induces plasma extravasation, possibly by tissue-specific mechanisms involving sympathetic and sensory nerves respectively, and we aimed to identify the mediators which contribute to this response in skin. 6-Hydroxydopamine (both hydrobromide and hydrochloride salts), dose dependently increased plasma extravasation into rat dorsal skin, however, when compared to bradykinin or the tachykinin NK_1 receptor agonist GR73632, high concentrations of 6-hydroxydopamine (1–10 μmol/site) were required. The response to 6-hydroxydopamine was not inhibited in chemically sympathectomised rats (6-hydroxydopamine, 300 mg/kg i.p. over 7 days) but was significantly reduced by co-administration with the histamine (H_1) and the 5-HT receptor antagonists mepyramine and methysergide and in skin sites pre-injected with compound 48/80 (4 μg, −18 h) to degranulate dermal mast cells. The response was not inhibited by co-injection of the tachykinin NK_1 receptor antagonist SR140333 or by the cyclo-oxygenase inhibitor indomethacin (5 mg kg^{-1} i.p., −30 min) except at the lowest dose of 6-hydroxydopamine (1 μmol/site). We conclude that 6-hydroxydopamine is not a potent or selective mediator of increased vascular permeability in rat skin but, at high concentrations, may induce oedema formation via release of vasoactive amines from mast cells, augmented by generation of prostaglandins.

Keywords: Sympathetic nerve; 6-Hydroxydopamine; Mast cell; Dorsal skin

1. Introduction

The contribution of the sympathetic nervous system to acute inflammatory processes, including oedema formation, was initially investigated by Engel (1941). More recent publications (Coderre et al., 1989; Green et al., 1993b) suggest that activation of sympathetic post-ganglionic nerve terminals promotes oedema formation in the rat knee joint. In addition, oedema formation in response to bradykinin, a potent mediator of inflammation and pain, was postulated to be sympathetic nerve-dependent in this site (Green et al., 1993a). Other workers have found little effect of sympathectomy in models of inflammation, including carrageenan-induced paw oedema in the rat (Donnerer et al., 1991) and, in our laboratory, sympathectomy was found to potentiate the effects of bradykinin in the rat knee (Cambridge and Brain, 1995). The longer term effects of sympathetic activation on disease outcome remains unknown, although increased plasma extravasation has been postulated to have (paradoxically) a protective effect in experimental arthritis (Coderre et al., 1991).

The precise mechanisms by which sympathetic stimulation may initiate oedema formation also remain poorly defined. Sympathetic post-ganglionic nerve terminals contain noradrenaline and neuropeptide Y which, as vasoconstrictors, could be expected to inhibit oedema formation (Green et al., 1993a) but they have also been shown to release purines and to synthesise prostaglandins (Gonzales et al., 1989) both of which produce vasodilation but not oedema formation per se. Therefore, unlike sensory nerve stimulation where release of substance P and calcitonin gene-related peptide (CGRP) produce a well characterised response (Lembeck and Holzer, 1979; Escott and Brain, 1993), the mediators of sympathetically induced responses are unclear (Lee et al., 1991).

In addition to observations in sympathectomised animals, additional evidence supporting the role of the sympathetic nerves in inflammation (Green et al., 1993b) has relied on the selective actions of 6-hydroxydopamine which

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has been extensively employed for its long-term neurotoxic effects on sympathetic and dopaminergic nerves (Thoenen and Tranzer, 1968). 6-Hydroxydopamine activates sympathetic nerve terminals (Sachs, 1971) to induce transmitter and co-transmitter release, and this acute response has been exploited in the study of neurogenic inflammation (Coderre et al., 1989). High doses (> 50 mM) of 6-hydroxydopamine have been found to produce significant plasma extravasation into the rat knee joint (Coderre et al., 1991; Cambridge and Brain, 1995). A lower dose (10 mM) was sufficient to induce leakage into the rat trachea although inhibition of this response in capsaicin (to deplete sensory neuropeptides)-treated, but not sympathectomised, rats points to sensory rather than sympathetic nerve involvement (Sulakvelidze et al., 1994). Previous work in our laboratory has shown that the high doses of 6-hydroxydopamine required to produce a significant response in the knee joint lead to severe systemic toxicity (Cambridge and Brain, 1995); therefore the relevance of these observations to physiological or pathological states must be questioned. The use of such high doses also increases the likelihood that non-specific actions of 6-hydroxydopamine contribute to its perceived pro-inflammatory effects.

As there is little information regarding the effects of 6-hydroxydopamine in tissues other than the joint we have undertaken a study of this drug using the rat skin oedema assay, a quantitative technique used extensively in the study of basic properties and interactions of inflammatory mediators (Brain and Williams, 1985). Our aims were to measure the response to 6-hydroxydopamine in rat dorsal skin, to compare the response to that produced by other mediators of inflammation, and to investigate possible secondary mechanisms involved in this response.

2. Materials and methods

2.1. Animals and anaesthesia

Male Wistar rats (200–350 g) were used in all experiments. For the skin oedema assay, for pre-injection of compound 48/80 and for the initial sympathectomy treatment with 6-hydroxydopamine, animals were anaesthetised with pentobarbitone (50 mg · kg^{-1} i.p., additional doses as required).

2.2. Drugs

6-Hydroxydopamine (hydrochloride and hydrobromide), bradykinin, Evans blue, histamine, and compound 48/80 were obtained from Sigma (Dorset, UK). Methysergide maleate was a gift from Sandoz (Middlesex, UK). The selective tachykinin NK_1 receptor agonist GR73632 (δ-Ava-Phe-Phe-Pro-MeLeu-Met-NH_2) was a gift from Dr D. Beattie (Glaxo, Herts, UK) and the selective tachykinin NK_1 receptor antagonist SR140333, ((*S*)1-{2-[3-(3,4-dichlorphenyl)-1-(3-iso-propoxyphenylacetyl)piperidin-3-yl]-ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octane, chloride) was a gift from Dr X. Emonds-Alt (Sanofi, France). Human αCGRP was a gift from Dr U. Ney (Celltech, Berks, UK). ^{125}I-Human serum albumin (^{125}I-HSA) was obtained from Amersham International (Bucks, UK). All drugs for intradermal injection were dissolved and diluted in Tyrode solution (composition, [mM] NaCl 136.9, KCl 2.7, NaH_2PO_4 0.42, $NaHCO_3$ 11.9, $MgCl_2$ 1.0, glucose 5.6) except for 6-hydroxydopamine which was dissolved and diluted in Tyrode solution containing 1% ascorbic acid to prevent oxidation. Indomethacin was dissolved in Na_2CO_3 with an equal volume of NaH_2PO_4 added to bring the pH to 7.4.

2.3. Skin oedema assay

The response to randomised intradermal injection of test agents (100 μl, 27 G needle) was measured in shaved dorsal skin of anaesthetised rats. Skin sites were marked out in duplicate, 1 on each side of the midline with a maximum of 24 sites in total per rat with each agent injected into 2 sites. Doses of test agents were chosen with reference to Brain and Williams (1985) and Richards et al. (1993). Plasma extravasation was measured by accumulation, over 30 min, of intravenously administered ^{125}I-human serum albumin (100 kBq, via tail vein) in the injected sites (Williams, 1976, Williams, 1979). Evans blue dye (25 mg · ml^{-1}, 0.2 ml/rat) was co-injected with the radioactivity as a visual marker of plasma extravasation. At the end of the experiment, a 1 ml blood sample was obtained by cardiac puncture and the rats killed by pentobarbitone overdose. The dorsal skin was removed and radioactivity in the skin sites (15 mm diameter) and plasma was counted in a gamma counter. Results are expressed as μl plasma/skin site (mean of the 2 replicate sites).

Preliminary experiments showed the response to the hydrochloride and hydrobromide compounds of 6-hydroxydopamine to be similar (see Results section) and the hydrobromide was then used for all subsequent studies.

2.4. Contribution of mast cell amines to 6-hydroxydopamine-induced plasma extravasation

The possibility that high concentrations of 6-hydroxydopamine may induce oedema formation by degranulating mast cells was initially investigated by co-injecting the histamine (H_1) receptor antagonist mepyramine and the 5-HT receptor antagonist methysergide with the test agents (2.8 and 1.9 nmol/site respectively).

Secondly the effect of prior degranulation of mast cells on the response to 6-hydroxydopamine was examined. Skin sites (total 8 per rat) were pre-injected with either compound 48/80 (16 μg ml^{-1}, 200 μl, 4 sites) or Tyrode solution (200 μl, 4 sites). Approximately 18 h later responses to intradermal injections of Tyrode solution, 6-hy-

droxydopamine, compound 48/80 (as a positive control) and histamine plus CGRP (as a negative control) were measured in both a Tyrode and compound 48/80 pre-injected site on each rat. Plasma extravasation was measured as for the skin oedema assay experiments described above.

2.5. Role of sensory nerve activation

To test the hypothesis that 6-hydroxydopamine may act via release of substance P from sensory nerves, the selective tachykinin NK_1 receptor antagonist SR140333 was co-injected (1 nmol/site) with test agents. Control sites on the same animals received test agents alone.

2.6. Pre-treatment with indomethacin

The contribution of cyclo-oxygenase products to plasma extravasation induced by 6-hydroxydopamine was measured by pre-treatment with indomethacin (5 $mg \cdot kg^{-1}$, i.p.,) 30 min prior to intradermal injection of test agents. Control rats were pre-treated with an equivalent volume of vehicle.

2.7. Chemical sympathectomy

The importance of selective activation of sympathetic nerves by 6-hydroxydopamine was investigated in rats chemically sympathectomised by chronic dosing with 6-hydroxydopamine. Animals were injected (i.p.) with 6-hydroxydopamine (50 $mg \cdot kg^{-1}$ on days 1 and 2 and 100 $mg \cdot kg^{-1}$ on days 6 and 7). This protocol has been shown to virtually abolish immunostaining for tyrosine hydroxylase in rat tracheal mucosa (Sulakvelidze et al., 1994) and a similar protocol was found to inhibit the hypertensive response to intravenous tyramine (Cambridge and Brain, 1995). Plasma extravasation in response to intradermal 6-hydroxydopamine, bradykinin ± CGRP, GR73632 ± CGRP, and compound 48/80 were measured in the skin oedema assay carried out on day 8.

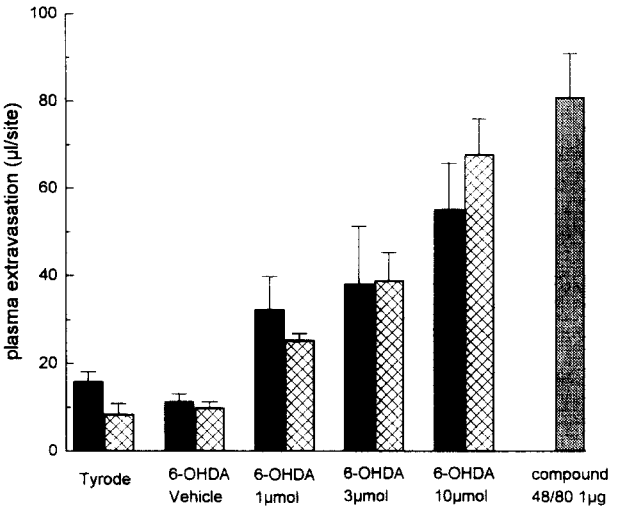

Fig. 1. Dose-response relationship (means ± S.E.M) for plasma extravasation measured by accumulation of ^{125}I-human serum albumin in rat dorsal skin in response to Tyrode solution, 6-hydroxydopamine vehicle (1% ascorbic acid), and 6-hydroxydopamine hydrobromide (solid bars, $n = 3$ rats) and 6-hydroxydopamine hydrochloride (cross hatched bars, $n = 3$ rats). The mean response to compound 48/80 (1 µg, grey bar) in the same animals is included for comparison.

2.8. Statistical analysis

Results are expressed as means ± S.E.M. Analysis of variance and a post-test to identify differences between treatment groups (Bonferroni's modified *t*-test) were carried out on log transformed data. $P < 0.05$ was considered significant.

Table 1
Effect of indomethacin (5 $mg\ kg^{-1}$ i.p., −30 min) or vehicle on plasma extravasation (measured by local accumulation of intravenously injected ^{125}I-human serum albumin) in response to intradermal injection of 6-hydroxydopamine (1–10 µmol/site) and other mediators of increased vascular permeability

Test agent	Plasma extravasation (µl/site)			
	Vehicle-pre-treated rats		Indomethacin-pre-treated rats	
	Mean	S.E.M.	Mean	S.E.M.
Tyrode solution	11.6	2.9	17.9	2.8
1% ascorbic acid (6-OHDA vehicle)	10.1	1.8	7.8	0.3
CGRP (10 pmol)	15.1	1.9	11.3	0.9
BK (3 nmol)	49.8	17.5	53.7	5.9
BK (3 nmol) + CGRP (10 pmol)	61.6	11.9	61.8	9.7
Compound 48/80	107.0	18.9	95.2	10.6
6-OHDA (1 µmol)	31.8	7.6	16.0 [a]	1.8
6-OHDA (3 µmol)	51.0	7.9	32.5	3.7
6-OHDA (10 µmol)	75.0	15.2	52.5	9.2
GR73632 (100 pmol) + CGRP (10 pmol)	56.8	11.9	53.2	9.7

No significant differences found between responses in control and treatment groups (ANOVA followed by Bonferroni's modified *t*-test, $n = 4$ per group).
[a] Response not significantly different ($P > 0.05$) from 6-hydroxydopamine (6-OHDA) vehicle in same animals.

3. Results

3.1. 6-Hydroxydopamine dose-response curve

Both the hydrobromide and hydrochloride salts of 6-hydroxydopamine, ($n = 3$ rats for each formulation) produced an equivalent dose-dependent increase in plasma extravasation which at 1, 3 and 10 μmol was significantly different to vehicle control (Fig. 1). Skin sites injected with 6-hydroxydopamine showed a characteristic pronounced blanching, suggestive of intense vasoconstriction, surrounded by an area of visible dye leakage. The animals also showed signs of sympathetic activation including piloerection, contraction of the nictitating membrane and stimulation of salivary glands.

3.2. Effect of co-injection of mepyramine and methysergide

The response to 6-hydroxydopamine (3 and 10 μmol/site) was significantly inhibited by co-injection with antagonists of mast cell amines at concentrations which also inhibited the response to compound 48/80 (1 μg) but did not inhibit the response to the tachykinin NK_1 receptor agonist GR73632 (100 pmol) co-injected with CGRP (10 pmol) ($n = 6$ rats) (Fig. 2a).

3.3. Effect of pre-treatment with compound 48/80 to degranulate dermal mast cells

In skin sites pre-treated with compound 48/80, responses to 6-hydroxydopamine and compound 48/40, but not histamine plus CGRP, were significantly reduced when compared to those in skin sites pre-treated with Tyrode solution (Fig. 2b) ($n = 4$ rats).

3.4. Effect of a tachykinin NK_1 receptor antagonist, SR140333

Co-injection of the tachykinin NK_1 receptor antagonist SR140333 (1 nmol) significantly attenuated plasma extravasation in response to the tachykinin NK_1 receptor agonist GR73632 (100 pmol) (36.4 ± 3.4 vs. 15.6 ± 2.23, $P < 0.05$, mean $\pm$ S.E.M., co-injected vs. agonist alone) and GR73632 (100 pmol) plus CGRP (10 pmol) (88.4 ± 9.52 vs. 30.2 ± 4.0, $P < 0.05$) but not 6-hydroxydopamine (100 μmol) (75.0 ± 15.17 vs. 56.5 ± 11.35) or 48/80 (1 μg) (100.2 ± 8.72 vs. 70.8 ± 8.1) ($n = 6$ rats).

3.5. Effect of a cyclo-oxygenase inhibitor, indomethacin

Systemic pre-treatment with the cyclo-oxygenase inhibitor indomethacin did not significantly inhibit plasma extravasation in response to any of the test agents (Table 1) when compared to controls animals. There is however a trend towards selective attenuation of the 6-hydroxydopamine response and a treatment effect of indomethacin

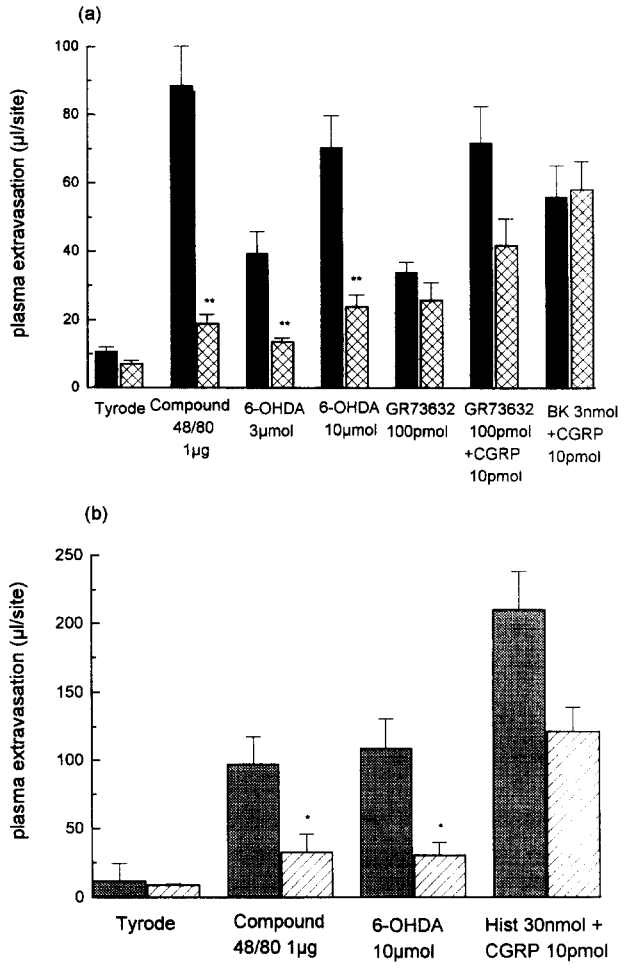

Fig. 2. (a) Effect of co-injection of the mast cell amine receptor antagonists mepyramine and methysergide (2.9 nmol/site and 1.8 nmol/site respectively) on plasma extravasation in response to intradermal injection of compound 48/80 (1 μg), 6-hydroxydopamine (3 and 10 μmol), and GR73632 (100 pmol) $\pm$ CGRP (10 pmol) and (3 nmol) + CGRP (10 pmol). Solid bars = agonist alone, hatched bars = co-injection with methysergide and mepyramine. Means $\pm$ S.E.M. ** $P < 0.01$, co-injected vs. control sites in same animals ($n = 6$). (b) Effect of local pre-treatment with compound 48/80 (4 μg/site, -24 h), on plasma extravasation in response to intradermal injection of Tyrode solution, compound 48/80 (1 μg), 6-hydroxydopamine (10 μmol) and histamine (30 nmol) + CGRP (10 pmol). Grey bars show the response in saline pre-injected sites and hatched bars the response in compound 48/80 pre-injected sites in the same animal ($n = 4$ rats). Means $\pm$ S.E.M. * $P < 0.05$, saline vs. compound 48/80 pre-treatment, ANOVA, followed by Bonferroni's modified t-test.

was seen at the lowest dose of 6-hydroxydopamine (1 μmol). This dose did not produce significant plasma extravasation compared to the ascorbic acid vehicle in the indomethacin-treated animals ($n = 4$ in each group).

3.6. Effect of chemical sympathectomy on the response to 6-hydroxydopamine and other mediators of increased vascular permeability.

Significant ($P < 0.05$) increases in plasma extravasation in response to 6-hydroxydopamine (1 and 3 μmol/site),

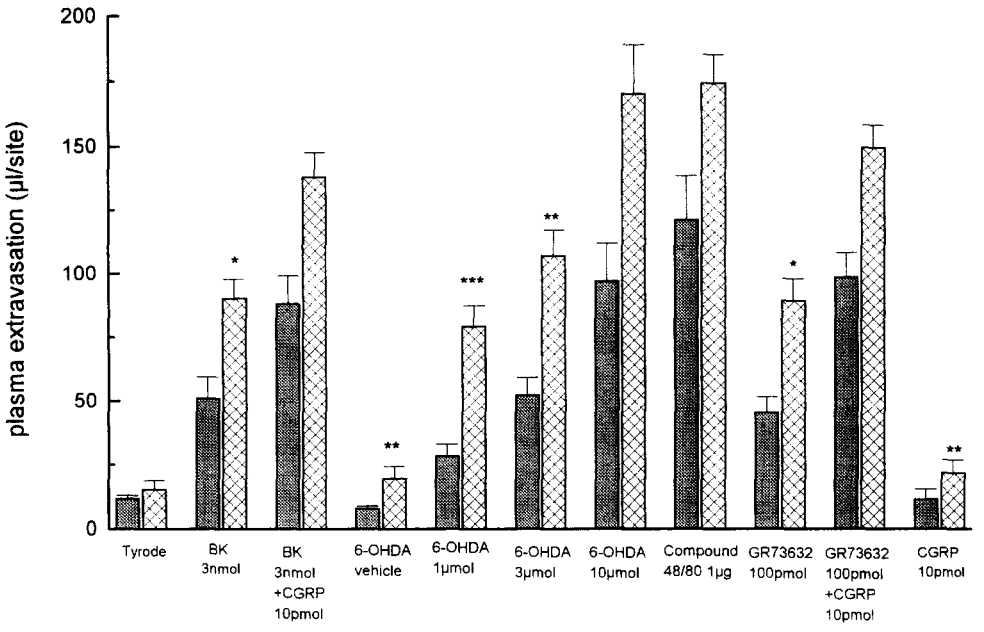

Fig. 3. Effect of chemical sympathectomy (6-hydroxydopamine, 50 mg · kg^{-1} on day 1 and 2, 100 mg · kg^{-1} on day 6 and 7) on plasma extravasation (measured on day 8) in response to 6-hydroxydopamine and mediators of increased vascular permeability. Grey bars represent the response in vehicle (1% ascorbic acid)-treated animals ($n = 6$) and hatched bars the response in sympathectomised animals ($n = 5$). Means ± S.E.M. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, vehicle vs. sympathectomised, ANOVA followed by Bonferroni's modified t-test.

bradykinin, GR73632, CGRP and 6-hydroxydopamine vehicle were noted in chemically sympathectomised rats ($n = 5$) compared to the control group ($n = 6$) (Fig. 3).

4. Discussion

As in the rat knee joint, high concentrations of 6-hydroxydopamine were found to produce plasma extravasation in rat skin (Cambridge and Brain, 1995). This effect could be significantly and selectively attenuated by co-injection with mepyramine and methysergide and by local pre-treatment with compound 48/80, thus mast cell activation plays a major part in this response. The pathway by which degranulation is triggered is unknown but may simply result from an irritant effect of such a high concentration of drug. The pro-inflammatory activity appears to be related to 6-hydroxydopamine itself, and not the reactive hydrobromide moiety, as a comparable response was obtained with the hydrochloride preparation of the drug. Acid pH also cannot account for the response as the vehicle effect was not greater than that observed for Tyrode solution at neutral pH.

Generation of prostaglandins via cyclo-oxygenase was not essential for oedema formation, although the response to 6-hydroxydopamine tended to be lower in indomethacin-treated animals and, at 1 µmol/site, 6-hydroxydopamine failed to produce a significant response compared to vehicle. This trend was not observed for the other mediators tested and may thus reflect a relatively greater contribution of cyclo-oxygenase products to the 6-hydroxydopamine response, at least at lower concentrations. The source of prostaglandins is unknown and was not been investigated and, although sympathetic post-ganglionic nerve terminals (Gonzales et al., 1989) have been shown to synthesise prostaglandins, many other cell types in the skin also have a similar capacity. Our results point to, at most, a moderate contribution of prostaglandins to the local effects of 6-hydroxydopamine, although whether this represents a selective action on sympathetic post-ganglionic nerve terminals requires further study.

Substance P has been shown to be the major sensory neuropeptide involved in neurogenic oedema formation in rat skin (Lembeck and Holzer, 1979) and plasma extravasation in response to stimulation of sensory nerves can be selectively blocked by the tachykinin NK_1 receptor antagonist SR140333 (Emonds-Alt et al., 1993). This drug had no effect on the response to 6-hydroxydopamine, and thus, unlike the trachea (Sulakvelidze et al., 1994), sensory nerve activation does not appear to be a major property of 6-hydroxydopamine in skin. When perfused directly onto the mucosa of the trachea a significant effect could be obtained at much lower concentrations of 6-hydroxydopamine than in either the knee joint (Cambridge and Brain, 1995) or the skin, therefore at lower concentrations more selective actions of 6-hydroxydopamine may be observed. As in our study, chemical sympathectomy did not inhibit plasma extravasation induced by 6-hydroxydopamine in this site (Sulakvelidze et al., 1994).

Our results obtained in the sympathectomised animals suggest that the pro-inflammatory effects of 6-hydroxydopamine are unrelated to a selective action on sympa-

thetic nerves. Interestingly sympathectomy increased plasma extravasation in response to bradykinin, the tachykinin NK_1 receptor agonist GR73632 and 1 and 3 μmol 6-hydroxydopamine. This finding is consistent with previous experiments carried out in our laboratory which showed that bradykinin-induced plasma extravasation in the rat knee was also increased in sympathectomised animals (Cambridge and Brain, 1995) although Coderre et al. (1989) previously reported inhibition of this response. Our findings also contrast to those of Khalil and Helme (1989) who found a decreased response to substance P perfused over blister bases after sympathectomy. The reason for this discrepancy is not obvious but may well reflect the different methodologies of intradermal injection vs. blister base perfusion. Intradermal injection is likely to produce acute tissue disruption within all the skin layers possibly activating sympathetic nerves and producing reflex vasoconstriction via release of noradrenaline and neuropeptide Y. Absence of this response in sympathectomised animals may mean that blood flow in the skin microarterioles is maintained promoting a relative increase in extravasation of plasma proteins from post-capillary venules. In blister base experiments the test agents are not present in deeper layers of the skin and there is less disruption of dermal tissue at the injection site, thus the pattern of reflex nerve activation is likely to be different. In agreement with our present findings, plasma extravasation in response to intravenous injection of both substance P, and a stable substance P analogue, was found to be increased in tissues of rats sympathectomised by chronic treatment with guanethidine (Mathison and Davison, 1994). Thus activation of sympathetic nerves in many sites, including the skin, appears to attenuate plasma extravasation in response to agents which increase vascular permeability, possibly by limiting blood flow to the site (Khalil and Helme, 1989). This idea is supported by the large enhancement in the response to 6-hydroxydopamine in sympathectomised animals where the indirect vasoconstrictor effect of the drug should be greatly diminished. The increased leakage induced by agents which normally do not increase vascular permeability, for example, calcitonin gene-related peptide, may also be indicative of loss of a local vasoconstrictor response induced by intradermal injection. Lack of local vasoconstriction at the injection sites could also allow agents, such as GR73632 or bradykinin, to reach the peripheral circulation more readily and thus produce an apparent increase in local responses by having a systemic effect on vascular permeability. Thus a systemic effect may also contribute to the increased responses in vehicle- or calcitonin gene-related peptide-injected sites where there is little oedema formation in normal animals. Further investigation of changes in blood pressure or peripheral resistance would be required to differentiate between local or systemic effects.

In conclusion our study suggests that 6-hydroxydopamine is not a useful tool for study of selective sympathetic nerve activation in the skin as the concentration required to produce responses comparable to known mediators of inflammation causes non-specific activation of mast cells. As similar concentrations are required to produce effects in the joint, the relevance of results obtained in this site should also be questioned. Further work is required to devise more appropriate techniques for the study of sympathetic nerve activation in inflammation.

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