*Br. J. Pharmacol.* (1994), **112**, 1181–1187 © Macmillan Press Ltd, 1994

# Cutaneous vasodilatation induced by nitric oxide-evoked stimulation of afferent nerves in the rat

[1]P. Holzer & M. Jocič

Department of Experimental and Clinical Pharmacology, University of Graz, Universitätsplatz 4, A-8010 Graz, Austria

**1** The site of action at which nitric oxide (NO) may contribute to neurogenic vasodilatation in the hindpaw skin of urethane-anaesthetized rats was examined by the use of $N^G$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthase.

**2** Skin blood flow was measured by laser Doppler flowmetry, and neurogenic vasodilatation was evoked either by topical application of mustard oil (5%) or antidromic electrical stimulation of the saphenous nerve (antidromic vasodilatation).

**3** L-NAME (60 $\mu$mol kg$^{-1}$, i.v.) attenuated the hyperaemia evoked by mustard oil in an enantiomer-specific manner but failed to reduce antidromic vasodilatation and the vasodilatation due to i.v. injected calcitonin gene-related peptide (CGRP) and substance P (0.1–1 nmol kg$^{-1}$ each), two proposed mediators of neurogenic vasodilatation.

**4** Pretreatment of rats with capsaicin (125 mg kg$^{-1}$, s.c. 2 weeks beforehand), to defunctionalize afferent neurones, reduced the hyperaemic response to mustard oil and prevented L-NAME from further decreasing the vasodilatation evoked by mustard oil.

**5** Intraplantar infusion of sodium nitroprusside (SNP, 0.15 nmol in 1 min), a donor of NO, induced hyperaemia which was significantly diminished by the CGRP antagonist $CGRP_{8-37}$ (50 nmol kg$^{-1}$, i.v.) and by capsaicin pretreatment. The ability of $CGRP_{8-37}$ to inhibit the vasodilator response to SNP was lost in capsaicin-pretreated rats.

**6** Taken together, these data indicate that NO does not play a vasorelaxant messenger role in neurogenic vasodilatation but can contribute to activation of, and/or transmitter release from, afferent nerve fibres in response to irritant chemicals.

**Keywords:** Nitric oxide; $N^G$-nitro-L-arginine methyl ester (L-NAME); capsaicin; mustard oil; sodium nitroprusside; calcitonin gene-related peptide (CGRP); substance P; afferent nerve stimulation; skin blood flow; antidromic vasodilatation

## Introduction

Electrical or chemical stimulation of afferent nerves in the skin causes an inflammatory reaction that begins with a marked rise of cutaneous blood flow. The chemical irritant mustard oil has long been used in the study of neurogenic inflammation because its effect to increase skin blood flow and vascular permeability involves, at least in part, afferent nerves (Bruce, 1910; Jancsó *et al.*, 1977; Lembeck & Holzer, 1979; Lynn & Shakhanbeh, 1988; Louis *et al.*, 1989). Calcitonin gene-related peptide (CGRP) and substance P have been established as primary mediators of neurogenic inflammation in the skin (Holzer, 1992), and experiments involving immunoneutralization of CGRP and substance P have indicated that both peptides contribute to the vasodilator effect of mustard oil in the rat hindpaw skin (Louis *et al.*, 1989).

A previous study from this laboratory (Lippe *et al.*, 1993) has shown that the hyperaemic response to mustard oil is significantly attenuated by $N^G$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthase (Gardiner *et al.*, 1990; Rees *et al.*, 1990). This finding as well as other observations (Hughes *et al.*, 1990; Khalil & Helme, 1992; Ralevic *et al.*, 1992) support the contention that neurogenic vasodilatation in the skin depends in part on the formation of NO as a vasorelaxant messenger. The precise role of NO remains enigmatic. In view of the reports that NO does not participate in the hyperaemic effect of CGRP (Ralevic *et al.*, 1992; Hughes & Brain, 1994) which is the chief mediator of antidromic and capsaicin-evoked vasodilatation (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993; Hughes & Brain, 1994) the present study was undertaken to examine systematically a possible participation of NO in the hyperaemic responses to antidromic nerve stimulation and to application of mustard oil, CGRP and substance P. The possible site of action of NO has also been evaluated. The latter study involved sodium nitroprusside (SNP), a donor of NO, which has been reported to dilate cerebral arterioles in part via release of CGRP (Wei *et al.*, 1992).

[1] Author for correspondence.

## Methods

### *Animal preparation*

The experiments in this study were approved by the Austrian Ministry of Science and Research. Female Sprague-Dawley rats (Versuchstierzuchtanstalt Himberg, Austria) weighing 180–220 g were anaesthetized with 1.5 g kg$^{-1}$ urethane injected s.c. and fitted with a tracheal cannula. A catheter in the left jugular vein was used for the continuous infusion of saline (1.5 ml h$^{-1}$), to avoid dehydration and for the i.v. injection of drugs. Mean arterial blood pressure (MAP) was recorded from a catheter inserted in the right carotid artery and connected to a pressure transducer. Rectal temperature was maintained at 37–38°C by automatic control of a heating pad.

### *Capsaicin pretreatment*

To destroy the function of fine afferent neurones, rats were pretreated s.c. with a total dose of 125 mg kg$^{-1}$ capsaicin 2 weeks before the experiments (Holzer *et al.*, 1992). This dose of capsaicin was given in 4 injections over 2 days (first day: 25 mg kg$^{-1}$ in the morning and 25 mg kg$^{-1}$ in the late after-

noon, second day: 25 mg $kg^{-1}$ in the morning and 50 mg $kg^{-1}$ in the late afternoon). The control rats received equal volumes (2 + 2 + 2 + 4 ml $kg^{-1}$) of vehicle (10% ethanol, 10% Tween 80 and 80% saline; vol/vol/vol). All injections of capsaicin and its vehicle were performed under deep ether anaesthesia. To counteract the respiratory impairment associated with the administration of capsaicin, the rats received an intraperitoneal injection of atropine (0.2 mg $kg^{-1}$), terbutaline (0.2 mg $kg^{-1}$) and aminophylline (20 mg $kg^{-1}$) 15 min before the first and third capsaicin and vehicle injections (Esplugues *et al.*, 1989).

### *Antidromic stimulation of the saphenous nerve*

Antidromic stimulation of the cut saphenous nerve was performed as described previously (Lembeck & Holzer, 1979; Delay-Goyet *et al.*, 1992; Escott & Brain, 1993). Briefly, the right saphenous nerve was carefully dissected in the leg and cut, and the peripheral stump placed on bipolar platinum electrodes and immersed in paraffin oil. Electrical stimulation was performed with pulses of 15 V strength and 1 ms duration delivered at 2 Hz for a period fo 30 s (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993).

### *Topical and intraplantar administration of substances*

Mustard oil (5% in paraffin oil, wt/wt) was applied with a cotton bud, to the dorsal side of the hindpaw close to the laser optic probe. For intraplantar administration of drugs a catheter fitted with an injection needle (outside diameter 0.3 mm) was inserted from the distal aspect of the hindpaw into the middle of the plantar subcutaneous space. Substances were administered by infusion of a volume of 15 µl over a period of 1 min with an infusion pump. Thus it was possible to record blood flow continuously, without injection artifacts, during and after the intraplantar administration of drugs.

### *Laser Doppler flowmetry*

Blood flow in the skin of the hind paw was recorded with a laser Doppler flowmeter (model MBF3D, Moor Instruments, Devon, U.K.). To this end, a stainless steel laser optic probe (model P1, Moor Instruments) was taped perpendicularly to the skin of the hindpaw, the topographical position depending on the type of experiment. In order to measure blood flow changes in response to mustard oil or electrical stimulation of the saphenous nerve, the laser probe was positioned on the medio-lateral aspect of the plantar side of the hindpaw. When the effect of intraplantar drugs was studied, the probe was placed on the centre of the plantar side of the hindpaw in close vicinity to the tip of the infusion needle. After completion of the experimental preparations an equilibration period of at least 20 min was allowed before the experiments commenced. Blood flow was monitored as flux, the time constant being 0.5 s, and changes in blood flow were expressed as percentage changes in flux (Escott & Brain, 1993) relative to the average values recorded during the 2 min-period immediately before the changes were induced.

### *Statistics*

All data are expressed as means ± s.e.mean. Statistical evaluation of the results was performed with the Mann-Whitney U test, when two independent groups of data were compared with each other, or with the Kruskal-Wallis H test followed by the Mann-Whitney U test, when more than two independent groups of data were compared with each other. The Wilcoxon test for pair differences was used when data obtained before and after drug treatment were compared with each other. Probability values $P<0.05$ were regarded as significant.

### *Substances and solutions*

The saline solution was 0.9% NaCl (wt/wt). Aminophylline (20 mg $ml^{-1}$; Sigma, Deisenhofen, Germany), atropine (0.2 mg $ml^{-1}$; Merck, Darmstadt, Germany) and terbutaline (0.2 mg $ml^{-1}$; Astra, Södertälje, Sweden) were dissolved in saline at the concentrations indicated. Capsaicin (Serva, Heidelberg, Germany) was dissolved in absolute ethanol, followed by addition of Tween 80 and saline, the final concentration being 12.5 mg $ml^{-1}$ capsaicin in 10% ethanol, 10% Tween 80 and 80% saline (v/v/v). The vehicle solution, without capsaicin, was prepared in the same manner. Mustard oil (allyl isothiocyanate; Fluka, Buchs, Switzerland) was diluted (5%, v/v) with paraffin oil. Urethane (Fluka, Buchs, Switzerland) was dissolved in water (25%, wt/wt).

D-NAME ($N^G$-nitro-D-arginine methyl ester) and L-NAME ($N^G$-nitro-L-arginine methyl ester) were obtained from Bachem (Bubendorf, Switzerland) and dissolved in saline to give solutions of 60 mM which were injected i.v. at a volume of 1 ml $kg^{-1}$. Sodium nitroprusside (SNP, 1 mM; Merck, Darmstadt, Germany) was also dissolved in saline. Rat α-CGRP (calcitonin gene-related peptide), human $CGRP_{8-37}$ and substance P (Bachem, Bubendorf, Switzerland) were dissolved in 0.02 M acetic acid and diluted with saline. The tachykinin $NK_1$ receptor antagonist RP-67,580 ([3aR,7aR]-7,7-diphenyl-2-[1-imino-2-(2-methoxyphenyl)ethyl] perhydroisoindol-4-one, Rhône-Poulenc Rorer, Vitry-sur-Seine, France) was dissolved in one part of 0.1 M HCl, to which 9 parts of distilled water were added to give a stock solution of 10 mM RP-67,580. For intravenous administration of RP-67,580, the stock solution was diluted five fold with Tyrode buffer solution (composition, mM NaCl 136.9, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, $NaH_2PO_4$ 0.4). The vehicle solution was prepared in the same manner.

## Results

### *Effect of intraplantar versus intravenous L-NAME on cutaneous blood flow*

Initial experiments were carried out to compare the effects of intraplantar and i.v. administration of L-NAME on cutaneous blood flow and mean arterial blood pressure (MAP). Intraplantar L-NAME (95 nmol) led, after a brief initial rise, to a 45% reduction of plantar blood flow (Table 1), an effect that became maximal after 19 ± 3 min ($n = 6$). This pronounced attenuation of blood flow was associated with a modest increase in MAP. I.v. injection of L-NAME, at a dose (60 µmol $kg^{-1}$) that had previously been found to be maximally effective in causing hypertension (Holzer *et al.*, 1993), caused a marked rise of MAP which was accompanied by a small increase, rather than a decrease, in cutaneous blood flow. Pretreatment of rats with capsaicin or its vehicle did not alter the ability of i.v. L-NAME to elevate MAP and blood flow (Table 1). The enantiomer D-NAME (60 µmol $kg^{-1}$, i.v.) did not change blood flow but led to a delayed and gradual rise of MAP (Table 1) whereas the hypertensive response to L-NAME was immediate in onset and sustained. Saline (1 ml $kg^{-1}$, i.v.) was without effect on cutaneous blood flow and MAP.

### *Effect of L-NAME and capsaicin pretreatment on mustard oil-induced hyperaemia*

Topical administration of mustard oil (5%) caused a rapid rise of cutaneous blood flow that reached a peak within a few minutes and then stayed roughly constant during the observation period of 15 min. This hyperaemic response was accompanied by a transient increase of MAP (Figure 1). The experiments designed to examine the effects of saline, D-NAME and L-NAME on the mustard oil-induced hyperaemia were initiated by applying mustard oil to the

**Table 1** Effect of saline, $N^G$-nitro-D-arginine methyl ester (D-NAME) and $N^G$-nitro-L-arginine methyl ester (L-NAME) on cutaneous blood flow (CBF) and mean arterial blood pressure (MAP)

| | | *CBF* (% of control) | | *MAP* (mmHg) | | |
|---|---|---|---|---|---|---|
| *Pretreatment* | *Acute treatment* | n | *after* | n | *before* | *after* |
| None | Saline intraplantar | 9 | 105.2 ± 8.5 | 9 | 107 ± 2.6 | 109 ± 2.9 |
| | L-NAME intraplantar | 6 | 54.4 ± 9.8** | 6 | 106 ± 6.6 | 118 ± 5.1* |
| | Saline i.v. | 23 | 96.8 ± 3.0 | 21 | 96 ± 3.2 | 96 ± 3.3 |
| | D-NAME i.v. | 20 | 106.2 ± 4.5 | 17 | 92 ± 2.2 | 105 ± 2.3** |
| | L-NAME i.v. | 25 | 135.7 ± 14.0* | 22 | 92 ± 2.6 | 141 ± 2.5** |
| Vehicle for capsaicin | L-NAME i.v. | 8 | 129.5 ± 12.7 | 8 | 103 ± 6.3 | 142 ± 4.2** |
| Capsaicin | L-NAME i.v. | 8 | 133.9 ± 12.9* | 8 | 103 ± 3.5 | 139 ± 5.5** |

The changes of CBF and MAP induced by intraplantar infusion of saline (15 µl given during a period of 1 min) and L-NAME (95 nmol) reflect the maximal changes that were observed in the period 0–30 min post-infusion. The changes induced by i.v. injection of saline (1 ml $kg^{-1}$), D-NAME and L-NAME (60 µmol $kg^{-1}$ each) refer to the changes measured 30 min post-injection. Rats were pretreated with capsaicin (125 mg $kg^{-1}$, s.c.) or its vehicle (10% ethanol, 10% Tween 80 and 80% saline; v/v/v) 2 weeks before the experiments. The values shown are means ± s.e.mean of *n* animals as indicated. $*P<0.05$; $**P<0.01$ versus before acute treatment (Wilcoxon test for pair differences).

right hindpaw and recording blood flow and MAP for a period of 15 min. Then saline (1 ml $kg^{-1}$), D-NAME or L-NAME (60 µmol $kg^{-1}$ each) was injected i.v. followed 15 min later by application of mustard oil to the left hindpaw and recording blood flow in this paw. The rise of blood flow and MAP evoked by application of mustard oil did not differ significantly between the right and left hindpaw when saline or D-NAME was injected i.v. in between the two applications of mustard oil. In contrast, L-NAME diminished the cutaneous vasodilator reaction to mustard oil by 54% (Figure 2). The average hypotensive reaction to mustard oil was also lessened by L-NAME but this change, which is most probably related to the sustained hypertension caused by L-NAME (Table 1), was not statistically significant.

Responses of rats pretreated with the vehicle for capsaicin did not differ from those of untreated rats with respect to basal cutaneous blood flow (data not shown), basal MAP (Table 1), the vasodilatation and transient hypertension due to mustard oil and the ability of L-NAME to attenuate the mustard oil-evoked hyperaemia (Figure 2). Capsaicin pretreatment caused a 47% reduction of the effect of mustard oil to increase cutaneous blood flow when compared with the respective effect in vehicle-pretreated rats (Figure 2). I.v. injection of L-NAME to capsaicin-pretreated animals failed to inhibit further the hyperaemic reaction to mustard oil (Figure 2). The transient rise of MAP evoked by mustard oil was totally absent in capsaicin-pretreated rats (Figure 2) whereas basal MAP (Table 1) and basal cutaneous blood flow (not shown) were not altered by capsaicin pretreatment.

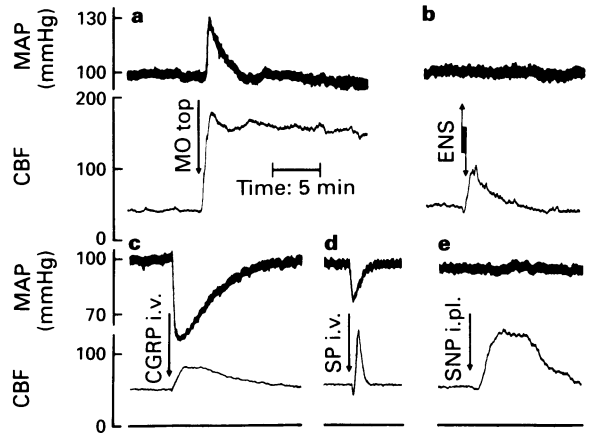


**Figure 1** Recordings of the effects of (a) mustard oil (MO, 5% in paraffin oil) applied topically (top) to the skin, (b) antidromic electrical stimulation of the saphenous nerve (ENS, pulses of 15 V and 1 ms delivered at 2 Hz for 30 s), (c) i.v. injection of calcitonin gene-related peptide (CGRP, 0.3 nmol $kg^{-1}$) and (d) substance P (SP, 0.3 nmol $kg^{-1}$) and (e) intraplantar (i.pl.) administration of sodium nitroprusside (SNP, 0.15 nmol in 15 µl infused over a period of 1 min) on cutaneous blood flow (CBF) in the rat hindpaw and mean arterial blood pressure (MAP). CBF was measured by laser Doppler flowmetry and expressed in arbitrary flux units. Each tracing is from a separate rat.

### *Effect of L-NAME on antidromic vasodilatation*

Antidromic vasodilatation was elicited by electrical stimulation of the saphenous nerve for a period of 30 s at 25 min-intervals. This procedure increased cutaneous blood flow (Figure 1) in a manner similar to that seen in other studies (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993). Fifteen minutes after the recording of two episodes of antidromic vasodilatation, saline (1 ml $kg^{-1}$) or L-NAME (60 µmol $kg^{-1}$) was injected i.v. and the cycle of electrical stimulation of the saphenous nerve continued after a wait of 10 min. As is shown in Table 2, antidromic vasodilatation did not change significantly after the i.v. administration of saline or L-NAME, and the hyperaemic response to electrical nerve stimulation stayed constant for a period of 60 min post-treatment.

### *Effect of L-NAME on the hyperaemic responses to CGRP and substance P*

The effect of L-NAME on the dermal hyperaemia evoked by CGRP and substance P was tested with a previously reported protocol (Holzer *et al.*, 1993). After priming the animals with three i.v. injections of rat α-CGRP or subtance P (300 pmol $kg^{-1}$) at 15-min intervals, to ensure reproducible

**Table 2** Effect of $N^G$-nitro-L-arginine methyl ester (L-NAME) on antidromic vasodilatation evoked by electrical stimulation of the saphenous nerve (pulses of 15 V and 1 ms delivered at 2 Hz for 30 s)

| | *Antidromic vasodilatation* (% of control) after | | |
|---|---|---|---|
| *Treatment* | 10 min | 35 min | 60 min |
| Saline | 91 ± 6.9 | 90 ± 6.5 | 101 ± 10.8 |
| L-NAME | 117 ± 15.1 | 111 ± 15.9 | 122 ± 22.0 |

Antidromic vasodilatation was evoked at 25-min intervals before and after the i.v. injection of saline (1 ml $kg^{-1}$) or L-NAME (60 µmol $kg^{-1}$). The vasodilator response measured 15 min before the injection of saline or L-NAME was taken as 100%, and the responses measured post-treatment were expressed as a percentage of that control response. The values shown are means ± s.e.mean of 5 rats in each group.

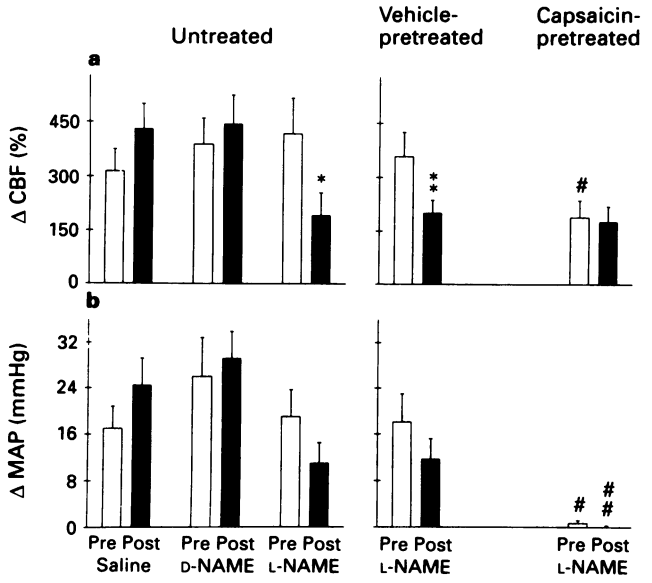


**Figure 2** Effect of i.v. injection of saline (1 ml $kg^{-1}$), $N^G$-nitro-D-arginine methyl ester (D-NAME) and $N^G$-nitro-L-arginine methyl ester (L-NAME; 60 μmol $kg^{-1}$ each) on the increment of cutaneous blood flow (ΔCBF) (a) and mean arterial blood pressure (ΔMAP) (b) evoked by topical application of mustard oil (5% in paraffin oil) to the rat hindpaw. The responses 'pre'-treatment were evoked by mustard oil application to the right hindpaw and are shown by the open columns. The responses 'post'-treatment were evoked by mustard oil application to the left hindpaw 15 min after the injection of saline, D-NAME or L-NAME and are shown by the solid columns. The experiments were carried out with untreated rats and rats that had been pretreated with vehicle (10% ethanol, 10% Tween 80 and 80% saline; v/v/v) or capsaicin (125 mg $kg^{-1}$, s.c.) 2 weeks before the experiments. The values shown are means with s.e.mean; $n = 6-8$. $^*P<0.05$; $^{**}P<0.01$ versus 'pre'-treatment (Wilcoxon test for pair differences); $^{\#}P<0.05$; $^{\#\#}P<0.01$ versus respective values in vehicle-pretreated rats (evaluated by the Mann-Whitney U test after the existence of a statistically significant difference between all groups had been revealed by the Kruskal-Wallis H test).

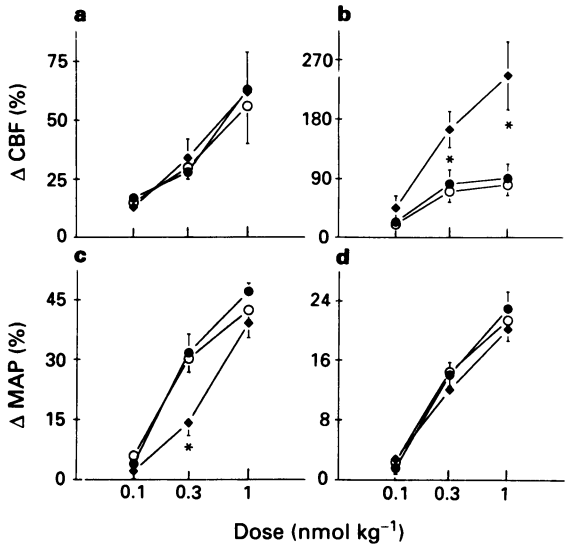


**Figure 3** Dose-dependent changes in cutaneous blood flow (Δ CBF) and mean arterial blood pressure (Δ MAP) evoked by i.v. injection of calcitonin gene-related peptide (CGRP) (a, c) and substance P (b, d) to rats treated with either saline (○), $N^G$-nitro-D-arginine methyl ester (●, D-NAME) or $N^G$-nitro-L-arginine methyl ester (◆, L-NAME). Saline (1 mg $kg^{-1}$), D-NAME and L-NAME (60 μmol $kg^{-1}$ each) were injected i.v. 15 min before the recording of the dose-response curves for CGRP and substance P was begun. The values shown are means with s.e.mean; $n = 5-8$. $^*P<0.05$ versus respective values recorded in rats treated with saline (evaluated by the Mann-Whitney U test after the existence of a statistically significant difference between rats treated with saline, D-NAME and L-NAME had been revealed by the Kruskal-Wallis H test).

vasodilator effects on repeated injection of the peptides (Holzer *et al.*, 1993), saline (1 ml $kg^{-1}$), D-NAME or L-NAME (60 μmol $kg^{-1}$ each) was given by i.v. injection. After a wait of 15 min, dose-response curves for the hyperaemic effects of CGRP or substance P were recorded by i.v. injection of increasing doses of CGRP and substance P (0.1–1 nmol $kg^{-1}$ in volumes of 1 ml $kg^{-1}$) at 15-min intervals. CGRP and substance P caused dose-dependent increases of cutaneous blood flow which were accompanied by dose-dependent decreases of MAP (Figures 1 and 3). The two peptides were roughly equipotent but their effects differed with respect to magnitude and duration (Figures 1 and 3). I.v. injection of the vehicle (saline, 1 ml $kg^{-1}$) was devoid of any effect on blood flow and MAP ($n = 12$, data not shown).

L-NAME failed to alter the potency of CGRP in augmenting blood flow in the skin when compared with the activity of CGRP in rats treated with saline. However, the ability of CGRP to lower MAP was attenuated by L-NAME to a minor but significant extent as deduced from a parallel rightward shift of the pertinent dose-response curve (Figure 3). In contrast, D-NAME was without influence on the CGRP-evoked hyperaemia and hypotension (Figure 3). The effects of substance P in causing cutaneous hyperaemia remained unchanged by D-NAME but were significantly enhanced by L-NAME (Figure 3). The hypotension which accompanied the hyperaemic effect of substance P was not modified by L- or D-NAME.

### *Effects of $CGRP_{8-37}$, RP-67,580 and capsaicin pretreatment on the cutaneous hyperaemia induced by sodium nitroprusside*

The haemodynamic effect of sodium nitroprusside (SNP) was explored by repeated intraplantar infusion (15 μl in 1 min) of the substance at 15 min-intervals. Initially two or three infusions of the drug were performed to establish reproducible blood flow responses. SNP caused a transient rise of cutaneous blood flow in the absence of any change in MAP (Figure 1). Preliminary experiments had established that the vasodilator effect of SNP was dose-dependent, 0.015 nmol SNP enhancing cutaneous blood flow by 15 ± 5% ($n = 7$, $P<0.05$) compared with a rise of +115 ± 15% ($n = 7$, $P<0.01$) evoked by 0.15 nmol SNP. The latter dose was chosen as test dose in the further experiments. Intraplantar administration of the vehicle (saline, 15 μl) enhanced blood flow to an insignificant extent (+12 ± 6%, $n = 9$, $P>0.05$) and had no effect on MAP.

The effect of $CGRP_{8-37}$ (50 nmol $kg^{-1}$; Delay-Goyet *et al.*, 1992) on the vasodilator response to SNP was tested by injecting this CGRP antagonist i.v. 2 min before administration of SNP. $CGRP_{8-37}$ led to a small but significant elevation of skin blood flow (+20 ± 4%, $n = 24$, $P<0.01$) and MAP (+5 ± 1 mmHg, $n = 24$, $P<0.01$) whereas blood flow and MAP did not change significantly after i.v. injection of the vehicle (saline, 1 ml $kg^{-1}$; $n = 7$). The SNP-induced hyperaemia was attenuated by 41% after administration of $CGRP_{8-37}$ but was left unaltered by the vehicle (Figure 4). The ability of intraplantar SNP to augment cutaneous blood flow and the effect of $CGRP_{8-37}$ to inhibit partially the SNP-induced hyperaemia did not differ between untreated rats and rats pretreated with the vehicle for capsaicin (Figure 4). Pretreatment of animals with capsaicin, however, decreased the vasodilator reaction to SNP by 53% when

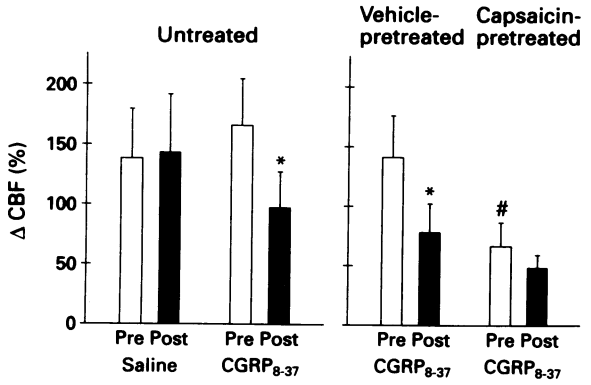


**Figure 4** Effect of i.v. injection of saline (1 ml $kg^{-1}$) and $CGRP_{8-37}$ (50 nmol $kg^{-1}$) on the increment of cutaneous blood flow (ΔCBF) evoked by intraplantar administration of sodium nitroprusside (SNP, 0.15 nmol infused over a period of 1 min). $CGRP_{8-37}$ was injected 2 min before SNP. The experiments were carried out with untreated rats and rats that had been pretreated with vehicle (10% ethanol, 10% Tween 80 and 80% saline; v/v/v) or capsaicin (125 mg $kg^{-1}$, s.c.) 2 weeks before the experiments. The values shown are means with s.e.mean; $n = 6-7$. $^{*}P<0.05$ versus 'pre'-treatment (Wilcoxon test for pair differences); $^{\#}P<0.05$ versus respective value in vehicle-pretreated rats (evaluated by the Mann-Whitney U test after the existence of a statistically significant difference between all groups had been revealed by Kruskal-Wallis H test).

compared with that found in vehicle-pretreated rats (Figure 4). $CGRP_{8-37}$ was no longer able to inhibit significantly the SNP-evoked hyperaemia in capsaicin-pretreated rats (Figure 4).

The substance P antagonist, RP-67,580 (2 μmol $kg^{-1}$, i.v.; Moussaoui *et al.*, 1993) caused a small elevation of MAP (+6 ± 2 mmHg, $n = 18$, $P<0.01$) and cutaneous blood flow (+10 ± 3%, $n = 18$, $P<0.01$) as measured 5 min post-treatment. However, RP-67,580 failed to alter significantly the SNP-evoked hyperaemia in the skin, the increase in blood flow before RP-67,580 being +110 ± 25% and that measured after RP-67,580 being +95 ± 30% ($n = 6$).

The specificity and effectiveness of $CGRP_{8-37}$ and RP-67,580 were evaluated by the i.v. injection of test doses of CGRP and substance P (0.3 nmol $kg^{-1}$) at 15 min-intervals. After establishing a reproducible hyperaemic response to these agonist doses, $CGRP_{8-37}$ was injected 2 min, and RP-67,580 5 min, before another application of the respective agonists. $CGRP_{8-37}$ reduced the CGRP-evoked increment of cutaneous blood flow from +80 ± 13% to +22 ± 5% ($n = 6$, $P<0.01$) but failed to affect that to substance P (+143 ± 19% before $CGRP_{8-37}$, +151 ± 19% after $CGRP_{8-37}$, $n = 5$). Conversely, RP-67,580 decreased the substance P-induced rise of cutaneous blood flow from +141 ± 28% to +13 ± 8% ($n = 6$, $P<0.01$) but did not alter that evoked by CGRP (+55 ± 7% before RP-67,580, +60 ± 7% after RP-67,580, $n = 5$). The effects of CGRP and substance P on MAP were also specifically inhibited by $CGRP_{8-37}$ and RP-67,580, respectively.

## Discussion

The present findings with the NO synthase inhibitor L-NAME confirm that the cutaneous hyperaemia evoked by topical mustard oil depends in part on the formation of endogenous NO (Lippe *et al.*, 1993). The inhibitory effect of L-NAME was enantiomer-specific as D-NAME which is said to be inactive on NO synthase (Gardiner *et al.*, 1990; Rees *et al.*, 1990) did not interfere with mustard oil-induced vasodilatation. D-NAME, however, was not totally inactive since it gave rise to a slowly and gradually developing hypertension, an unexplained effect that has been noted previously (Holzer *et al.*, 1993). L-NAME was given systemically since this route of administration did not diminish, but rather enhanced, cutaneous blood flow whereas local application of the drug reduced blood flow as has been observed in other studies (Hughes *et al.*, 1990; Lawrence & Brain, 1992). The use of s.c. L-NAME was regarded as impractible, therefore, given that local L-NAME could inhibit vasodilator responses solely by virtue of its vasoconstrictor effect.

The vascular responses to topical mustard oil are thought to arise in part from stimulation of afferent nerve fibres and release of vasodilator transmitter substances from their peripheral endings (Bruce, 1910; Jancsó *et al.*, 1977; Lembeck & Holzer, 1979; Louis *et al.*, 1989). Pretreatment of rats with a neurotoxic dose of capsaicin (Holzer, 1991) confirmed that a component of the vasodilator response to mustard oil was mediated by fine afferent nerve fibres. This observation is consistent with other findings indicating that the ability of mustard oil to augment vascular permeability in the skin is reduced but not abolished by pretreatment of adult animals with capsaicin (Jancsó *et al.*, 1977; Lynn & Shakhanbeh, 1988). In contrast, the transient rise of MAP evoked by mustard oil was abolished by capsaicin pretreatment, which suggests that the pressor response to mustard oil is another reflection of the involvement of capsaicin-sensitive afferent nerve fibres in cardiovascular reflexes activated by noxious stimuli (Holzer, 1992).

The combination of capsaicin pretreatment and i.v. administration of L-NAME established that it is the neurogenic component of the mustard oil-evoked hyperaemia that depends on the formation of NO. This conclusion is in keeping with the hypothesis that, while CGRP and substance P are the primary neurogenic mediators of mustard oil-induced vasodilatation (Louis *et al.*, 1989), NO is a secondary vasorelaxant messenger of neurogenic vasodilatation in the skin (Lippe *et al.*, 1993). However, this hypothesis was not supported by the inability of L-NAME to inhibit the increase of cutaneous blood flow elicited by antidromic stimulation of the saphenous nerve. NO has likewise been ruled out as a contributor to antidromic vasodilatation in the pulp and lip of the rat, which is not reduced but augmented by L-NAME (Kerezoudis *et al.*, 1993). The chief, if not exclusive, mediator of antidromic vasodilatation evoked by saphenous nerve stimulation (Delay-Goyet *et al.*, 1992; Escott & Brain, 1993) or local application of capsaicin (Hughes & Brain, 1991; 1994) is CGRP. It was not unexpected, therefore, to find that the cutaneous hyperaemia due to CGRP was also independent of NO as has been noted in other studies (Ralevic *et al.*, 1992; Hughes & Brain, 1994). However, L-NAME caused a slight depression of the hypotensive effect of CGRP, an effect that reflects the participation of NO in the vasodilator action of CGRP in certain non-cutaneous vascular beds (Gardiner *et al.*, 1991; Holzer *et al.*, 1993). In contrast, the ability of substance P to lower MAP remained unaltered by L-NAME (this study) and $N^{G}$-nitro-L-arginine (Santicioli *et al.*, 1993) but was attenuated by $N^{G}$-monomethyl-L-arginine (Whittle *et al.*, 1989). These discrepant data as well as the ability of L-NAME to enhance the vasodilator activity of substance P in the hind-paw skin in a way similar to that seen in the pulp and oral mucosa (Kerezoudis *et al.*, 1993) are not understood, and it was beyond the scope of the present study to explore the interaction between L-NAME and substance P in more detail. Here it was important to know that the hyperaemic action of substance P, another supposed neurogenic mediator of the mustard oil-evoked hyperaemia (Louis *et al.*, 1989), did not depend on the formation of NO.

The data discussed thus far indicate that NO participates in the neurogenic component of the vasodilator response to the chemical irritant mustard oil, but has no role in the vasodilatation elicited by electrical nerve stimulation and in the vasodilatation evoked by the neuropeptides CGRP and substance P. Provided that antidromic vasodilatation and the

neurogenic component of the mustard oil-induced hyperaemia arise from the same neurovascular transmission process it would appear, therefore, that NO is not a vasorelaxant messenger of neurogenic vasodilatation but is involved in the mustard oil-evoked excitation of, or peptide release from, afferent nerve fibres. An identical conclusion has been reached with regard to the hyperaemic action of another chemical irritant, capsaicin, in the rabbit skin (Hughes & Brain, 1994). The emerging hypothesis, that NO plays an important regulatory role in peptide release from afferent nerve fibres in response to chemical irritants, raises the question as to the cellular location of the NO synthase that is stimulated by mustard oil and capsaicin. As discussed by Hughes & Brain (1994) it seems most probable that NO is formed either in cells adjacent to afferent nerve fibres or in the nerve fibres themselves. This question cannot be settled on the basis of the available data, but it is worth considering that NO can be formed in mast cells (Salvemini *et al.*, 1991), which could be a non-neural source of NO generated in response to chemical irritants, and in afferent neurones themselves (Morris *et al.*, 1992; Verge *et al.*, 1992; Vizzard *et al.*, 1994). Important in the latter context is the finding that the bradykinin- and capsaicin-induced production of guanosine 3':5'-cyclic monophosphate in cultured primary afferent neurones is blocked by an inhibitor of NO synthase (Bauer *et al.*, 1993), which demonstrates that NO can be formed in stimulated afferent neurones and may play an important role in the chemical activation of sensory neurones and, further on, release of peptide transmitters from them.

The proposed role of NO in the chemical activation of neurogenic vasodilator responses was substantiated by the observation that the hyperaemia caused by the NO donor, SNP, was in part inhibited by the specific CGRP antagonist, $CGRP_{8-37}$ and by capsaicin-induced defunctionalization of afferent nerve fibres. This and the finding that $CGRP_{8-37}$ was no longer able to inhibit the vasodilator effect of SNP in capsaicin-pretreated animals indicate that SNP can dilate blood vessels in the rat skin by releasing CGRP from afferent nerve fibres, a conclusion that also holds true for the SNP-evoked dilatation of cerebral arterioles in the cat (Wei *et al.*, 1992). It is important to consider that the mechanism of SNP-evoked hyperaemia may be subject to species and tissue differences, since the vasodilator effect of SNP in the rabbit skin was not antagonized by $CGRP_{8-37}$ (Hughes & Brain, 1994). Unlike CGRP, substance P does not seem to play a role in the hyperaemic response to SNP in the rat skin, because the tachykinin $NK_1$ receptor antagonist, RP-67,580 (Moussaoui *et al.*, 1993), failed to antagonize SNP. RP-67,580 was chosen because the microvascular effects of substance P appear to be mediated by $NK_1$ receptors and RP-67,580 is a potent and selective antagonist at the rat variant of the $NK_1$ receptor (Maggi *et al.*, 1993; Moussaoui *et al.*, 1993). The significance of the small hyperaemic and hypertensive effects of RP-67,580 and $CGRP_{8-37}$, which were observed here, and their possible physiological implications await further clarification.

In conclusion, the present study has shown that NO or a related product of the L-arginine:NO pathway participates in the neurogenic component of the cutaneous vasodilator response to the irritant mustard oil. NO, however, does not play a vasorelaxant messenger role but contributes to the irritant-elicited excitation of, or release of vasodilator peptides from, afferent nerve fibres. The source of NO generated in response to chemical irritants remains to be identified.

The authors are indebted to Dr Rainer Amann for reading the manuscript, to Wolfgang Schluet for organizational assistance and to Irmgard Russa for secretarial help. Financial support for the study was provided by the Austrian Science Foundation (grant P9473-MED).

## References

BAUER, M.B., SIMMONS, M.L, MURPHY, S. & GEBHART, G.F. (1993). Bradykinin and capsaicin stimulate cyclic GMP production in cultured rat dorsal root ganglion neurons via a nitrosyl intermediate. *J. Neurosci. Res.*, **36**, 280–289.

BRUCE, N.A. (1910). Über die Beziehung der sensiblen Nervenendigungen zum Entzündungsvorgang. *Arch. Exp. Path. Pharmakol.*, **63**, 424–433.

DELAY-GOYET, P., SATOH, H. & LUNDBERG, J.M. (1992) Relative involvement of substance P and CGRP mechanisms in antidromic vasodilatation in the rat skin. *Acta Physiol. Scand.*, **146**, 537–538.

ESCOTT, K.J. & BRAIN, S.D. (1993). Effect of a calcitonin gene-related peptide antagonist ($CGRP_{8-37}$) on skin vasodilatation and oedema induced by stimulation of the rat saphenous nerve. *Br. J. Pharmacol.*, **110**, 772–776.

ESPLUGUES, J.V., WHITTLE, B.J.R. & MONCADA, S. (1989). Local opioid-sensitive afferent sensory neurones in the modulation of gastric damage induced by Paf. *Br. J. Pharmacol.*, **97**, 579–585.

GARDINER, S.M., COMPTON., A.M., KEMP, P.A. & BENNETT, T. (1990). Regional and cardiac haemodynamic effects of $N^G$-nitro-L-arginine methyl ester in conscious, Long Evans rats. *Br. J. Pharmacol.*, **101**, 625–631.

GARDINER, S.M., COMPTON, A.M., KEMP, P.A., BENNETT, T., FOULKES, R. & HUGHES, B. (1991). Haemodynamic effects of human α-calcitonin gene-related peptide following administration of endothelin-1 or $N^G$-nitro-L-arginine methyl ester in conscious rats. *Br. J. Pharmacol.*, **103**, 1256–1262.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol. Rev.*, **43**, 143–201.

HOLZER, P. (1992). Peptidergic sensory neurons in the control of vascular functions: mechanisms and significance in the cutaneous and splanchnic vascular beds. *Rev. Physiol. Biochem. Pharmacol.*, **121**, 49–146.

HOLZER, P., LIPPE, I.TH. & AMANN, R. (1992). Participation of capsaicin-sensitive afferent neurons in gastric motor inhibition caused by laparotomy and intraperitoneal acid. *Neuroscience*, **48**, 715–722.

HOLZER, P., LIPPE, I.TH., JOCIČ, M., WACHTER, CH., ERB, R. & HEINEMANN, A. (1993). Nitric oxide-dependent and -independent hyperaemia due to calcitonin gene-related peptide in the rat stomach. *Br. J. Pharmacol.*, **110**, 404–410.

HUGHES, S.R. & BRAIN, S.D. (1991). A calcitonin gene-related peptide (CGRP) antagonist ($CGRP_{8-37}$) inhibits microvascular responses induced by CGRP and capsaicin in skin. *Br. J. Pharmacol.*, **104**, 738–742.

HUGHES, S.R. & BRAIN, S.D. (1994). Nitric oxide-dependent release of vasodilator quantities of calcitonin gene-related peptide from capsaicin-sensitive nerves in rabbit skin. *Br. J. Pharmacol.*, **111**, 425–430.

HUGHES, S.R., WILLIAMS, T.J. & BRAIN, S.D. (1990). Evidence that endogenous nitric oxide modulates oedema formation induced by substance P. *Eur. J. Pharmacol.*, **191**, 481–484.

JANCSÓ, G., KIRÁLY, E. & JANCSÓ-GÁBOR, A. (1977). Pharmacologically induced selective degeneration of chemosensitive primary sensory neurones. *Nature*, **270**, 741–743.

KEREZOUDIS, N.P., OLGART, L. & EDWALL, L. (1993). Differential effects of nitric oxide synthesis inhibition on basal blood flow and antidromic vasodilation in rat oral tissues. *Eur. J. Pharmacol.*, **241**, 209–219.

KHALIL, Z. & HELME, R.D. (1992). The quantitative contribution of nitric oxide and sensory nerves to bradykinin-induced inflammation in rat skin microvasculature. *Brain Res.*, **589**, 102–108.

LAWRENCE, E. & BRAIN, S.D. (1992). Responses to endothelins in the rat cutaneous microvasculature: a modulatory role of locally-produced nitric oxide. *Br. J. Pharmacol.*, **106**, 733–738.

LEMBECK, F. & HOLZER, P. (1979). Substance P as neurogenic mediator of antidromic vasodilation and neurogenic plasma extravasation. *Naunyn-Schmied. Arch. Pharmacol.*, **310,** 175–183.

LIPPE, I.TH., 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.

LOUIS, S.M., JAMIESON, A., RUSSELL, N.J.W. & DOCKRAY, G.J. (1989). The role of substance P and calcitonin gene-related peptide in neurogenic plasma extravasation and vasodilatation in the rat. *Neuroscience*, **32,** 581–586.

LYNN, B. & SHAKHANBEH, J. (1988). Substance P content of the skin, neurogenic inflammation and numbers of C-fibres following capsaicin application to a cutaneous nerve in the rabbit. *Neuroscience*, **24,** 769–776.

MAGGI, C.A., PATACCHINI, R., ROVERO, P. & GIACHETTI, A. (1993). Tachykinin receptors and tachykinin receptor antagonists. *J. Auton. Pharmacol.*, **13,** 23–93.

MORRIS, R., SOUTHAM, E., BRAID, D.J. & GARTHWAITE, J. (1992). Nitric oxide may act as a messenger between dorsal root ganglion neurones and their satellite cells. *Neurosci. Lett.*, **137,** 29–32.

MOUSSAOUI, S.M., MONTIER, F., CARRUETTE, A., BLANCHARD, J.C.,LADURON, P.M. & GARRET, C. (1993). A non-peptide $NK_1$-receptor antagonist, RP 67580, inhibits neurogenic inflammation postsynaptically. *Br. J. Pharmacol.*, **109,** 259–264.

RALEVIC, V., KHALIL, Z., DUSTING, G.J. & HELME, R.D. (1992). Nitric oxide and sensory nerves are involved in the vasodilator response to acetylcholine but not calcitonin gene-related peptide in rat skin microvasculature. *Br. J. Pharmacol.*, **106,** 650–655.

REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101,** 746–752.

SALVEMINI, D., MASINI, E., PISTELLI, A., MANNAIONI, P.F. & VANE, J. (1991). Nitric oxide: a regulatory mediator of mast cell reactivity. *J. Cardiovasc. Pharmacol.*, **17** (Suppl. 3), S258–S264.

SANTICIOLI, P., GIULIANI, S. & MAGGI, C.A. (1993). Failure of L-nitroarginine, a nitric oxide synthase inhibitor, to affect hypotension and plasma protein extravasation produced by tachykinin NK-1 receptor activation in rats. *J. Auton. Pharmacol.*, **13,** 193–199.

VERGE, V.M.K., XU, Z., XU, X.-J., WIESENFELD-HALLIN, Z. & HÖKFELT, T. (1992). Marked increase in nitric oxide synthase messenger RNA in rat dorsal root ganglia after peripheral axotomy: in situ hybridization and functional studies. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 11617–11621.

VIZZARD, M.A., ERDMAN, S.L., ERICKSON, V.L., STEWART, R.J., ROPPOLO, J.R. & DE GROAT, W.C. (1994). Localization of NADPH diaphorase in the lumbosacral spinal cord and dorsal root ganglia of the cat. *J. Comp, Neurol.*, **339,** 62–75.

WEI, E.P., MOSKOWITZ, M.A., BOCCALINI, P. & KONTOS, H.A. (1992). Calcitonin gene-related peptide mediates nitroglycerin and sodium nitroprusside-induced vasodilation in feline cerebral arterioles. *Circ. Res.*, **70,** 1313–1319.

WHITTLE, B.J.R., LOPEZ-BELMONTE, J. & REES, D.D. (1989). Modulation of the vasodepressor actions of acetylcholine, bradykinin, substance P and endothelin in the rat by a specific inhibitor of nitric oxide formation. *Br. J. Pharmacol.*, **98,** 646–652.

*(Received December 20, 1993*
*Revised March 28, 1994*
*Accepted April 12, 1994)*