Cutaneous blood flow responses in the forearms of Raynaud's patients induced by local cooling and intradermal injections of CGRP and histamine.

S. D. BRAIN¹, R. G. PETTY², J. D. LEWIS³ & T. J. WILLIAMS⁴

¹Pharmacology Group, Biosciences Division, King's College, Manresa Road, London SW3 6LX, ²Section of Vascular Biology, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, ³Department of Vascular Surgery, Northwick Park Hospital, Watford Road, Harrow, Middlesex HA1 3UJ and ⁴Department of Applied Pharmacology, National Heart and Lung Institute, Dovehouse Street, London SW3 6LY

1 The cutaneous responses of the forearm to local cold exposure and intradermal injection of CGRP and other vasoactive mediators were compared in primary Raynaud's sufferers and normal volunteers.

2 Skin responses in the forearm were measured in terms of erythema reddening and skin blood flow. Visual responses were recorded by tracing and then area calculated by computerised planimetry. Skin blood flow was measured using a laser Doppler blood flow meter.

3 Cooling (5–6° C for 2 min) of a 1 cm² area of the forearm caused a localised reactive hyperaemia response in normal volunteers, measured using the laser Doppler blood flow meter. The peak response in Raynaud's patients was significantly smaller than that of normal volunteers.

4 The cutaneous responses of Raynaud's patients and normal volunteers to intradermal injections of CGRP, histamine and PGE₂ were not significantly different.

5 The results suggest that Raynaud's sufferers do not exhibit a diminished response to CGRP in the cutaneous microvasculature and can respond normally to histamine with an axon reflex mediated flare.

Keywords Raynaud's CGRP blood flow reactive hyperaemia

Introduction

In patients with Raynaud's syndrome there is an intermittent arteriolar vasospasm. However, the mechanisms that lead to the digital ischaemia and pain that Raynaud's patients experience after exposure to the cold or stress are unclear. The potent vasodilator calcitonin gene-related peptide (CGRP) has been suggested to be present in a large number of unmyelinated cutaneous fibres in human skin (Gibbons *et al.*, 1987; Salahuddin *et al.*, 1988). Axons containing CGRP are found in the dermis around arterioles

and larger blood vessels. These fibres have the dual function of transmitting sensory information centrally and secreting vasoactive peptides peripherally (Weihe & Hartschuh, 1988). In this study, we have examined the possibility that the lack of reflex vasodilatation observed in Raynaud's patients may be due to either a lack of responsiveness to CGRP in the cutaneous microvasculature, or a defect in local axon reflexes.

Intradermal injection (i.d.) of low femtomole

Correspondence: Dr S. D. Brain, Pharmacology Group, Biosciences Division, King's College (Chelsea Campus), Manresa Road, London SW3 6LX

doses of CGRP into the skin of normal individuals causes a persistent local reddening which is associated with increased blood flow (Brain *et al.*, 1985, 1986). In contrast, histamine, substance P and vasoactive intestinal peptide (VIP) stimulate the classical triple response as described by Thomas Lewis (Anand *et al.*, 1983; Foreman *et al.*, 1983; Lewis, 1927). The triple response consists of a wheal, local reddening and an axon reflex flare, which can spread for up to several centimetres around the injection site. CGRP causes local reddening without a wheal and flare, except at high doses (Brain *et al.*, 1985; Piotrowski & Foreman, 1986). Wheal and flare responses are short in duration and usually fade by 1 h after injection. In contrast the local reddening induced by CGRP is very persistent, lasting for 3–4 h with a 10 pmol dose (Brain *et al.*, 1986; Brain & Williams, 1988). The skin response to CGRP is similar to that induced by vasodilator prostaglandins but longer in duration. The potency of CGRP in increasing blood flow in human skin has led us to suggest that CGRP is the substance released, as a result of an axon reflex, to mediate the increased blood flow observed in the flare component of the triple response (Brain *et al.*, 1986). Furthermore infusion of CGRP via the brachial artery stimulates increased forearm blood flow in normal volunteers (McEwan *et al.*, 1988; Thom *et al.*, 1987).

It has been reported that a local cold stress test to the nail bed reveals functional differences between normal subjects and patients with Raynaud's syndrome at the level of the micro-circulation (Mahler *et al.*, 1987). We have used a localised cooling probe to show that Raynaud's patients exhibit altered reactive hyperaemia in the forearm, the site chosen in this study to compare skin responses to intradermal CGRP, PGE₂ and histamine of normal volunteers to those observed in Raynaud's patients.

Two sections of this study have previously been presented in abstract form to the British Pharmacological Society (June 1989, see Brain *et al.*, 1989a) and to the Bayliss and Starling Society (September 1989, see Brain *et al.*, 1989b).

Methods

Patients and volunteers

Eight patients with primary Raynaud's (two male and six females, age range 26–39 years) and eight age, sex and race matched volunteers were studied. The Raynaud's sufferers had normal arterial digital blood pressure (> 25 mm Hg),

with no evidence of any underlying disorder, but with a delayed recovery after whole arm cold stress test. All studies were conducted after a minimum acclimatisation period of 15 min in a room with temperature maintained at 23–26° C. The study was approved by the Clinical Research Centre and Northwick Park Hospital Ethics Committees and all patients and volunteers gave informed consent.

Measurement of skin blood flow

Blood flow was measured using a Perimed PF2 laser Doppler blood flow meter (Holloway & Watkins, 1977; Brain *et al.*, 1986; Brain & Williams, 1988). The probe was secured on the forearm for each reading. Blood flow is measured by the laser Doppler as follows; monochromatic light from a 2 mW helium-neon laser is led by an optical fibre to the probe head. The light reaches a small volume of skin (1–2 mm³). Moving red blood cells in the illuminated area are reflected, the light inducing a Doppler shift in frequency. The backscattered light is picked up by two optical fibres attached to photodetectors. The Doppler shifted part of the signal is then isolated and converted into a voltage output signal, read as flux. The flux is a measure of the number of moving red blood cells detected, multiplied by the mean cell velocity. It is therefore expressed as arbitrary units (the flux value) and not as an absolute measure of blood flow. However, an increase in flux is linearly related to an increase in cutaneous blood flow. Measurements were taken with the machine at a high sensitivity setting (4 Hz, gain 10) when the reactive hyperaemia studies were being performed and a less sensitive setting (4 Hz, gain 3) when investigating the effect of injected mediators.

Measurement of reactive hyperaemia after localised cooling

Cutaneous blood flow was measured for 1–3 min at a predetermined site on the volar surface of the forearm in six Raynaud's patients and six matched volunteers. The laser probe was then removed and a cooled probe (4–6° C, 1 cm diameter) was placed in contact with the site for 2 min. The cold probe was then removed and the laser Doppler probe replaced at the same site. Blood flow was then measured at the cooled sites for the following five minutes. A typical result from the local reactive hyperaemia test is shown in Figure 1.

Figure 1 Example of a reactive hyperaemia response in a normal volunteer (Normal) and a Raynaud's sufferer (Raynaud's). Blood flow was measured for 2 min at the chosen site on the forearm. This site was then cooled ($5-6^{\circ}\text{C}$ for 2 min), shown by the solid black block. Blood flow was then measured at the site for a further 5 min after cooling (see Methods).

Cutaneous responses to intradermal injections

Agents under test were made up in sterile pyrogen-free saline using aseptic techniques and injected intradermally in 50 μl volumes using a 27 gauge needle. Injections were made into the volar surface of the human forearm according to a randomised pattern. Injections were carried out at 1 min intervals. Blood flow (laser Doppler) and visual responses were measured at 10 min, 1 h, 2 h, 3 h and 4 h after injections. Visual responses were recorded by tracing onto transparent plastic sheets. The area of visual responses was then calculated by computerised planimetry. Blood flow was measured by placing the laser probe on the skin (taking care not to place the probe on the injection point). The subject remained still and quiet whilst readings were being taken. The mean of three consecutive readings (each for 15 s) was calculated for each time point and results expressed as flux.

Statistics

The significance of differences between treatments in normal volunteers compared with Raynaud's patients was assessed after logarithmic transformation of the data, by Bonferroni's modified *t*-test; using the standard error estimate from the analysis of variance to account for the fact that multiple tests were performed.

Results

Reactive hyperaemia responses of five out of six Raynaud's sufferers, after localised cooling, were absent or attenuated when compared with controls; (peak blood flow: normal volunteers before cold test 13.2 ± 4.8 flux, after cold test 57.8 ± 20.6 flux; Raynaud's patients before cold test 12.2 ± 4.6 flux, after cold test 28.0 ± 24.9 flux: (mean $\pm$ s.d., $n = 6$). The peak blood flow response was significantly greater in the normal volunteers when compared with the Raynaud's patients ($P = 0.0046$). Figure 1 shows the response of a normal volunteer and the response of a Raynaud's volunteer, who showed a total lack of reactive hyperaemia response.

Responses to CGRP (10 pmol) and PGE_2 (10 pmol) and saline are shown in Figure 2. Both CGRP and PGE_2 caused localised erythema. In each case the area of erythema was associated with increased blood flow as shown in Figure 2. The response to CGRP was longer lasting than that to PGE_2 for both groups, as previously shown in normal individuals (Brain *et al.*, 1986). Similar time courses of responses to both CGRP and PGE_2 were observed in Raynaud's patients when compared with normal volunteers. There is no significant difference in the responses between patients and control groups at any time point.

Histamine stimulated a triple response. The response consisted of a wheal, flare and local reddening response. Blood flow was measured in the flare area as shown in Figure 3. The area of the flare was also calculated. Responses of normal volunteers and Raynaud's sufferers were not significantly different in terms of either blood flow measured in the flare area or in the area of the flare.

Discussion

The results presented in this paper suggest that Raynaud's patients exhibit a normal axon reflex flare response to intradermal histamine. The flare is mediated by C-fibre nerves which contain

Figure 2 The effect of CGRP and PGE₂ in Raynaud's patients and normal volunteers. Blood flow results measured in response to i.d. CGRP (10 pmol), PGE₂ (10 pmol) and saline (50 μ l) are shown on the left hand side. The ordinate shows the flux. The visual responses (local reddening) are shown on the right hand side, the ordinate shows the area in mm². Responses were measured up to 4 h in normal volunteers (closed symbols) and Raynaud's sufferers (open symbols). Results are mean $\pm$ s.e. mean of $n = 8$ humans in each group. No statistically significant differences were observed between responses in normal volunteers and Raynaud's patients.

CGRP and the flare has been suggested to be due to the action of CGRP released in extremely low concentrations from C-fibre nerve endings (Brain *et al.*, 1985, 1986). The observation that Raynaud's patients and normal volunteers respond similarly to intradermal CGRP, together with the presence of the axon reflex flare to histamine, suggests that C-fibre neurogenic responses in the skin of Raynaud's patients can occur. However, the Raynaud's patients have an abnormality in their response to the cold which

is observed locally in skin in this study.

An altered reactive hyperaemia in response to localised cooling (1 cm diameter area at 5–6°C for 2 min) of the forearm was observed in Raynaud's sufferers through the use of a laser Doppler blood flow meter to measure skin blood flow. Previously it has been shown that localised cooling of the nail bed capillaries of Raynaud's patients causes a constriction and cessation of blood flow as observed by direct microscopy (Mahler *et al.*, 1987). Raynaud's phenomenon is

Figure 3 The histamine-induced flare in Raynaud's patients (shaded columns) and normal volunteers (open columns). Blood flow, shown as flux is in the left graph. Blood flow was measured in the flare area at 10 min after i.d. histamine (HA, 500 pmol) and at a corresponding site after i.d. saline (Sal, 50 µl). The right graph shows the area of the flare response to histamine (HA). No flare was observed in response to i.d. saline. Results are mean $\pm$ s.e. mean, $n = 8$ experiments in each group.

primarily observed as a digital vasospasm, but our results suggest that cutaneous blood flow in the forearm can also be altered by cold exposure in Raynaud's patients. A localised cooling test, similar to that used in this study could be useful in the quantification of the severity of Raynaud's conditions, and for evaluating medical treatment by vasoactive drugs. The test is less stressful for the Raynaud's sufferer than the widely used whole arm cooling technique.

The mechanisms underlying the reactive hyperaemia response are poorly understood. The results presented here suggest that local microvascular mechanisms are responsible for the reactive hyperaemia observed in response to localised cooling of the human forearm in the normal volunteer. Consequently it is suggested that the lack of reactive hyperaemia observed in Raynaud's sufferers, in response to localised cooling of the forearm, is due to a reduced response of dilator mechanisms in the cutaneous microvascular bed. Previously sympathetic hyperactivity has been thought to be responsible for the lack of reactive hyperaemia observed in Raynaud's, especially as the cutaneous vessels of the hands and feet are supplied only by constrictor fibres (Barcroft & Swan, 1953). However, as discussed above, the cutaneous micro-

vasculature is richly innervated with C-fibre nerves which contain dilator peptides such as CGRP. It has also been suggested that a local histaminergic mechanism is important for the maintenance of blood flow and that this mechanism is deficient in Raynaud's (Lafferty *et al.*, 1983). An intact sensory nerve network is essential for the axon reflex flare dilator component in the cutaneous response to intradermal histamine. In this study the flare in response to intradermal histamine was similar in Raynaud's patients and normal volunteers. This suggests that the neurogenic dilator response, to histamine at least, is normal in Raynaud's patients.

The results show that CGRP induces the typical sustained local reddening response in Raynaud's sufferers previously observed in normal volunteers. This demonstrates that the cutaneous microvasculature in Raynaud's sufferers exhibits a normal dilator response to CGRP. The Raynaud's patients also responded in a similar manner to normal volunteers, in this study, after intradermal injection of the prostanoid vasodilator PGE₂. Previously another prostanoid vasodilator, PGI₂, has been shown to be beneficial after systemic treatment in certain types of Raynaud's phenomenon (Belch *et al.*, 1983). In a recent study the effects of intra-

venous PGI₂ and intravenous CGRP were compared (Shawket *et al.*, 1989). A similar increase in skin blood flow in the hands and faces of Raynaud's patients and normal volunteers was observed after intravenous PGI₂. In contrast, although CGRP caused an increased blood flow in the face and hands of Raynaud's patients, increased blood flow was observed only in the face of normal volunteers. The authors suggest that the skin in the hands of patients with Raynaud's phenomenon is supersensitive to CGRP, due to a lack of endogenous CGRP release. Our results cannot support this. However, the increased skin blood flow observed by Shawket and co-workers (1989) after intravenous administration of CGRP may reflect a supersensitivity of larger vessels which innervate the cutaneous microvasculature. It has also been demonstrated that CGRP induces a potent vasodilator response when injected intradermally into the fingers of Raynaud's sufferers (Bunker

et al., 1989), although no comparison with the response of normal volunteers was made.

In conclusion the results suggest that Raynaud's patients do not exhibit a diminished response to either the vasodilator neuropeptide CGRP or the vasodilator peptide PGE₂ in the cutaneous vasculature. Furthermore the patients respond normally to histamine with an axon-reflex mediated flare. These combined results suggest that the function of nociceptive C-fibres is normal in Raynaud's patients. However, it is possible that the selective response of C-fibres to the cold is abnormal in Raynaud's patients and this will be the subject of further studies. The results are in keeping with a suggested role for CGRP in the therapy of Raynaud's phenomenon.

This study was supported by the Halley Stewart Trust and the Medical Research Council.

References

- Anand, A., Bloom, S. R. & McGregor, G. P. (1983). Topical capsaicin pretreatment inhibits axon reflex vasodilatation caused by somatostatin and vasoactive intestinal polypeptide in human skin. *Br. J. Pharmacol.*, **78**, 665–669.
- Barcroft, H. & Swan, H. J. C. (1953). *Sympathetic control of human blood vessels*, pp 79–87. London: Edward Arnold & Co.
- Belch, J. F., Drury, J. K., Capell, H., Forkes, C. D., Newman, P., McKenzie, F., Lieberman, P. & Prentice, C. R. M. (1983). Intermittent epoprostenol (prostacyclin) infusion in patients with Raynaud's syndrome. *Lancet*, **ii**, 313–315.
- Brain, S. D. & Williams, T. J. (1988). Substance P regulates the vasodilator activity of calcitonin gene-related peptide. *Nature*, **335**, 73–75.
- Brain, S. D., Petty, R. G., Lewis, J. & Williams, T. J. (1989a). The effect of intradermal CGRP and histamine on blood flow in the forearm of normal volunteer and Raynaud's sufferers. *Br. J. clin. Pharmacol.*, **28**, 736P–737P.
- Brain, S. D., Petty, R. G., Lewis, J. & Williams, T. J. (1989b). The effect of intradermal CGRP on cutaneous blood flow in Raynaud's patients. *Regulatory Peptides*, **26**, 60.
- Brain, S. D., Williams, T. J., Tippins, J. R., Morris, H. R. & Macintyre, I. (1985). Calcitonin gene-related peptide (CGRP) is a potent vasodilator. *Nature*, **313**, 54–56.
- Bunker, C. B., Foreman, J. C., O'Shaughnessy, D., Reavley, C. & Dowd, P. M. (1989). Calcitonin gene-related peptide in Raynaud's phenomenon. *Regulatory Peptides*, **26**, 51.
- Foreman, J. C., Jordan, C. C., Oehme, P. & Renner, H. (1983). Structure-activity relationships for some substance P-related peptides that cause wheal and flare reactions in human skin. *J. Physiol.*, **335**, 449–465.
- Gibbons, I. L., Wattchow, D. & Coventry, B. (1987). Two immunohistochemically identified populations of calcitonin gene-related peptide (CGRP)-immunoreactive axons in human skin. *Brain Res.*, **414**, 143–148.
- Holloway, G. A. & Watkins, D. M. (1977). Laser Doppler measurement of cutaneous blood flow. *J. invest. Dermatol.*, **69**, 306–309.
- Lafferty, K., Detrafford, J. C., Roberts, V. C. & Cotton, L. T. (1983). On the nature of Raynaud's phenomenon: The role of histamine. *Lancet*, **ii**, 313–315.
- Lewis, T. (1927). *The blood vessels of the human skin and their response*. London: Shaw & Sons.
- Mahler, F., Saner, H., Boss, C. & Annaheim, M. (1987). Local cold exposure test for capillaroscopic examination of patients with Raynaud's Syndrome. *Microvascular Research*, **33**, 422–427.
- McEwan, J. R., Benjamin, N., Larkin, S., Fuller, R. W., Dollery, C. T. & Macintyre, I. (1988). Vasodilatation by calcitonin gene-related peptide and by substance P: a comparison of their effects on resistance and capacitance vessels of human forearms. *Circulation*, **77**, 1072–1080.
- Piotrowski, W. & Foreman, J. C. (1986). Some effects of calcitonin gene-related peptide in human skin and on histamine release. *Br. J. Dermatol.*, **114**, 37–46.
- Salahuddin, M. J., Karanth, S. S., Ghatei, M. A., Springall, D. R., Polak, J. M. & Bloom, S. R. (1988). Distribution of three sensory neuropeptides in human skin. *Regulatory Peptides*, **22**, 428.
- Shawket, S., Dickerson, C., Hazleman, B. & Brown,

- M. J. (1989). Selective suprasensitivity to calcitonin gene-related peptide in the hands in Raynaud's patients. *Lancet*, *ii*, 1354–1357.
- Thom, S., Hughes, A. D., Goldberg, P., Martin, G., Schachter, M. & Sever, P. S. (1987). The actions of calcitonin gene related peptide and vasoactive intestinal peptide as vasodilators in man *in vivo* and *in vitro*. *Br. J. clin. Pharmac.*, **24**, 139–144.
- Weihe, E. & Hartschuh, W. (1988). Multiple peptides in cutaneous nerves: regulators under physiological conditions and a pathogenic role in skin disease. *Seminars in Dermatology*, **7**, 284–300.

(Received 13 February 1990,
accepted 3 August 1990)