Investigations into the mechanism of vasoconstrictor action of the topical steroid betamethasone-17-valerate in the rat

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- 1 The effect of topical betamethasone upon skin blood flow was investigated in the rat. Two types of vasodilator stimuli were used; local heating to the surface of the skin and intradermal application of inflammatory agents. Blood flow was measured by laser doppler velocimetry.
- 2 Topical betamethasone-17-valerate (1 g with an 18 h pretreatment) significantly inhibited the heat-induced vasodilatation in the rat skin, as also did systemically administered betamethasone (1 mg kg⁻¹, 3 h pretreatment).
- 3 Angiotensin converting enzyme (ACE) inhibitors (captopril, 5 mg kg⁻¹ and enalapril, 1 mg kg⁻¹, 30 min pretreatments) were the only drugs out of several different types of systemically administered inhibitors and antagonists that were tested which also inhibited the heat-induced vasodilatation. Aprotinin (100,000 KIU kg⁻¹, 5 min pretreatment) a serine protease inhibitor, significantly potentiated the heat-induced response.
- 4 Bradykinin (50 nmol per site), des-Arg⁹-bradykinin (5 nmol per site), substance P (0.1 nmol per site) and capsaicin (1 µmol per site) induced an increase in skin blood flow.
- 5 Topical betamethasone treatment resulted in a significant inhibition of the vasodilator response to des-Arg⁹-bradykinin, whereas captopril treatment inhibited the responses to substance P, capsaicin, bradykinin and des-Arg⁹-bradykinin.
- 6 Intradermal application of captopril (10–100 µg) also caused a dose-dependent inhibition of the heat-induced vasodilatation.
- 7 These results suggest that topical betamethasone may be acting in a manner similar to that of the ACE inhibitors to produce an inhibition of the flow responses in the skin and that this effect may be brought about by interfering with the action of vasodilator peptide(s) or protein(s).

Keywords: Topical steroids; vasoconstriction; skin blood flow; ACE inhibitors

Introduction

Topical steroids have been used in the treatment of skin disease for many years. Their effectiveness and the availability of efficient assay systems have aided their ever-increasing popularity. Although these drugs are used extensively in the treatment of skin disease their mechanism of action remains unknown.

One of the properties of the topical steroids is their ability to induce a blanching of the skin after topical application, otherwise described as vasoconstriction. In fact, it is upon this property that the most commonly used assay system for determination of anti-inflammatory potency is based (McKenzie & Stoughton, 1962). Although this blanching is used as a marker of anti-inflammatory potency, no direct link between these properties has ever been made. It is not known exactly how this blanching is brought about or what role, if any, it may have to play in providing the anti-inflammatory effects of the topical steroids.

Vasodilatation is one of the primary features of inflammation and its importance was outlined by Williams & Peck in 1977, when describing the two-mediator hypothesis. This theory indicates that oedema is dependent upon two factors, firstly the presence of a vasodilator and secondly an agent which includes venular leakage, both of which are normally present at a site of inflammation. They also stated that these two factors when present together will synergize to produce a greater oedema than when either is given alone. Thus, it seems correct to assume that vasodilatation is an important factor in the development and presentation of an inflammatory response.

As indicated earlier, topical steroids will cause a vasoconstriction of the skin as shown by blanching, although whether this occurs through the induction of a vasoconstrictor substance or through the inhibition of formation or enhanced breakdown of a vasodilator(s) is unknown. It seems plausible that the vasoconstrictor property of the steroids contributes to the anti-inflammatory behaviour of these drugs, since vasodilatation plays such an active role in the development of an inflammation. If we can gain an understanding of exactly how this vasoconstriction is produced then its relevance to the anti-inflammatory effects of the topical steroids may be elucidated.

Laser Doppler velocimetry (LDV) is one of the techniques widely employed to measure both basal and stimulus-induced changes in cutaneous blood flow (Bircher *et al.*, 1990). In 1983, Amantea *et al.* used the LDV technique to measure the effects of steroids upon human skin blood flow. They compared basal flow to flow after treatment with the steroid, fluocinonide, and found no measurable decrease in flow although blanching was visible to the naked eye. Therefore they went on to investigate the effect of topical steroid treatment upon topical administration of aqueous methyl nicotinate, a powerful vasodilator. Here again topical steroid treatment had no effect. This group concluded that laser Doppler velocimetry did not provide a clear-cut system with which the vasoconstrictor effects of the topical steroids could be investigated. However, we have further developed and adapted this technique to measure rat skin basal blood flow and stimulus-induced increases in blood flow to provide a system with which the effects of topical steroid upon skin blood flow may be investigated. Preliminary accounts of these results have been presented to the British Pharmacological Society (Ahluwalia & Flower, 1991; 1992).

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Methods

Male Wistar rats (250–350 g) were anaesthetized with a mixture of hypnorm and hypnovel in sterile water in a ratio of 1:1:2, 4 ml kg⁻¹, their backs shaved and a depilatory cream applied to remove all remaining hair. Rats were left for a minimum of 1 h, during which time recovery from anaesthesia was allowed, before any experimental procedure was carried out. Rats were anaesthetized and skin blood flow measured on the back by the LDV technique (Perimed, Periflux PF3). The effect of topical and systemic steroid and a variety of enzyme inhibitors and receptor antagonists upon two different types of stimuli were investigated: (i) heat-induced changes in blood flow and (ii), chemical vasodilator-induced changes in blood flow.

Local heating in rat skin

Prepared rats were anaesthetized and basal blood flow measured by LDV at a marked site. Heat, 44°C, was applied for 20 min to this site after which blood flow was again measured. The heat was supplied by a thermostatically controlled heated disc which was coupled to the LDV. This measurement was repeated in duplicate at sites of measured distances apart upon opposite sides on the back of the rat, approximately 5 cm from the base of the tail. To investigate the effect of steroid upon this heat response, 1 g betamethasone-17-valerate was rubbed into one side of the back of the rat for approximately 2–3 min. After 18 h the effect of heat upon skin blood flow on the steroid-treated site was again measured and compared to the response observed on the untreated side. Vehicle controls were also carried out. The effects upon heat-induced vasodilatation of systemically administered betamethasone (0.1–1 mg kg⁻¹, s.c.) with 1 and 3 h pretreatment periods and a variety of enzyme inhibitors and receptor antagonists, with varying pretreatment periods, were also investigated. Saline controls were performed. All doses of blocking agents used in these experiments have previously been shown to be effective in several studies.

Intradermal vasodilators in rat skin

Prepared rats were anaesthetized and basal blood flow measured at a marked site. Once a stable basal flow had been obtained, vasodilators at different concentrations were injected intradermally (50 µl) 2–3 mm from the site at which flow was being measured and the vasodilator effect observed. The following agents were studied: bradykinin (BK) 50.0 nmol per site, des-Arg⁹-BK (5.0 nmol per site), substance P (SP) (0.1 nmol per site) and capsaicin (1 µmol per site) and saline control. There were 6 available sites on the back of the rat, 3 on each side. For the series of experiments investigating the effect of topical betamethasone-17-valerate upon the dilator effects of the aforementioned agonists, 3 of the stimuli could be tested on any one rat. Control responses were measured on both sides prior to topical betamethasone application. After 18 h the responses to the vasodilators on the treated side were compared to the responses to these agonists on the untreated side of the rat back.

We then investigated the effect of the angiotensin converting enzyme (ACE) inhibitor, captopril (5 mg kg⁻¹, i.v. with a 30 min pretreatment) upon the dilator responses to each of the intradermal stimuli. Control responses were carried out on one side of the back of the rat, captopril administered and then the responses repeated on the opposite side of the rat back.

Intradermal captopril in rat skin

Prepared rats were anaesthetized and basal blood flow measured at a marked site. The heat stimulus was then applied and the peak flow response measured. This was then repeated on the contralateral side of the back of the rat. Once both

controls had been established captopril (10–100 µg) was injected, in 50 µl of saline, intradermally approximately 2–3 mm from the site at which flow was measured. Basal flow was measured for the following 5 min, after which the heat stimulus was reapplied and the response measured. This was repeated on the opposite side, but with an intradermal injection of saline alone.

Materials

Betamethasone sodium phosphate was produced by Glaxo. Topical betamethasone-17-valerate (Betnovate) and vehicle cream were generous gifts from Glaxo, UK. Indomethacin, atropine, pentolinium, captopril, enalapril, aprotinin, bradykinin, des-Arg⁹-bradykinin, substance P and capsaicin were purchased from Sigma, UK. Methysergide maleate was purchased from Sandoz, UK, mepyramine maleate from May and Baker Ltd, UK.

All peptides were dissolved in sterile water and kept at –20°C until used. Indomethacin was dissolved in 1% sodium bicarbonate in saline. Methysergide, mepyramine, captopril, enalapril, aprotinin, atropine, pentolinium, N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) were all dissolved in saline and made up freshly each day.

Data and statistical analysis

All the results are expressed as mean ± s.e.mean with *n* representing the number of animals. Flow is measured by LDV in an arbitrary unit called the Perfusion Unit (PU), where:

Blood flow (PU) =
no. of red blood cells × speed at which the cells are moving

The response to heat or intradermal vasodilator was measured as the maximal change in flow from basal flow and this response after drug treatment was expressed as a percentage of the response before drug treatment. Student's paired and un-paired *t* tests were used to determine the levels of significance with a **P* < 0.05 being considered as significant.

Results

Local heating

Basal blood flow in the rat skin measured 19.7 ± 1.03 PU (*n* = 32). Local heating of rat skin gave a peak flow of 60.5 ± 5.2 PU (*n* = 32), therefore causing approximately a 40 PU increase in blood flow. Topical treatment of the skin with betamethasone-17-valerate significantly attenuated the responses to the heat stimulus, whereas the vehicle cream had no significant inhibitory effect (Figure 1). Systemic betamethasone at both 0.1 mg kg⁻¹ with a 3 h pretreatment and 1 mg kg⁻¹ with a 1 h pretreatment had no significant inhibitory effects upon the heat-induced vasodilatation, the response after steroid treatment being 117.9 ± 35.9%, *n* = 3, and 95.1 ± 15.5%, *n* = 5, of the response before treatment, respectively. However a 3 h pretreatment of betamethasone of 1 mg kg⁻¹ inhibited the dilator response to the heat stimulus, the response being 44.9 ± 17.6%, *n* = 4 of the response before betamethasone. This was significant at the 5% level when compared to the response after a 3 h pretreatment with saline (114.3 ± 7.6%, *n* = 3).

Table 1 shows the effects of a range of antagonists and enzyme inhibitors upon the heat-induced vasodilatation. Only the ACE inhibitors, captopril and enalapril, significantly inhibited the heat-induced increase in flow and aprotinin, a serine protease inhibitor, significantly potentiated the heat response.

Intradermal vasodilatation

All of the dilators used caused increases in flow within the first 5 min after injection. The changes in flow induced by each of the stimuli were approximately the same and the particular doses used here were chosen from dose-response curves to each of the stimuli. The change in flow in response to BK (50 nmol) was 24.3 ± 3.3 PU ($n = 6$), des-Arg⁹-BK (5 nmol) 18.7 ± 4.6 PU ($n = 4$), SP (0.1 nmol) 19.5 ± 2.2 PU ($n = 6$) and capsaicin (1 μ mol) 23.6 ± 6.2 PU ($n = 8$). We found that after topical betamethasone treatment only the dilator response to des-Arg⁹-BK was significantly inhibited at the 5% level by the paired Student's *t* test as compared to the response on the untreated side, see Figure 2. An interesting observation was that although the response to capsaicin after steroid treatment remained unaffected, the response on the untreated side was reduced, although the two were not significantly different.

Unlike topical steroid treatment, systemic administration of captopril resulted in significant inhibition of the flow

Figure 1 Inhibition of the response to heat-induced vasodilatation by topical application of betamethasone-17-valerate cream ($n = 7$) or vehicle cream ($n = 3$) (1 g rubbed in to one side of the back of the rat), with an 18 h pretreatment represented by the hatched columns with the response on the untreated control side represented by the solid columns. The results are expressed as mean (the response after drug treatment is expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (comparison to untreated side of rat skin) is represented by * $P < 0.05$ (Student's paired *t* test). The control changes in blood flow in response to heat were 44.1 ± 9.7 PU and 40.1 ± 9.5 PU on the steroid-treated and untreated sides respectively and 33.2 ± 14.5 PU on the vehicle-treated and 32.8 ± 11.6 PU on the untreated side prior to topical treatment measured by laser Doppler velocimetry.

Figure 2 Effect of topical application of betamethasone-17-valerate upon the response to bradykinin (BK, 50 nmol per site, $n = 8$), des-Arg⁹-bradykinin (DA⁹BK, 5 nmol per site, $n = 6$), capsaicin (Cap, 1 μ mol per site, $n = 8$) and substance P (SP, 0.1 nmol per site, $n = 6$) shown by the hatched columns. All of the doses of vasodilators used gave similar control peak changes in blood flow of 18.7–24.3 PU, as measured by laser Doppler velocimetry. Values are means (response after drug treatment is expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (compared to responses upon untreated side of rat skin shown by the filled columns) represented by * $P < 0.05$ (Student's paired *t* test).

responses to all the intradermal stimuli (see Figure 3). Systemically administered saline had no effect upon the dilator responses.

Intradermal captopril

Captopril (10–100 μ g) injected intradermally caused a dose-dependent inhibition of the heat-induced vasodilatation as shown in Figure 4. Captopril alone had no effect upon basal blood flow in the skin when injected intradermally as was the case with systemic captopril administration.

Discussion

The aim of this study was to investigate the mechanisms involved in topical steroid-induced blanching or vasoconstriction. It is well known that steroid when applied topically will induce a blanching of the skin after several hours (McKenzie & Stoughton, 1962). In accordance with others (Amantea *et al.*, 1983) no measurable change in basal skin blood flow

Table 1 Effect of a range of different drug types upon the increase in blood flow shown in response to local heating (44°C) of the rat skin

Drug treatment	% of control heat-induced change in blood flow	
Saline (i.v. 30 min)	99.6 $\pm$ 6.4	($n = 4$)
Indomethacin (5 mg kg ⁻¹ , i.v. 30 min)	104.2 $\pm$ 21.4	($n = 5$)
Atropine (1 mg kg ⁻¹ , i.v. 30 min)	90.7 $\pm$ 21.4	($n = 3$)
L-NMMA (100 mg kg ⁻¹ , i.v. 30 min)	107.8 $\pm$ 13.3	($n = 5$)
L-NAME (10 mg kg ⁻¹ , i.v. 15 min)	136.8 $\pm$ 22.1	($n = 5$)
Methysergide (6 mg kg ⁻¹ , i.p. 15 min)	159.8 $\pm$ 26.1	($n = 4$)
Mepyramine (6 mg kg ⁻¹ , i.p. 15 min)	98.5 $\pm$ 15.3	($n = 4$)
Pentolinium (5 mg kg ⁻¹ , i.v. 30 min)	84.7 $\pm$ 18.9	($n = 7$)
Captopril (5 mg kg ⁻¹ , i.v. 30 min)	32.3 $\pm$ 8.6***	($n = 8$)
Enalapril (1 mg kg ⁻¹ , i.v. 30 min)	24.4 $\pm$ 5.3***	($n = 5$)
Aprotinin (100,000 KIU kg ⁻¹ , i.v. 5 min)	240.5 $\pm$ 51.5*	($n = 5$)

Results are expressed as mean (the response after drug treatment being expressed as a % of the response before drug treatment) $\pm$ s.e.mean.

n = number of animals.

Statistical significance (Student's *t* test): * $P < 0.05$; *** $P < 0.001$, compared to saline control.

Figure 3 Inhibitory effects of systemic captopril (5 mg kg^{-1} , 30 min pretreatment) with saline controls upon the dilator responses to bradykinin (BK, 50 nmol per site) ($n = 7$, i.v. saline $n = 7$), des-Arg⁹-bradykinin (DA⁹BK, 5 nmol per site) ($n = 7$, i.v. saline $n = 5$), substance P (SP, $0.1 \text{ nmol per site}$) ($n = 9$, saline i.v. $n = 8$) and capsaicin (Cap $1 \mu\text{mol per site}$) ($n = 3$, saline i.v. $n = 3$) in the rat skin. Values are means (response after drug treatment is expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (comparison to the responses in saline-treated animals shown by solid columns) represented by ** $P < 0.01$ and *** $P < 0.001$.

Figure 4 Inhibitory effects of intradermally administered captopril of doses of 10, 30 and $100 \mu\text{g}$ per site ($n = 5$ animals in each case) upon local heat-induced (44°C) vasodilatation in the rat skin. Values are means (responses after drug treatment are expressed as a % of the response before drug treatment) with s.e.mean shown by vertical bars and statistical significance (comparison to saline control, $n = 15$ animals) represented by * $P < 0.05$ (Student's unpaired t test).

after steroid treatment was found. We next investigated the effect of steroid application upon a stimulus-induced increase in flow. The stimulus chosen was one of local skin heating. The reasons for this choice were manifold. Firstly, heat at 44°C provided a non-pathological response causing no injury, the threshold for thermal injury being $45\text{--}46^\circ\text{C}$ (Rocha e Silva, 1964; Sevt, 1964; Saria, 1984). Also the response to this heat stimulus is both reproducible and completely reversible by cooling. Thirdly, the technique is non-invasive, an ideal setting for investigating skin blood flow. Finally, a mild heat stimulus of vasodilatation seemed an appropriate stimulus to use as it resembled that seen in an inflammatory response, where the vasodilatation is insufficient to produce oedema unless there is accompanying permeability changes. Topical steroid administration will reduce the flow response after induced reactive hyperaemia (Bisgaard *et al.*, 1986), however, this does not provide a system in which extrapolation to the inflammatory situation can be made, as is we believe, the case in our model.

The topical betamethasone treatment used for these studies has been shown to be sufficient to inhibit oedema formation by a range of different inflammatory mediators in the rat (Ahluwalia & Flower, 1992) and is comparable to the doses used in the vasoconstriction assay in man of Marks & Sawyer (1986). We found that although basal flow in the rat skin appeared to be unaffected by topical betamethasone treatment, the increase in flow produced in response to heat was significantly attenuated. This suggests that the vasoconstrictor effects of the steroid, in this case, are probably not brought about through the production of a vasoconstrictor substance but, rather, that the steroid is affecting the processing of a substance(s) responsible for the vasodilator response to heat and consequently only becomes apparent when applying the stimulus. It seems also that this inhibition of induced flow is a general feature of steroids, as systemic administration of steroid also inhibited the response to heat.

Table 1 shows the effects of a variety of enzyme inhibitors and receptor antagonists upon basal flow and heat-induced vasodilatation. The reason for looking at the effects of these antagonists and inhibitors was to find an agent which produced a similar pattern of inhibition of the increase in flow as the topical steroid. This could provide some indication of the mediator involved in the heat response and the system in which the steroids are exerting their inhibitory effects. Many of the mediators known to play various roles in the inflammatory response are not involved in this system; from the data we can exclude the prostanoids, nitric oxide (endothelium-derived relaxing factor), the cholinergic system, histamine and 5-hydroxytryptamine (5-HT). The ACE inhibitors were the only drugs that inhibited the heat response. The classical action of these inhibitors is to inhibit ACE resulting in a decrease in the conversion of angiotensin I to the active mediator of the renin-angiotensin-system, the vasoconstrictor, angiotensin II. Also ACE inhibition will induce an increase in the presence of the vasodilator bradykinin, by inhibiting its breakdown. These actions however do not explain the effects seen in this model and, in fact, are exactly the contrary to that observed i.e. the resultant effect of ACE inhibition would normally itself be vasodilatation whereas here it appears that ACE inhibitors result in an inhibition of vasodilatation. These findings suggest that the ACE inhibitors are acting on a peptide(s) or protein(s) other than BK or angiotensin II. Indeed these inhibitors can influence the activity of other proteolytic enzymes such as tripeptide aminopeptidases (Sach & Marks, 1982). The only other agent that we tested that had any significant effect upon the test response was aprotinin, the serine protease inhibitor. Aprotinin caused a significant potentiation of heat-induced vasodilatation. This when combined with the ACE inhibitor effects suggest that the heat-induced increase in flow may be brought about by a released peptide or protein, the processing of which may be altered by both serine protease and ACE inhibitors.

In situations of thermal injury it has been shown that a variety of vasodilators are released which contribute to the pathology of the injury. These include bradykinin (Rocha e Silva & Antonio, 1960; Rocha e Silva & Rosenthal, 1961) and substance P (Saria, 1984). Although our model is not one of thermal injury it is conceivable that these mediators may also be involved in this heat response. Thus, we investigated the effect of steroid and captopril treatment upon the vasodilator effects of both BK, SP, des-arg⁹-BK and capsaicin. Des-Arg⁹-BK is an active metabolite of BK, which is active at B₁ receptors. B₁ receptors have been found to be present only in situations of stress such as shock or inflammation. Capsaicin produces a vasodilatation by inducing the release of the contents of the sensory neurone, one of which is SP.

We found that topical steroid inhibited only the response to des-Arg⁹-BK; however, systemic captopril significantly inhibited the vasodilator responses to all of the stimuli, suggesting some non-specificity in the inhibitory effects of this

drug. B₁ receptors as indicated earlier are not normally constitutively present but are induced in the presence of inflammatory stimuli by a process which depends upon new protein synthesis (Bouthillier *et al.*, 1987). It has been shown that the induction of B₁ receptors, by various cytokines, in the rabbit isolated aorta is prevented by exposure to the steroid, dexamethasone (Deblois *et al.*, 1988). Possibly the topical steroid, here, is acting at the level of the B₁ receptor to produce its inhibitory effect upon the response to des-Arg⁹-BK. Captopril introduced intradermally at the site of the heat stimulus also inhibited the response, while saline administration on the contralateral side had no effect, implying that there was no systemic component to the observed captopril effects here. This indicates that the inhibitory effect of captopril in this system is not merely an artifact due to

peripheral vasodilatation and hence recruitment of flow away from the skin, but rather a local action within the skin microcirculation.

Thus it appears that the heat-induced vasodilatation may be brought about by the activation of a peptide(s) or protein(s), the processing of which can be manipulated by both ACE inhibitors and serine protease inhibition. Since, both the ACE inhibitors and topical betamethasone appear to have similar resultant effects, it is conceivable that the skin blood flow effects of topical betamethasone are also brought about via the processing of this same unknown substance.

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