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# Evidence for $ET_A$ and $ET_B$ receptors in rat skin and an investigation of their function in the cutaneous microvasculature

E. Lawrence, L. Siney, P. Wilsoncroft, *G.A. Knock, [1]G. Terenghi, *J.M. Polak & [2]S.D. Brain

Pharmacology Group, Biomedical Sciences Division, King's College, Manresa Road, London, SW3 6LX and *Histochemistry Department, RPMS Hammersmith Hospital, DuCane Road, London W12 0NN

**1** The relative contribution of $ET_A$ and $ET_B$ receptors in the response of rat skin to endothelins was investigated by use of the selective $ET_B$ agonist IRL-1620 and the selective $ET_A$ antagonist BQ-123.

**2** Binding data suggest the presence of $ET_A$ and $ET_B$ receptors as preincubation with [$Ala^{3,11,18}Nle^7$]-endothelin-1 reduced ET-1 binding by approximately 40%.

**3** Intradermal injection of endothelin-1 (ET-1, 1-10 pmol/site) and ET-3 (3-100 pmol/site) induced a dose-dependent decrease in local blood flow assessed by $^{133}Xe$ clearance at test sites in rat skin.

**4** The endothelin analogue [$Ala^{3,11,18}Nle^7$]-ET-1 (30-1000 pmol/site) induced significant vasoconstriction ($P<0.05$) at the highest doses used and the selective $ET_B$ receptor agonist, IRL-1620 [Suc-[$Glu^9$,$Ala^{11,15}$] endothelin (8-21)], (0.01-100 pmol/site) acted in a potent manner to induce a significant ($P<0.01$) dose-dependent decrease in $^{133}Xe$ clearance.

**5** Co-injection with the selective $ET_A$ receptor antagonist, BQ-123 (1 nmol/site), completely abolished the vasoconstriction to ET-1 and partially to ET-3, but had no effect on IRL-1620-induced vasoconstriction. In addition, IRL-1620 responses were not altered at sites treated with submaximal doses of a nitric oxide synthase inhibitor or a prostaglandin synthase inhibitor.

**6** ET-1 and IRL-1620 (100 fmol-1 pmol/site) did not induce oedema formation as measured by [$^{125}I$]-albumin accumulation in the presence or absence of the vasodilator, calcitonin gene-related peptide (CGRP). ET-1 (1-3 pmol/site) inhibited substance P-induced oedema formation and this effect, suggested to be secondary to a vasoconstrictor effect, was significantly reversed by BQ-123 (1 nmol/site).

**7** The findings in this study indicate that there are $ET_A$ and $ET_B$ receptors in rat skin and agents which activate either receptor act to mediate a decrease in cutaneous blood flow, but have no effect on increased microvascular permeability.

**Keywords:** $ET_A$ receptor; $ET_B$ receptor; endothelin; cutaneous microvasculature; blood flow; IRL-1620

## Introduction

The endothelin isopeptides have numerous pharmacological activities and may be important in the regulation of a variety of biological functions, including vascular resistance, gene expression, and cell growth (see Miller *et al.*, 1993). The cDNA encoding at least two distinct endothelin receptor subtypes has been cloned (Arai *et al.*, 1990; Sakurai *et al.*, 1990). The $ET_A$ receptor has binding affinity: ET-1 > ET-3 (Arai *et al.*, 1990) while the $ET_B$ receptor is non-isopeptide-selective (Sakurai *et al.*, 1990).

The recent development and availability of specific agonists and antagonists has allowed the functions of the $ET_A$ and $ET_B$ receptors to be described. The $ET_A$ receptor is known to be responsible for vasoconstriction, while the $ET_B$ receptor on endothelial cells mediates endothelium-dependent vasodilatation (Randall *et al.*, 1989; Takayanagi *et al.*, 1991). More recently an $ET_B$-like receptor present on some smooth muscle cells has been shown to mediate vasoconstriction *in vitro* (Harrison *et al.*, 1992; Moreland *et al.*, 1992; see Davenport & Maguire, 1994) in addition to evidence that $ET_B$ receptors are implicated in pressor responses in pithed rats (Williams *et al.*, 1991). BQ-123 (*cyclo*[D-Asp-Pro-D-Val-Leu-D-Trp]) is a selective $ET_A$ receptor antagonist (Ihara *et al.*, 1992), [$Ala^{3,11,18}Nle^7$]-ET-1 is a selective $ET_B$ receptor ligand (Hunt *et al.*, 1991) and IRL-1620 (Suc-[$Glu^9$, $Ala^{11,15}$] endothelin (8-21)) is a selective agonist at the $ET_B$ receptor in porcine lung membrane and guinea-pig trachea (Takai *et al.*, 1992) and in the rat aorta (Karaki *et al.*, 1993). We have previously shown that local treatment of rat skin with ET-1 and ET-3 induces vasoconstriction (Lawrence & Brain, 1992), which can be antagonized by BQ-123 and the non-peptide mixed $ET_A/ET_B$ antagonist, bosentan (Lawrence & Brain, 1994).

In the present study selective ligands, BQ-123, [$Ala^{3,11,18}NLe^7$]-ET-1 and IRL-1620 have been used to learn more about the receptors present in rat skin and to determine their roles in mediating blood flow and permeability changes in the rat cutaneous microvasculature.

## Methods

In the binding experiments two dorsal skin samples were taken from each of three rats. Following removal of excess hair with a razor blade, skin samples were placed on cork mats, embedded in mounting medium (Tissue-Tek, Miles Inc, U.S.A.) and frozen unfixed in melting dichlorodifluoromethane (Arcton 12, ICI Runcorn, U.K.) suspended over liquid nitrogen. All six samples were used for this study, and ligand binding experiments were carried out according to a protocol previously used for receptor studies in skin (Knock *et al.*, 1993). The presence of ET receptor subtypes was investigated by incubating consecutive sections with 200 pM [$^{125}I$]-ET-1 (Amersham International, Amersham) in the presence of increasing concentrations of unlabelled ET-1 and ET-3 ($10^{-11}M-10^{-9}M$), or by incubating sections as normal, following preincubation in the presence of the $ET_B$-specific agonist, [$Ala^{3,11,18}$, $Nle^7$]ET-1 ($10^{-7}M$).

Local clearance of $^{133}Xe$ (Amersham International, Amersham, 0.36 MBq per site) was measured in the dorsal skin of male Wistar rats (180-220 g) following intradermal (i.d.) in-

[1] Present address: Blond McIndoe Centre, Queen Victoria Hospital, East Grinstead, Sussex RH19

[2] Author for correspondence.

**Table 1** Competitive inhibition of [$^{125}$I]-endothelin-1 ([$^{125}$I]ET-1) binding sites in rat skin microvessels by unlabelled ET-1 and ET-3

| *Peptide* | *Specific binding remaining (% of maximum) (n=6 blocks from 3 rats)* |
|---|---|
| *Unlabelled ET-1* | |
| $10^{-7}$M | 2.19 ± 1.05 |
| $10^{-9}$M | 7.84 ± 1.56 |
| $10^{-11}$M | 84.21 ± 9.39 |
| Zero | 100 |
| *Unlabelled ET-3* | |
| $10^{-7}$M | 17.29 ± 1.59 |
| $10^{-9}$M | 65.43 ± 6.77 |
| $10^{-11}$M | 89.41 ± 4.12 |
| Zero | 100 |
| [Ala$^{3,11,18}$Nle$^{7}$]-ET-1 | |
| Preinc $10^{-7}$M | 61.17 ± 5.21 |

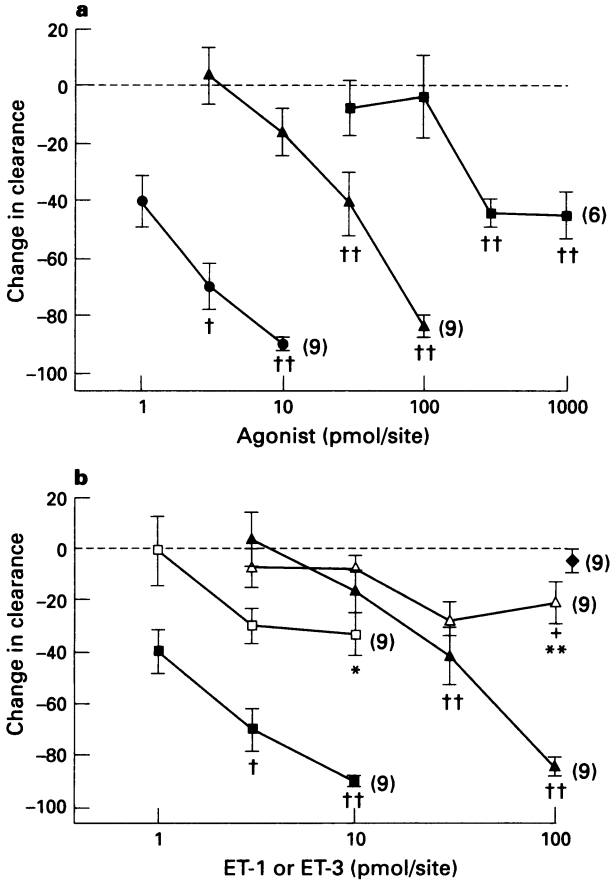


**Figure 1** The effect of endothelin-1 (ET-1, ●), ET-3 (▲) and [Ala$^{3,11,18}$,Nle$^{7}$]-ET-1 (■) on local $^{133}$Xe clearance in rat dorsal skin (a), and the response to ET-1 (■) and ET-3 (▲) in the absence and presence of BQ-123 (1 nmol/site; □, △) is shown in (b). The effect of BQ-123 alone (◆) is also shown. The results are expressed as change in local clearance at test sites compared with at control (Tyrode-injected) sites in the same rat, measured over 15 min. Values are the mean ± s.e.mean for the number of rats shown in parentheses. †$P<0.05$ cf. Tyrode control; ††$P<0.01$ cf. Tyrode control; *$P<0.05$ cf. + BQ-123; **$P<0.01$ cf + BQ-123.

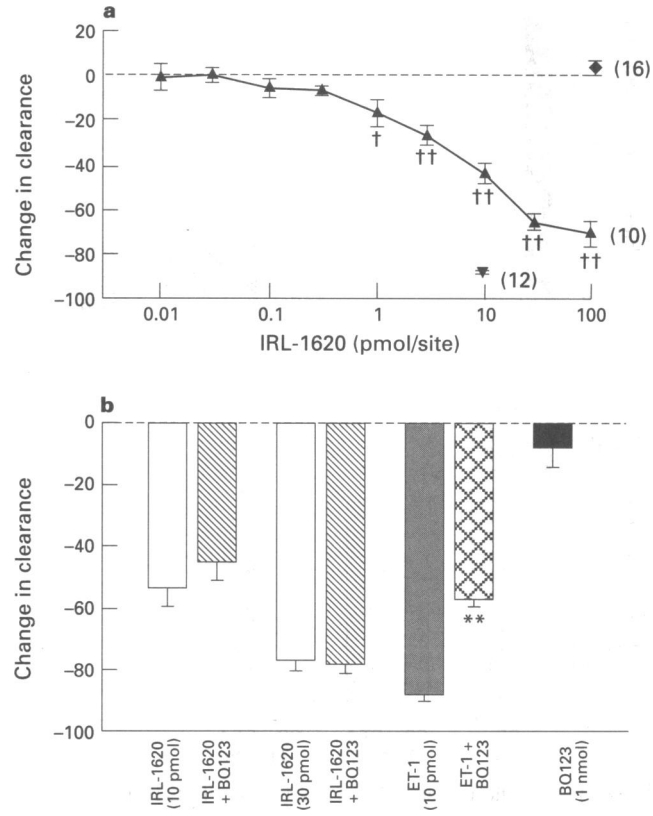


**Figure 2** The effect of IRL-1620 on local $^{133}$Xe clearance in rat dorsal skin in the absence and presence of BQ-123. The effect of IRL-1620 (▲), endothelin-1 (ET-1, 10 pmol, ▼) and vehicle (0.1% ammonium bicarbonate, ◆) is shown in (a). The effect of BQ-123 on ET-1 (10 pmol) and IRL-1620 (10 pmol and 30 pmol) responses is shown in (b). Results are expressed as mean ± s.e.mean for at least 8 experiments. †$P<0.05$ cf. Tyrode; ††$P<0.01$ cf. Tyrode; **$P<0.001$ cf. ET-1 alone.

jection of multiple test agents; this technique has been described in detail (Williams, 1976; Lawrence & Brain, 1992). Briefly, rats were anaesthetized with pentobarbitone (Sagatal, May & Baker, 50 mg kg$^{-1}$ body weight). Each test agent or combination of test agents was made up in Tyrode solution and an equal quantity of $^{133}$Xe was mixed with 1 ml sample of each test agent. Test or control agent was rapidly injected i.d. in duplicate and in random order according to a balanced site pattern and a 100 ml aliquot of each solution was counted to determine the total radioactivity present. After a clearance period of 15 min the rat was killed, skin sites punched out and radioactivity counted. Changes in blood flow were expressed as percentage change in clearance at test sites compared with clearance at control sites injected with Tyrode solution; a decrease in clearance indicates that a decrease in local blood flow due to vasoconstriction has occurred, while an increase in clearance indicates that an increase in local blood flow due to vasodilatation of microvessels has occurred.

In a separate series of experiments the effects of agents on local oedema formation were measured in rat skin as extravasation of intravenously injected $^{125}$I-labelled human serum albumin (Amersham International, Amersham, 2.5 mCi per

**Table 2** The effects of $N^G$-nitro-L-arginine methyl ester (L-NAME) and indomethacin on IRL-1620-induced responses in the rat dorsal skin

| *Agent* | *IRL 1620* (pmol/site) | | | |
|---|---|---|---|---|
| | 0 | 1.0 | 3.0 | 30.0 |
| None | – | −17.0±5.4 (8) | −23.9±4.1 (14) | −67.3±3.3 (12) |
| L-NAME (100 nmol/site) | −10.8±2.8 (12) | −23.7±8.0 (6) | −24.9±3.3 (12) | −60.0±3.7 (12) |
| Indomethacin (3 nmol/site) | −20.0±6.9 (9) | −22.3±3.7 (6) | −26.5±4.7 (6) | −72.7±2.1 (6) |

Results are mean±s.e.mean % decrease in $^{133}Xe$ clearance for the number of experiments shown in parentheses.

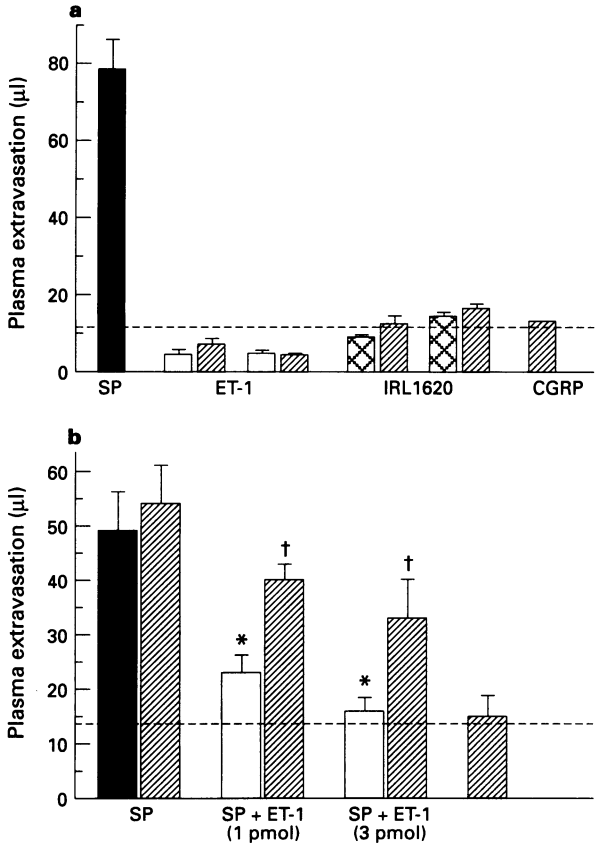


**Figure 3** The effect of endothelin-1 (ET-1) and IRL-1620 on oedema formation in rat skin measured over 30 min as $^{125}I$-labelled human serum albumin accumulation, (a) shows ET-1 (100 fmol/site and 1 pmol/site, open columns) and IRL-1620 (100 fmol/site and 1 pmol/site, cross-hatched columns) alone and in combination with the vasodilator CGRP (10 pmol/site, hatched columns). The effect of substance P alone (100 pmol/site, solid column) is also shown. (b) Shows the effect of BQ-123 on the inhibitory effect of ET-1 on substance P (SP, 100 pmol/site)-induced oedema. Substance P alone is shown by the solid column and substance P plus ET-1 by the open columns. The effect of co-injected BQ-123 is shown by the hatched column. The dashed line represents the level of oedema formation in control sites which received Tyrode alone. Results are expressed as mean±s.e.mean of 6 rats. *$P<0.05$ cf. substance P, †$P<0.05$ cf. substance P plus ET-1.

rat) accumulated at skin sites over 30 min in response to the i.d. agents made up in 100 ml Tyrode solution as previously described (Brain & Williams, 1989). Oedema formation was measured as radioactivity in skin sites and expressed as plasma volume calculated from the counts in 1 ml of plasma.

Test agents used were human ET-1 and human ET-3 obtained from Bachem (U.K.) Ltd. (Essex); [$Ala^{3,11,18}$,$Nle^7$]-ET-1, BQ-123 and IRL-1620 were from Peninsula Laboratories (Lancashire); human α-calcitonin gene-related peptide (CGRP) was a gift from Dr U. Ney, Celltech (Bucks); $N^G$-nitro-L-arginine methyl ester (L-NAME), substance P and indomethacin were from Sigma Chemical Company Ltd. (Dorset).

Results are expressed as mean±s.e.mean. The significant difference between intradermal treatments was assessed with Bonferroni's modified *t* test, which uses the standard error estimate for the analysis of variance to allow comparison of multiple sites. $P<0.05$ was accepted as statistically significant.

## Results

[$^{125}I$]-ET-1 binding sites were localized by autoradiography to microvessels of rat skin in the superficial dermis. Binding was inhibited by both ET-1 and ET-3 in a concentration-dependent manner. Competition studies gave an apparently biphasic curve for ET-3 as competitor, consistent with previous results (Knock *et al.*, 1993), suggesting the presence of both ET-1 selective ($ET_A$) and non-selective ($ET_B$) subtypes. Indeed at the concentration of $10^{-9}$M, ET-1 inhibited binding by >90%, whereas ET-3 inhibited binding by only approximately 40% (Table 1). Similarly, binding was not completely abolished by preincubation with $10^{-7}$M [$Ala^{3,11,18}$,$Nle^7$]-ET-1, but was reduced by approximately 40% (Table 1).

The greater potency of ET-1 as a constrictor in rat skin compared with ET-3 is shown in Figure 1a. [$Ala^{3,11,18}$,$Nle^7$]-ET-1 induced vasoconstriction only at the highest doses used (Figure 1a). BQ-123 (1 nmol/site) had no effect on local $^{133}Xe$ clearance when injected alone (Figure 1b). However, it caused attenuation of the vasoconstriction induced by the highest dose of ET-1 (Figure 1b, $P<0.05$). BQ-123 also significantly decreased the vasoconstriction induced by the highest dose of ET-3 (Figure 1b). IRL-1620 induced a dose-dependent vasoconstriction in rat skin (Figure 2a), BQ-123 (1 nmol/site) did not alter IRL-1620-induced vasoconstriction (10–30 pmol/site, Figure 2b).

The decrease in $^{133}Xe$ clearance induced by the $ET_B$ selective agonist, IRL-1620 (at concentrations between 1–30 pmol/site), was not significantly altered when sites were simultaneously treated with submaximal doses of the NO synthase inhibitor, L-NAME (100 nmol/site; Lawrence & Brain, 1992) or the prostaglandin inhibitor, indomethacin (3 nmol/site; Brain *et al.*, 1985) (Table 2).

ET-1 and IRL-1620 (100 fmol–1 pmol/site) did not induce oedema formation when injected alone or in combination with the vasodilator CGRP (Figure 3a) which was used to act in a functional manner to antagonize the constrictor activity of ET-1 and IRL-1620. Under such conditions, oedema formation may be more easily observed (see Brain *et al.*, 1989). Substance P (100 pmol/site) did cause a significant increase in oedema formation and thus acted as a positive control. In further experiments ET-1 (1 and 3 pmol/site) inhibited substance P-induced oedema formation which was significantly reversed by BQ-123. However, BQ-123 had no effect on substance P-induced oedema formation in the absence of ET-1 and did not itself stimulate oedema (Figure 3b).

## Discussion

The results of this study indicate that the rat dorsal skin has both $ET_A$ and $ET_B$ receptor subtypes, a result consistent with similar results observed in human skin (Knock *et al.*, 1993). The heterogeneity of the receptors was demonstrated by reduced binding with different ET isoforms and by the inhibition of labelled ET-1 binding with an $ET_B$-specific agonist.

There is increasing evidence that the microcirculation of animals is particularly sensitive to the effects of endothelins (Brain *et al.*, 1988; Boric *et al.*, 1990; Lamping *et al.*, 1992). More recently, it has been demonstrated that small arteries and veins in man are also sensitive to endothelins, causing a vasoconstriction that is interestingly not mediated entirely by $ET_A$ receptors (Riezebos *et al.*, 1994). Further, it has been shown that in human skin microcirculation, ET-1 but not ET-3 induced pronounced vasoconstrictor activity at the injection site and neurally-mediated vasodilatation in the surrounding area (Wenzel *et al.*, 1994) suggesting that in this preparation, ET-1 is primarily involved in the regulation of vascular tone by activation of $ET_A$ receptors. There does appear, therefore, to be differences between species and between vascular beds. It is now widely accepted that $ET_B$ receptors are important in some vasoconstrictor responses (see Davenport & Maguire, 1994). In the present study, functional data using the selective $ET_A$ antagonist BQ-123 suggest that ET-1 and ET-3 also act, at least in part, via $ET_A$ receptors to mediate vasoconstriction in the cutaneous microvasculature. The finding that the $ET_B$-selective agonist, IRL-1620, induced a decrease in blood flow which is not affected by BQ-123 provides evidence that $ET_B$ receptors also mediate vasoconstriction in the rat cutaneous microvasculature. The results of the present study are further evidence that $ET_B$ receptors can mediate vasoconstriction *in vivo*.

In addition, the possibility that vasodilator responses mediated via $ET_B$ receptors occur was also studied. It has previously been shown that the vasodilatation induced in the intact preconstricted rat aorta by IRL-1620 was endothelium-dependent and due to release of nitric oxide (Karaki *et al.*, 1993). Despite finding that a basal level of nitric oxide is important for maintaining blood flow in the rat skin microvasculature, we have been unable to demonstrate endothelin-stimulated release of vasodilator quantities of nitric oxide (Lawrence & Brain, 1992). IRL-1620 is a very selective and potent $ET_B$ agonist (Takai *et al.*, 1992) and since there was no effect on blood flow at sites simultaneously treated with L-NAME, compared to those treated with IRL-1620 alone, it would appear that the $ET_B$ receptor in rat skin does not release nitric oxide. Furthermore, it has previously been reported that, in addition to nitric oxide, $ET_B$-mediated vasodilatation may be due to prostacyclin release (Fozard & Part, 1992). The results of the present study, however, are in contrast to this finding and suggest that vasodilator prostaglandins do not contribute to the observed response to IRL-1620 in rat skin. The residual component of the response to ET-3 following treatment with BQ-123 is compatible with the suggestion that further subtypes of endothelin receptors exist which are selective for ET-3 > ET-1 (Harrison *et al.*, 1992). In this context the cloning of a third distinct receptor, $ET_C$, which is selective for ET-3 (Karne *et al.*, 1993) is of relevance.

There is conflicting evidence on the function of endothelin in microvascular permeability and the route of administration appears to be important. ET-1 injected i.v. induces oedema formation in the human forearm (Dahlof *et al.*, 1990) but when injected i.d. reduces oedema formation induced by agents which increase microvascular permeability in rat and rabbit skin (Brain *et al.*, 1989). Further Filep *et al.* (1991, 1993) have shown that endothelin-1 enhances microvascular permeability induced in various vascular beds following systemic treatment of rats and that this is mediated at least in part via the $ET_A$ receptor. These authors have provided evidence that the oedema formation is not merely a secondary effect to changes in systemic blood pressure, but related to local production of platelet activating factor (Filep *et al.*, 1991).

In the present study oedema formation was not observed when ET-1 and IRL-1620 were injected alone, or with a vasodilator dose of CGRP (to counteract vasoconstrictor activity). Thus it would appear that the phenomenon observed by Filep and coworkers after systemic administration of endothelins is not observed after intradermal injection of endothelins in skin. The results from the present study support the concept that ET-1 released in low concentrations in the cutaneous microcirculation is more potent as a vasoconstrictor than as a mediator of microvascular permeability and that the decrease in blood flow can lead to an inhibition of oedema formation as previously reported (Brain *et al.*, 1989).

In conclusion, we present evidence that $ET_B$ receptors, in addition to $ET_A$ receptors, exist in rat skin and our results suggest that $ET_B$ receptors can mediate vasoconstriction but, since IRL-1620 had no effect on blood flow in constricted sites, not vasodilatation in the rat cutaneous microvasculature. In addition our experiments suggest that neither $ET_A$ nor $ET_B$ receptors are involved in mediating increases in microvascular permeability in the rat cutaneous microvasculature.

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