Role and mechanism of endothelin-B receptors in mediating ET-1-induced vasoconstriction in pig skin

CHO Y. PANG,1,2,3 JIANRONG ZHANG,1 HUAI XU,1 JOAN E. LIPA,1,2 CHRISTOPHER R. FORREST,1,2 AND PETER C. NELIGAN1,2

1The Hospital for Sick Children Research Institute and Departments of 2Surgery and 3Physiology, University of Toronto, Toronto, Ontario, Canada M5G 1X8

Pang, Cho Y., Jianrong Zhang, Huai Xu, Joan E. Lipa, Christopher R. Forrest, and Peter C. Neligan. Role and mechanism of endothelin-B receptors in mediating ET-1-induced vasoconstriction in pig skin. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R1066–R1074, 1998.—We investigated the functional importance and signal transduction pathways of endothelin (ET)-B receptors in mediating ET-1-induced vasoconstriction in pig skin. Skin vasoconstriction was studied by monitoring the perfusion pressure of isolated perfused pig skin flaps (6 × 16 cm) at a constant flow rate. Intra-arterial infusion of the ET A receptor agonist ET-1, the ET B receptor agonists sarafotoxin 6C (S6c) and BQ-3020, or the thromboxane A2 mimetic U-46619 (n = 4 or 5) caused a concentration-dependent skin vasoconstriction. The vasoconstrictor potency of ET-1 (EC 50 3.1 × 10−10 M) was lower (P < 0.05) than that of S6c (EC 50 1.8 × 10−9 M) and similar to that of BQ-3020 (EC 50 2.6 × 10−9 M). The vasoconstrictor potency of ET-1, S6c, and BQ-3020 was at least 300-fold higher than that of U-46619 (EC 50 0.9 × 10−6 M). The skin vasoconstrictor effect of ET-1 (10−9–10−8 M) was partially inhibited by 10−5 M BQ-123, an ET A receptor antagonist. Further inhibition was achieved with the combination of 10−5 M BQ-123 and BQ-788 (an ET A receptor antagonist) or with an ET A receptor antagonist (10−5 M bosentan or PD-145065) (n = 5; P < 0.05). In addition, the skin vasoconstrictor effect of the ET B receptor agonist BQ-3020 was completely blocked by 5 × 10−6 M BQ-788 and partially inhibited by 5 × 10−6 M of the phospholipase C (PLC) inhibitor 2-nitro-4-carboxyl-N,N-diphenylcarbamate (NCDC), an L-type Ca2+ channel antagonist (nifedipine), a protein kinase C (PKC) inhibitor (cherythrine), or removal of Ca2+ from the perfusate (n = 4 or 5; P < 0.05). The vasoconstrictor effect of S6c was also partially blocked by 5 × 10−4 M of NCDC, nifedipine, or cherythrine or by removal of Ca2+ from the perfusate (n = 4 or 5; P < 0.01). We conclude that ET B receptors play a central role in mediating ET-1-induced vasoconstriction in pig skin, and the mechanism probably involves L-type Ca2+ channels, PLC, and PKC.

ENDOTHELINS (ET-1, ET-2, ET-3) are a family of structurally related 21-amino acid isopeptides (19). The ETs are also structurally and functionally related to mouse vasoactive intestinal contractor (38), sarafotoxins (5, 23), and ribotoxin (4). Two ET receptor subtypes, termed ET A and ET B, have been cloned, sequenced, and characterized from the bovine and rat lung, respectively (1, 40). ET A receptors are selective for ET-1 and ET-2 over ET-3, and ET B receptors are nonsensitive for the ET isopeptides. Subsequently, the human ET A and ET B receptors have also been cloned (18, 31, 33, 39). More recently, an ET C receptor subtype selective for ET-3 has been cloned from dermal melanophores of the clawed toad Xenopus laevis (22), but a mammalian homologue has not been identified.

The ET A receptor mediates vasoconstriction and is widely localized in vascular smooth muscle cells (1, 28). The ET B receptor is localized in endothelial cells and is associated with vasodilator activity through the release of endothelium-derived relaxing factors, nitric oxide, and/or prostacyclin (12, 40, 42). However, ET B receptors are also present in vascular smooth muscle, mediating a contractile effect (14, 30). Thus it has been suggested that the endothelial ET B receptor, mediating vasodilation, and the smooth muscle cell ET B receptor, mediating vasoconstriction, be subclassified as ET B1 and ET B2 receptors, respectively (14). The tissue ET A and ET B receptor populations and their functional importance seem to be dependent on species and location (8, 11).

Of particular interest to us is the relative importance of ET A and ET B receptors in the mediation of ET-1-induced skin vasoconstriction. It has been demonstrated that ET-1 is a potent and long-acting vasoconstrictor in the skin of the rat, rabbit, pig, and human (6, 9, 26, 35). Autoradiography has demonstrated the presence of ET A and ET B receptors in microvessels of rat and human skin (24, 27). There is general consensus that ET A receptors are involved in skin vasoconstriction by ET-1 in animal and human skin (16, 27, 35, 43), but the role of ET B receptors in the mediation of ET-1-induced vasoconstriction is unclear and the mechanism has not been studied in animal or human skin. We hypothesized that ET B receptors may participate in ET-1-induced skin vasoconstriction in the pig. Therefore, the objectives of this project were to investigate the functional importance of ET B receptors and the postreceptor signal transduction pathways linked to ET B receptors in the mediation of ET-1-induced skin vasoconstriction. The pig isolated perfused skin flap model was chosen for this project because the pig is the only laboratory animal whose skin vasculature closely resembles that of the human (31), and an isolated perfused pig skin flap model has already been established for in vitro study of skin vascular contraction and relaxation and mechanism of action in response to intra-arterial drug infusion (34, 35, 37). With the use of this unique isolated perfused pig skin flap model we have demonstrated that both ET A and ET B receptors are functionally important in the mediation of ET-1-induced skin vasoconstriction. In addition, we have demonstrated for the first time that L-type Ca2+ channels, phospholipase C (PLC), and protein kinase C (PKC) are involved in ET B receptor-mediated skin vasoconstriction.

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MATERIALS AND METHODS

Surgical Procedures

Castrated pigs (20.3 ± 1.7 kg; mean ± SD) were used. Skin flaps were harvested under general anesthesia induced by intramuscular ketamine (25 mg/kg) and intravenous pentobarbital sodium (20–25 mg/kg). General anesthesia was maintained by intravenous infusion of isotonic saline (2 ml/min) containing pentobarbital sodium (0.5 mg/kg). A 6 × 16-cm skin flap based on the deep circumflex iliac neurovascular bundle was outlined on both sides of the buttock. The marked skin flaps were incised and completely undermined with all musculocutaneous blood vessels (perforators) tied and/or cauterized carefully. The circumflex iliac neurovascular bundle was dissected and formed the vascular pedicle (4–5 cm) of the island buttock skin flap. All side branches of blood vessels of the pedicle were ligated with 3-0 silk sutures and cauterized. Finally, the proximal end of the neurovascular pedicle of the flap was tied with a 2-0 silk suture and then transected. The arterIALIZED island buttock skin flap was freed and used for in vitro perfusion. The pig was killed with an overdose of intravenous pentobarbital sodium (100 mg/kg). This animal protocol was approved by The Hospital for Sick Children Animal Care Committee.

Skin Flap Preparation for In Vitro Perfusion

The skin flap was wrapped around a plastic tube (22 cm in length and 1.2 cm in diameter), and the longitudinal edges of the flap were sewn together with 3-0 silk sutures to form a tubed flap. From our previous experience, inclusion of this tube in the tubed skin flap significantly reduced edema formation during 4–5 h of in vitro perfusion and water retention was reduced to <10%. The circumflex iliac artery and one of its veins were cannulated with a 20- and 18-gauge angiocatheter, respectively, for skin flap perfusion.

Skin Flap Perfusion Technique

Modified Krebs-Henseleit buffer of the following composition (mM) was used for perfusate: 100 NaCl, 4.60 KCl, 1.10 NaH2PO4, 1.20 MgSO4, 2.25 CaCl2, 30 NaHCO3, 11 glucose, and 2 d-mannitol. Bovine serum albumin (Cohn fraction V) was added to the buffer (65 g/l), which was stirred and filtered (Whatman 44) before use. Ca2+-free buffer contained 2 mM EGTA.

The commercially available Two Ten Perfuser (MX International, Aurora, CO) equipped with two reservoirs and a pump with adjustable rates (model 7014, Cole Palmer Instrument, Niles, IL) was used as a perfusion apparatus. The perfusate was equilibrated in the reservoirs with 95% O2-5% CO2 at 38°C and pH 7.35–7.40. The body temperature of young pigs was equilibrated in the reservoirs with 95% O2-5% CO2 at 38–40 mmHg. Drugs to be tested were infused into the perfusate through a sidearm shortly before the perfusate entered the arterial angiocatheter of the skin flap. A thermistor probe (YSI series 400, Yellow Springs Instrument, Yellow Springs, OH) connected to a microcomputer thermometer (series 084 202, Cole Parmer Instrument) was positioned on the surface of the longitudinal midpoint of the skin flap for continuous monitoring of surface skin temperature, which was kept at ~34°C.

A baseline perfusion pressure of 38–40 mmHg was selected because our past experiments revealed that the pig skin flap was well perfused and oxygenated, with less than 10% water retention (edema formation) and 2–3 mmHg increase in baseline perfusion pressure over a period of 3–4 h of perfusion (34, 35). The basal perfusion pressure used by other investigators for perfusion of rabbit ears was 35.8 ± 3.5 mmHg (36).

From our past experience, we also noticed that the weights of the 6 × 16-cm buttock skin flaps in pigs weighing 17–22 kg were quite uniform (51 ± 3 g). A pump rate of ~2.0 ml/min would produce a baseline perfusion pressure of 38–42 mmHg.

In all the studies reported here, a 45-min stabilization period was allowed to establish a steady baseline perfusion pressure at a constant flow rate. Unless otherwise stated, drugs used as inhibitors or antagonists were infused continuously, starting 45 min before infusion of an agonist.

Chemicals

Unless otherwise stated, reagents and drugs were purchased from Sigma Chemical (St. Louis, MO), Porcine BQ-123 [cyclo(-Val-Leu-Asp-Asp-Pro)], BQ-3020 (N-acetyl-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Lle-Trp-Thr-Asp-Glu-Cys-Leu-Asn-Phe-His-Glu-Asp-Lle-Trp-OH), and sarafotoxin 6c (56C; H,N-Cys-Thr-Cys-Asn-Asp-Met-Thr-Asp-Glu-Cys-Leu-Asn-Phe-Cys-His-Glu-Asp-Lle-Trp-OH) were purchased from Bachem California (Torrance, CA). BQ-788 (N-cis,2,6-dimethylpipеридинокарбонил-л-γ-метил-Leu-d-1-метоксикarbонyl-Trp-d-Nle) and U-46619 (9,11-dideoxy-9-α,11α-methano-epoxy prostaglandin F2α) were purchased from Peptides International (Louisville, KY) and Clayman Chemicals (Ann Arbor, MI), respectively.

The following drugs were kindly donated to us: bosentan [4-tert-butyl-N-(6-(2-hydroxy-ethyl)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidine-4-yl)-benzene-sulfonamide] (Dr. M. Clozel, Hoffmann-La Roche and PD-145065 [Ac-cycloheptene-10,11-di-hydroglycine=Leu=Asp-Lle-Lle-Trp] (Dr. A. Doherty, Parke-Davis).

Purified water (Milli-Q Water System) was used for making solutions and perfusion buffer. ET-1 stock solution (10−4 M) was made with 0.1% acetic acid and stored at ~80°C until use. S6c and chelerythrine chloride (1,2-dimethoxy-12-methyl-[1,3]benzodioxol[5,6]-benzanthrinium chloride) were dissolved in perfusion buffer containing albumin protein. BQ-123, BQ-3020, BQ-788, bosentan, nifedipine (C17H11NO2), PD-145065, U-46619, 1,2-bis(2-aminophenox)-ethane-N,N,N′,N″-tetraacetic acid acetoxymethyl ester (BAPTA-AM), and 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC) were each dissolved in 200 μl of DMSO before being added to the buffer containing albumin protein. Vehicle containing the same amount of DMSO did not affect the baseline perfusion pressure of the isolated perfused skin flaps.

Experimental Protocol

Protocol 1: Comparison of vasoconstrictor potency of ET-1, S6c, BQ-3020, and U-46619. Cumulative concentration-dependent vasoconstrictor effects of ET-1 (5 × 10−10–10−8 M), S6c (5 × 10−10–10−7 M), BQ-3020 (5 × 10−10–10−7 M), and U-46619 (5 × 10−7–10−5 M) on perfusion pressure were studied in isolated perfused pig skin flaps. ET-1 is an ETα receptor agonist. S6c and BQ-3020 are selective ETα receptor agonists and U-46619 is a thromboxane A2 mimetic. Skin flaps were exposed to each concentration of ET-1, S6c, and...
BQ-3020 for 30 min and U-46619 for 15 min. In our preliminary study, we observed that the maximal vasoconstrictor effect of U-46619 was expressed within 15 min. The vasoconstrictor effect of ET-1, BQ-3020, and S6c began to peak at ~30 min. Cumulative concentration-dependent curves were plotted, and the concentration of each agonist that caused a half-maximal (i.e., EC50) and maximal (i.e., Emax) increase in perfusion pressure was estimated. The apparent affinity (pD2), defined as the negative log molar concentration that caused a half-maximal increase in perfusion pressure, was calculated for each agonist.

Protocol 2: Study of the contribution of ETA and ETB receptors in the mediation of ET-1-induced skin vasoconstriction. The cumulative concentration-dependent effect of ET-1 (10^-9 – 10^-8 M) on perfusion pressure in isolated perfused pig skin flaps was investigated in the absence or presence of 10^-5 M of 1) BQ-123 (a selective ETA receptor antagonist), 2) BQ-123 and BQ-788 (a selective ETB receptor antagonist), 3) bosentan (a nonpeptide ET Ar receptor antagonist), or 4) PD-145065 (a peptide ETar receptor antagonist). To confirm that ETB receptors are involved in ET-1-induced vasoconstriction, the concentration-dependent vasoconstrictor effect of ET-1 was studied again in the absence and presence of 10^-5 M of BQ-788.

In a separate study, the selective antagonistic action of bosentan and PD-145065 on ETAr receptors in pig skin was tested. Specifically, the increase in perfusion pressure induced by norepinephrine (10^-7 and 10^-6 M) was studied in the absence and presence of 10^-5 M of bosentan and PD-145065.

Protocol 3: Study of the postreceptor signal transduction pathways in ETB receptor-mediated skin vasoconstriction. The vasoconstrictor action of BQ-3020 mediated by ETB receptors was investigated in this study. Specifically, the cumulative concentration-dependent increase in perfusion pressure induced by BQ-3020 (10^-9 – 10^-7 M) in isolated perfused skin flaps was studied in the absence and presence of 10^-5 M BQ-123, 10^-7 M BQ-788, and 5 x 10^-6 M BQ-788. The effect of BQ-3020 on skin perfusion pressure was also studied in the absence and presence of 5 x 10^-6 M NCDC, a PLC inhibitor. In a separate study, the cumulative concentration-dependent effect of BQ-3020 (10^-9 – 10^-7 M) on perfusion pressure was investigated in skin flaps perfused with normal buffer, Ca^2+-free buffer, and Ca^2+-free buffer containing 5 x 10^-6 M of chelerythrine, a PKC inhibitor. In our preliminary study, we examined the basal perfusion pressure of three skin flaps that were perfused with normal buffer (control) for 45 min followed by perfusion with Ca^2+-free buffer for another 45 min. We did not observe any significant change in basal perfusion pressure when these skin flaps were perfused with Ca^2+-free buffer compared with the control.

In another study, the cumulative concentration-dependent effect of BQ-3020 (10^-9 – 10^-7 M) on perfusion pressure in isolated perfused skin flaps was studied with normal buffer and normal buffer containing 5 x 10^-6 M of nifedipine (an L-type Ca^2+ channel antagonist), 5 x 10^-6 M of nifedipine and chelerythrine, or 5 x 10^-6 M of BAPTA-AM (an intracellular Ca^2+ chelator). The intracellular signal transduction pathways linked to the ETB receptor in the mediation of cutaneous vasoconstriction were further investigated by studying the concentration-dependent (10^-9 – 10^-8 M) vasoconstrictor effect of S6c in the absence and presence of 5 x 10^-6 M of NCDC, nifedipine, or chelerythrine, and also in Ca^2+-free buffer with or without 5 x 10^-6 M chelerythrine.

Fig. 1. Cumulative concentration-dependent increase in perfusion pressure induced by endothelin-1 (ET-1), sarafotoxin 6c (S6c), BQ-3020, and U-46619 in isolated perfused pig skin flaps. n = number of experiments.

**RESULTS**

Comparison of Skin Vasoconstrictor Potency of ET-1, S6c, BQ-3020, and U-46619

ET-1, S6c, BQ-3020, and U-46619 caused a concentration-dependent increase in skin perfusion pressure in isolated perfused pig skin flaps (Fig. 1). Emax in perfusion was significantly (P < 0.01) higher for ET-1 than for S6c, BQ-3020, and U-46619 (Table 1). Unlike ET-1, BQ-3020, and U-46619, Emax obtained with S6c was not sustained at the maximal dose and Emax decreased after the skin flap was exposed to 10^-8 M S6c for 30 min (Fig. 1).

<table>
<thead>
<tr>
<th>Drug</th>
<th>pD2</th>
<th>Emax, mmHg</th>
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<tbody>
<tr>
<td>Endothelin-1 (n = 5)</td>
<td>8.513 ± 0.014†</td>
<td>254 ± 9*</td>
</tr>
<tr>
<td>Sarafotoxin 6c (n = 4)</td>
<td>8.766 ± 0.024*</td>
<td>126 ± 8†</td>
</tr>
<tr>
<td>BQ-3020 (n = 4)</td>
<td>8.607 ± 0.028‡</td>
<td>118 ± 10†</td>
</tr>
<tr>
<td>U-46619 (n = 5)</td>
<td>6.063 ± 0.075‡</td>
<td>113 ± 6†</td>
</tr>
</tbody>
</table>

Values are means ± SE (n is no. of experiments). Means in the same column without a common symbol are significantly different (1-way ANOVA and Duncan’s multiple-range test) [P < 0.05 for pD2; P < 0.01 for maximum increase in perfusion pressure (Emax)]. Apparent affinity (pD2) is defined as negative log molar concentration that caused a 50% increase in perfusion pressure.
value for BQ-3020 was similar to those for ET-1 and S6c (Table 1).

Contribution of ETA and ETB Receptors in the Mediation of ET-1-Induced Skin Vasconstriction

The concentration-dependent increase in perfusion pressure induced by ET-1 in pig skin flaps was partially inhibited \((P < 0.05)\) by \(10^{-5} \text{M} \) BQ-123 (an ETA receptor antagonist), reducing \(E_{\text{max}}\) at \(10^{-8} \text{M}\) of ET-1 by 45% compared with the control (Fig. 2). Further significant \((P < 0.05)\) inhibition of ET-1-induced increase in perfusion pressure was achieved either by a combined treatment of \(10^{-5} \text{M} \) BQ-123 and \(10^{-5} \text{M} \) BQ-788 (an ETB receptor antagonist) or by a nonselective nonpeptide ETA/B receptor antagonist \((10^{-5} \text{M} \) bosentan), reducing the maximal increase in perfusion by 65 and 56% at \(10^{-8} \text{M}\) of ET-1, respectively, compared with the control. However, \(10^{-5} \text{M} \) PD-145065 (a nonselective peptide ETA/B receptor antagonist) was most potent in blocking the concentration-dependent increase in perfusion pressure induced by ET-1 \((P < 0.05)\) compared with other treatment groups, reducing the maximal increase in perfusion pressure by 76% at \(10^{-8} \text{M}\) ET-1 compared with the control (Fig. 2). The contribution of ETB receptors in the mediation of ET-1-induced skin vasconstriction was further investigated. Specifically, the ETB receptor antagonists BQ-788 alone significantly blocked \((P < 0.01)\) the increase in perfusion pressure induced by ET-1 (Fig. 3).

Perfusion of skin flaps with \(10^{-5} \text{M} \) BQ-123, BQ-788, bosentan, or PD-145065 alone for 45 min did not affect the basal perfusion pressure. In addition, \(10^{-5} \text{M} \) bosentan and \(10^{-5} \text{M} \) PD-145065 did not have any significant effect on the increase in perfusion pressure induced by different concentrations of norepinephrine (Fig. 4).

Postreceptor Signal Transduction Pathways in ETB Receptor-Mediated Skin Vasconstriction

The concentration-dependent increase in perfusion pressure induced by the ETB receptor agonist BQ-320 was inhibited \((P < 0.01)\) by the ETB receptor antagonist BQ-788 in a concentration-dependent manner (Fig. 5). The increase in perfusion induced by BQ-320 was completely blocked by \(5 \times 10^{-8} \text{M} \) BQ-788. ETA receptor antagonist BQ-123 did not inhibit the BQ-320-induced increase in perfusion pressure. Pretreatment of BQ-123 or BQ-788 alone for 45 min did not affect the basal perfusion pressure.
The concentration-dependent increase in perfusion pressure in pig skin flaps induced by BQ-3020 was significantly (P < 0.01) reduced by the PLC inhibitor NCDC (Fig. 6) or by removing Ca\(^{2+}\) from the perfusion buffer, and this perfusion pressure was further reduced (P < 0.05) in Ca\(^{2+}\)-free buffer containing 5 \times 10^{-6} \text{ M} chelerythrine, a PKC inhibitor (Fig. 7). Pretreatment with chelerythrine for 45 min did not change the basal perfusion pressure.

Treatment with 5 \times 10^{-6} \text{ M} nifedipine, an L-type Ca\(^{2+}\) channel antagonist, or with 5 \times 10^{-6} \text{ M} BAPTA-AM, an intracellular Ca\(^{2+}\) chelator, significantly (P < 0.05) attenuated the concentration-dependent increase in perfusion pressure induced by BQ-3020 in pig skin flaps (Fig. 8). Further attenuation of the BQ-3020-induced increase in perfusion pressure (P < 0.05) was achieved by the combined treatment of 5 \times 10^{-6} \text{ M} nifedipine and 5 \times 10^{-6} \text{ M} chelerythrine (Fig. 8). Pretreatment with nifedipine, BAPTA-AM, or chelerythrine alone for 45 min did not affect the basal perfusion pressure.

Treatment with 5 \times 10^{-6} \text{ M} NCDC (a PLC inhibitor), 5 \times 10^{-6} \text{ M} nifedipine, or 5 \times 10^{-6} \text{ M} chelerythrine...
significantly (P < 0.01) reduced the concentration-dependent increase in perfusion pressure induced by the selective ETB receptor agonist S6c (Fig. 9). Pretreatment with NCDC, nifedipine, and chelerythrine alone for 45 min did not affect the basal perfusion pressure of the skin flap. The increase in perfusion pressure induced by S6c was significantly (P < 0.01) inhibited when Ca\(^{2+}\) was removed from the buffer, and further inhibition was seen in Ca\(^{2+}\)-free buffer containing 5 \(\times\) 10\(^{-6}\) M chelerythrine (Fig. 10).

**DISCUSSION**

Other investigators have studied the role of ETA and ETB receptors in the mediation of ET-1-induced vasoconstriction in rat and human skin by intradermal injection of ET\(_{AB}\) and ETB receptor agonists and antagonists (27, 43). Here, we used isolated perfused pig skin flaps (6 \(\times\) 16 cm) to investigate the functional importance and mechanism of ETB receptors in the mediation of ET-1-induced skin vasoconstriction. This approach is unique in that pig skin vascular anatomy closely resembles that of the human (10), and this isolated perfused skin flap model allowed drugs to be delivered by continuous intra-arterial infusion through a direct cutaneous artery of the skin flap; thus the intraluminal effects of these drugs on vascular contraction can be studied in a concentration-dependent manner. In addition, this is the first study of the postreceptor pathways linked to ETB receptors in the mediation of skin vasoconstriction. With the use of this pig skin flap model, we have observed that 1) both ETA and ETB receptor subtypes are functionally important in the mediation of ET-1-induced skin vasoconstriction and 2) L-type Ca\(^{2+}\) channels, PLC, and PKC are involved in ETB receptor-mediated skin vasoconstriction.

**Functional Importance of ETA and ETB Receptors in the Mediation of ET-1-Induced Vasoconstriction in Pig Skin**

It has been reported that intradermal injection of ET-1, ET-3, or the selective ETB receptor agonist IRL-1620 induced a dose-dependent decrease in skin blood flow in the rat assessed by \(^{133}\)Xe clearance at skin test sites. Concomitant intradermal injection of the ETA receptor antagonist BQ-123 blocked the vasoconstrictor effect of ET-1 but BQ-123 did not block IRL-1620-induced skin vasoconstriction. In addition, radioligand binding activity studied by autoradiography indicated that ~40% of ET-1 binding sites were of the ETB subtype. These observations indicate that both ETA and ETB receptors mediate extraluminal ET-1-induced skin vasoconstriction in the rat (25). There are several lines of evidence in the present study to indicate that both ETA and ETB receptors also play a central role in vasoconstriction in pig skin induced by intraluminal ET-1. Specifically, intra-arterial infusion of the ET\(_{AB}\) receptor agonist ET-1 and the ETB receptor agonists S6c and BQ-3020 caused skin vasoconstriction in a concentration-dependent manner, with vasoconstrictor potency at least 300-fold higher than that of U-46619 (Fig. 1). In addition, the ET-1-induced vasoconstriction in pig skin was partially inhibited by the selective ETA receptor antagonist U-46619 and further inhibition of ET-1-induced skin vasoconstriction could be achieved by the combined treatment of BQ-123 and the selective ETB receptor antagonist BQ-788 or by a nonselective...
ET<sub>A/B</sub> receptor antagonist, bosentan or PD-145065 (Figs. 2 and 3). It is unlikely that bosentan and PD-145065 may have a nonspecific inhibitory effect on ET-1-induced vasoconstriction because they did not inhibit the vasoconstrictor effect of varying concentrations of norepinephrine (Fig. 4). Similarly, it is also unlikely that the ET<sub>B</sub> receptor agonist BQ-3020 could have produced nonspecific skin vasoconstriction, because its vasoconstrictor effect was completely blocked by the selective ET<sub>B</sub> receptor antagonist BQ-788, and the ET<sub>A</sub> receptor antagonist BQ-123 had no antagonistic effect (Fig. 5).

Although we have not documented the relative proportions of ET<sub>A</sub> and ET<sub>B</sub> receptors and their binding activities in the microvasculature of pig skin, results from the current functional study discussed thus far clearly indicate that ET<sub>A</sub> and ET<sub>B</sub> receptors exist in pig skin vasculature and they both play an important role in mediating ET-1-induced skin vasoconstriction.

It was previously observed that the vasoconstrictor potency of S6c was significantly higher than ET-1 in isolated perfused rabbit pulmonary artery rings, and prolonged activation of the ET<sub>B</sub> receptor subtype by S6c produced tachyphylaxis (25). Similar events might have occurred in the present study. Specifically, the vasoconstrictor potency (pD<sub>2</sub>) of S6c was slightly but significantly higher than ET-1 (Table 1), and the concentration-dependent vasoconstrictor effect of S6c began to decrease after the skin vasculature was exposed to 10<sup>-8</sup> M S6c for ~30 min (Figs. 1 and 9). The mechanism of tachyphylaxis is unclear. In addition, it is not known why tachyphylaxis occurs in S6c-induced (Figs. 1 and 9), but not in BQ-3020-induced (Figs. 5–8), skin vasoconstriction. Other investigators have also observed that S6c but not BQ-3020 caused tachyphylaxis in rabbit pulmonary arteries (20, 25). It is likely that tachyphylaxis is the result of ET<sub>B</sub> receptor desensitization caused by receptor downregulation. We speculate that tachyphylaxis occurs in S6c- but not in BQ-3020-induced skin vasoconstriction because S6c is more potent than BQ-3020 at high concentrations (Fig. 1).

It is of interest to note that the vasoconstrictor effect of ET-1 (10<sup>-9</sup>–10<sup>-8</sup> M) was not completely blocked by 10<sup>-5</sup> M BQ-123 and 10<sup>-5</sup> M PD-145065 (Fig. 2), and the reason is unknown. It could be the result of an increase in basal perfusion pressure due to blockade of the endothelial ET<sub>B</sub> receptors causing inhibition of synthesis of endothelium-derived relaxing factors nitric oxide/PGI<sub>2</sub>. However, we demonstrated previously that NO synthase inhibitors, N<sup>ω</sup>-nitro-L-arginine and N<sup>ω</sup>-monomethyl-L-arginine, did not affect the basal perfusion pressure in the same pig skin flap model (35). Other investigators also observed that BQ-123 and BQ-788 did not completely block the ET-1-induced renal vasoconstriction in the pig (7). There is the possibility that ET receptors insensitive to BQ-123 and/or BQ-788 (7, 21, 41) may exist in pig skin vasculature.

Mechanism of ET<sub>B</sub> Receptor-Mediated Skin Vasoconstriction

The intracellular signal transduction pathways of ET<sub>A</sub> and ET<sub>B</sub> receptors have been studied by transfection and stable expression of individual receptor cDNAs in Chinese hamster ovary cells. The pathways for these two receptor subtypes were common in that stimulation of either the ET<sub>A</sub> or ET<sub>B</sub> receptor subtype resulted in a rapid phosphatidylinositol hydrolysis and increased release of intracellular free Ca<sup>2+</sup> and arachidonic acid (2, 32). On the other hand, evidence obtained from rat tracheas indicated that ET<sub>A</sub> and ET<sub>B</sub> receptors are linked to different signal transduction pathways (17). Specifically, ET-1 but not S6c induced the accumulation of inositol 1,4,5-trisphosphate and release of intracellular Ca<sup>2+</sup>, and these responses were blocked by the ET<sub>A</sub> receptor antagonist BQ-123. In contrast, S6c-induced tracheal smooth muscle contraction was almost entirely dependent on the influx of extracellular Ca<sup>2+</sup> through non-L-type Ca<sup>2+</sup> channels (17). Furthermore, it has also been reported in rabbit saphenous veins that ET<sub>A</sub> and ET<sub>B</sub> receptors seemed to be linked to separate signal transduction pathways, with the ET<sub>A</sub> but not the ET<sub>B</sub> receptor associated with activation of PKC (15). The postreceptor signal transduction pathways for the ET<sub>A</sub> and ET<sub>B</sub> receptor in skin vasculature have not been investigated. In the present studies, we used selective ET<sub>B</sub> receptor agonists BQ-3020 and S6c as probes to investigate the postreceptor pathways associated with ET<sub>B</sub> receptor-mediated vasoconstriction. We observed that removal of Ca<sup>2+</sup> from perfusion buffer or pretreatment with the PLC inhibitor NCDC, the L-type Ca<sup>2+</sup> channel blocker nifedipine, or the intracellular Ca<sup>2+</sup> chelator BAPTA-AM partially inhibited BQ-3020-induced skin vasoconstriction (Figs. 6–8). We also demonstrated that the combined treatment of the PKC inhibitor chelerythrine and Ca<sup>2+</sup>-free perfusate or chelerythrine and nifedipine caused a greater reduction in vasoconstriction induced by BQ-3020 than by Ca<sup>2+</sup>-free perfusate or nifedipine alone, respectively (Figs. 7 and 8). These observations led us to speculate that L-type Ca<sup>2+</sup> channels and PLC- and PKC-linked pathways are most likely involved in ET<sub>B</sub> receptor-mediated skin vasoconstriction. This speculation was confirmed by further observations that the PLC inhibitor NCDC, the L-type Ca<sup>2+</sup> channel inhibitor nifedipine, removal of Ca<sup>2+</sup> from the buffer, or the PKC inhibitor chelerythrine also partially reduced the concentration-dependent vasoconstrictor effect of the selective ET<sub>B</sub> receptor agonist S6c in pig skin flaps (Figs. 9 and 10).

Inhibition of vasoconstriction induced by BQ-3020 or S6c was more pronounced in Ca<sup>2+</sup>-free buffer containing chelerythrine than in Ca<sup>2+</sup>-free buffer alone (Figs. 7 and 10). This observation implies that the postreceptor vasoconstrictor mechanism of BQ-3020 and S6c most likely involves a PKC component that is independent of Ca<sup>2+</sup> influx.
Possibility of Species Difference in the Role of ET_b Receptors in the Mediation of Skin Vasconstriction

So far the relative functional importance of the ETA and ET_b receptor in the mediation of skin vasoconstriction in human skin remains unclear. ETA and ET_b receptors were identified in microvessels in human skin biopsies by autoradiography, and it was speculated that both receptor subtypes may be involved in ET-1-induced vasoconstriction in human skin (24). It has also been demonstrated in humans that dorsal hand vein infusion of S6c caused local venoconstriction (16). On the other hand, it has been demonstrated in humans that the intradermal injection of ET-1 but not ET-3 caused a decrease in skin blood flow assessed by laser-Doppler flowmetry and intradermal injection of the nonselective ET_A/B receptor antagonist PD-145065 did not cause any additional inhibition of ET-1-induced skin vasoconstriction compared with the selective ETA receptor antagonist PD-147953. These observations were interpreted to indicate that the vasoconstrictor effect of ET-1 in human skin is primarily by activation of ET_A receptors (43), and this is different from pig skin in which both ETA and ETB are functionally involved in ET-1-induced vasoconstriction. However, in the aforementioned human study, all drugs were given intravascularly by intradermal injection and nonspecific vascular reactivities were reported in saline and drug injection (43). Therefore, further studies are required to study the role of ETA and ETB receptors in the mediation of ET-1-induced vasoconstriction in human skin.

In summary, with the use of an in vitro skin perfusion technique, we have for the first time demonstrated that both ETA and ETB receptors play an important functional role in the mediation of ET-1-induced vasoconstriction in pig skin and the postreceptor signal transduction pathways of ETB receptors most likely involve L-type Ca^{2+} channels, PLC, and PKC.

Perspectives

Several peripheral vascular disease processes, such as diabetes microangiopathy, Buerger's disease, Raynaud's disease, and scleroderma, are known to predispose to skin vasospasm. These diseases are also known to be associated with elevated circulating levels of ET-1; thus a pathological role for ET-1 has been postulated in these peripheral vascular diseases (13). In addition, there is experimental evidence to indicate that ET-1 is also associated with skin ischemia in surgical trauma (29) and burns (3). In clinical conditions associated with elevated circulating and/or tissue levels of ET-1, ET receptors may be an important therapeutic target, because blocking these receptors or their signal transduction pathways would alleviate skin vasospasm. Therefore, this area of research would likely contribute to the development of an effective pharmacological intervention for ET-1-related ischemic skin diseases.

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Address for reprint requests: C. Y. Pang, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada MSG 1X8.

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