Vasoconstrictor effect of endothelin-1 in human skin: role of ET<sub>A</sub> and ET<sub>B</sub> receptors

JOAN E. LIPA,1,2 PETER C. NELIGAN,1,2 THERESA M. PERREAUT,3 RONALD H. LEVINE,2 ROBERT J. KNOWLTON,2 AND CHO Y. PANG1,2,4

1Research Institute, The Hospital for Sick Children and Departments of Surgery and Physiology, University of Toronto, Toronto, Ontario M5G 1X8; and 3Division of Newborn Medicine, The Montreal Children’s Hospital, Montreal, Quebec, H3H 1P3 Canada

Lipa, Joan E., Peter C. Neligan, Therese M. Perreault, Ronald H. Levine, Robert J. Knowlton, and Cho Y. Pang. Vasoconstrictor effect of endothelin-1 in human skin: role of ET<sub>A</sub> and ET<sub>B</sub> receptors. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H359–H367, 1999.—The aim of this project was to investigate the role of ET<sub>A</sub> and ET<sub>B</sub> receptors in the mediation of endothelin (ET)-1-induced vasoconstriction in human skin. This information should provide important insights into the design of pharmacological intervention against skin vasospasm induced by ET-1 in peripheral vascular disease or surgical trauma. Vasoconstriction in response to intra-arterial drug infusion in isolated perfused human skin flaps (8 × 18 cm) derived from dermoflap specimens was assessed by studying changes in skin perfusion and perfusion pressure under constant flow rate in each drug treatment (n = 4). It was observed that ET-1 (10<sup>-10</sup> to 10<sup>-8</sup> M) and norepinephrine (NE, 10<sup>-8</sup> to 10<sup>-5</sup> M) caused skin vasoconstriction in a concentration-dependent manner, with the vasocostrictor potency of ET-1 ~200-fold higher than NE. The ET<sub>A</sub>-receptor antagonist BQ-123 but not the ET<sub>B</sub>-receptor antagonist BQ-788 blocked the vasoconstrictor effect of ET-1. This observation was confirmed by studying skin perfusion using the dermofluorometry technique. In addition, ET<sub>B</sub>-receptor agonists BQ-3020 and sarafotoxin S6c (10<sup>-9</sup> to 10<sup>-6</sup> M) did not evoke skin vasoconstriction. BQ-3020 also did not elicit skin vasoconstriction even in the presence of 10<sup>-5</sup> M of N<sup>ω</sup>-nitro-L-arginine methyl ester and indomethacin. Furthermore, results from saturable and competitive ET-1 radioligand membrane receptor binding assays revealed that high-affinity and capacity binding sites are predominantly the ET<sub>A</sub> receptor subtype in endothelin-denuded skin arteries and veins of 0.5–1.5 mm diameter, with an ET<sub>A</sub>-to-ET<sub>B</sub> receptor ratio of 83:17 in arteries (n = 5) and 78:22 in veins (n = 7). Results from the present functional and radioligand receptor binding studies clearly indicate that ET-1 is a very potent vasoconstrictor in human skin and its vasoconstrictor effect is primarily mediated by ET<sub>A</sub> receptors, with no significant participation from ET<sub>B</sub> receptors.

vasoconstriction

THE ENDOTHELINS (ETs) are a family of 21-amino acid peptides that include ET-1, ET-2, ET-3, and the sarafotoxins (17). ET-1 is the predominant isopeptide in the cardiovascular system. It is synthesized and released by the endothelium, and it plays an important role in the regulation of vascular tone (17, 45). Two major classes of ET receptor subtypes exist. Specifically, ET<sub>A</sub> membrane receptors have a higher affinity for ET-1 and ET-2 than for ET-3 and are present in vascular smooth muscle cells to mediate vasoconstriction (1). On the other hand, ET<sub>B</sub> receptors are located on endothelial cells and have equal affinity for all ET isopeptides (32). Stimulation of endothelial ET<sub>B</sub> receptors results in synthesis and release of nitric oxide (16), prostacyclin (10), or both (22) to evoke vasodilation. More recently, ET<sub>B</sub> receptors have also been identified in vascular smooth muscle cells, and these receptors mediate vasoconstriction. ET<sub>B</sub>-receptor-mediated vasoconstriction has been observed in human internal mammary and pulmonary resistance arteries and human saphenous veins (26, 34, 44), in dog and pig coronary arteries (33, 36, 40), and in rabbit pulmonary arteries and jugular and saphenous veins (13, 14, 27, 37, 38). However, the contribution of ET<sub>B</sub> receptors to vasoconstriction is variable and appears to be dependent on species, vessel type, and vessel size (7, 12, 26, 27, 38). It is important to point out that ET<sub>B</sub> receptors also appear to function as “clearance receptors” for ET-1 in vivo. For example, it has been observed that selective ET<sub>B</sub> receptor blockade caused a decrease in ET-1 uptake by the lung and kidney and ET-1 clearance from the circulation in the rat (11).

Of particular interest to us is the relative functional importance of ET<sub>A</sub> and ET<sub>B</sub> receptors in the mediation of vasoconstriction in human skin. Several peripheral vascular disease processes such as diabetic microangiopathy, Buerger’s disease, Raynaud’s disease, and scleroderma are known to predispose the skin to 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 (9, 31). In addition, experimental evidence is available to indicate that ET-1 is associated with skin ischemia in surgical trauma (25, 39). In clinical conditions associated with elevated circulating and/or skin tissue levels of ET-1, ET receptors may be an important therapeutic target, since blocking these receptors would potentially alleviate skin vasospasm. However, development of an effective pharmacological intervention requires identification of the ET-receptor subtype(s) mediating ET-1-induced skin vasoconstriction. Ideally, use of an ETA instead of an ET<sub>B</sub>-receptor antagonist would exclude the potential detrimental effect arising from blockade of endothelial ET<sub>B</sub> receptors, which mediate the vasodilatory effect of ET-1.

The relative functional importance of ET<sub>A</sub> and ET<sub>B</sub> receptors in mediating ET-1-induced vasoconstriction...
receptor subtypes are involved in vasoconstriction (18). Human skin biopsies has led to speculation that both ETA and ET B receptors in mediating ET-1-was used to investigate the relative functional import-
ance of ETA and ET B receptors in the mediation of ET-1-induced vasoconstriction in human skin is primarily mediated by ET A receptors. Specifically, it was reported in humans that intradermal injection of ET-1 but not ET-3 caused a decrease in skin blood flow assessed by laser Doppler flowmetry, and there was no difference between the ET A-receptor antagonist PD-147953 and the ET A/ET B-receptor antagonist PD-145065 in the attenuation of skin vasoconstriction induced by intradermal injection of ET-1 (43). The aim of the present study, therefore, was to elucidate the role of ET receptor subtypes in the mediation of ET-1-induced vasoconstriction in human skin. To this end, the isolated perfused human skin flap model (19–21) was used to investigate the relative functional importance of ETA and ET B receptors in mediating ET-1-induced vasoconstriction, and the radioligand receptor binding assay technique was used to assess the distribution and binding activity of ETA and ET B receptors in endothelium-denuded arteries and veins from human skin specimens. Results obtained from these studies should provide important insights into the pharmacological intervention of ET-1-induced skin vasospasm.

MATERIALS AND METHODS

Source of Human Skin

The skin pannus excised from patients undergoing dermolipectomy serves no purpose to the patient and is normally disposed of by incineration. A clinical protocol was approved for the design of skin flaps from these skin panni for in vitro skin perfusion experiments and membrane vascular receptor binding assays. At the end of each experiment, the skin specimen was disposed of in the normal manner in accordance with the Department of Pathology at St. Joseph’s Health Centre.

Sixty-one skin specimens were accepted for this project; 27 were used for skin perfusion experiments and harvesting of skin blood vessels, and the remaining skin specimens were used only for harvesting of blood vessels for ET-1 radioligand membrane receptor binding assays. The median age of the patients was 41 yr (range 23–77 yr), and 89% of these patients were female. The skin specimens accepted for this project did not have scars, lesions, or infection, and the patients were not known to have any systemic disease.

Skin Flap Model for In Vitro Skin Perfusion Experiments

The anatomy and design of the human skin flap model derived from the excised abdominal skin pannus have been described by us previously (19–21). The following modifications were made for the present project. The skin pannus was divided along its midline; one-half was used for the design of a skin flap for in vitro perfusion, and the remaining one-half was used for harvesting of skin blood vessels for ET-1 radioligand membrane receptor binding assays. Because the edges of the skin flap were not sutured and could not be cauterized while enough to stop leakage during perfusion, a length of Plexiglas 1 cm wide was placed on the skin surface and undersurface at the edges of all sides of the skin flap. These lengths of Plexiglas were pressed together with screws to provide compression of dermal vessels at the cut edges of the skin flap to prevent leakage during skin perfusion. The leakage from the undersurface was controlled by cautery and vascular clips (Ligacips; Ethicon), and no measurement was taken to quantify leakage. The resulting width and length of the skin flap were 8 × 18 cm, respectively. This length of the skin flap was always longer than the maximum length of skin that could be perfused; therefore, there was practically no leaking at the distal end of the skin flap.

The vascular pedicle in the proximal end of the skin flap consisted of a paired perforator artery and vein (0.5–1.5 mm diameter), which were cannulated with 22- or 24-gauge angiocatheters, depending on vessel size. Perfusion buffer containing 10 U/ml of heparin was gently instilled through the arterial catheter with a 3-ml syringe until venous flow was observed, thus confirming that the vascular system of the skin flap was satisfactorily flushed to start the perfusion experiment. All experiments in this study were initiated within 2 h after excision of skin specimens at room temperature. It was previously demonstrated that the skin flap was thereafter metabolically and physiologically stable for at least 5 h of in vitro perfusion (21).

Skin Perfusion Technique

The skin flap with its cannulated arterial and venous perfuser was placed on skin side up on an aluminum mesh and was then subsequently connected to the commercially available MX Amber perfuser apparatus (model TwoTen; MX International, Aurora, CO). The perfusate consisted of modified Krebs-Henseleit buffer with the following composition (in mM): 100 NaCl, 4.6 KCl, 1.1 NaH2PO4, 1.2 MgSO4-7H2O, 2.25 CaCl2, 30 NaHCO3, 11 glucose, and 2 bovine serum albumin. Bovine serum albumin (BSA, Cohn fraction V) was added to the buffer for a final concentration of 6.5%. The buffer was then filtered and looped (Whatman no. 40) and equilibrated within the reservoir chamber of the perfusion apparatus with 95% O2-5% CO2 at 37.1 ± 0.1°C, pH 7.40 ± 0.01, and PO2 444 ± 17 mmHg. The PO2 of perfusate collected at the venous outflow was 175 ± 25 mmHg, indicating adequate supply of oxygen to the skin flap. A transcutaneous PO2 detector was not available to measure the PO2 of perfused tissue. An adjustable-rate pump (model 7014; Cole-Parmer Instrument, Vernon Hills, IL) was used to deliver perfusate, which passed through a bubble trap in the reservoir, to the arterial catheter of the skin flap. A three-way connector linked the tubing from the pump to the flap and allowed for parallel connection of a pressure transducer (AB high-performance pressure transducer; Data Instruments, Lexington, MA). The transducer output continuously displayed the perfusion pressure on a digital monitor (Trendicator II 621A digital strain gauge; Doric Scientific, San Diego, CA) and a chart recorder (Lineacorder WR3101; Graphitec).

The buffer flow rate was adjusted (3.1 ± 0.2 ml/min) to achieve a stable baseline perfusion pressure of ~50 mmHg (46.0 ± 1.6 mmHg). A baseline of 50 mmHg was chosen because results from previous studies with this perfusion skin flap model revealed it to provide good tissue perfusion with minimal leakage and edema formation (~10%; see Refs. 19–21). In addition, a perfusion pressure of 50 mmHg, when Krebs buffer with an albumin concentration of 65 g/l is used, is equivalent to the perfusion pressure of ~90 mmHg in whole blood perfusion (2). A 45-min stabilization period was allowed at the beginning of each experiment, and the surface tempera-
demonstrated a concentration-dependent increase in perfusion pressure (i.e., percentage of baseline perfusion pressure). Skin flap was standardized by its baseline perfusion pressure which remained constant, a change in perfusion pressure in response to drug administration is indicative of a change in vascular resistance of the skin flap. The change in perfusion pressure in each skin flap was standardized by its baseline perfusion pressure (i.e., percentage of baseline perfusion pressure).

**Criteria for Rejection of Skin Flap**

A skin flap was deemed to be vascularly reactive if it demonstrated a concentration-dependent increase in perfusion pressure to the drug under test, e.g., ET-1 and norepinephrine (NE). At the end of the last dose of each experiment, acetylcholine (ACH, 10^{-5} M) was used to test endothelium-dependent relaxation. In the event that little or no vasoconstriction was detected in a test drug, NE (5 x 10^{-7} M) was infused to ensure that the vascular contractility was intact in the skin flap. Infusion of 1 ml of 6.2% wt/vol sulfan blue dye (Imperial Chemical Industry) was carried out to characterize the extent (length and width) of skin flap perfusion. Any skin flap that did not respond to vasoconstrictor and vasodilator drugs and showed <6 cm length in skin perfusion was not included in this study.

**Surface Dermofluorometry Technique for Assessment of Skin Perfusion**

The dermofluorometry technique for indirect assessment of in vivo dermal perfusion has been validated against the radioactive microsphere technique in the pig (41). Dermofluorometry has also been applied to the present isolated perfused human skin flap model in vitro (19, 20). In the present study, dermofluorometry was used to corroborate the observation of skin vascular reactivities assessed by measurement of perfusion pressure. Specifically, confluent circles of 1-cm diameter were marked along the longitudinal midline of the skin flap surface. After the 45-min stabilization period, the background fluorescence in each circular skin area was measured (fluorescence units) using a dermofluorometer (Fluorescan unit; Santa Barbara Technology, Santa Barbara, CA). Fluorescin dye (fluorescite; Alcon Canada) with a final concentration of 3 x 10^{-5} M was then infused for 4 min, and fluorescence in each circular area was measured again. A washout period of 15 min was allowed, and the background fluorescence was taken again. After the perfusion pressure had stabilized subsequent to drug infusion for study of skin vascular reactivity, fluorescein dye infusion was repeated, and skin fluorescence was measured again. The difference in fluorescence units for each circular skin area between the background and postfluorescein dye infusion was defined as the net fluorescence unit for that area. The total dye fluorescence is the sum of all net fluorescence units measured from all circular skin areas along the midline of the skin flap.

**ET-1 Radioligand Membrane Receptor Binding Assay**

Source of blood vessels. Assessment of ET-1 binding site activity was performed on membranes of skin arteries and veins (0.5–1.5 mm diameter) dissected from human skin specimens. The techniques for membrane preparation and receptor binding assays were similar to those described previously for pulmonary blood vessels (29). The excised vessels were opened longitudinally, the endothelium was removed by gently scraping the intimal surface with a scalp blade, and they were then rinsed with cold (4°C) buffered physiological salt solution (HPSS, pH 7.4, with the following composition (in mM): 20 HEPES, 135 NaCl, 2.68 KCl, 1.8 CaCl_2, and 2.05 MgCl_2. Arteries and veins were stored separately at −80°C. To confirm that the endothelium was indeed removed, endothelium-intact (control) and endothelium-denuded specimens of artery and vein were randomly sampled and submitted in 10% formaldehyde solution to the Histopathology Laboratory of the Department of Pathology at The Hospital for Sick Children for hematoxylin and eosin sections. The basophilic nuclei of endothelial cells could be seen clearly along the luminal surface in control arteries and veins but were absent in denuded vessels, thus confirming satisfactory removal of endothelial cells.

Membrane preparation. Pooled samples of arteries and veins from skin specimens were pulverized separately while frozen and then were homogenized separately in 5 vol of 0.25 M sucrose and 10 mM HPSS, pH 7.4, at 4°C with a Polytron at a speed of ~14,000 rpm with six bursts of 20 s separated by 10-s cooling intervals. The homogenate was centrifuged at 16,000 g for 30 min at 4°C. The resulting supernatant was filtered through a 100-µm mesh filter (Nytex) and centrifuged at 100,000 g for 60 min at 4°C. The pellet was washed with HPSS and centrifuged again at 100,000 g for 60 min at 4°C. The resulting membranes were denatured in HPSS to a concentration of 20–40 µg protein/ml and stored at −20°C in a volume of 1 ml to be thawed just before use in a binding assay. Protein concentration was determined by the Bradford method, using BSA as a standard.

**Saturable ET-1 Radioligand Receptor Binding Assay**

ET-1 saturable receptor binding assay was carried out in duplicate on two separate membrane preparations of arteries and veins. Membrane suspensions were diluted with buffer B (HPSS solution containing 1 µM aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 0.2% BSA, pH 7.4) to a concentration of 2 µg protein/tube and were incubated with varying concentrations (0–1.5 nM) of [3H]iodotyrosyl ET-1 (ET-1) in a final volume of 250 µl. The reaction mixture was incubated for 2 h at 25°C as described previously (29). Incubation was terminated by rapid vacuum filtration of the membrane suspension through Whatman GF/B filters precoated with polyethyleneimine and 0.2% BSA. Radioactivity associated with the filters (i.e., bound [3H]iodotyrosyl ET-1 (ET-1)) was measured in a gamma counter. Nonspecific binding was defined as the binding of ET-1 to membranes in the presence of a saturating concentration of 100 mM unlabeled ET-1. Specific binding was calculated as the total binding minus the nonspecific binding.

**Competitive Radioligand Receptor Binding Assay**

Experiments were performed in duplicate with membrane preparations from arteries and veins as described previously (14). Briefly, 100 µl of [125I]ET-1 (60 pM) and 100 µl of membrane (2 µg protein) in buffer B were incubated with 50 µl of varying concentrations of BQ-123 (ET_A receptor antagonist, 0.1 nM to 1 µM) or with varying concentrations of BQ-788 (ET_A receptor antagonist, 0.1 nM to 1 µM) and 1 µM BQ-123. Experimental conditions and the method for determination of nonspecific and specific binding were the same as those in saturable receptor binding assays except that 0.1 mM of cold ET-1 was used for the study of nonspecific binding.

**Biochemicals**

Unless otherwise stated, all chemicals were purchased from Sigma Chemical (St. Louis, MO). ET-1, the ETA receptor antagonist, was purchased from Peptide Institute (Osaka, J apan). BQ-3020, N-acetyl-[11,15Ala]ET-1 (6–21), and S6c were pur-
chased from Bachem California (Torrance, CA). BQ-123, cyclo-
(d-Trp-d-Asp-Pro-d-Val-Leu) sodium salt, and BQ-788 [N-3-
2,6-dimethylpiperidinocarbonyl-L-Mel-Leu-d-Trp(COOCH3)-d-
-Nle] sodium salt were purchased from Peptides International
(Louisville, KY). 125I-ET-1 (2,000 Ci/mol), was purchased from
Amersham Life Science (Oakville, Ontario, Canada). ET-1 was
dissolved in 0.1% acetic acid and was stored at −70°C for no
longer than 30 days until just before use. BQ-3020 was
dissolved in 200 µl of dimethyl sulfoxide (DMSO). The remaining
peptides were dissolved in distilled water immediately before use.
Indomethacin (Indo), a cy-
oxynugenase inhibitor, was dissolved in 200 µl ethanol before
mixing with buffer solution. The final maximum concentra-
tion of DMSO or ethanol in perfusion studies was <0.04%;
this concentration did not affect the baseline perfusion pres-
sure. Injectable vials of NE were purchased from Sanofi
Winthrop (Markham, Ontario, Canada). Distilled Milli Q
water was used to make solutions and buffers. Fresh solu-
tions were made on the day of perfusion. Ascorbic acid was
added to the stock solution of NE (1 g/l) to suppress oxidation
of NE. On the day of the experiment, the drugs were prepared
and stored at 4°C before use.

Experimental Protocols

Protocol 1: to investigate the vasoconstrictor potency of
ET-1, BQ-3020, S6c, and NE in isolated perfused human skin.
The cumulative concentration-dependent effect of ET-1 (10
−10 to 10
−6 M), BQ-3020, S6c (ETB agonists, 10
−9 to 10
−6 M), and
NE (10
−8 to 10
−5 M) on perfusion pressure in isolated
perfused human skin flaps (n = 4) was studied, allowing 30 or
10 min for each concentration of peptide drug and NE,
respectively, to achieve its effect. Resultant perfusion pres-
sure measurements were expressed as a percentage of the
baseline perfusion pressure (−50 mmHg). For each experi-
ment, the concentration of drug required to elicit a half-
maximal increase in perfusion pressure (EC50), the apparent
affinity (pD2), and the maximal increase in perfusion pres-
sure were calculated. The pD2 value is defined as the negative
logarithm of the EC50. The cumulative concentration-depen-
dent effect on perfusion pressure of BQ-3020 (10
−4 to 10
−3 M)
was also studied (n = 4) in the presence of 10
−9 M N?-nitro-L-
arginine methyl ester (L-NAME, a nitric oxide synthase
inhibitor) and 10
−4 M Indo (a cytoxynugenase inhibitor).
L-NAME and Indo infusion started 45 min before BQ-3020
infusion.

Protocol 2: to investigate the ET-receptor subtypes in the
mediation of ET-1-induced vasooconstriction in isolated
perfused human skin. The cumulative concentration-dependent
effect of ET-1 (5 × 10
−10 to 10
−8 M) on skin perfusion pressure
in isolated perfused human skin flaps (n = 4) was studied in
the absence or presence of a selective ETB receptor antagonist
(5 × 10
−6 M BQ-123) or a selective ETB receptor antagonist
(10
−6 M BQ-788). Resultant perfusion pressure measure-
ments were expressed as a percentage of the baseline perfu-
sion pressure.

In a separate study, the dermofluorometry technique was
used to assess skin perfusion in the perfused skin flap.
Dermofluorometry was conducted on each skin flap at two
experimental time points. The first time point was at the end
of the stabilization period, before any ET-1 was administered;
this was referred to as the baseline. The second time point
was after administration of a treatment. Treatment groups
(n = 4) were 1) vehicle, 2) 5 × 10
−9 M ET-1, 3) 5 × 10
−9 M ET-1
in the presence of a selective ETB receptor antagonist (5 ×
10
−6 M BQ-123), or 4) 5 × 10
−9 M ET-1 in the presence of a
selective ETB receptor antagonist (10
−6 M BQ-788).

Protocol 3: to investigate ET-1 binding activity and ETB-
and ETB-receptor subtype distribution in endothelium-denuded
skin arteries and veins. Separate membrane preparations were
obtained from endothelium-denuded arteries and veins dis-
sected from skin specimens, and saturable 125I-ET-1 radioli-
gand receptor binding assays were performed on these mem-
brane preparations.

In a separate study, competitive 125I-ET-1 radioligand
binding assays were performed on membranes obtained from
endothelium-denuded skin arteries and veins in the presence
of varying concentrations (0.1 nM-1 µM/tube) of the ETB-
receptor antagonist BQ-123 or varying concentrations of the
ETB-receptor antagonist BQ-788 and 1 µM BQ-123.

Graphical and Statistical Analysis

A least-squares best-fit program (Graph Pad Prism) was
used on a microcomputer for 1) plotting line graphs for
concentration-dependent effects of drugs on perfusion pres-
sure and calculation of the maximal increase in perfusion
pressure and drug concentration required to produce EC50; 2)
plotting line graphs for data obtained from saturable radioli-
gand receptor binding assays using a one-site binding model
to determine the equilibrium dissociation constant (binding
affinity) and the maximum number of binding sites (binding
capacity); and 3) plotting of line graphs for data obtained
from competitive radioligand receptor binding assays using a
one-site competition model to determine the inhibition po-
tency of the ET-1 receptor antagonist BQ-123, the affinity
of ETB receptors for BQ-123, and the percent binding capacity
for ETB receptors. All data are expressed as means ± SE.
For each experimental protocol, specific statistical analysis
and number of observations are described in the legend of Figs.
1–5 and Tables 1 and 2. Statistical significance was set at P <
0.05 for all tests.

RESULTS

Vasoconstrictor Potency of ET-1

Both ET-1 and NE caused a cumulative concentration-
dependent increase in skin perfusion pressure (Fig. 1). When
10
−5 M ACh was infused at the end of these experi-
ments, the vasoconstrictor effect was reduced by 62 ± 8%.
Thus the constrictor and dilator properties of ET-1 are
lowered by dilator response to ACh.

Fig. 1. Cumulative concentration-dependent vasoconstrictor effects of endothelin (ET)-1 (●), norepinephrine (●), BQ-3020 (▲), sarafotoxin S6c (□), and BQ-3020 in the presence of 10
−5 M N?-nitro-L-
arginine methyl ester (L-NAME) and indomethacin (Indo) (★). Values are means ± SE; n = 4 groups. Baseline perfusion pressure was 46.8 ± 1.6 mmHg.
were intact in the vasculature of these skin flaps. There was no significant difference in maximal increase in perfusion pressure between ET-1 and NE. However, there was a significant (P < 0.01) difference between ET-1 and NE in the dose required for half-maximal increase in skin perfusion pressure, and the calculated apparent affinity was also significantly (P < 0.01) different (Table 1). The skin vasoconstrictor potency of ET-1 was >200-fold that of NE.

ETB-receptor agonists BQ-3020 and S6c did not evoke a vasoconstrictor effect (Fig. 1). Even in the presence of 10\textsuperscript{-5} M L-NAME and Indo, BQ-3020 failed to evoke a significant increase in perfusion pressure. Forty-five minutes of pretreatment of L-NAME and Indo did not change the baseline perfusion pressure of the skin flaps.

Effect of the ET\textsubscript{A}-Receptor Antagonist BQ-123 and the ET\textsubscript{B}-Receptor Antagonist BQ-788 on ET-1-Induced Skin Vasoconstriction

The cumulative concentration-dependent increase in skin perfusion induced by ET-1 (Fig. 2) was not changed significantly in skin flaps pretreated with the selective ET\textsubscript{B}-receptor antagonist BQ-788 (10\textsuperscript{-6} M). However, in skin flaps pretreated with the selective ET\textsubscript{A}-receptor antagonist BQ-123 (5 \times 10\textsuperscript{-6} M), the ET-1-induced increase in perfusion pressure was completely blocked.

ET-1 Binding Activity and ET\textsubscript{A} and ET\textsubscript{B} Receptor Distribution in Membrane Preparations of Endothelium-Denuded Skin Arteries and Veins

ET-1 binding activity. Analysis of the saturation equilibrium binding results indicates that the binding of \textsuperscript{125}I-ET-1 (0–1.5 nM) to two pooled membrane preparations derived from endothelium-denuded skin arteries and veins was concentration dependent (Fig. 4). In arterial and venous membranes, over the concentration range tested, a one-site fit was preferred to a two-site fit model, suggesting that a single class of high-affinity binding sites was present (Fig. 4). The data on equilibrium dissociation constant (binding affinity) for skin arteries (61.2 ± 17.7 pM) and veins (51.6 ± 17.1 pM) and maximum number of binding sites (binding capacity) for skin arteries (2.30 ± 0.17 pmol/mg protein) and veins (2.55 ± 0.27 pmol/mg protein) indicate that the \textsuperscript{125}I-ET-1 binding sites in the skin arteries and veins are of high binding affinity.

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**Table 1.** \(E_{\text{max}}\), EC\textsubscript{50}, and pD\textsubscript{2} for endothelin-1 and norepinephrine in isolated perfused human skin

<table>
<thead>
<tr>
<th>Drug</th>
<th>(E_{\text{max}})</th>
<th>EC\textsubscript{50}, M</th>
<th>pD\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endothelin-1</td>
<td>645 ± 89</td>
<td>6.37 ± 0.56 \times 10^{-9}\textsuperscript{a}</td>
<td>8.20 ± 0.04\textsuperscript{a}</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>500 ± 35</td>
<td>1.46 ± 0.12 \times 10^{-6}</td>
<td>5.84 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 preparations. \(E_{\text{max}}\), maximal increase in perfusion pressure; EC\textsubscript{50}, concentration required to produce half-maximal increase in perfusion pressure; pD\textsubscript{2}, apparent affinity. Comparisons between means for endothelin-1 and norepinephrine were performed using the Wilcoxon rank-sum test. *P < 0.05.
Distribution of \( \text{ET}_A \) and \( \text{ET}_B \) receptors. BQ-123 competed monophasically for binding of \( ^{[125I]} \text{ET-1} \) to membrane preparations from endothelium-denuded skin arteries (5 preparations) and veins (7 preparations) in a concentration-dependent manner, and each of the competition binding curves was well described by a one-site model (Fig. 5). The maximal competitive displacements of \( ^{[125I]} \text{ET-1} \) binding to membrane preparations from skin arteries and veins were 83 ± 2% (\( n = 5 \)) and 78 ± 2% (\( n = 7 \)), respectively (Table 2). The remaining binding of \( ^{[125I]} \text{ET-1} \) in each case was displaced by the \( \text{ET}_B \)-receptor antagonist BQ-788. The mean inhibition concentration and the dissociation constant of BQ-123 were calculated and are shown in Table 2. There was no significant difference in mean inhibition concentration, dissociation constant, or percentage binding to \( \text{ET}_A \) receptor between arterial and venous membrane preparations. The data indicate that BQ-123 displaced \( ^{[125I]} \text{ET-1} \) binding with high affinity in both artery and vein membrane preparations.

**DISCUSSION**

**Major Findings from the Present Studies**

We have investigated the vasoconstrictor activity of ET-1 in human skin, using the in vitro skin perfusion technique and saturable and competitive ET-1 radioligand membrane receptor binding assays. We observed that 1) intra-arterial infusion of ET-1 caused a cumulative concentration-dependent skin vasoconstriction with...
In the present study, the ETA-receptor antagonist albumin also induced vasodilation to a lesser extent specific action in blocking the local vasoconstrictor agonist PD-145065 caused a slight skin vasodilation. ETA/ETB-receptor antagonist BQ-123 but not by the ETB-receptor antagonist BQ-788; 3) ETB-receptor agonists BQ-3020 and S6c did not evoke skin vasoconstriction at 10⁻⁹ to 10⁻⁶ M concentrations; and 4) the ET-1 binding sites in the endothelium-denuded human skin arteries and veins are predominantly high-affinity and high-capacity ETA receptors with an ETA-to-ETB Receptor ratio of 83:17 and 78:22, respectively. These new observations clearly indicate that ET-1 is an extremely potent vasoconstrictor in human skin, and ETA receptors are primarily responsible for ET-1-induced skin vasoconstriction.

**Skin Reaction to Intra-Arterial Infusion of ET-1 and ETₐ- or ETₐ/ ET₌-Receptor Antagonists**

It was reported that intradermal injection of ET-1 in human skin caused a small area of intense pallor or with vasoconstriction at the site of injection and a much larger area of surrounding flare (erythema) associated with increase in skin blood flow (6, 43). The latter was described as “axon flare,” which was partially histamine dependent, and this phenomenon was thought to be relevant to the local response to injury (6). Axon reflex flare was not seen in our skin flap model in which ET-1 was infused intra-arterially, and ET-1 caused a concentration-dependent increase in perfusion pressure (Fig. 1).

It was also observed by other investigators in humans that intradermal injection of the ETA-receptor antagonist PD-147953 and the ETₐ/ET₌-receptor antagonist PD-145065 caused a slight skin vasodilation. However, it is uncertain whether this observation was a specific action in blocking the local vasoconstrictor effect of ET-1 because intradermal injection of saline or albumin also induced vasodilation to a lesser extent (43). In the present study, the ETA-receptor antagonist BQ-123 alone infused intra-arterially did not cause any significant effect on the basal tone in isolated perfused human skin. Intra-arterial BQ-123 also did not have any significant effect on the basal tone of isolated perfused pig lungs (29).

**Vasoconstrictor Potency of ET-1 in Human Skin**

It was reported that the vasoconstrictor potency of ET-1 as assessed by intradermal injection was ~100 times higher than NE and phenylephrine in rabbit and rat skin, respectively (4, 23). We previously demonstrated in isolated perfused pig skin flaps that the vasoconstrictor potency of ET-1 by continuous intra-arterial infusion was ~300-fold of NE (28). Using a similar in vitro skin perfusion technique in the present study, we observed that the vasoconstrictor potency of ET-1 in human skin was ~200-fold of NE (Table 1). In both isolated perfused pig and human skin flap models, we observed that the onset and reversal of the skin vasoconstriction effect of ET-1 were very slow. It required 30–40 min for ET-1 to achieve its maximal vasoconstrictor effect, and complete return to the baseline perfusion pressure was not seen even at 2 h after cessation of ET-1 infusion. Taken together, these observations indicate that ET-1 is a potent and long-acting skin vasoconstrictor in laboratory animals and humans.

**Functional Importance of ETA and ETB Receptor Subtypes in the Mediation of ET-1-Induced Vasoconstriction in Human Skin**

It was 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 133Xe clearance at test sites, and concomitant injection of the ETA-receptor antagonist BQ-123 blocked the vasoconstrictor effect of ET-1 but not IRL-1620. In addition, radioligand binding activity studied by autoradiography indicated that ~40% of ET-1 binding sites were of the ETB subtype. These observations were taken together to indicate that both ETA and ETB receptors mediate ET-1-induced vasoconstriction in rat skin (24). So far, the relative functional importance of ETA and ETB receptors in the mediation of ET-1-induced vasoconstriction in the human skin is unclear. There is evidence to indicate that ETB receptors may also mediate vasoconstriction in human skin. ETA and ETB receptors were identified in microvessels in human skin biopsies by autoradiography, and it was speculated that both receptor subtypes are involved in ET-1-induced vasoconstriction in human skin (18). In addition, it was demonstrated in humans that intra-arterial infusion of the ETB-receptor agonist S6c caused a reduction in forearm blood flow, and dorsal hand vein infusion of S6c caused local venoconstriction (15). Meanwhile, it was demonstrated in humans that 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 ETA/ETB-receptor antagonist PD-145065 did not cause additional attenuation of the ET-1-induced decrease in skin blood flow 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 primary by activation of ETA receptors (43). However, ETB-receptor agonist was not used to confirm these findings. In addition, all drugs used in this study were given extraluminally by intradermal injection. In the present functional study, drugs were infused intra-arterially in stepwise increments in concentration, and skin perfusion and perfusion pressure were monitored. We observed that the nonselective ETA/ETB agonist ET-1 elicited a cumulative concentration-dependent increase in perfusion, but selective ETB-receptor agonists BQ-3020 and S6c did not evoke any significant increase in perfusion pressure over the range of 10⁻⁹ to 10⁻⁶ M concentrations (Fig. 1). BQ-3020 also did not have any significant effect on perfusion pressure even in the presence of a nitric oxide synthase inhibitor (l-NAME) and cyclooxygenase inhibitor (Indo; Fig. 1). We have also demonstrated that the ETA-receptor antagonist BQ-123, but not the ETB-receptor antagonist BQ-788, blocked the ET-1-induced perfusion pressure (Fig. 2). Furthermore, using the dermofluorometry technique, we have also demonstrated that ET-1 significantly reduced skin perfusion compared with the vehicle-treated control, and this skin vasoconstrictor ef-
fect of ET-1 was completely blocked by the ET<sub>A</sub>-receptor antagonist BQ-123 (Fig. 3). Again, the ET<sub>B</sub>-receptor antagonist BQ-788 did not attenuate the skin vasoconstrictor effect of ET-1 (Fig. 3). Our findings from functional studies discussed thus far clearly demonstrated that ET<sub>A</sub> receptors but not ET<sub>B</sub> receptors are the primary mediators of ET-1-induced vasoconstriction in human skin. In addition, results from competitive radioligand membrane receptor binding assays revealed that the ET<sub>A</sub>-to-ET<sub>B</sub> receptor ratio was 83:17 for endothelium-denuded skin arteries and 78:22 for endothelium-denuded skin veins (Table 2). These results corroborated findings from our functional studies that ET<sub>A</sub> receptors are predominantly responsible for mediating the vasoconstrictor effect of ET-1 in human skin. The postreceptor mechanism responsible for ET-1-induced vasoconstriction in human skin vasculature has not been studied. However, we previously observed in isolated perfused pig skin that L-type Ca<sup>2+</sup> channels, phospholipase C, and protein kinase C are involved in ET-1-induced skin vasoconstriction (28).

ET-1 has been implicated in cutaneous vasoconstriction and dermal fibrosis in the early stage of scleroderma (42). ET<sub>A</sub> receptors are known to mediate ET-1-induced cell proliferation (5). Here, we have demonstrated that ET-1-induced vasoconstriction is primarily mediated by ET<sub>A</sub> receptors in human skin. Therefore, it is tempting to speculate that ET<sub>A</sub> rather than ET<sub>B</sub> receptors are involved in the pathogenesis of scleroderma skin.

In summary, using the in vitro skin perfusion technique, we have demonstrated that ET-1 is a very potent skin vasoconstrictor in human skin, and its skin vasoconstrictor effect is primary mediated by ET<sub>A</sub> receptors, with no significant participation from ET<sub>B</sub> receptors. Results from ET-1 radioligand membrane receptor assays also reveal that the predominant ET-1 binding sites in endothelium-denuded human skin arteries and veins are high-affinity ET<sub>A</sub> receptors. These findings provide important insights into the pharmacological intervention of skin vasoconstriction associated with elevated circulating levels of ET-1 in peripheral vascular disease and surgical trauma.

Limitations of the Present Studies

Recent results from other laboratories indicated that BQ-788 may not be a potent and selective ET<sub>B</sub>-receptor antagonist in human tissues (30). This does not seem to be an important limitation in the present study. Specifically, BQ-123, a potent and selective ET<sub>A</sub>-receptor antagonist in human tissue (35), completely blocked the skin vasoconstrictor effect of ET-1 (Fig. 2), and two ET<sub>B</sub>-receptor agonists (BQ-3020, S6c) of different chemical structure did not elicit skin vasoconstriction (Fig. 1). Furthermore, BQ-788 completely displaced the residual <sup>125</sup>I-ET-1 binding, which was not displaced by 10<sup>-6</sup> M BQ-123 in membrane preparations of skin arteries and veins (Fig. 5). Therefore, our assessment of functional importance of ET<sub>B</sub> receptors in human skin was most likely valid.

In humans, ET<sub>A</sub> receptors are primarily responsible for ET-1-induced vasoconstriction in conduit pulmonary arteries (12), but ET<sub>B</sub> receptors mediate ET-1-induced vasoconstriction in pulmonary resistance arteries of 150- to 200-µm diameter (26). This observation suggests that the importance of ET<sub>B</sub> receptors in the mediation of ET-1-induced vasoconstriction may vary with the anatomic location of the blood vessel. In the present studies, saturable and competitive ET-1 radioligand receptor assays were performed on membranes of endothelium-denuded skin arteries and veins of 0.5- to 1.5-mm diameter; thus, it can be argued that these assay results may not truly reflect the ET<sub>A</sub> and ET<sub>B</sub> receptor distribution in skin microvessels, which plays an important role in regulation of skin vascular resistance. However, results from our functional studies with isolated perfused human skin flaps (8 × 18 cm), which incorporate the entire skin microvasculature, clearly indicated that ET<sub>A</sub> receptors primarily mediate ET-1-induced skin vasoconstriction. Therefore, it is most likely that the ET-1 binding sites in the skin microvessels are also predominantly ET<sub>A</sub> receptors as we have demonstrated in small endothelium-denuded skin arteries and veins. Another example of human tissue in which ET<sub>A</sub> receptors also play the predominant role in mediating ET-1-induced vasoconstriction in microvessels has been documented: ET-1-induced vasoconstriction in resistance arteries of human subcutaneous fat (250–350 µm diameters) are also primarily mediated by ET<sub>A</sub> receptors (8).

Oxygenated buffer instead of blood was used to perfuse skin in this study. There is the possibility that the microvascular hemodynamic may not be exactly the same in these two techniques, and blood perfusion is more physiological. However, it is unlikely that the functional importance of ET<sub>A</sub> and ET<sub>B</sub> receptors in the regulation of vascular resistance in the skin could be different between buffer and blood perfusion.

In conclusion, results obtained from the present functional and radioligand receptor binding studies clearly indicate that ET-1 is a very potent vasoconstrictor in human skin, and its vasoconstrictor effect is primarily mediated by ET<sub>A</sub> receptors, with no significant participation from ET<sub>B</sub> receptors.

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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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