British Journal of Pharmacology (2005) 146, 903–912
www.nature.com/bjp

Functional characterization and expression of endothelin receptors in rat carotid artery: involvement of nitric oxide, a vasodilator prostanoid and the opening of K^+ channels in ET_B-induced relaxation

[1]Carlos R. Tirapelli, [2]Debora A. Casolari, [2]Alvaro Yogi, [2]Augusto C. Montezano, [2]Rita C. Tostes, [3]Eurode Legros, [3]Pedro D'Orléans-Juste & *,[4]Ana M. de Oliveira

[1]Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, University of São Paulo (USP), SP, Brazil; [2]Department of Pharmacology, Institute of Biomedical Sciences, USP, São Paulo, SP, Brazil; [3]Department of Pharmacology, Medical School, Institut de pharmacologie de Sherbrooke, Universite de Sherbrooke, QC, Canada and [4]Department of Physics and Chemistry, Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, USP, Ribeirão Preto, SP, Brazil

1 We aimed to functionally characterize endothelin (ET) receptors in the rat carotid artery. mRNA and protein expressions of both ET_A and ET_B receptors, evaluated by reverse transcription–polymerase chain reaction (RT–PCR) and Western immunoblotting, were detected in carotid segments. Immunohistochemical assays showed that ET_B receptors are expressed in the endothelium and smooth muscle cells, while ET_A receptors are expressed only in the smooth muscle cells. In endothelium-denuded vessels, levels of ET_B receptor mRNA were reduced.

2 Vascular reactivity experiments, using standard muscle bath procedures, showed that ET-1 induces contraction in endothelium-intact and -denuded carotid rings in a concentration-dependent manner. Endothelial removal enhanced ET-1-induced contraction. BQ123 and BQ788, selective antagonists for ET_A and ET_B receptors, respectively, produced concentration-dependent rightward displacements of the ET-1 concentration–response curves.

3 IRL1620, a selective agonist for ET_B receptors, induced a slight vasoconstriction that was abolished by BQ788, but not affected by BQ123. IRL1620-induced contraction was augmented after endothelium removal.

4 ET-1 concentration dependently relaxed phenylephrine-precontracted rings with intact endothelium. The relaxation was augmented in the presence of BQ123, reduced in the presence of BQ788 and completely abolished after endothelium removal. IRL1620 induced vasorelaxation that was abolished by BQ788 and endothelium removal, but not affected by BQ123.

5 Preincubation of intact rings with N^G-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), indomethacin or tetraethylammonium (TEA) reduced IRL1620-induced relaxation. The combination of L-NAME, indomethacin and TEA completely abolished IRL1620-induced relaxation while sulfaphenazole did not affect this response. 4-aminopyridine (4-AP), but not apamin, glibenclamide or charybdotoxin, reduced IRL1620-induced relaxation.

6 The major finding of this work is that it firstly demonstrated functionally the existence of both ET_A and ET_B vasoconstrictor receptors located on the smooth muscle of rat carotid arteries and endothelial ET_B receptors that mediated vasorelaxation *via* NO–cGMP pathway, vasodilator cyclooxygenase product(s) and the activation of voltage-dependent K^+ channels.
British Journal of Pharmacology (2005) **146,** 903–912. doi:10.1038/sj.bjp.0706388;
published online 5 September 2005

Keywords: Carotid artery; ET_B receptor; relaxation; endothelin-1

Abbreviations: 4-AP, 4-aminopyridine; BQ123, c(DTrp–Dasp–Pro–Dval–Leu); BQ788, [*N-cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl1-D-1methoxycarbonyltryptophanyl-D-norleucine]; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; ET-1, endothelin-1; IRL1620, {succinyl-[Glu9,Ala11,15]-ET-1(8–210}; L-NAME, *NG*-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; TEA, tetraethylammonium

Introduction

Endothelin-1 (ET-1) is a 21-amino-acid peptide produced by the endothelium, belonging to a family of potent vasoconstrictors (Yanagisawa *et al*., 1988). The actions of ETs are mediated by two receptors named ET_A and ET_B. The ET_A receptor is restricted to vascular smooth muscle and mediates

*Author for correspondence at: Laboratório de Farmacologia, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, CEP 14040-903, Ribeirão Preto, SP, Brazil; E-mail: amolive@usp.br

vasoconstriction (Haynes & Webb, 1993). ET_B receptors were initially described on vascular endothelium, mediating relaxation *via* production of nitric oxide (NO) (Hirata *et al*., 1993) and (or) prostacyclin (PGI_2) (Filep *et al*., 1991). Some studies have shown that in addition to ET_B receptors mediating vasorelaxation (Matsuda *et al*., 1993; Schilling *et al*., 1995) there are also ET_B receptors causing contraction located on vascular smooth muscle (Ihara *et al*. 1991; Deng *et al*., 1995).

Although the functions of ET receptors are well characterized, it has been described that these receptors possess different distribution and functional importance depending on the species and blood vessel. In this line, Caló *et al*. (1996) demonstrated that the rabbit carotid artery is a pure ET_A system, while the rabbit pulmonary artery contains contractile ET_A and ET_B receptors which contribute by approximately 20 and 80%, respectively, to the contraction induced by ETs. In addition, they also found that the rabbit jugular vein is a pure contractile ET_B system. It has been demonstrated previously that human saphenous vein contains both endothelin ET_A and ET_B contractile receptors (White *et al*., 1994). ETs were also described to contract the human isolated umbilical artery *via* stimulation of an ET_A receptor, while both ET_A and ET_B receptors mediate the contraction in the human umbilical vein (Bogoni *et al*., 1996). Similarly, ET_A and ET_B receptors located in the vascular smooth muscle were described to mediate the contractile effect induced by ET in the pig coronary artery (Elmoselhi & Grover, 1997).

Endothelial ET_B receptor mediating relaxation was described in the isolated rat basilar artery (Schilling *et al*., 1995), rat aorta (Fujitani *et al*., 1993) and guinea-pig aorta (Matsuda *et al*., 1993). On the other hand, the selective agonists for ET_B receptors alanine $^{[1,3,11,15]}$ET-1 and sarafotoxin failed to induce relaxation in rat renal artery, further suggesting that ET_B receptors mediating relaxation do not appear to be present in this vascular bed (Clark & Pierre, 1995).

The presence of ET_A and ET_B receptors mRNA was previously identified in the carotid artery of Sprague–Dawley rat (Wang *et al*., 1995; 1996). It was also noted by these authors that the levels of ET_A and ET_B receptors mRNA were elevated in rat carotid arteries after balloon angioplasty. Additionally, the presence of ET_A receptors was demonstrated in the medial layer of rat carotid arteries by use of autoradiography (De Oliveira *et al*., 1995). The authors suggested that in addition to the ET_A receptors a small amount of ET_B receptors could be expressed in this vascular bed. Furthermore, Viswanathan *et al*. (1997) confirmed the presence of ET_A receptors in the medial layer of rat carotid arteries and also verified that ET_A receptor expression was increased in the media of these arteries after balloon angioplasty.

The endothelinergic system has been postulated to have a pathophysiological role in a wide range of diseases (for reviews, please refer to Rubanyi & Polokoff, 1994; D'Orléans-Juste *et al*., 2002). Thus, the study of the physiological expression and function of ET receptors may provide valuable information on the contribution of ETs to the arterial response to injuries. The function of ET receptors in blood vessels could have important implications for the design of ET receptor antagonists for use in vascular diseases in which ETs have been implicated. Although the expression of ET receptors has been described in rat carotid, to our knowledge, there are no reports describing the functionality of these receptors in this vascular bed. The aims of the present study were to attempt a functional characterization of the ET receptors in the rat carotid artery and also to investigate the mechanisms underlying ET_B-induced relaxation. In addition, RT–PCR and Western immunoblotting were performed to detect the mRNA and protein expression of ET_A and ET_B receptors. Immunohistochemical assays were conducted to localize these receptors.

Methods

Reverse transcriptase–polymerase chain reaction

RT–PCR was performed in rat carotid arteries with intact or denuded endothelium. The endothelium was removed mechanically by gently rolling the lumen of the vessels on a thin wire. Endothelial removal was confirmed by RT–PCR for eNOS.

Total cell RNA was isolated from carotid arteries using Trizol Reagent (Gibco BRL, Life Technologies, Rockville, MD, U.S.A.). After DNA digestion (RQ1 DNAse RNAse-free, Promega Corporation, Madison, WI, USA), total RNA (20 ng per sample) was used for RT in the presence of an RNase inhibitor (RnasIn®, Promega Corporation), 200 U of Moloney murine leukemia virus RT (Gibco BRL) and 1 μg of oligo (dT)12–18 primer at 37°C for 60 min, according to manufacturer specifications. The cDNA products were isolated by phenol–chloroform extraction, precipitated with ethanol, resuspended in 120 μl TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) and stored at −20°C until required for the PCR. PCR primers were designed on the basis of published rat cDNA sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ET_A/ET_B receptors, and are as follows: ET_A, antisense primer CTGTGCTGCTCGCCCTTGTA, sense primer GAAGTCGTCCGTGGGCATCA (216-bp fragment); ET_B, antisense primer CACGATGAGGACAATGAGAT, sense primer TTACAAGACAGCCAAAGACT (565-bp fragment); GAPDH, antisense primer CACCACCCTGTTGCTG TA, sense primer TATGATGACATCAAGAAGGTGG (219-bp fragment), eNOS left: TGACCCTCACCGATACAACA, right: CTGGCCTTCTGCTCATTTTC. GAPDH was used as an internal control for the coamplification. In order to identify the optimal amplification conditions, a series of pilot studies were performed using a thermal cycler with a temperature gradient in the annealing step (Eppendorf Mastercycler gradient, Eppendorf-Netheler-Hinz, Hamburg, Germany), various amounts of RT products from 2 to 200 ng RNA, and 20–35 cycles of PCR amplification. The following conditions were selected for PCR in a volume of 50 μl: RT products from 20 ng of RNA, 2.5 U Taq polymerase (Gibco BRL), 30 cycles for ET_A receptor gene, 36 for ET_B receptor gene, 26 cycles for eNOS gene, and 24 cycles for the GAPDH gene. Amplification was carried out using an initial denaturing cycle at 94°C for 5 min and the subsequent cycles as follows: denaturation, 30 s at 94°C; annealing, 30 s at 58°C (ET_B), 66°C (ET_A), 62°C (GAPDH) or 60°C (eNOS), and extension, 45 s at 72°C. PCR products (10 μl per lane) were electrophoresed using 1% agarose gel containing ethidium bromide (0.5 μg ml^{-1}). The identity of cDNA products has been confirmed by DNA sequence analysis. The gel was subjected to ultraviolet light and photographed. The band intensities

were measured using a software package (Kodak Digital Science, Eastman Kodak Company, New Haven, CT, U.S.A.) and the signals are reported relatively to the intensity of GAPDH amplification in each co-amplified sample.

Western immunoblotting

Total protein was extracted from carotid rings with endothelium. The Bradford assay was used to determine protein concentration. Denaturation of proteins was performed in 2 × Laemmli sample buffer by heating to 95°C for 5 min, cooled on ice and followed by a quick spin. Total protein (20 μg) was separated by electrophoresis on 10% SDS polyacrylamide gel and transferred to methanol-activated PVDF membrane (Amersham) in Tris-Glycine buffer containing 20% of methanol. Membranes were blocked on TBST with 8% nonfat dry milk and incubated with rabbit polyclonal antiserum (1 : 200) raised against rat ET_BR (AER-002) and (1 : 100) rat ET_AR (AER-001) (Alomone Labs). COX-1 was used as internal control and detected with rabbit polyclonal antiserum (1/750) (160109) (Cayman). As the second antibody, the donkey polyclonal antiserum against rabbit IgG coupled to horse-radish peroxidase (NA9340V) (Amersham) was used. Visualization of protein bands was carried out with the enhanced chemiluminescence's ECL detection system (Amersham). Densitometric analysis was performed with a densitometer (Gel Doc, Bio-Rad) to determine protein level.

Immunohistochemistry

Carotid arteries were fixed in Methacarn (60% methanol, 30% chloroform, and 10% acetic acid), and paraffin-embedded longitudinal sections (7 μm) were incubated with 3% H_2O_2 and a Pierce solution to block endogenous peroxidase and biotin, respectively. Then, sections were incubated (humidified box, 4°C) with primary polyclonal antibodies against rat ET_A and ET_B receptors (1 : 10 dilution; Alomone Labs Ltd – Jerusalem, Israel) and with a biotin-conjugated secondary anti-rabbit antibody (1 : 1000, Vector Laboratories Inc., Burlingame, CA, U.S.A.) and streptavidin-conjugated peroxidase (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA, U.S.A.). Color was developed by the addition of DAB (Sigma). Sections were lightly stained in hematoxylin, dehydrated with alcohol and xylene, and scored by an independent observer unaware of the groups and treatments of the rats. To evaluate background reaction, procedures were also performed in sections incubated only with the secondary antibody (indirect technique) or in the absence of antibodies (direct technique).

Vascular reactivity studies

Male Wistar rats weighting between 400 and 450 g (100–120 days old) were anesthetized and killed by aortic exsanguination in accordance with standards and policies of the University of Sao Paulo's Animal Care and Use Committee.

The carotid artery was quickly removed, cleaned of adherent connective tissues and cut into 5-mm-length rings. Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (Letica Scientific Instruments) to measure tension in the vessels. The rings were placed in 5-ml organ chambers containing Krebs solution, pH 7.4, gassed with 95% O_2/5% CO_2, and maintained at 37°C. The composition of Krebs solution was as follows (mM): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 15.0; Glucose, 5.5; $CaCl_2$, 2.5. The rings were stretched until an optimal basal tension of 1.0 g, which was determined by length–tension relationship experiments, and then were allowed to equilibrate for 60 min, with the bath fluid being changed every 15–20 min. In some rings, the endothelium was removed mechanically by gently rolling the lumen of the vessel on a thin wire. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μM) in the presence of contractile tone induced by phenylephrine (0.1 μM). For studies of endothelium-intact vessels, the ring was discarded if relaxation with acetylcholine was not 80% or greater. For studies of endothelium-denuded vessels, the rings were discarded if there was any degree of relaxation.

Effects of endothelial removal on ET-1-induced contraction

To verify the influence of the endothelium on the contraction induced by ET-1, concentration–response curves for this peptide (10^{-12}–3×10^{-8} mol l^{-1}) were obtained in endothelium-intact and -denuded rings.

Effects of antagonists on ET-1-induced contraction

Initial studies showed that consecutive reproducible concentration–response curves for ET-1 could not be obtained (data not shown). The contraction induced by the peptide was much smaller following the construction of the first curve, further indicating tachyphylaxis. Therefore, for a given preparation, contractile responses to ET-1 could not be studied in the absence and subsequent presence of an antagonist. Hence, antagonists were added 30 min prior to the construction of the curve and the responses were compared with those observed in time-matched control experiments. Both the selective ET_A (BQ123; Ihara *et al*., 1992) and ET_B (BQ788; Ishikawa *et al*., 1994) receptor antagonists were tested. After incubation with the antagonists, concentration–response curves for ET-1 (10^{-12}–10^{-7} mol l^{-1}) were obtained. Four concentrations of BQ123 (0.01, 0.1, 1 and 3 μM) and three concentrations of BQ788 (0.3, 1 and 3 μM) were tested in endothelium-denuded rings to construct a Schild plot.

IRL1620-induced contraction

To verify whether the selective ET_B receptor agonist IRL1620 (Takai *et al*., 1992), could induce contraction, cumulative concentration–response curves for this peptide (10^{-10}–3×10^{-7} mol l^{-1}) were obtained in endothelium-intact and denuded rings. The contractile responses of IRL1620 were also analyzed in the presence of BQ123 (3 μM) and BQ788 (3 μM).

ET-1 and IRL1620-induced relaxation

Endothelium-intact and -denuded rings were precontracted with phenylephrine, used at concentrations of 0.1 and 0.03 μM, respectively, to induce contractions of similar magnitude. After reaching a stable and sustainable contraction, ET-1 (10^{-14}–3×10^{-11} mol l^{-1}) or IRL1620 (10^{-10}–3–10^{-8} mol l^{-1})

were added cumulatively to the organ bath. In endothelium-intact rings, experiments were conducted in the absence and presence of BQ123 (3 μM) and BQ788 (3 μM). As would be expected owing to their ET receptor specificity, neither BQ123 nor BQ788 changed the resting tension of the tissues or the contractile response induced by phenylephrine.

The mechanisms underlying the relaxant effect induced by IRL1620 were studied in endothelium-intact rings. These mechanisms were evaluated by experiments performed in the presence of N^G-nitro-L-arginine-methyl-ester (NO synthase inhibitor, L-NAME, 100 μM), indomethacin (cyclooxygenase inhibitor, 10 μM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (guanylyl cyclase inhibitor, ODQ, 1 μM), sulfaphenazole (selective blocker of cytochrome P450 2C9, 10 μM; Bussemaker *et al*., 2003). The concentrations of the channel blockers tetraethylammonium chloride (nonselective K^+ channel blocker, TEA, 10 mM), apamin (selective blocker of low-conductance Ca^{2+}-activated channels, 1 μM), glibenclamide (selective blocker of ATP-sensitive K^+ channels, 3 μM), charybdotoxin (selective blocker of large-conductance Ca^{2+}-activated K^+ channels, 0.1 μM) and 4-aminopyridine (selective blocker of voltage-dependent K^+ channels, 4-AP, 1 mM) were used as described by Nelson & Quayle (1995). All drugs were incubated for 30 min before the experimental procedures. Relaxation was expressed as percentage change from the phenylephrine-contracted levels. Since we noted that L-NAME and ODQ enhanced phenylephrine-induced contraction, the rings with intact endothelium exposed to these compounds were precontracted with phenylephrine 0.03 μM, to induce a magnitude of contraction similar to that found in the intact rings not exposed to the inhibitors.

Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, ODQ, glibenclamide, 4-AP, ET-1 (Sigma, St Louis, MO, U.S.A.), L-NAME, TEA, sulfaphenazole (Sigma/RBI, Natick, MA, U.S.A.), indomethacin (Calbiochem, U.S.A.), apamin, BQ123, BQ788, IRL1620 (American Peptide Company, Sunnyvale, CA, U.S.A.) and charybdotoxin (Alomone Labs, Jerusalem, Israel). Glibenclamide and ODQ were prepared as stock solutions in ethanol and DMSO, respectively. Indomethacin was dissolved in Tris buffer (pH 8.4). The other drugs were dissolved in distilled water. The bath concentration of ethanol or DMSO did not exceed 0.5%, which was shown to have no effects *per se* on the basal tonus of the preparations or on the agonist-mediated contraction or relaxation.

Data analysis

Contractions were recorded as changes in the displacement (g) from baseline. Relaxation was expressed as percentage change from the phenylephrine-contracted levels. Agonist concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 2.01; GraphPad Software Inc., San Diego, CA, U.S.A.). Agonist potencies and maximum response are expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximum response) and E_{max} (maximum effect elicited by the agonist), respectively. Concentration ratios (CRs) were determined from EC_{50} values in the presence and absence of the antagonists. The concentration–response curves to the agonist in the presence or absence of the antagonists were analyzed by plotting the negative logarithm of the ratio of concentrations of the agonist that produced the same effect (50% contraction) in the presence and absence of the antagonist minus 1[log(CR−1)] against the negative logarithm of the concentration of antagonists (i.e., Schild plot analysis; Arunlakshana & Schild, 1959). The intercept on the abscissa yields the pA_2 value (negative logarithm of the concentration of antagonist that induces a two-fold rightward shift of the concentration–response curve to the agonist). Statistically significant differences were calculated by one-way analysis of variance (ANOVA) or Student's *t*-test. $P<0.05$ was considered as statistically significant.

Results

Effects of endothelium removal on ET-1-induced contraction

Cumulative concentrations of ET-1 induced contraction of both endothelium-intact and -denuded rat carotid arteries in a concentration-dependent manner (Figure 1). Removal of functional endothelium lead to an enhancement in the E_{max} values (0.63 ± 0.02 g) when compared to intact rings (0.44 ± 0.02 g) ($P<0.05$, Student's *t*-test). Conversely, no differences in the pD_2 values between denuded (8.95 ± 0.12) and intact rings (8.72 ± 0.05) were found.

ET_A and ET_B receptor mRNA expression

The results obtained by RT–PCR show that rat carotid arteries with endothelium express mRNA for both ET_A and ET_B receptors. Similarly, mRNAs for ET_A and ET_B receptors were observed in endothelium-denuded carotid rings. However, endothelial denudation reduced the levels of mRNA for ET_B receptors (Figure 2a). Endothelial denudation was confirmed

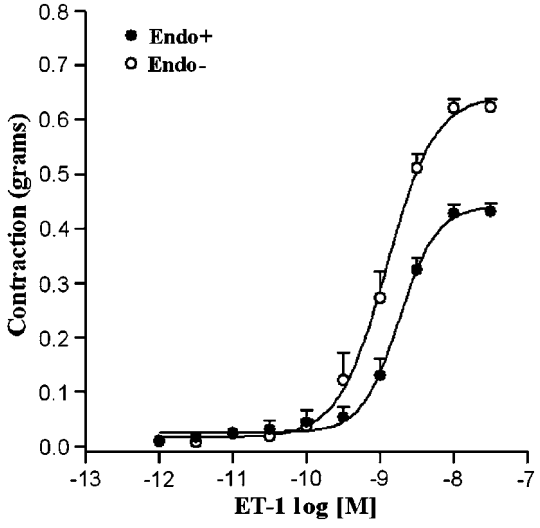

Figure 1 Concentration–response curves for ET-1 obtained in isolated rat carotid rings in the presence (Endo+) and absence (Endo−) of endothelium. Values are means±s.e.m.; $n=9$ for Endo+ and $n=6$ for Endo− rings.

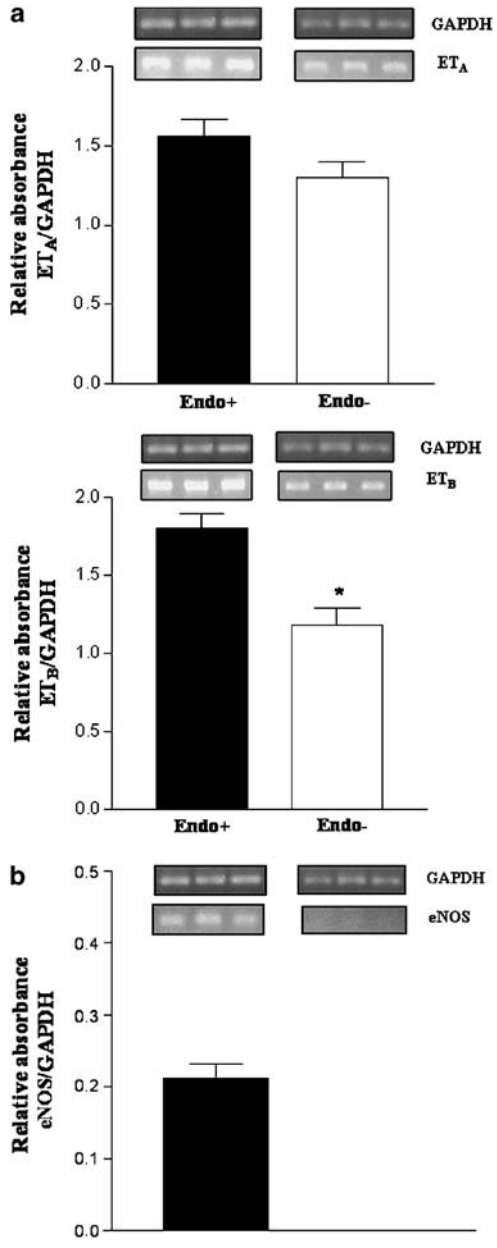

Figure 2 Representative RT–PCR products of 20 ng total RNA extracted from carotid arteries of Wistar rats. The bar graphs show the relative absorbance values of ET_A and ET_B receptor bands in endothelium intact (Endo+) or denuded (Endo−) rings (a) and the relative absorbance values of eNOS bands (b). ET_A and ET_B values were normalized to the corresponding GAPDH values, used as internal standard. Results are reported as means±s.e.m. and are representative of three experiments.

by the absence of mRNA for eNOS (Figure 2b) and Von Willebrand factor (data not shown).

Expression and localization of ET_A and ET_B receptors in the rat carotid artery

Western immunoblotting assays detected the protein expression of ET_A and ET_B receptors in the rat carotid. The expression of ET_A receptors was not significantly different from that found for ET_B receptors (Student's *t*-test) (Figure 3). The immunohistochemical studies revealed intense staining for

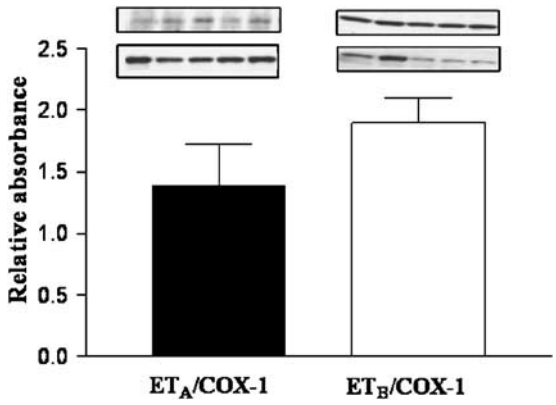

Figure 3 Representative Western immunoblotting products of 20 μg total protein extracted from endothelium-intact carotid arteries of Wistar rats. The bar graphs show the relative absorbance values of ET_A and ET_B receptor bands. Values were normalized by the corresponding COX-1 bands, used as internal standard. Results are reported as means±s.e.m. and are representative of five experiments.

ET_A and ET_B immunoreactivities in smooth muscle cells. In endothelial cells, positive immunostaining for ET_B, but not for ET_A, receptors was detected (Figure 4).

Effects of antagonists on ET-1-induced contraction

BQ123 (0.1, 1 and 3 μM) produced concentration-dependent rightward displacements of the ET-1 concentration–response curves with reduction of the maximal response (Figure 5a). Schild analysis yielded pA_2 value for BQ123 of 7.07±0.33 and a slope of 0.36±0.10, which was significantly different from the unity. Similarly, BQ788 (1 and 3 μM) displaced the curves for ET-1 to the right (Figure 5b). Schild analysis yielded pA_2 values for BQ788 of 6.20±0.10 and a slope of 0.71±0.10, which was significantly different from unity. The combination of BQ123 and BQ788 resulted in a greater antagonism than that exerted by the antagonists when added individually. The pD_2 of ET-1 concentration–response curves, as well as the E_{max} values observed in the absence and presence of BQ123 and BQ788, is given in Table 1.

IRL1620-induced contraction

The cumulative application of the selective ET_B agonist IRL1620 induced a small contraction of intact rings, which was blocked by BQ788 but not BQ123 (Figure 6). The contraction induced by IRL1620 was greater in endothelium-denuded than in intact rings. The pD_2 of IRL1620, as well as the E_{max} values observed in the absence and presence of BQ123 and BQ788, are given in Table 2.

ET-1 and IRL1620-induced relaxation

Figure 7a shows that preincubation of the rings with BQ788 (E_{max}: 23.45±1.66%; $n=5$) reduced ET-1-induced relaxation (E_{max}: 50.30±2.51%; $n=7$) ($P<0.01$), while BQ123 enhanced the relaxation (E_{max}: 65.79±6.02%; $n=8$) ($P<0.01$). Denudation of the endothelium abolished the relaxation induced by ET-1 (E_{max}: 7.53±3.07%; $n=5$) ($P<0.01$). The pD_2 values of ET-1 concentration–response curves in the presence of BQ788

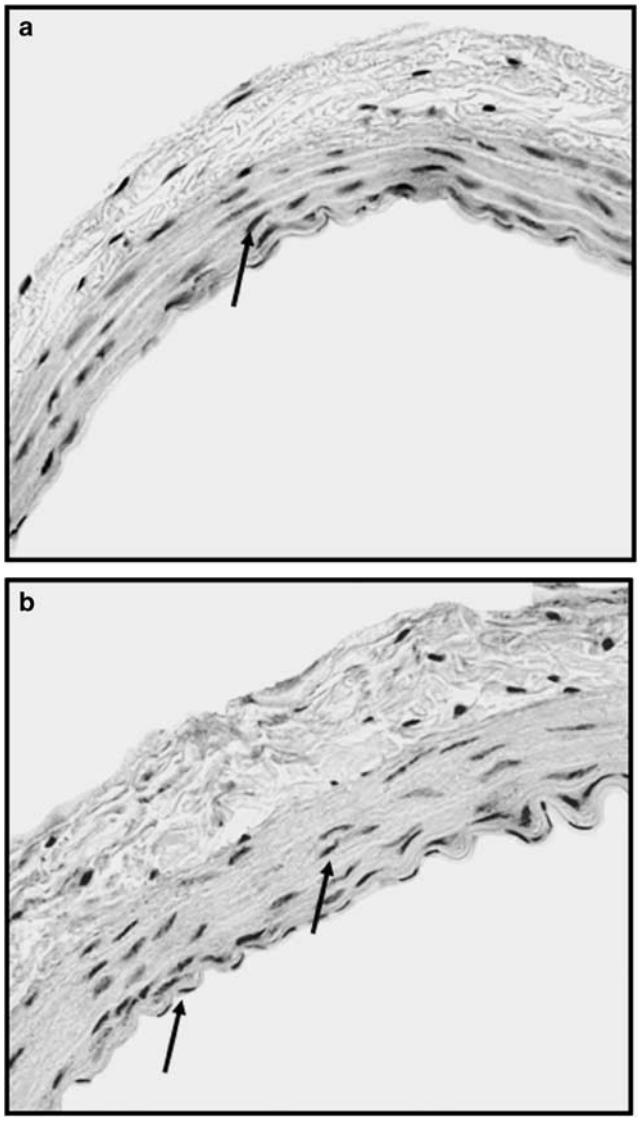

Figure 4 Representative immunohistochemical photomicrographs of ET_A (a) and ET_B (b) receptors in rat carotid artery sections. Arrows indicate expression of ET_A receptor in smooth muscle cells and ET_B in both endothelial and smooth muscle cells.

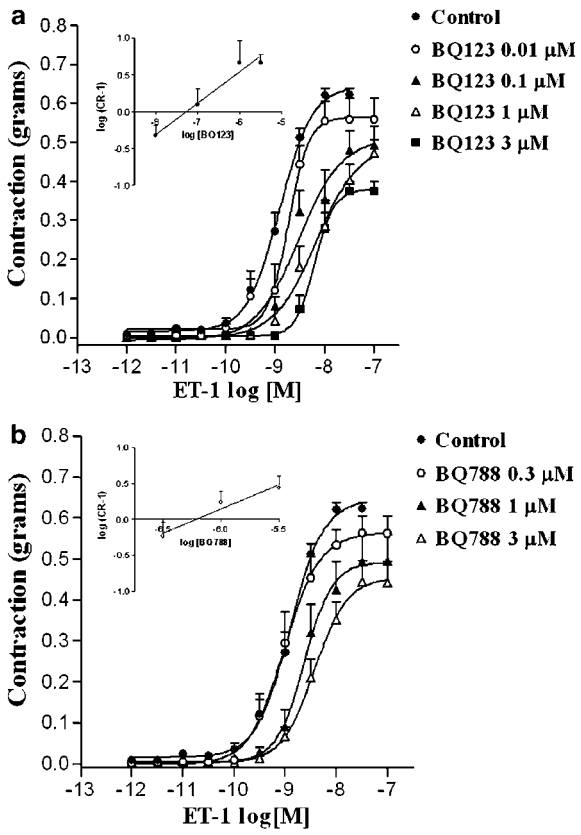

Figure 5 Concentration–response curves for ET-1 obtained in endothelim-denuded rat carotid rings in the absence or presence of different concentrations of BQ123 (a) or BQ788 (b). Values are means ± s.e.m. Six preparations were used in each group.

Table 1 E_{max} and pD_2 values for ET1-induced contraction of endothelium-denuded rat carotid rings in the absence or presence of different concentrations of BQ123 and BQ788

Groups	E_{max} *(g)*	*pD_2*
ET-1	0.63 ± 0.02	8.95 ± 0.12
ET-1 + BQ123 (0.01 μM)	0.56 ± 0.06	8.76 ± 0.12
ET-1 + BQ123 (0.1 μM)	0.49 ± 0.05*	8.46 ± 0.13*
ET-1 + BQ123 (1 μM)	0.45 ± 0.04*	8.03 ± 0.22**
ET-1 + BQ123 (3 μM)	0.37 ± 0.03**	8.15 ± 0.08**
ET-1 + BQ788 (0.3 μM)	0.56 ± 0.04	9.00 ± 0.11
ET-1 + BQ788 (1 μM)	0.50 ± 0.07	8.59 ± 0.08*
ET-1 + BQ788 (3 μM)	0.44 ± 0.05*	8.46 ± 0.10*
ET-1 + BQ123 (3 μM) + BQ788 (3 μM)	0.08 ± 0.02**	—

Values are means ± s.e.m. Six preparations were used in each group. *$P<0.05$ vs control (ET-1); **$P<0.01$ vs control (ANOVA).

(13.28 ± 0.16) or BQ123 (13.26 ± 0.19) were not significantly different from that found in the absence of the antagonists (12.85 ± 0.36).

The relaxation induced by IRL1620 in endothelium-intact rings (E_{max}: 45.32 ± 3.34%; $n=8$) was significantly reduced ($P<0.01$) in the presence of BQ788 (3 μM) (E_{max}: 10.93 ± 3.96%; $n=6$), but not in the presence of BQ123 (3 μM) (E_{max}: 46.42 ± 6.11%; $n=5$). Denudation of the endothelium abolished ($P<0.01$) the relaxation induced by IRL1620 (E_{max}: 2.35 ± 3.80%; $n=6$). No differences were found between the pD_2 values of endothelium-intact rings in the absence (pD_2: 8.76 ± 0.24) or presence of BQ123 (pD_2: 9.08 ± 0.14) (Figure 7b).

In order to verify the mechanisms underlying ET_B-induced relaxation, endothelium-intact rings were exposed to a variety of drugs. When added alone, L-NAME or indomethacin reduced IRL1620-induced relaxation to a similar extent. The combination of these two compounds showed further suppression than that observed with either L-NAME or indomethacin alone. However, even when added together, these compounds were not able to abolish IRL1620-induced relaxation. TEA also reduced the relaxation induced by IRL1620. The combination of TEA, L-NAME and indomethacin completely abolished IRL1620-induced relaxation. Also, the guanylyl cyclase inhibitor ODQ reduced ET_B-induced relaxation. However, sulfaphenazole, a selective blocker of CPY2C9, did

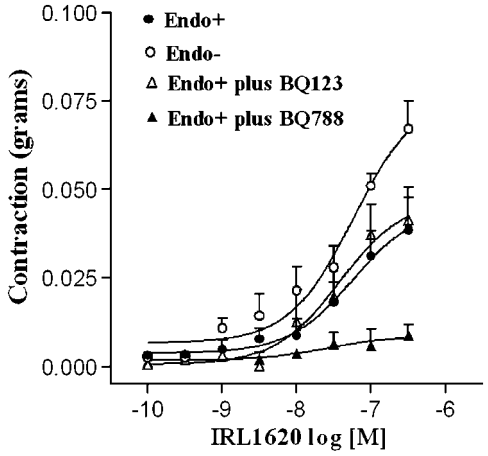

Figure 6 Concentration–response curves for IRL1620 obtained in endothelium-denuded (Endo−) and endothelium-intact (Endo+) rat carotid rings in the absence or presence of BQ123 (3 μM) and BQ788 (3 μM). Values are means±s.e.m. of 5–7 independent preparations.

Table 2 E_{max} and pD_2 values for IRL1620-induced contraction of endothelium-denuded (Endo−) and endothelium-intact (Endo+) rat carotid in the absence or presence of BQ123 and BQ788

Groups	E_{max} *(g)*	pD_2	n
Endo+	0.040±0.009	7.27±0.26	7
Endo−	0.067±0.008*	8.03±0.21	6
Endo+ plus BQ123 (3 μM)	0.041±0.009	7.43±0.21	5
Endo+ plus BQ788 (3 μM)	—	—	5

Values are means±s.e.m. of *n* preparations. *$P<0.05$ vs Endo+ (ANOVA).

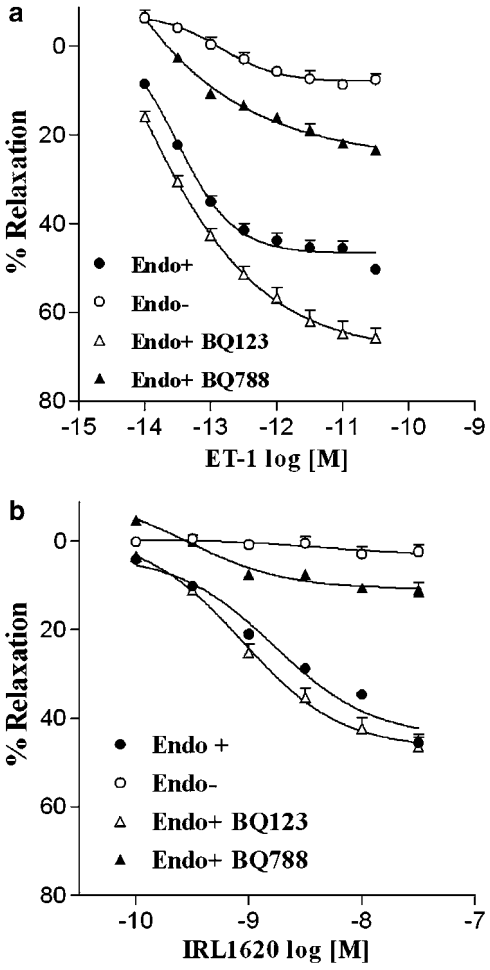

Figure 7 Relaxation responses induced by ET-1 (a) and IRL1620 (b) on rat carotid rings precontracted with phenylephrine. The concentration–response curves for both agonists were obtained in endothelium-denuded (Endo−) and endothelium-intact rings (Endo+) in the absence or presence of BQ123 (3 μM) and BQ788 (3 μm). Values are means±s.e.m. of 5–8 independent preparations.

not alter IRL1620-induced relaxation. Finally, the vasodilatory response induced by IRL1620 was reduced by 4-AP, whereas apamin, glibenclamide or charybdotoxin had no effect in this response (Figure 8). The mean pD_2's of the relaxant IRL1620 concentration–response curves, as well as the E_{max} values, in the absence or presence of the previously mentioned inhibitors, are given in Table 3.

Discussion

In the present study, mRNA expression of both ET_A and ET_B receptors was detected in carotid segments. Endothelial removal reduced mRNA expression of ET_B, but not ET_A, receptors, further indicating the presence of mRNA for ET_B receptors in the vascular endothelium. By using Western immunoblotting, we demonstrated the protein expression of both ET_A and ET_B receptors in rat carotid artery with endothelium. Immunohistochemical assays showed that ET_B receptors are expressed in the endothelium and smooth muscle cells of rat carotid, while ET_A receptors are expressed exclusively in the smooth muscle cells.

A pharmacological characterization of the receptors mediating the contractile effects of ETs in endothelium-denuded rat carotid arteries has been attempted by use of the classical criteria recommended by Schild, namely the order of potency of agonists and the affinities of competitive antagonists. The selective antagonist for ET_A receptors BQ123 produced a rightward displacement of ET-1-induced curves in a concentration-dependent manner. Interestingly, this effect was followed by a decrease in E_{max}, as described previously (Zellers *et al*., 1994; Bogoni *et al*., 1996; Dieye & Gairard, 2000), resulting in a Schild plot slope less than unity. Some possibilities have been postulated to explain this: (1) tachyphylaxis to the agonist, (2) equilibrium conditions between the antagonist and receptors have not been attained, (3) the interaction between the antagonist and receptors is not reversible, (4) the antagonist is not competitive and (5) heterogeneous receptor population are observed (Kenakin, 1981; 1992). We noted that two consecutive curves for ET-1 could not be obtained in the same tissue. Thus, stimulation with ET-1 was performed in parallel vessels to avoid tachyphylaxis. The interaction between the ET-1 antagonist and its receptors is reversible and BQ123 acts as a competitive antagonist (Ihara *et al*., 1992). Finally, BQ123 was left in

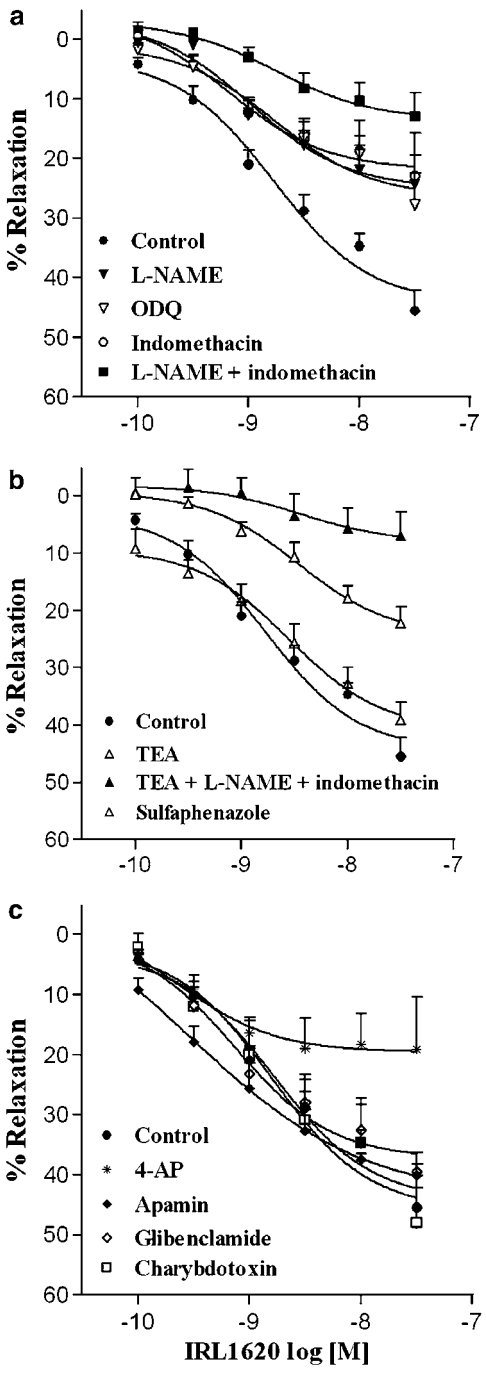

Figure 8 Relaxation responses induced by IRL1620 on endothelium-intact rat carotid rings precontracted with phenylephrine. The concentration–response curves were obtained in the absence (control) or in the presence of L-NAME (100 μM), ODQ (1 μM), indomethacin (10 μM) (a), TEA (10 mM), sulfaphenazole (10 μM) (b), 4-AP (1 mM), glibenclamide (3 μM), apamin (1 μM) or charybdotoxin (0.01 μM) (c). The rings were pre-incubated with the drugs for 30 min. Values are means $\pm$ s.e.m. of 6–8 independent preparations.

Table 3 E_{max} and pD_2 values for IRL1620-induced relaxation of endothelium-intact rat carotid in the absence (Control) or presence of different drugs

Groups	E_{max} *(% relaxation)*	pD_2	n
Control	45.52 $\pm$ 3.34	8.76 $\pm$ 0.24	8
L-NAME (100 μM)	26.29 $\pm$ 5.07*	9.01 $\pm$ 0.16	7
ODQ (1 μM)	27.79 $\pm$ 5.27*	9.11 $\pm$ 0.06	7
INDO (10 nM)	22.93 $\pm$ 7.16*	8.87 $\pm$ 0.60	6
L-NAME (100 μM) + INDO (10 μM)	13.57 $\pm$ 3.78**	8.75 $\pm$ 0.10	6
TEA (10 mM)	24.02 $\pm$ 3.90*	8.63 $\pm$ 0.18	7
L-NAME (100 μM) + INDO (10 μM) + TEA (10 mM)	7.24 $\pm$ 3.70**	—	6
Sulfaphenazole (10 μM)	40.01 $\pm$ 3.10	8.55 $\pm$ 0.20	5
4-AP (1 mM)	18.62 $\pm$ 6.95**	8.48 $\pm$ 0.42	8
Apamin (1 μM)	41.25 $\pm$ 1.15	9.07 $\pm$ 0.20	6
Glibenclamide (3 μM)	42.90 $\pm$ 3.88	8.91 $\pm$ 0.26	7
Charybdotoxin(0.1 μM)	47.99 $\pm$ 7.87	8.79 $\pm$ 0.14	6

Values are means $\pm$ s.e.m. of n preparations. *$P<0.05$ vs Endo+; **$P<0.01$ vs Endo+ (ANOVA).

contact with the carotids for 30 min, which is a period of incubation sufficient to achieve the equilibrium conditions (Ihara *et al*., 1992; Clark & Pierre, 1995). Thus, four of the five possibilities raised were excluded. Schild plots yielding slopes of less than unity have also been obtained with BQ123 in other tissues (Schoeffter *et al*., 1993; Riezebos *et al*., 1994), and such findings were interpreted as an indicative of receptor heterogeneity. In our experiments, the most plausible explanation for a slope less than unity is a heterogeneous population of ET receptors mediating contraction. In support of this proposal, BQ788, a selective antagonist for ET_B receptors, produced a rightward displacement of the concentration–response curves for ET-1. Furthermore, a combination of BQ123 and BQ788 resulted in a greater antagonism than that exerted by the antagonists when added individually, further supporting the idea that ET_A and ET_B receptors are involved in ET-1-induced contraction.

IRL1620, a selective agonist for the ET_B receptors (Takai *et al*., 1992), evoked contractions of rat carotid arteries, albeit in only approximately 70% of the preparations tested. BQ788, but not BQ123, inhibited IRL1620-induced contraction, confirming that the contraction is mediated by ET_B receptors. Removal of the endothelium significantly enhanced IRL1620-induced contraction, further suggesting that the endothelium counteracts the contraction induced by ET_B receptors. The reason(s) why IRL1620 did not contract all the arteries tested is unclear, albeit it is unlikely related to tissue damage, since phenylephrine added to the tissues at the end of the concentration–response curves to the ET_B agonist produced a contractile response similar in magnitude to that obtained at the beginning of the experiment. This result is in line with previous observations of White *et al*. (1994) in human saphenous vein showing that the endothelin ET_B receptor-selective agonists [$Ala^{1,3,11,15}$]endothelin-1 and sarafotoxin S6c only produced contractions in 50% of the preparations tested. The authors suggested that this response occurs because the proportion of ET_A and ET_B receptors varies among vessels and/or along the length of an individual vessel. IRL1620 produced about 10% of the total contraction induced by ET-1 in both endothelium-intact and -denuded carotid rings. A small contraction induced by IRL1620 was also reported in rat (Sharifi & Schiffrin, 1996) and porcine (Elmoselhi & Grover, 1997) arteries. Our data indicate that in the rat carotid ET-1 acts predominantly on the ET_A receptor to induce contraction, while ET_B receptors play a minor role.

Apart from their potent vasoconstrictor responses, ETs have also been shown to produce endothelium-dependent relaxation (Matsuda *et al*., 1993; Zellers *et al*., 1994) *via* ET_B receptors (Auguet *et al*., 1993; Fujitani *et al*., 1993). ET-1 induced endothelium-dependent relaxation that was increased in the presence of BQ123, but reduced by BQ788, indicating that ET-1-induced relaxation is mediated by endothelial ET_B receptors. These data also suggest that ET_A receptors in the smooth muscle cells counteract the relaxant response induced by the activation of endothelial ET_B receptors. Relaxation induced by ET-1, in a similar concentration range of that employed in the present study, has been described previously (Zellers *et al*., 1994). Frelin & Guedin (1994) hypothesized, regarding the response of the ET-1-induced biphasic action (vasodilation followed by constriction), that ET-1 first stimulates endothelial ET_B receptors and occupies all these receptors and then diffuses into the media to act on receptors on smooth muscle.

IRL1620-induced relaxation was not altered by BQ123, but reduced in the presence of BQ788, confirming that the endothelium-dependent relaxation induced by IRL1620 is mediated by ET_B receptors. IRL1620 induced relaxation in a low concentration when compared to that necessary to induce contraction. A possible explanation for this observation is that ET_B receptors found on the vascular smooth muscle possess far less affinity for ET_B receptors selective agonists than the supersensitive receptor moiety located on the endothelium (Sokolovsky *et al*., 1992).

ET_B receptors were described to mediate relaxation *via* production of NO (Hirata *et al*., 1993) and vasodilator cyclooxygenase product(s) (Filep *et al*., 1991). We found that L-NAME as well as indomethacin partially, but significantly, reduced ET_B-mediated relaxation, further indicating that activation of NO synthase and vasodilator cyclooxygenase product(s) plays a role in ET_B-mediating relaxation. The selective inhibitor of guanylyl cyclase enzyme, ODQ (Garthwaite *et al*., 1995), reduced the vasorelaxant action of IRL1620, confirming the involvement of the NO–cGMP pathway in ET_B-mediated vasorelaxation, as observed previously (Fujitani *et al*., 1993). When L-NAME and indomethacin were simultaneously added, further additional inhibitory effect on IRL1620-induced relaxation was observed. However, the relaxant response was not completely abolished, indicating that factors unrelated to the production of vasodilator cyclooxygenase product(s) or NO synthase activation also participate in this response.

TEA, a nonselective blocker of K^+ channels, significantly reduced IRL1620-induced relaxation. When added simultaneously, L-NAME, indomethacin and TEA completely abolished ET_B-mediated relaxation, indicating that cyclooxygenase product(s), NO and K^+ channels are involved in this response. Sulfaphenazole, a specific inhibitor of cytochrome P450 2C9 (CYP2C9) epoxygenase, a putative EDHF candidate (Archer *et al*., 2003), had no effect on IRL1620-induced relaxation, suggesting that CYP2C9 metabolites of arachidonic acid could not account for the ET_B-mediated relaxation in this vascular bed. The lack of effect of sulfaphenazole is in agreement with the nonsignificant effect of apamin and charybdotoxin, because epoxyeicosatrienoic acids have been reported to exert their effects through activation of BKC_a channels on smooth muscle cells (Archer *et al*., 2003).

We found that 4-AP, but not apamin, glibenclamide or charybdotoxin, reduced IRL1620-induced relaxation, indicating that the activation of K_V channels plays a role in ET_B-mediated relaxation. Although a rise in Ca^{2+} is known to occur upon activation of endothelial ET_B receptors, Ca^{2+}-activated K^+ channels do not participate in this response in the rat carotid, which is in agreement with previous studies in rat isolated extrapulmonary and intrapulmonary arteries (Higashi *et al*., 1997), and in the perfused lung (Hasunuma *et al*., 1990; Muramatsu *et al*., 1999), where activation of K_{ATP} channels was described. Our data show that ET_B-induced relaxation is reduced by the K^+ channel blockers TEA and 4-AP, suggesting that ET-1 may cause membrane hyperpolarization by opening K^+ channels as described previously in the rat mesenteric artery (Nakashima & Vanhoutte, 1993a, b).

In summary, the present study shows that rat carotid artery contains a heterogeneous population of contractile ET_A and ET_B receptors located in the vascular smooth muscle and endothelial ET_B receptors that mediated vasorelaxation *via* NO–cGMP pathway, vasodilator cyclooxygenase product(s) and the activation of K_V channels. The study of the expression and function of ET receptors may provide valuable information on the contribution of ETs to the arterial response to injuries since the endothelinergic system has a pathophysiological role in a wide range of diseases.

We are most grateful to Professor Lusiane M. Bendhack (Faculty of Pharmaceutical Sciences-USP) for the kind gift of ODQ and Marcio A.F. de Godoy for helpful scientific discussion. Our thanks are also expressed to Mirian C.C. de Melo, Juliana A. Vercesi, Mayara S. Gomes and Eleni L.T. Gomes for their technical assistance. This work was supported by FAPESP (Brazil) and the Canadian Institutes for Health Research Sciences.

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(Received April 1, 2005
Revised May 5, 2005
Accepted August 5, 2005
Published online 5 September 2005)