British Journal of Pharmacology (2009), 157, 568–580

0007-1188/09
www.brjpharmacol.org

THEMED SECTION: ENDOTHELIUM IN PHARMACOLOGY

RESEARCH PAPER

Increased endothelin-1 reactivity and endothelial dysfunction in carotid arteries from rats with hyperhomocysteinemia

CR de Andrade[1], PF Leite[2], AC Montezano[3], DA Casolari[3], A Yogi[3], RC Tostes[3], R Haddad[4], MN Eberlin[5], FRM Laurindo[2], HP de Souza[6], FMA Corrêa[1], AM de Oliveira[7]

[1]*Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil,* [2]*Heart Institute (InCor), School of Medicine, University of São Paulo, São Paulo, SP, Brazil,* [3]*Department of Pharmacology, Institute of Biomedical Sciences, USP, São Paulo, SP, Brazil,* [4]*School of Medical Sciences, UNICAMP, Campinas, SP, Brazil,* [5]*Institute of Chemistry, UNICAMP, Campinas, SP, Brazil,* [6]*Emergency Medicine Department, School of Medicine, University of São Paulo, São Paulo, SP, Brazil, and* [7]*School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil*

Background and purpose: There are interactions between endothelin-1 (ET-1) and endothelial vascular injury in hyperhomocysteinemia (HHcy), but the underlying mechanisms are poorly understood. Here we evaluated the effects of HHcy on the endothelin system in rat carotid arteries.
Experimental approach: Vascular reactivity to ET-1 and ET_A and ET_B receptor antagonists was assessed in rings of carotid arteries from normal rats and those with HHcy. ET_A and ET_B receptor expression was assessed by mRNA (RT-PCR), immunohistochemistry and binding of [^{125}I]-ET-1.
Key results: HHcy enhanced ET-1-induced contractions of carotid rings with intact endothelium. Selective antagonism of ET_A or ET_B receptors produced concentration-dependent rightward displacements of ET-1 concentration response curves. Antagonism of ET_A but not of ET_B receptors abolished enhancement in HHcy tissues. ET_A and ET_B receptor gene expressions were not up-regulated. ET_A receptor expression in the arterial media was higher in HHcy arteries. Contractions to big ET-1 served as indicators of endothelin-converting enzyme activity, which was decreased by HHcy, without reduction of ET-1 levels. ET-1-induced Rho-kinase activity, calcium release and influx were increased by HHcy. Pre-treatment with indomethacin reversed enhanced responses to ET-1 in HHcy tissues, which were reduced also by a thromboxane A_2 receptor antagonist. Induced relaxation was reduced by BQ788, absent in endothelium-denuded arteries and was decreased in HHcy due to reduced bioavailability of NO.
Conclusions and implications: Increased ET_A receptor density plays a fundamental role in endothelial injury induced by HHcy. ET-1 activation of ET_A receptors in HHcy changed the balance between endothelium-derived relaxing and contracting factors, favouring enhanced contractility.
British Journal of Pharmacology (2009) **157,** 568–580; doi:10.1111/j.1476-5381.2009.00165.x; published online 9 April 2009

This article is part of a themed section on Endothelium in Pharmacology. For a list of all articles in this section see the end of this paper, or visit: http://www3.interscience.wiley.com/journal/121548564/issueyear?year=2009

Keywords: hyperhomocysteinemia; carotid artery; endothelin-1; vasoconstriction; ET_A receptors; relaxation, ET_B receptors; nitric oxide; calcium

Abbreviations: BQ123, c(DTrp – Dasp-Pro-Dval-Leu); BQ788, [N-cis-2,6-dimethyl-piperidinocarbonyl-L-J-methylleucyl1-D-1methoxycarbonyl tryptophanyl-D-norleucine]; ECE, endothelin-converting enzyme; ET-1, endothelin-1; HHcy, hyperhomocysteinemia; IRL1620, {succinyl-[Glu9,Ala11,15]-ET-1(8-210)}; L-NAME, N^G-nitro-L-arginine methyl ester; SQ29548, ([1S-[1alpha-2alpha(Z)3alpha,4alpha]]-7-[3-[(phenylamino)carbonyl]hydrazino] methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid); TEA, tetraethylammonium; TXA_2, thromboxane A_2

Correspondence: Ana Maria de Oliveira, Universidade de São Paulo, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Laboratório de Farmacologia, Avenida do Café s/n, CEP 14040-903, Ribeirão Preto, SP, Brazil, E-mail: amolive@usp.br
Received 28 February 2008; revised 21 August 2008; accepted 11 December 2008

Introduction

Hyperhomocysteinemia (HHcy) is considered to be an independent risk factor for cardiovascular disease (Pisciotta *et al.*, 2005). Many recent clinical studies have observed a positive association between increased plasma homocysteine and atherosclerosis (Weiss *et al.*, 2003; Au-Yeung *et al.*, 2004; Dusitanond *et al.*, 2005). Experiments in animals demonstrated that homocysteine could be a pathogenic factor responsible for arterial damage, such as cell proliferation, increased matrix formation and endothelial injury (Tyagi *et al.*, 1998; Pruefer *et al.*, 1999; Chen *et al.*, 2000). The underlying molecular mechanisms remain conjectural and are likely to be multifactorial.

The adverse vascular effects of homocysteine on endothelial function appear to be an increased oxidant stress with a depletion of biologically active NO (de Andrade *et al.*, 2006). Although decreased NO bioavailability has generally been considered the leading process, recent evidence suggests that raised concentrations of endothelin might have a role in such vascular injury. There is a growing body of evidence suggesting that the endothelin system is affected by homocysteine (Drunat *et al.*, 2001; 2002; Demuth *et al.*, 2002). In particular, the treatment of human endothelial cells with non-cytotoxic concentrations of homocysteine leads to a dose-dependent decrease in both mRNA levels and endothelin-1 (ET-1) secretion (Demuth *et al.*, 1999).

ET-1, the predominant member of the endothelin peptide family, is mainly synthesized by endothelial and vascular cells from a large precursor, prepro-ET-1, which is converted through enzymatic pathways into ET-1, a 21-amino-acid polypeptide (see Masaki, 1995). ET-1 has potent vasoconstrictor, mitogenic and pro-inflammatory properties and is implicated in numerous cardiovascular diseases (Yanagisawa *et al.*, 1988; Tostes and Muscara, 2005). We previously demonstrated 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 responsible for ET-1-induced vasorelaxation via the NO-cGMP pathway, vasodilator cyclooxygenase product(s) and the activation of voltage-dependent K^+ (K_V) channels (Tirapelli *et al.*, 2005).

Despite evidence showing an interaction between HHcy and ET-1 on endothelial function, the underlying mechanisms were not fully clarified. Particularly, we aimed to investigate the effects of HHcy on endothelin receptors and the interactions of HHcy with mediators of vascular reactivity.

Methods

Homocysteine diet-induced HHcy

All animal procedures and the experimental protocols were carried out in accordance with the standards and policies of the University of São Paulo Animal Care and Use Committee, Brazil. Male Wistar rats (total 51) were provided by the Central Biotery of University of São Paulo – Campus Ribeirão Preto, and housed at the Biotery of Faculty of Pharmaceutical Sciences of Ribeirão Preto (FCFRP-USP), and kept on a 12 h light/12 h dark lighting schedule, at 23 ± 2°C, with food and water *ad libitum*. HHcy was induced by feeding rats a diet rich in homocysteine (equivalent to 1 g·kg^{-1}) for 15 days, as previously described (de Andrade *et al.*, 2006). After the 15 days, the HHcy group of rats weighed less (312 ± 5 g) than the age-matched control diet group (338 ± 6 g), although they were the same weights at the start of the diet (HHcy, 301 ± 9 g; control, 298 ± 12 g; $n < 15$ for both groups).

Determination of plasma homocysteine levels

Blood samples from control and HHcy rats were collected into EDTA and centrifuged at 3000× *g* for 20 min. In order to minimize the release of homocysteine from blood cells, iced tubes were used to collect blood and centrifugation was carried out at 4°C. Plasma was then stored at –70°C until assayed. Total homocysteine concentration was measured by mass spectrometry using the Q-TRAP system (de Andrade *et al.*, 2006; Haddad *et al.*, 2006).

Functional study of carotid rings

Animals were anaesthetized and killed by aortic exsanguination. The arteries were prepared for organ bath studies as described previously (de Andrade *et al.*, 2006). The composition of the Krebs solution was (mmol·L^{-1}): NaCl, 118.4; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; glucose, 11.6; $CaCl_2$, 1.9.

The rings were stretched to an optimal basal tension of 1 g, which was determined by length–tension relationship experiments. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (1 μmol·L^{-1}) in the presence of contractile tone induced by phenylephrine (0.1 μmol·L^{-1}), the ring was discarded if relaxation with acetylcholine was less than 80%. For studies using endothelium-denuded vessels, the rings were discarded if there was any degree of relaxation.

Effects of HHcy on ET-1-induced contraction

To delineate the effect of HHcy on the contraction induced by ET-1, concentration–response curves for this peptide (1 pmol·L^{-1}–30 nmol·L^{-1}) were obtained in intact and denuded endothelium carotid rings from control and HHcy groups, in the absence or presence of BQ123 (1 μmol·L^{-1}), a selective ET_A antagonist (Ihara *et al.*, 1992; Tirapelli *et al.*, 2005), and BQ788 (100 nmol·L^{-1}), a selective ET_B antagonist (Ishikawa *et al.*, 1994; Tirapelli *et al.*, 2005). Contractile curves for the selective ET_B receptor agonist {succinyl-[Glu9,Ala11,15]-ET-1(8-210)} (IRL1620) (Takai *et al.*, 1992) were also obtained in intact-endothelium and denuded-endothelium rings.

The measurement of the contraction induced by big ET-1 served as an indicative of functional endothelin-converting enzyme (ECE) activity and concentration–response curves for this peptide (1 pmol·L^{-1}–300 nmol·L^{-1}) were obtained in the absence or in the presence of phosphoramidon (a non-selective ECE inhibitor; 10 mmol·L^{-1}, 30 min).

The curves for ET-1 were performed in endothelium-intact rings, in the absence or after incubation for 30 min with the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester

(L-NAME, 100 μmol·L^{-1}) or a cyclooxygenase inhibitor, indomethacin (10 μmol·L^{-1}). In addition, endothelium-intact rings were pre-incubated for 30 min with AH6809 (antagonist of $PGF_{2\alpha}$ receptors, 10 μmol·L^{-1}) or SQ29548 [PGH_2/thromboxane A_2 (TXA_2) receptor antagonist, 1 μmol·L^{-1}].

To analyse the relative contribution of intracellular calcium (Ca^{2+}) to the ET-1 contractile response, the normal Krebs solution was replaced by a Ca^{2+}-free solution. The rings were exposed to this solution for 1 min and then were stimulated with ET-1 (1 nmol·L^{-1}). In addition, the role of extracellular Ca^{2+} mobilization was investigated by performing concentration–response curves to Ca^{2+} in the presence of ET-1. Endothelium-intact rings were first contracted for approximately 90 min with phenylephrine (0.1 μmol·L^{-1}) to deplete the intracellular Ca^{2+} stores in Ca^{2+}-free solution containing EGTA (1 mmol·L^{-1}) and then rinsed in Ca^{2+}-free solution (without EGTA) containing ET-1 (1 nmol·L^{-1}).

Rho-kinase participation in the ET-1-induced response was determined in the presence of increased concentrations of Y-27632 (Rho-kinase selective inhibitor) (Chitaley and Webb, 2002). The inhibitor was added 30 min prior to the data being collected for the construction of the curve and the responses were compared with those observed in time-matched control experiments.

Contractions were recorded as changes in the displacement (mN·mg tissue^{-1}) from baseline.

Effects of HHcy on ET-1-induced relaxations

Endothelium-intact and endothelium-denuded rings were pre-contracted with Phe (0.1 and 0.03 μmol·L^{-1}, respectively, to induce contractions of similar magnitude). After a stable and sustainable contraction was obtained, the selective ET_B receptor agonist, IRL1620 (100 pmol·L^{-1}–30 nmol·L^{-1}) was added cumulatively to the organ bath.

In the endothelium-intact rings, the same experiments were conducted in the absence and presence of BQ123 (1 μmol·L^{-1}) and BQ788 (100 nmol·L^{-1}). The effects of IRL1620 were also evaluated in the absence or after incubation for 30 min with L-NAME (100 μmol·L^{-1}), indomethacin (10 μmol·L^{-1}) and tetraethylammonium chloride (TEA, non-selective K^+ channel blocker 10 mmol·L^{-1}, Nelson and Quayle (1995). Relaxation was expressed as the percentage change from the phenylephrine-contracted levels. As we noted that L-NAME enhanced phenylephrine-induced contraction, the rings with intact endothelium exposed to these compounds were pre-contracted with phenylephrine 0.03 μmol·L^{-1}, to induce a magnitude of contraction similar to that found in the intact endothelium rings not exposed to the inhibitors. Relaxation was expressed as the percentage change from the phenylephrine-contracted levels.

RT-PCR

The RT-PCR was carried out with rat carotid arteries with intact endothelium, as previously described by Tirapelli *et al*. (2006). Total cell RNA was isolated from carotid arteries using Trizol Reagent. After DNA digestion, total RNA was used for RT in the presence of an RNase inhibitor (RNase OUT®, Gibco BRL), 400 U of Moloney murine leukemia virus RT (Gibco BRL) and 1 μg of oligo (dT)12-18 primer at 21°C for 10 min, 42°C for 60 min and 99°C for 10 min, according to manufacturer's specifications. 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 (5′-3′): ET_A, antisense primer CTGTGCTGCTC GCCCTTGTA, sense primer GAAGTCGTCCGTGGGCATCA (216 bp fragment) and ET_B, antisense primer CACGATGAG GACAATGAGAT, sense primer TTACAAGACAGCCAAAGACT (565 bp fragment); GAPDH, anti-sense primer CACCACCCT GTTGCTGTA, sense primer TATGATGACATCAAGAAGGTGG (219-bp fragment). PCR products (10 μL per lane) were electrophoresed using 1% agarose gel containing ethidium bromide (0.5 μg·mL^{-1}). 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, USA), and the signals are reported relative to the intensity of the GAPDH amplification in each co-amplified sample.

Immunohistochemistry

Carotid arteries were fixed in Methacarn (60% methanol, 30% chloroform and 10% acetic acid). The 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, the 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), with a biotin-conjugated secondary anti-rabbit antibody (1:1000, Vector Laboratories Inc, Burlingame, CA, USA) and with streptavidin-conjugated peroxidase (Vectastain ABC kit, Vector Laboratories Inc, Burlingame, CA, USA). Colour was developed by the addition of DAB (Sigma). Sections were lightly stained in haematoxylin, dehydrated with alcohol and xylene. To evaluate the background reaction, the procedures were also performed on sections incubated only with the secondary antibody (indirect technique) or in the absence of antibodies (direct technique).

Autoradiography

Carotid segments were frozen in isopentane at −30°C, and kept frozen at −70°C. Cross sections (16 μm) of the vessels were cut in a cryostat as previously described (De Oliveira *et al*., 1995; Viswanathan *et al*., 1997).

[^{125}I]ET-1 (74 TBq·mmol^{-1}, Amersham Biosciences Corp, Piscataway, NJ, USA) was used as the ligand. The binding assay used was identical to that previously described (De Oliveira *et al*., 1995; Viswanathan *et al*., 1997). To characterize ET receptor subtypes, consecutive sections were incubated with [^{125}I]ET-1, with or without single 10 μmol·L^{-1} concentrations of the ET_A receptor antagonist BQ123, and the ET_B receptor specific ligand BQ788 (RBI, Natick, MA, USA). Sections were dried after washing and exposed to a pellicle sensitive to radiation (Amersham Corp., Arlington Heights, IL, USA), along with 16-mm-thick sections of ^{14}C (ARC-140B, Art Incorporated, St Louis, MO, USA). Films were developed in ice-cold Kodak D-19 developer (Eastman Kodak Co., Rochester, NY, USA) for 4 min, fixed in Kodak rapid fixer (with a hardener)

for 4 min at room temperature, and then rinsed in water for 10 min. The films with autoradiographic images of the binding and the standards were used to measure the mean optical densities from different areas of interest on the images. The mean optical density measurements per unit area were obtained from both the standard and the sample images. The mean optical density measurements from images of the plastic standards and their corresponding disintegrations min^{-1} per mg of plastic values were used to generate standard curves by nonlinear fitting using the computerized NIH Image 1.4 analysis system (Nazarali *et al.*, 1989). These standard curves were used to obtain the disintegrations $min^{-1}{\cdot}mg^{-1}$ of plastic values for the samples. After correcting for the specific activity of the ligand, the values of $fmol{\cdot}mg^{-1}$ of protein were obtained. The quantification program that we used initially provided the optical density values per unit area, and statistically significant differences were obtained in these values (results not shown) whenever significant differences were obtained using the $fmol{\cdot}mg^{-1}$ protein values.

Measurement of ET-1 by ELISA

Rat carotids (5–6 mm in length) were isolated. The tissues were transferred to an organ bath containing 2.5 mL of Krebs solution at 37°C that was continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The arteries were placed in the Krebs solution for 30 min. Next, the supernatant were systematically collected and stored at −70°C for later determination of ET-1 using commercially available enzyme immunoassay kits (Amersham). For each experimental group, control samples (for the estimation of basal release) were collected.

Levels of nitrogen oxides in vascular homogenates

Nitrite and nitrate levels were measured in supernatants from total carotid artery homogenates prepared under liquid N_2. Following a centrifugation at 3000× *g* for 10 min, supernatants were assayed for nitrite or nitrate through chemiluminescence, using a Nitric Oxide Analyser (Sievers, Model 280), as described (Leite *et al.*, 2003; de Andrade *et al.*, 2006).

Lucigenin-amplified chemiluminescence assays

Lucigenin assays were performed as previously described (Brandes *et al.*, 1997; Li *et al.*, 1998; de Andrade *et al.*, 2006), with a final lucigenin concentration of 5 $\mu mol{\cdot}L^{-1}$. Also as previously described, we used two cell permeable superoxide dismutase (SOD) mimetics, polyethylene glycol (PEG)-SOD and manganese (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), and a superoxide scavenger, Tiron, to confirm the presence of superoxide in the assays.

Data and statistical analysis

Contractions were recorded as changes in the displacement ($mN{\cdot}mg\ tissue^{-1}$) from baseline. Relaxation was expressed as the percentage change from the phenylephrine-contracted levels. Agonist concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA, USA). Agonist potencies were expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximum response), and maximum response was expressed as E_{max} (maximum effect elicited by the agonist). For Rho-kinase experiments, using the inhibitor Y-27632, E_{max} and pD_2 were calculated as the % maximal fall in the ET-1 response, induced by Y-27632.

The results were reported as mean ± SEM. Statistically significant differences were calculated by one-way ANOVA or Student's *t*-test. *Post hoc* comparisons were performed using Bonferroni's multiple comparison test. $P < 0.05$ was considered as statistically significant.

Drugs

The following drugs were used: DL-homocysteine-thiolactone (Acrós Organics/Fischer Scientific, WI, USA); phenylephrine hydrochloride, acetylcholine hydrochloride, ET-1, PEG-SOD and Tiron (Sigma, St. Louis, MO, USA); L-NAME, TEA (Sigma/RBI, Natick, MA, USA); big ET-1, indomethacin, MnTBAP (Calbiochem, USA); BQ123, BQ788, IRL1620, (American Peptide Company, Sunnyvale, CA, USA). Indomethacin was dissolved in Tris buffer (pH: 8.4); the other drugs were dissolved in distilled water.

Results

Plasma homocysteine measurements

Treatment for 15 days with the homocysteine-rich diet (1 $g{\cdot}kg\ body\ weight^{-1}$) induced a 10-fold increase in plasma homocysteine levels (control: 6.2 ± 0.5 vs. HHcy: 62.7 ± 6.9 $\mu mol{\cdot}L^{-1}$; $P < 0.01$, Student's *t*-test).

Effect of HHcy on vascular reactivity to ET-1

Initially, we investigated a possible influence of HHcy on the ET-1-induced contraction. This ET-1-induced contraction was significantly increased in arterial rings from HHcy rats ($P < 0.05$, ANOVA; Figure 1). This difference was endothelium-dependent, as after removal of endothelium, both sets of rings were equally responsive to ET-1. Note that the ET-1-induced contractions in arteries from the HHcy group were unaffected by removal of endothelium (Figure 1). The pD_2 values were not altered.

This enhanced contraction was not evoked by alterations in ET_B receptors, as IRL1620, a selective agonist for ET_B receptors ($100\ nmol{\cdot}L^{-1}$–$300\ nmol{\cdot}L^{-1}$), was not able to induce more than a slight contraction in control or HHcy rat arterial rings (E_{max} values, control: 8.23 ± 0.10 vs. HHcy: 9.48 ± 0.15 $mN{\cdot}mg\ tissue^{-1}$).

Effects of HHcy on ET_A and ET_B receptors

In carotids from control rats, BQ123 (1 $\mu mol{\cdot}L^{-1}$) produced a concentration-dependent rightward displacement of the ET-1 contractile response with reduction on E_{max} values. Furthermore, such displacement and E_{max} reduction were more pronounced in the arteries from the HHcy rats (Figure 2, Table 1).

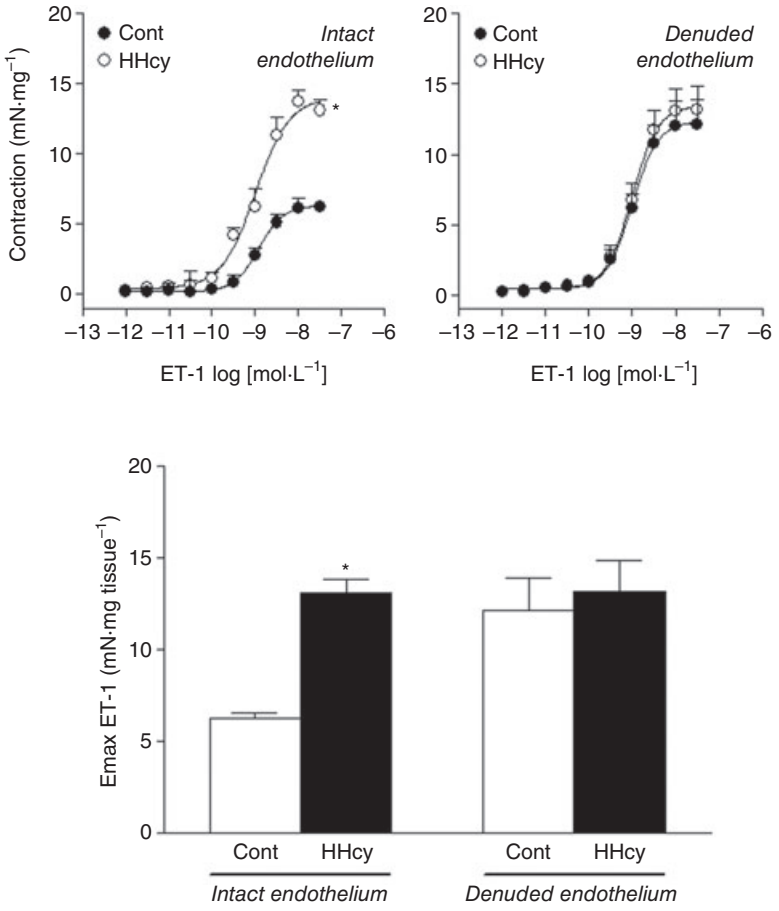

Figure 1 Concentration–response curves for ET-1 obtained in endothelium-intact and endothelium-denuded and isolated carotid rings from control (Cont) and HHcy rats. Values are means ± SEM; $n = 8$ for intact endothelium and $n = 7$ for denuded rings. *Compared with respective Cont group (one-way ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$). ET-1, endothelin-1; HHcy, hyperhomocysteinemia.

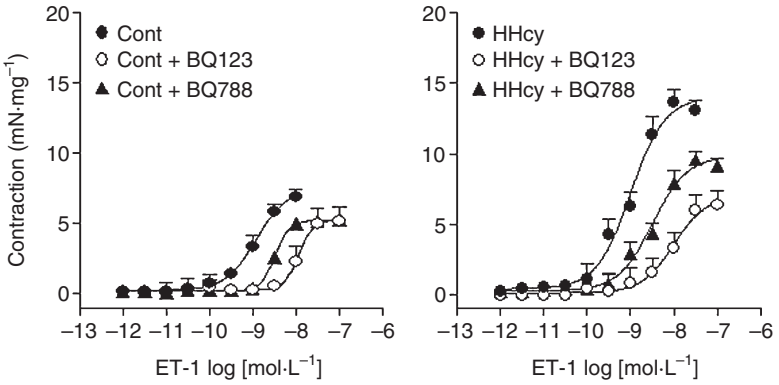

Figure 2 Concentration–response curves for ET-1 obtained in endothelium-intact carotid rings from control (Cont) and HHcy rats in the absence or presence of BQ123 (1 μmol·L^{-1}) or BQ788 (0.1 μmol·L^{-1}), ET_A and ET_B antagonists respectively. The concentration–response curves in both sets of rings were significantly shifted by either antagonist. Changes in pD2 and E_{max} values are summarized in Table 1. Data shown here are means ± SEM, from seven preparations in each group. ET-1, endothelin-1; HHcy, hyperhomocysteinemia.

Similarly, BQ788 (0.1 μmol·L^{-1}) right-shifted the ET-1 concentration–response curves and reduced the E_{max} in arteries from both control and HHcy groups, but without differences between these two groups (Figure 2, Table 1).

The RT-PCR analysis showed that rat carotid arteries express mRNA for both ET_A and ET_B receptors and that HHcy did not alter the gene expression for these receptors (Figure 3). The immunohistochemical analysis revealed the presence of ET_A and ET_B receptors in arterial smooth muscle cells. In endothelial cells, positive immunostaining for ET_B, but not for ET_A receptors, was detected. The ET_A receptor expression on the media layer of arteries from HHcy rats was higher than in

Table 1 Effect of HHcy on the E_{max} (mN·mg^{-1}) and pD_2 values for endothelin-1 in rat carotid arteries in the absence or presence of BQ123 (1 μmol·L^{-1}) or BQ788 (0.1 μmol·L^{-1})

	Control		*HHcy*	
	E_{max}	pD_2	E_{max}	pD_2
	7.29 ± 0.65	8.92 ± 0.09	13.82 ± 0.07	8.99 ± 0.06
BQ123 (1 μmol·L^{-1})	5.15 ± 0.99*	7.94 ± 0.10*	6.35 ± 0.09*	8.00 ± 0.09*
BQ788 (0.1 μmol·L^{-1})	5.28 ± 0.33*	8.48 ± 0.09*	9.25 ± 0.05*	8.46 ± 0.08*

Values are means ± SEM of $n = 7$ preparations.
*Compared with respective control in the absence of antagonists ($P < 0.05$; one-way ANOVA).

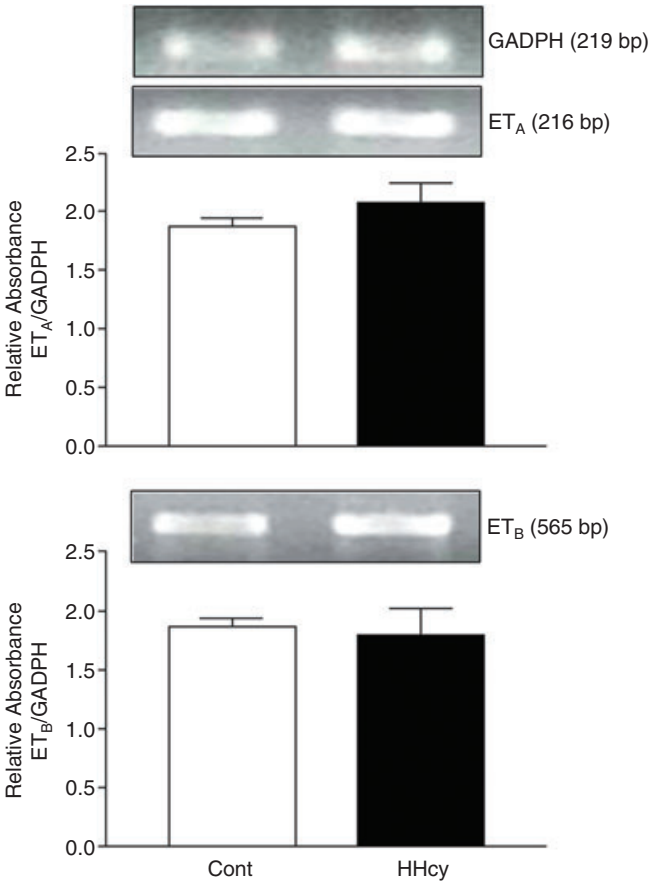

Figure 3 Representative RT-PCR products of 2 μg total RNA extracted from carotid arteries of control (Cont) and HHcy rats. The bar graphs show the relative absorbance values of ET_A and ET_B receptor bands in endothelium-intact rings. ET_A and ET_B values were normalized to the corresponding GAPDH values, used as internal standard. Results are reported as means ± SEM from five experiments. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HHcy, hyperhomocysteinemia.

arteries from control rats (Figure 4). HHcy also resulted in increased binding of [^{125}I]-ET-1 in the media layer (Figure 5). In addition, ET-1 binding was displaced by unlabelled ET-1 and by the ET_A antagonist BQ123, but not by the ET_B-specific ligand BQ788. These observations on ET-1 binding indicate that there was a 15% enhancement of ET_A receptor density in the arteries obtained from HHcy rats, relative to that in control arteries.

Effects of HHcy on ET-1 production

The ET-1 precursor peptide, big ET-1, induced contractions of endothelium-intact rat carotid arteries obtained from the control and HHcy group (Figure 6). HHcy induced a decrease in E_{max} and pD_2 values for big ET-1 in arteries with intact endothelium. To confirm that the contraction to big ET-1 was because it was converted to ET-1, the ECE activity was being assayed, the ECE inhibitor, phosphoramidon, was used. As expected, phosphoramidon reduced the contractile response to big ET-1 in arteries from control rats (Figure 6).

In addition, HHcy did not alter prepro-ET-1 gene expression in rat carotid arteries (control: 1.09 ± 0.09 vs. HHcy: 0.96 ± 0.13 relative absorbance). Indeed, the basal release of ET-1 did not differ in carotid rings from both groups (control: 136 ± 36 vs. HHcy 140 ± 50 pg mL·mg tissue^{-1}).

Role of Rho-kinase activity on ET-1-induced responses

The ET-1-induced E_{max} was reduced in a concentration-dependent manner by the selective Rho-kinase inhibitor, Y-27632, in carotid arteries obtained from the control and HHcy rats. The maximal fall in the ET-1 response was 12 ± 1% in control arteries and 13 ± 3% in HHcy arteries. The pD_2 values for Y-27632 were 6.74 ± 0.11 in control and 6.15 ± 0.21 in HHcy arteries. These pD_2 values were significantly different ($P < 0.05$, Student's *t*-test) between control and HHcy tissues.

Contribution of intracellular and extracellular Ca^{2+} to the changes in ET-1 vascular reactivity

In Ca^{2+}-free medium, ET-1-induced contractions reached a peak and then returned to baseline levels. The magnitude of the peak response to ET-1 (1 nmol·L^{-1}) was enhanced in the arteries with intact endothelium obtained from HHcy rats (1.34 ± 0.23 mN·mg tissue^{-1}) when compared with the control rats (0.38 ± 0.11 mN·mg tissue^{-1}, $P < 0.01$, Student's *t*-test). Similarly, $CaCl_2$-induced contraction was greater in carotid rings obtained from HHcy rats (6.86 ± 0.14 mN·mg tissue^{-1}) than in those from control rats (2.82 ± 0.34 mN·mg tissue^{-1}, $P < 0.001$, Student's *t*-test).

Contribution of NO and endothelial cyclooxygenase metabolites to the ET-1 response

Incubation with L-NAME significantly enhanced the E_{max} induced by ET-1 in arterial segments obtained from control,

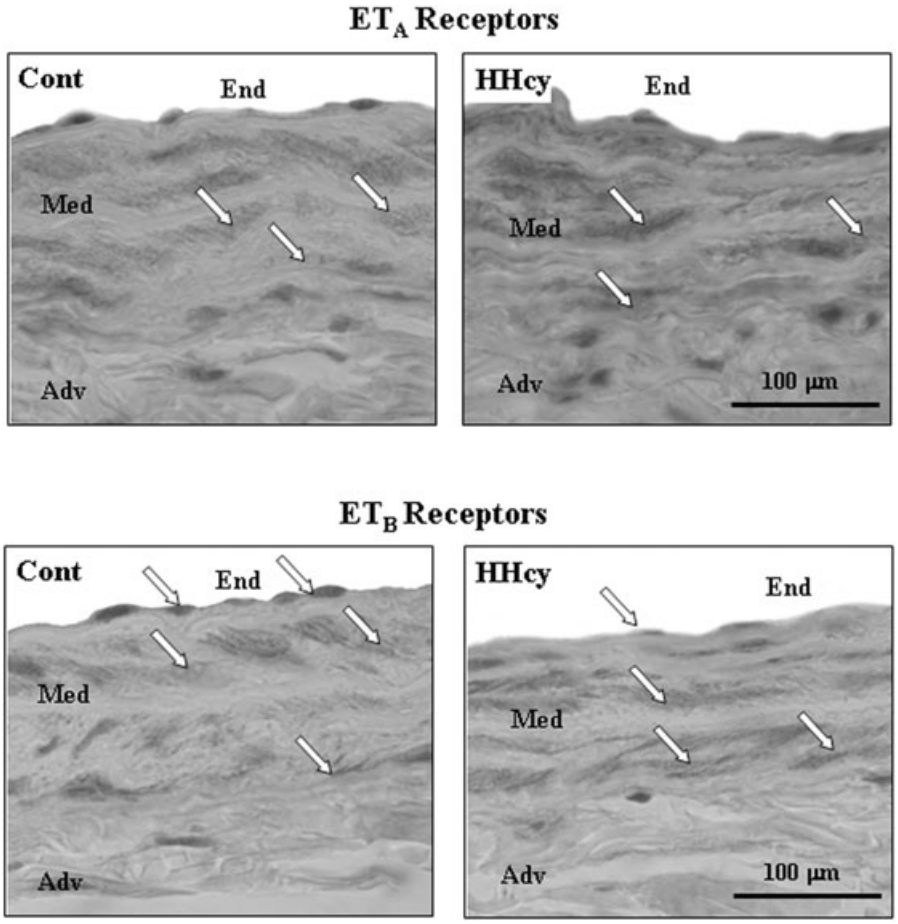

Figure 4 Representative immunohistochemical photomicrographs of ET_A and ET_B receptors in rat carotid artery sections. Arrows indicate expression of ET_A receptor in smooth muscle cells and ET_B in both endothelial and smooth muscle cells (magnification: × 400). Cont, control; HHcy, hyperhomocysteinemia.

but not in those obtained from HHcy rats (Figure 7). Indomethacin did not alter E_{max} values for ET-1 in the endothelium-intact carotid rings obtained from control, whereas in the arteries obtained from HHcy rats, such E_{max} values were reduced, compared with the values obtained when the inhibitor was absent (Figure 7).

The PGH_2/TXA_2 receptor antagonist, SQ29548, reduced the ET-1 E_{max} in arteries from HHcy group (Figure 7). No changes in pD_2 values were detected (data not shown). On the other hand, pre-incubation with AH6809 did not produce changes in the concentration–response curves for ET-1 (data not shown).

IRL1620-induced relaxation

The selective agonist at ET_B receptors, IRL1620, induced relaxation in endothelium-intact rings from both groups. This relaxation was significantly reduced by BQ788, but not by BQ123. Moreover, HHcy impaired this IRL1620-induced relaxation (Figure 8 top). There were no differences in the pD_2 values of IRL1620 in endothelium-intact rings in the absence or presence of antagonists. Removal of the endothelium abolished this relaxation response in arteries from both groups.

In order to verify the mechanisms underlying ET_B-induced relaxation, endothelium-intact rings were exposed to L-NAME or indomethacin. Both inhibitors reduced IRL1620-induced relaxation in the arteries obtained from control, but not in the arteries from the HHcy rats, and abolished the differences between the groups in the IRL1620 responses. TEA also reduced the relaxation induced by IRL1620 (Figure 8 bottom). The mean pD_2 of the relaxant IRL1620 concentration–response curves, as well as the E_{max} values, in the presence or absence of the previously mentioned inhibitors, are given in Table 2.

Carotid arteries obtained from HHcy rats also had reduced nitrite levels (control: 3.42 ± 0.08 vs. HHcy: 2.86 ± 0.21 µmol·L^{-1}, protein^{-1} $P < 0.05$, Student's *t*-test), but not in the nitrate levels (control: 10.41 ± 5.74 vs. HHcy: 11.71 ± 4.28 µmol·L^{-1} protein^{-1}). In parallel, our data indicated an increased output of superoxide from carotid segments, as assessed by chemiluminescence, in HHcy rats (control: 1375 ± 199 vs. HHcy: 2468 ± 305 cpm mg tissue). MnTBAP (5 µmol·L^{-1}) and PEG-SOD (200 U·mL^{-1}), cell permeable SOD mimetics and Tiron, a superoxide scavenger (1 mmol·L^{-1}), abolished superoxide generation.

Discussion and conclusions

The present findings demonstrate that a diet rich in homocysteine thiolactone caused a 10-fold increase in plasma

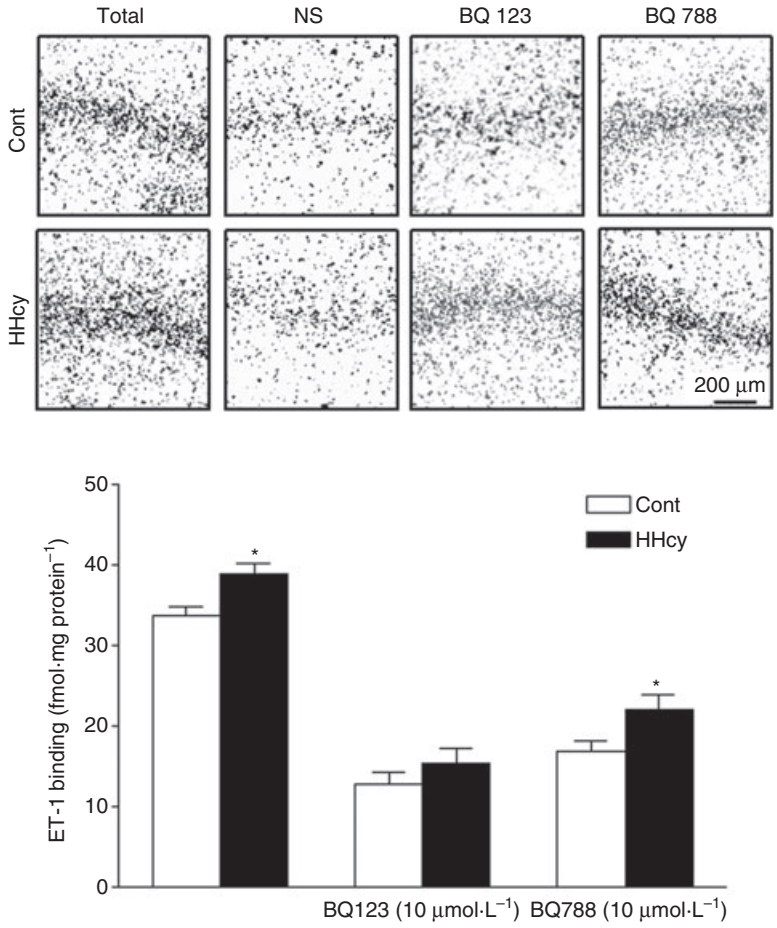

Figure 5 Autoradiography of [H^3]-ET-l binding in rat carotid artery. Representative autoradiography of ET_A and ET_B receptors in rat carotid artery sections: total binding, non-specific binding (NS) and in the presence of BQ123 (10 μmol·L^{-1}) or BQ788 (10 μmol·L^{-1}). Summary data are shown below as means ± SEM from three experiments. *$P < 0.05$, Student's *t*-test; different from Cont groups. Cont, control; ET-1, endothelin-1; HHcy, hyperhomocysteinemia.

homocysteine in rats and, although this model does not exactly mimic the human condition, it provides important information about the vascular consequences of HHcy in rats (Refsum *et al.*, 1998; Hill *et al.*, 2002; Bonaventura *et al.*, 2004, de Andrade *et al.*, 2006).

Our present results also showed that vascular ET-1 receptor density was enhanced in rat arteries exposed to high concentrations of homocysteine and this increased density played an active role in endothelium-dependent vascular contraction. However, thus far there has been little information regarding the interactions of ET-1 in HHcy. We therefore carried out experiments on isolated carotid arteries from control and HHcy rats. Interestingly, we observed that high plasma levels of homocysteine led to a significant enhancement of vascular reactivity to ET-1.

ET-1 is produced by a variety of cells but predominantly by endothelial cells (Luscher and Barton, 2000). Endothelial dysfunction is accepted as one of the earliest process in the development of vascular diseases such as atherosclerosis (Traupe *et al.*, 2003) and two mechanisms have been described as underlying the endothelial dysfunction in HHcy: endothelial injury and the reduced bioavailability of NO (Powers *et al.*, 2003; de Andrade *et al.*, 2006). The present

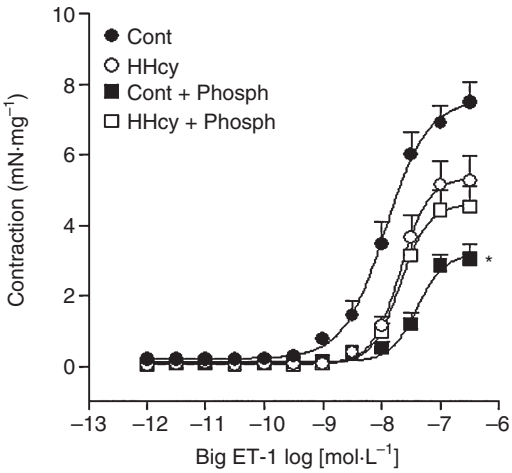

Figure 6 Concentration–response curves for big ET-1 obtained in endothelium-intact carotid rings from control (Cont) and HHcy rats, in the absence or presence of phosphoramidon (Phosph; 10 mmol·L$^{-1)}$. Values are means ± SEM from five preparations in each group. *Compared with respective Cont group in the absence of inhibitor (one-way ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$). HHcy, hyperhomocysteinemia.

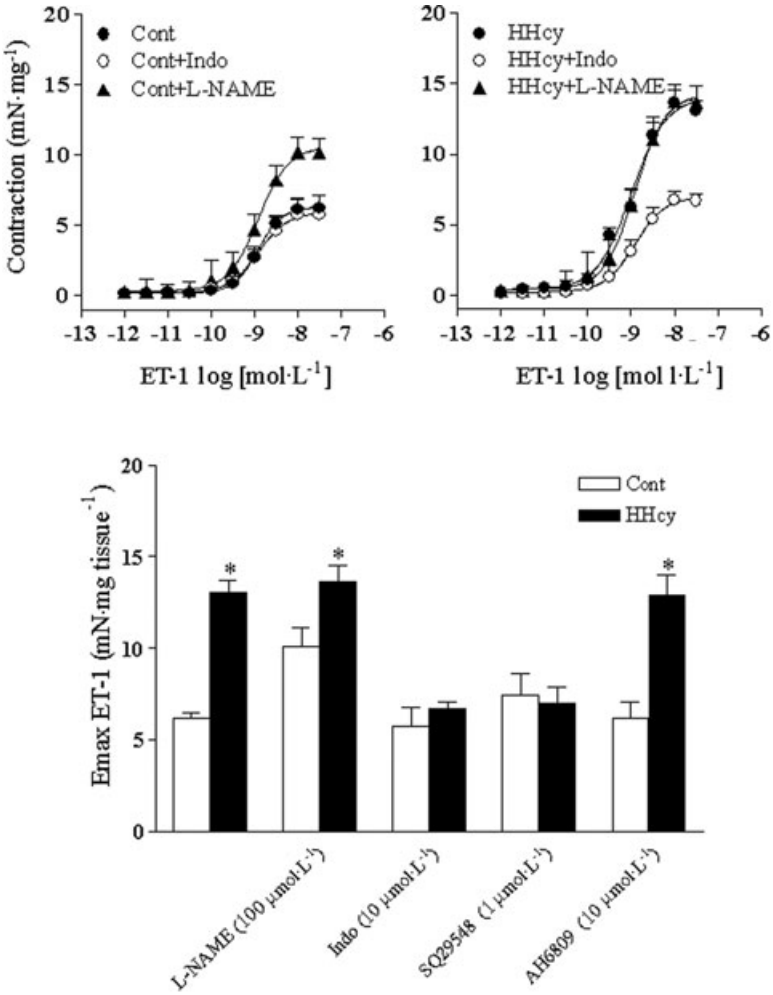

Figure 7 Concentration–response curves for ET-1 obtained in endothelium-intact carotid rings from control (Cont) (left-hand graph) and HHcy (right-hand graph) rats in the absence or presence of L-NAME (100 µmol·L^{-1}), indomethacin (Indo) (10 µmol·L^{-1}), inhibitors of nitric oxide synthase and cyclooxygenase respectively. In the lower graph are shown the effects on E_{max} for ET-1 of L-NAME (100 µmol·L^{-1}), Indo (10 µmol·L^{-1}), SQ29548 (TXA_2 receptor antagonist; 1 µmol·L^{-1}) or AH6809 ($PGF_{2\alpha}$ receptor antagonist, 10 µmol·L^{-1}). Values are means ± SEM of five to seven independent preparations. *Compared with respective Cont group (one-way ANOVA followed by Bonferroni's multiple comparison test, $P < 0.05$). ET-1, endothelin-1; HHcy, hyperhomocysteinemia; L-NAME, N^G-nitro-L-arginine methyl ester; SQ29548.

report addressed changes in ET_A/ET_B receptor density, intracellular transduction signalling (Ca^{2+} and Rho-kinase pathways), ET-1 synthesis and/or bioavailability, participation of prostanoids and the relaxing factors released after activation of ET_B receptors.

We first evaluated whether endothelial cells modulated ET-1 responses in arteries from HHcy rats. HHcy enhanced ET-1 responses only in arteries with intact endothelium, which reinforces the role of the endothelium in the enhanced ET-1 vascular responsiveness.

Although HHcy had no effects on either ET_A or ET_B receptor gene expression, autoradiographical or immunohistochemical assays showed that ET_A receptor expression is significantly enhanced in arteries obtained from HHcy rats, suggesting that the number of ET_A receptors is one of the factors involved in the enhanced reactivity to ET-1 in HHcy rats. This differs from *in vitro* experiments where high homocysteine levels increased ET-1 mRNA (Drunat *et al.*, 2002), and changes in ET-1 release have previously been associated with an increase in vascular resistance.

It should be pointed out that the effects we observed also involved a reduction of ECE activity. Altered ECE activity might lead to changes in ET-1 production, which could alter the density of endothelin receptors, thus promoting differences in the vascular responsiveness to exogenous ET-1. Exposure of the ECE to increased superoxide, which can occur in specific microenvironments, could decrease ECE activity (Lopez-Ongil *et al.*, 2000). We have previously observed increased generation of O_2^- in arteries from HHcy rats (de Andrade *et al.*, 2006), which emphasizes the role that this reactive oxygen species has in the observed changes. The precise nature of such interaction requires further study.

Enhanced contractility to ET-1, triggered by HHcy, also involves the activation of intracellular signalling pathways. Basal peripheral and systemic vascular tone depends on Rho-associated kinase pathways (Büssemaker *et al.*, 2007), which negatively regulate the vascular bioavailability of NO by increasing superoxide concentrations. In this study, the effect of the Rho-kinase inhibitor (Y-27632) on HHcy-treated rats clearly indicated that the contraction evoked by ET-1 required

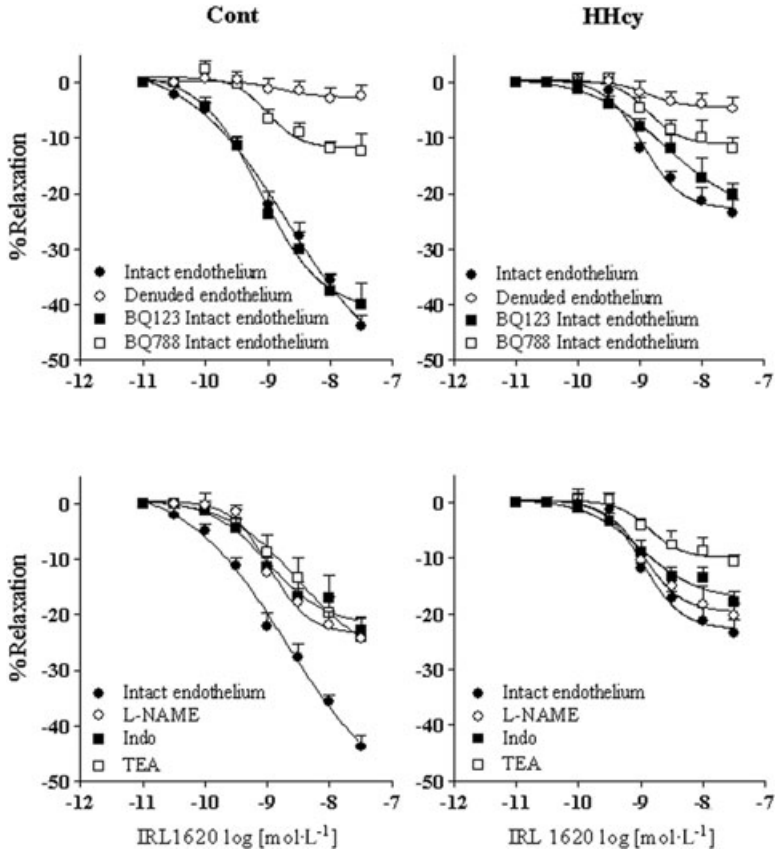

Figure 8 Relaxation responses induced by IRL1620 (ET_B agonist) on rat carotid rings pre-contracted with phenylephrine. Top: the concentration–response curves for IRL1620 were obtained in endothelium-denuded and endothelium-intact rings, in the absence or presence of BQ123 and BQ788. Bottom: the concentration–response curves were obtained in the absence or in the presence of L-NAME (100 μmol·L^{-1}), indomethacin (10 μmol·L^{-1}) and TEA (10 mmol·L^{-1}), from Cont and HHcy rats. Values are means ± SEM of five to seven independent preparations. BQ123; BQ788, [N-cis-2,6-dimethyl-piperidinocarbonyl-L-J-methylleucyl1-D-1methoxycarbonyl tryptophanyl-D-norleucine]; Cont, control; ET-1, endothelin-1; HHcy, hyperhomocysteinemia; IRL1620, TEA, tetraethylammonium.

Table 2 Relaxation (%) induced by IRL1620 in the presence of BQ123 (1 μmol·L^{-1}), BQ788 (0.1 μmol·L^{-1}), L-NAME (100 μmol·L^{-1}), indomethacin (10 μmol·L^{-1}) or TEA (10 mmol·L^{-1})

	Control	*HHcy*
	IRL1620 E_{max} (%)	
Intact endothelium	44 ± 2	24 ± 2*
Denuded endothelium	2.5 ± 2.1	4.7 ± 2.1
BQ123 (1 μmol·L^{-1})	40 ± 4	20 ± 2†
BQ788 (0.1 μmol·L^{-1})	12 ± 3	12 ± 2
L-NAME (100 μmol·L^{-1})	24 ± 1	20 ± 2
Indomethacin (10 μmol·L^{-1})	23 ± 2	18 ± 2
TEA (10 mmol·L^{-1}).	24 ± 3	11 ± 1
	pD_2 (–log EC_{50})	
Intact endothelium	8.68 ± 0.22	8.95 ± 0.08
Denuded endothelium	8.81 ± 0.26	8.86 ± 0.09
BQ123 (1 μmol·L^{-1})	9.11 ± 0.06	8.59 ± 0.08
BQ788 (0.1 μmol·L^{-1})	9.01 ± 0.12	8.87 ± 0.07
L-NAME (100 μmol·L^{-1})	8.95 ± 0.08	8.90 ± 0.07
Indomethacin (10 μmol·L^{-1})	8.98 ± 0.15	8.89 ± 0.12
TEA (10 mmol·L^{-1}).	8.49 ± 0.07	8.86 ± 0.10

Values are means ± SEM of n = 5–7 preparations.
*Compared with arteries from control ($P < 0.05$; one-way ANOVA).
†Compared with respective control in the absence of inhibitors ($P < 0.01$; one-way ANOVA).

the activation of Rho-kinase pathway. Rho/Rho-kinase signalling, as well as Ca^{2+} mobilization, is thought to play important roles in vasoconstriction and may contribute to the aetiology of cardiovascular diseases both in experimental animals and humans (Jin *et al.*, 2004; Nakakuki *et al.*, 2005). In line with these previous observations, increased O_2^- levels can modify the contraction of vascular smooth muscle through activation of the Rho-kinase pathway (Jin *et al.*, 2004).

Changes in phosphoinositide turnover and transmembrane Ca^{2+} influx in vascular smooth muscle cells have been described (Okatani *et al.*, 2001). The present study showed a significant increase in vascular sensitivity to extracellular Ca^{2+} in HHcy rats, which is in agreement with the suggestion that HHcy increases ET-1-stimulated influx of Ca^{2+}. Furthermore, we found that the potentiating effect of HHcy on ET-1-induced contraction may also involve increased release of intracellular Ca^{2+}. Interestingly, there is a previous report that HHcy induced mobilization of intracellular Ca^{2+} pools and activated a variety of kinases in the mouse hippocampus (Robert *et al.*, 2005). This suggests a major role for Rho-kinase in HHcy-induced changes in vascular reactivity, in addition to its role in changing intracellular and extracellular Ca^{2+}.

Additionally, L-NAME significantly enhanced the ET-1-induced response in carotid arteries from control, but not in

those from HHcy rats, in the presence of endothelium, which suggests that the enhanced responsiveness of HHcy carotid arteries to ET-1 was partially triggered by impaired NO release. Indomethacin, by contrast, significantly inhibited the E_{max} induced by ET-1 in arteries from HHcy rats. The contribution of endothelial prostanoids derived from the cyclooxygenase pathway on the modulation of the contraction triggered in HHcy rats has been previously reported (Ungvari *et al*., 2000; Bagi *et al*., 2001). On analysing the possible prostanoid(s) involved in the enhanced reactivity to ET-1, we found that SQ29548, a competitive antagonist of PGH_2/TXA_2 receptors, reduced the E_{max} evoked by ET-1 in carotid rings obtained from HHcy rats, but not in carotid rings obtained from control rats. Thus, our data corroborate previous findings, namely that HHcy enhances the production of TXA_2 (Ungvari *et al*., 2000; Bagi *et al*., 2002). We discounted the contribution of $PGF_{2\alpha}$, as AH6809, an antagonist of $PGF_{2\alpha}$ receptors, had no effect on such contractions.

Finally, increased generation of reactive oxygen species in the arteries obtained from HHcy rats may lead to NO inactivation, thus reducing NO bioavailability, and increasing the formation of peroxynitrite and secondary nitro-oxidative species (de Andrade *et al*., 2006). Indeed, IRL1620-induced relaxation was decreased in the arteries obtained from HHcy rats, possibly due to changes in the NO and cyclooxygenase pathways. The decreased bioavailability of NO could provide an additional mechanism by which HHcy induces enhanced ET-1 vascular reactivity.

In summary, this is the first study using rat carotid rings to investigate the molecular mechanisms of ET-1-induced vascular dysfunction in rats with HHcy. Observations made in HHcy arteries provide direct evidence that ET_A receptors not only modulate the contractile response to ET-1, but also increase the generation of potent constrictor factors such as TXA_2 and the Rho-kinase pathway. Staining for ET_A receptors in the media layer reinforces the premise that ET_A is a major player in the vascular system in rats with HHcy. This increase in ET_A receptors, combined with redox imbalance, appear to play an important role in ET-1-induced vascular contraction, and are probably key mediators of the long-term vascular structural adaptation to raised homocysteine concentrations.

Acknowledgments

We are most grateful to Juliana A. Vercesi, Miriam C. C. de Melo, Mayara Santos Gomes (FCFRP-USP), Laura Ventura (Incor, FMUSP) and Eleni L. T. Gomes (FMRP-USP), for their technical assistance. This work was supported by FAPESP-Brazil (01/05549-3 and 02/8045-9) and by FAPESP Grant 04/13683-0 to FRML.

Conflict of interest

The authors state no conflict of interest.

References

de Andrade CR, Fukada SY, Olivon VC, de Godoy MA, Haddad R, Eberlin MN *et al.* (2006). Alpha1D-adrenoceptor-induced relaxation on rat carotid artery is impaired during the endothelial dysfunction evoked in the early stages of hyperhomocysteinemia. *Eur J Pharmacol* **543**: 83–91.

Au-Yeung KK, Woo CW, Sung FL, Yip JC, Siow YL, Karmin O (2004). Hyperhomocysteinemia activates nuclear factor-kappaB in endothelial cells via oxidative stress. *Circ Res* **94**: 28–36.

Bagi Z, Ungvari Z, Szollar L, Koller A (2001). Flow-induced constriction in arterioles of hyperhomocysteinemic rats is due to impaired nitric oxide and enhanced thromboxane A(2) mediation. *Arterioscler Thromb Vasc Biol* **21**: 233–237.

Bagi Z, Ungvari Z, Koller A (2002). Xanthine oxidase-derived reactive oxygen species convert flow-induced arteriolar dilation to constriction in hyperhomocysteinemia: possible role of peroxynitrite. *Arterioscler Thromb Vasc Biol* **22**: 28–33.

Bonaventura D, Tirapelli CR, Haddad R, Hoehr NF, Eberlin MN, de Oliveira AM (2004). Chronic methionine load-induced hyperhomocysteinemia enhances rat carotid responsiveness for angiotensin II. *Pharmacology* **70**: 91–99.

Brandes RP, Barton M, Philippens KM, Schweitzer G, Mugge A (1997). Endothelial-derived superoxide anion in pig coronary arteries; evidence from lucigenin chemiluminescence and histochemical techniques. *J Physiol* **500**: 331–342.

Büssemaker E, Pistrosch F, Förster S, Herbrig K, Gross P, Passauer J *et al.* (2007). Rho kinase contributes to basal vascular tone in humans: role of endothelium-derived nitric oxide. *Am J Physiol Heart Circ Physiol* **293**: H541–H547.

Chen C, Halkos ME, Surowiec SM, Conklin BS, Lin PH, Lumsden AB (2000). Effects of homocysteine on smooth muscle cell proliferation in both cell culture and artery perfusion culture models. *J Surg Res* **88**: 26–33.

Chitaley K, Webb RC (2002). Nitric oxide induces dilation of rat aorta via inhibition of rho-kinase signaling. *Hypertension* **39**: 438–442.

De Oliveira AM, Viswanathan M, Capsoni S, Heemskerk FM, Correa FM, Saavedra JM (1995). Characterization of endothelinA receptors in cerebral and peripheral arteries of the rat. *Peptides* **16**: 139–144.

Demuth K, Atger V, Borderie D, Benoit MO, Sauvaget D, Lotersztajn S *et al.* (1999). Homocysteine decreases endothelin-1 production by cultured human endothelial cells. *Eur J Biochem* **263**: 367–376.

Demuth K, Drunat S, Girerd X, Moatti N, Paul JL, Safar M *et al.* (2002). Homocysteine is the only plasma thiol associated with carotid artery remodeling. *Atherosclerosis* **165**: 167–174.

Drunat S, Moatti N, Paul JL, Cogny A, Benoit MO, Demuth K (2001). Homocysteine-induced decrease in endothelin-1 production is initiated at the extracellular level and involves oxidative products. *Eur J Biochem* **268**: 5287–5294.

Drunat S, Moatti N, Demuth K (2002). Homocysteine decreases endothelin-1 expression by interfering with the AP-1 signaling pathway. *Free Radic Biol Med* **33**: 659–668.

Dusitanond P, Eikelboom JW, Hankey GJ, Thom J, Gilmore G, Loh K *et al.* (2005). Homocysteine-lowering treatment with folic acid, cobalamin, and pyridoxine does not reduce blood markers of inflammation, endothelial dysfunction, or hypercoagulability in patients with previous transient ischemic attack or stroke: a randomized substudy of the VITATOPS trial. *Stroke* **36**: 144–146.

Haddad R, Sparrapan R, Eberlin MN (2006). Desorption sonic spray ionization for (high) voltage-free ambient mass spectrometry. *Rapid Commun Mass Spectrom* **20**: 2901–2905.

Hill CH, Mecham R, Starcher B (2002). Fibrillin-2 defects impair elastic fiber assembly in a homocysteinemic chick model. *J Nutr* **132**: 2143–2150.

Ihara M, Noguchi K, Saeki T, Fukuruda T, Tsuchida S, Kimura S *et al.* (1992). Biological profiles of highly potent novel endothelin antagonists selective for ETA receptor. *Life Sci* **50**: 247–255.

Ishikawa K, Ihara M, Noguchi K, Mase T, Mino N, Saeki T *et al.* (1994). Biochemical and pharmacological profile of a newly-developed potent and selective endothelin B receptor antagonist, BQ-788. *Proc Natl Acad Sci USA* **20**: 4892–4896.

Jin L, Ying Z, Webb RC (2004). Activation of Rho/Rho kinase signaling pathway by reactive oxygen species in rat aorta. *Am J Physiol Heart Circ Physiol* **287**: H1495–H500.

Leite PF, Danilovic A, Moriel P, Dantas K, Marklund S, Dantas AP *et al*. (2003). Sustained decrease in superoxide dismutase activity underlies constrictive remodeling after balloon injury in rabbits. *Arterioscler Thromb Vasc Biol* **23**: 2197–2202.

Li Y, Zhu H, Kuppusamy P, Roubaud V, Zweier JL, Trush MA (1998). Validation of lucigenin (bis-N- methylacridinium) as a chemiluminescent probe for detecting superoxide anion radical for detecting superoxide anion radical production by enzymatic and cellular sources. *J Biol Chem* **273**: 2015–2023.

Lopez-Ongil S, Senchak V, Saura M, Zaragoza C, Ames M, Ballermann B *et al*. (2000). Superoxide regulation of endothelin-converting enzyme. *J Biol Chem* **275**: 26423–26427.

Luscher TF, Barton M (2000). Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs. *Circulation* **102**: 2434–2440.

Masaki T (1995). Possible role of endothelin in endothelial regulation of vascular tone. *Annu Rev Pharmacol Toxicol* **35**: 235–255.

Nakakuki T, Ito M, Iwasaki H, Kureishi Y, Okamoto R, Moriki N *et al*. (2005). Rho/Rho-kinase pathway contributes to C-reactive protein-induced plasminogen activator inhibitor-1 expression in endothelial cells. *Arterioscler Thromb Vasc Biol* **25**: 2088–2093.

Nazarali AJ, Gutkind JS, Saavedra JM (1989). Calibration of 125I-polymer standards with 125I-brain paste standards for use in quantitative receptor autoradiography. *J Neurosci Methods* **30**: 247–253.

Nelson MT, Quayle JM (1995). Physiological roles and properties of potassium channels in arterial smooth muscle. *Am J Physiol* **268**: C799–C822.

Okatani Y, Wakatsuki A, Reiter RJ (2001). Melatonin counteracts potentiation by homocysteine of KCL-induced vasoconstriction in human umbilical artery: relation to calcium influx. *Biochem Biophys Res Commun* **280**: 940–944.

Pisciotta L, Cortese C, Gnasso A, Liberatoscioli L, Pastore A, Mannucci L *et al*. (2005). Serum homocysteine, methylenetetrahydrofolate reductase gene polymorphism and cardiovascular disease in heterozygous familial hypercholesterolemia. *Atherosclerosis* **179**: 333–338.

Powers RW, Majors AK, Cerula SL, Huber HA, Schmidt BP, Roberts JM (2003). Changes in markers of vascular injury in response to transient hyperhomocysteinemia. *Metabolism* **52**: 501–507.

Pruefer D, Scalia R, Lefer AM (1999). Homocysteine provokes leukocyte-endothelium interaction by downregulation of nitric oxide. *Gen Pharmacol* **33**: 487–498.

Refsum H, Ueland PM, Nygard O, Vollset SE (1998). Homocysteine and cardiovascular disease. *Annu Rev Med* **49**: 31–62.

Robert K, Pages C, Ledru A, Delabar J, Caboche J, Janel N (2005). Regulation of extracellular signal-regulated kinase by homocysteine in hippocampus. *Neuroscience* **133**: 925–935.

Takai M, Umemura K, Yamasaki K, Watanabe T, Fujitani Y, Oda K *et al*. (1992). A potent and specific agonist, Suc-[Glu9 Ala11,15]-endothelin-1 (8-21), IRL 1620, for ETB receptor. *Biochem Biophys Res Commun* **184**: 953–959.

Tirapelli CR, Casolari DA, Yogi A, Montezano AC, Tostes RC, Legros E *et al*. (2005). 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. *Br J Pharmacol* **146**: 903–912.

Tirapelli CR, Casolari DA, Montezano AC, Yogi A, Tostes RC, Legros E *et al*. (2006). Ethanol consumption enhances endothelin-1-induced contraction in the isolated rat carotid. *J Pharmacol Exp Ther* **318**: 819–827.

Tostes RC, Muscara MN (2005). Endothelin receptor antagonists: another potential alternative for cardiovascular diseases. *Curr Drug Targets Cardiovasc Haematol Disord* **5**: 287–301.

Traupe T, Ortmann J, Munter K, Barton M (2003). Endothelial therapy of atherosclerosis and its risk factors. *Curr Vasc Pharmacol* **1**: 111–121.

Tyagi SC, Smiley LM, Mujumdar VS, Clonts B, Parker JL (1998). Reduction-oxidation (Redox) and vascular tissue level of homocyst(e)ine in human coronary atherosclerotic lesions and role in extracellular matrix remodeling and vascular tone. *Mol Cell Biochem* **181**: 107–116.

Ungvari Z, Sarkadi-Nagy E, Bagi Z, Szollar L, Koller A (2000). Simultaneously increased TxA(2) activity in isolated arterioles and platelets of rats with hyperhomocysteinemia. *Arterioscler Thromb Vasc Biol* **20**: 1203–1208.

Viswanathan M, De Oliveira AM, Johren O, Saavedra JM (1997). Increased endothelin ET(A) receptor expression in rat carotid arteries after balloon injury. *Peptides* **18**: 247–255.

Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y *et al*. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **332**: 411–415.

Weiss N, Heydrick SJ, Postea O, Keller C, Keaney JF Jr, Loscalzo J (2003). Influence of hyperhomocysteinemia on the cellular redox state – impact on homocysteine-induced endothelial dysfunction. *Clin Chem Lab Med* **41**: 1455–1461.

Themed Section: Endothelium in Pharmacology

Endothelium in pharmacology: 30 years on: *J. C. McGrath*

Role of nitroso radicals as drug targets in circulatory shock: *E. Esposito & S. Cuzzocrea*

Endothelial Ca^{2+}-activated K^+ channels in normal and impaired EDHF–dilator responses – relevance to cardiovascular pathologies and drug discovery: *I. Grgic, B. P. Kaistha, J. Hoyer & R. Köhler*

Endothelium-dependent contractions and endothelial dysfunction in human hypertension: *D. Versari, E. Daghini, A. Virdis, L. Ghiadoni & S. Taddei*

Nitroxyl anion – the universal signalling partner of endogenously produced nitric oxide?: *W. Martin*

A role for nitroxyl (HNO) as an endothelium-derived relaxing and hyperpolarizing factor in resistance arteries: *K. L. Andrews, J. C. Irvine, M. Tare, J. Apostolopoulos, J. L. Favaloro, C. R. Triggle & B. K. Kemp-Harper*

Vascular K_{ATP} channels: dephosphorylation and deactivation: *P. Tammaro*

Ca^{2+}/calcineurin regulation of cloned vascular K_{ATP} channels: crosstalk with the protein kinase A pathway: *N. N. Orie, A. M. Thomas, B. A. Perrino, A. Tinker & L. H. Clapp*

Understanding organic nitrates – a vein hope?: *M. R. Miller & R. M. Wadsworth*

Increased endothelin-1 reactivity and endothelial dysfunction in carotid arteries from rats with hyperhomocysteinemia: *C. R. de Andrade, P. F. Leite, A. C. Montezano, D. A. Casolari, A. Yogi, R. C. Tostes, R. Haddad, M. N. Eberlin, F. R. M. Laurindo, H. P. de Souza, F. M. A. Corrêa & A. M. de Oliveira*

Mechanisms of U46619-induced contraction of rat pulmonary arteries in the presence and absence of the endothelium: *C. McKenzie, A. MacDonald & A. M. Shaw*

This issue is available online at http://www3.interscience.wiley.com/journal/121548564/issueyear?year=2009