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RESEARCH PAPER

Chronic ethanol intake modulates vascular levels of endothelin-1 receptor and enhances the pressor response to endothelin-1 in anaesthetized rats

CR Tirapelli[1,2], E Legros[1], I Brochu[1], J-C Honoré[1], VL Lanchote[3], SA Uyemura[3], AM de Oliveira[4] and P D'Orléans-Juste[1]

[1]*Department of Pharmacology, Faculty of Medicine and Health Sciences, University of Sherbrooke, Sherbrooke, Quebec, Canada;* [2]*Department of Psychiatry Nursing and Human Sciences, College of Nursing of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, SP, Brazil;* [3]*Department of Clinical, Toxicological and Food Science Analysis, Faculty of Pharmaceutical Sciences, University of São Paulo (USP), Ribeirão Preto, SP, Brazil and* [4]*Laboratory of Pharmacology, Department of Physics and Chemistry, Faculty of Pharmaceutical Sciences, University of São Paulo (USP), Ribeirão Preto, SP, Brazil*

Background and purpose: The contribution of endothelin-1 (ET-1) to vascular hyper-reactivity associated with chronic ethanol intake, a major risk factor in several cardiovascular diseases, remains to be investigated.
Experimental approach: The biphasic haemodynamic responses to ET-1 (0.01–0.1 nmol kg^{-1}, i.v.) or to the selective ET_B agonist, IRL1620 (0.001–1.0 nmol kg^{-1}, i.v.), with or without ET_A or ET_B antagonists (BQ123 (c(DTrp-Dasp-Pro-Dval-Leu)) at 1 and 2.5 mg kg^{-1} and BQ788 (*N-cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl1-D-1methoxycarbonyltryptophanyl-D-norleucine) at 0.25 mg kg^{-1}, respectively) were tested in anaesthetized rats, after 2 weeks' chronic ethanol treatment. Hepatic parameters and ET receptor protein levels were also determined.
Key results: The initial hypotensive responses to ET-1 or IRL1620 were unaffected by chronic ethanol intake, whereas the subsequent pressor effects induced by ET-1, but not by IRL1620, were potentiated. BQ123 at 2.5 but not 1 mg kg^{-1} reduced the pressor responses to ET-1 in ethanol-treated rats. Conversely, BQ788 (0.25 mg kg^{-1}) potentiated ET-1-induced increases in mean arterial blood pressure in control as well as in ethanol-treated rats. Interestingly, in the latter group, increases in heart rate, induced by ET-1 at a dose of 0.025 mg kg^{-1} were enhanced following ET_B receptor blockade. Finally, we observed higher levels of ET_A receptor in the heart and mesenteric artery and a reduction of ET_B receptor protein levels in the aorta and kidney from rats chronically treated with ethanol.
Conclusions and implications: Increased vascular reactivity to ET-1 and altered protein levels of ET_A and ET_B receptors could play a role in the pathogenesis of cardiovascular complications associated with chronic ethanol consumption.
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Keywords: chronic ethanol consumption; blood pressure; ET-1; ET_A receptors

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BQ123, c(DTrp-Dasp-Pro-Dval-Leu); BQ788, *N-cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl1-D-1methoxycarbonyltryptophanyl-D-norleucine; ET-1, endothelin-1; IRL1620, {succinyl-[Glu9,Ala11,15]-ET-1(8-210}

Introduction

Chronic ethanol intake constitutes an important cardiovascular risk factor in the general population (Kurihara *et al*., 2004). However, the mechanisms involved in these deleterious properties of chronic ethanol consumption have yet to be fully investigated (Strogatz *et al*., 1991; Kurihara *et al*., 2004). Enhanced secretion of hormones and neurotransmitters, stimulation of the sympathetic nervous system (Chan *et al*., 1985), as well as a myogenic mechanism involving alteration of contractile properties of vascular smooth muscle (Chan and Sutter, 1982), are triggered in conditions of chronic ethanol intake. Furthermore, other reports investigating the chronic effects of ethanol on the cardiovascular system suggest that exacerbation of vascular responsiveness to constrictor agents (Strickland and Wooles, 1988; Hatton *et al*., 1992) or impairment of vascular relaxation (Utkan *et al*., 2001) contribute to the enhanced blood pressure associated with chronic ethanol consumption.

Correspondence: Dr P D'Orléans-Juste, Department of Pharmacology, Faculty of Medicine and Health Sciences, Université de Sherbrooke, 3001, 12th Avenue North, Sherbrooke, Québec, Canada J1H 5N4.
E-mail: pedro.dorleans-juste@usherbrooke.ca
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We have recently provided evidence that chronic ethanol intake enhances endothelin-1 (ET-1)-induced contraction in the isolated rat carotid artery, a consequence of a reduced expression of vasodilator endothelial ET_B receptors (Tirapelli *et al*., 2006b). Thus, based on the above-mentioned study, we hypothesized that chronic ethanol consumption could alter the ET-1 pathway, also *in vivo*.

ET-1 is a 21 amino-acid peptide, belonging to a family of potent vasoconstrictors (Yanagisawa *et al*., 1988). This property of ETs involves the activation of two receptor types, ET_A and ET_B (Alexander *et al*., 2007). The ET_A receptor is restricted to vascular smooth muscle and activation of this G-protein-coupled receptor triggers vasoconstriction (Haynes and Webb, 1993). ET_B receptors were initially described on vascular endothelium, mediating relaxation through production of nitric oxide (NO) (Hirata *et al*., 1993) or prostacyclin (PGI_2) (Filep *et al*., 1991; Schilling *et al*., 1995; Matsuda *et al*., 1999) or causing contraction via the same receptor population localized in vascular smooth muscle (Ihara *et al*., 1991; Tirapelli *et al*., 2005).

ET-1 has potent vasoconstrictor, mitogenic and pro-inflammatory properties, which may contribute to the progression of several cardiovascular disorders (D'Orléans-Juste *et al*., 2002; Tostes and Muscara, 2005). Interestingly, some reports have described that ethanol consumption induces an increase in ET-1 production. Tsuji *et al*. (1992) observed that ethanol increased the production of ET-1 and ET-2 in human cultured umbilical vein. Furthermore, Nanji *et al*. (1994) reported increased plasma ET-1 levels in rats treated with ethanol, suggesting that chronic ethanol consumption alters the ET-1 pathway.

In the present study, we therefore aimed to determine whether and how chronic ethanol consumption affects the ET system in rats. We compared the effect of ethanol intake for 2 weeks on the haemodynamic responses to ET-1 in anaesthetized rats and assessed putative alterations in the expression of both, ET_A and ET_B receptors in the heart, mesenteric artery, aorta, liver and kidney. Our results highlight a possible correlation between ET-1-induced increase in blood pressure and enhanced levels of ET receptors in resistance vessels after chronic treatment with ethanol, suggesting a biochemical modification underlying the development of the pathological ethanol-induced vascular hyper-responsiveness.

Methods

Ethanol treatment

Experiments were performed in accordance with the principles and guidelines of the Canadian Council on Animal Care, using male Wistar rats obtained from Charles River (St Constant, QC, Canada). The rats, initially weighing 300–350 g (90–100 days old), were randomly divided into two groups: control and ethanol-treated animals. Control rats received tap water *ad libitum*, whereas rats from the ethanol group received 20% (v/v) ethanol in their drinking water (Tirapelli *et al*., 2006a, b). All animals had free access to Purina Lab Chow^R. To avoid a considerable loss of animals, the ethanol-treated group was submitted to a brief and gradual adaptation period. The animals received 5% ethanol in their drinking water during the first week, 10% in the second and 20% in the third week. At the end of the third week, the experimental stage was initiated and lasted for 2 weeks.

Blood ethanol and serum measurements of glucose, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total proteins and bilirubin

Blood samples were collected for analysis of blood ethanol content at the end of the second week of ethanol feeding. All samples were collected during the morning period, from the carotid artery of anaesthetized rats using heparinized syringes. The samples were analysed as previously described (Tirapelli *et al*., 2006a) using a CG-17A gas chromatography (Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and an HSS-4A headspace sampler (Shimadzu). Injections were made in the split mode onto a Supelcowax 10 (Supelco, Bellefonte, PA, USA) column (30 m × 25 mm i.d. and 25 μm film thickness). Calibration standards were prepared in the same headspace vials (0.10–3.16 mg mL^{-1}) and results were expressed as milligrams of ethanol per millilitre of blood.

For glucose, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total proteins and bilirubin measurements, blood was collected from the carotid artery of anaesthetized rats. The samples were centrifuged at 8000–10 000 *g* for 10 min at room temperature. The serum was assayed using commercial kits (Labtest Diagnóstica, São Paulo, São Paulo, Brazil) with the auto-analyzer ABBOTT (model ABAA VP) and results were expressed as units per litre, grams per litre or milligrams per litre.

In vivo *experimental procedures*

The experimental approaches used in this study are in accordance with Gobeil *et al*. (1996) and Gendron *et al*. (2005) with slight modifications. Briefly, rats were anaesthetized with ketamine/xylazine (87/13 mg kg^{-1}) injected intramuscularly. A polyethylene catheter (PE-50) filled with heparin/saline solution was inserted into the common right carotid artery to monitor the mean arterial blood pressure (MAP) and heart rate (HR) with a transducer linked to a blood pressure analyzer (Micro-Med, Louisville, KY, USA). Another catheter (PE-50) was inserted into the left jugular vein to administer pharmacological agents (agonists and antagonists). Dose–response curves to ET-1 (0.01–0.1 nmol kg^{-1}), IRL1620 ({succinyl-[Glu9,Ala11,15]-ET-1(8-210}) (0.001–1 nmol kg^{-1}), a selective endothelin ET_B receptor agonist, phenylephrine (1.5–48 μg kg^{-1}) or acetylcholine (1.5–48 μg kg^{-1}) were performed in control and ethanol-treated rats. On the basis of results obtained in our dose–response curves, doses of 0.01 and 0.025 nmol $kg^{-1}$of ET-1 were used to investigate the mechanisms underlying the increase in blood pressure induced by chronic ethanol consumption. Because there was a loss of responsiveness to ET-1 after repeated administrations, parallel assays were needed in which the following antagonists were injected i.v. 5 min before ET-1: the endothelin ET_A receptor antagonist

c(DTrp-Dasp-Pro-Dval-Leu) (BQ123) (1 or 2.5 mg kg^{-1}) and the endothelin ET_B receptor antagonist BQ788 (*N-cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl1-D-1methoxy-carbonyltryptophanyl-D-norleucine) (0.25 mg kg^{-1}). The doses of antagonists were based on previous reports (McMurdo *et al*., 1993; Honore *et al*., 2002).

Western immunoblotting

Total protein was extracted from heart, kidney, arterial mesenteric bed, aorta and liver. The Bradford assay was used to determine protein concentration. The assay was performed as previously described (Tirapelli *et al*., 2005). In brief, proteins were denatured in 2 × Laemmli sample buffer by heating to 95 °C for 5 min, cooled on ice and followed by centrifugation (12 000 *g*; 5 min; 15 °C). Total protein (30–50 μg) was separated by electrophoresis on 10% SDS polyacrylamide gel and transferred to methanol-activated polyvinylidene fluoride membrane (Amersham, Little Chalfont, Buckinghamshire, UK) in Tris-glycine buffer containing 20% of methanol. Membranes were blocked on Tris-buffered saline Tween-20 with 8% non-fat dry milk and incubated with rabbit polyclonal antiserum (1:200) raised against rat ET_B (AER-002) or (1:200) rat ET_A receptors (AER-001) (Alomone Labs, Jerusalem, Israel). GAPDH was used as an internal control and detected with rabbit polyclonal antiserum (1/400), (sc-25778) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). As second antibody, the goat anti-rabbit IgG coupled to horseradish peroxidase (sc-2004) (Santa Cruz Biotechnology Inc.) 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, Piscataway, New Jersey, USA) to determine level of protein expression. As a final specificity control, antibodies were mixed with antigenic peptides used to raise either the ET_A or ET_B antiserum, prior to incubation with total protein-containing membranes, as recommended by Alomone Labs.

Statistical analysis

The results (mean ± s.e.mean) were compared using Student's *t*-test or one-way ANOVA (followed by Bonferroni's multiple comparison test) as indicated in the text and legends. Results of statistical tests with $P<0.05$ were considered as significant.

Drugs

ET-1 and IRL1620 were purchased from Peptide International (Louisville, Kentucky, USA), phenylephrine and acetylcholine from Sigma Co (Oakville, Ontario, Canada). BQ123 and BQ788 were synthesized by Dr Witold Neugebauer (Department of Pharmacology, University of Sherbrooke). Drugs were dissolved in phosphate-buffered saline (pH 7.4) except for BQ123 and BQ788, which were diluted in 10% dimethylsulphoxide and subsequently in phosphate-buffered saline. The concentration of dimethylsulphoxide in the final solution had no effects *per se* on basal cardiovascular parameters or on the pharmacological effects of agonists or antagonists used in the present study.

Results

Body weight, blood ethanol measurements and serum levels of glucose, AST, ALT, alkaline phosphatase, total proteins and bilirubin

Results for body weight, blood ethanol measurements and serum levels of glucose, AST, ALT, alkaline phosphatase, total proteins and bilirubin are summarized in Table 1. As expected, ethanol plasma levels were increased only in animals given ethanol in drinking water. Furthermore, there was a reduction in body weight in the ethanol-treated rats compared with that in control animals. On the other hand, the values of the other metabolic variables measured did not differ between the groups.

Effect of chronic ethanol consumption on basal MAP and HR

The baseline MAP of ethanol-treated rats (105 ± 1.6 mm Hg, $n=48$) was higher that that of the control group (85 ± 0.7 mm Hg, $n=50$) ($P<0.05$; Student's *t*-test). Likewise, the basal values of the systolic blood pressure and diastolic blood pressure were higher in the ethanol group (118 ± 2 mm Hg and 92 ± 1.6 mm Hg, respectively) when compared with control group (103 ± 1.1 mm Hg and 74 ± 0.7 mm Hg, respectively) ($P<0.05$; Student's *t*-test). On the other hand, no HR changes were observed between control (253 ± 3.4 b.p.m., $n=50$) and ethanol-treated rats (247 ± 3.7 b.p.m., $n=48$).

Effect of chronic ethanol consumption on the depressor or pressor response induced by ET-1, IRL1620, phenylephrine and acetylcholine

In anaesthetized rats, bolus intravenous injection of ET-1 or IRL1620 produced a transient fall in blood pressure followed by a sustained pressor response. Figure 1 shows the maximal decrease and increase in MAP induced by ET-1 (0.01–0.1 nmol kg^{-1}) or IRL1620, a selective endothelin ET_B receptor agonist (0.001–1.0 nmol kg^{-1}). We observed that the decrease in MAP induced by ET-1 and IRL1620 did not

Table 1 Body weight, blood ethanol levels and serum levels of glucose, AST, ALT, total proteins, alkaline phosphatase, total bilirubin and direct bilirubin obtained from control and ethanol-treated rats

	Control	*Ethanol*	n
Body weight (g)	466 ± 7	415 ± 7	32
Blood ethanol (mg mL^{-1})	ND	1.9 ± 0.2*	11
Glucose (mg L^{-1})	1064 ± 77	1090 ± 59	12
AST (U L^{-1})	128 ± 12	98 ± 7	15
ALT (U L^{-1})	68 ± 3	62 ± 8	15
Total proteins (g L^{-1})	73 ± 2	74 ± 2	14
Alkaline phosphatase (U L^{-1})	186 ± 17	177 ± 15	16
Total bilirubin (mg L^{-1})	4 ± 0.2	4 ± 0.2	13
Direct bilirubin (mg L^{-1})	2 ± 0.1	2 ± 0.2	14

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ND, non-detectable.
*Significantly different compared with control group ($P<0.05$, Student's *t*-test).
Numbers in the last column indicate the number of replicates. Values shown are means ± s.e.mean.

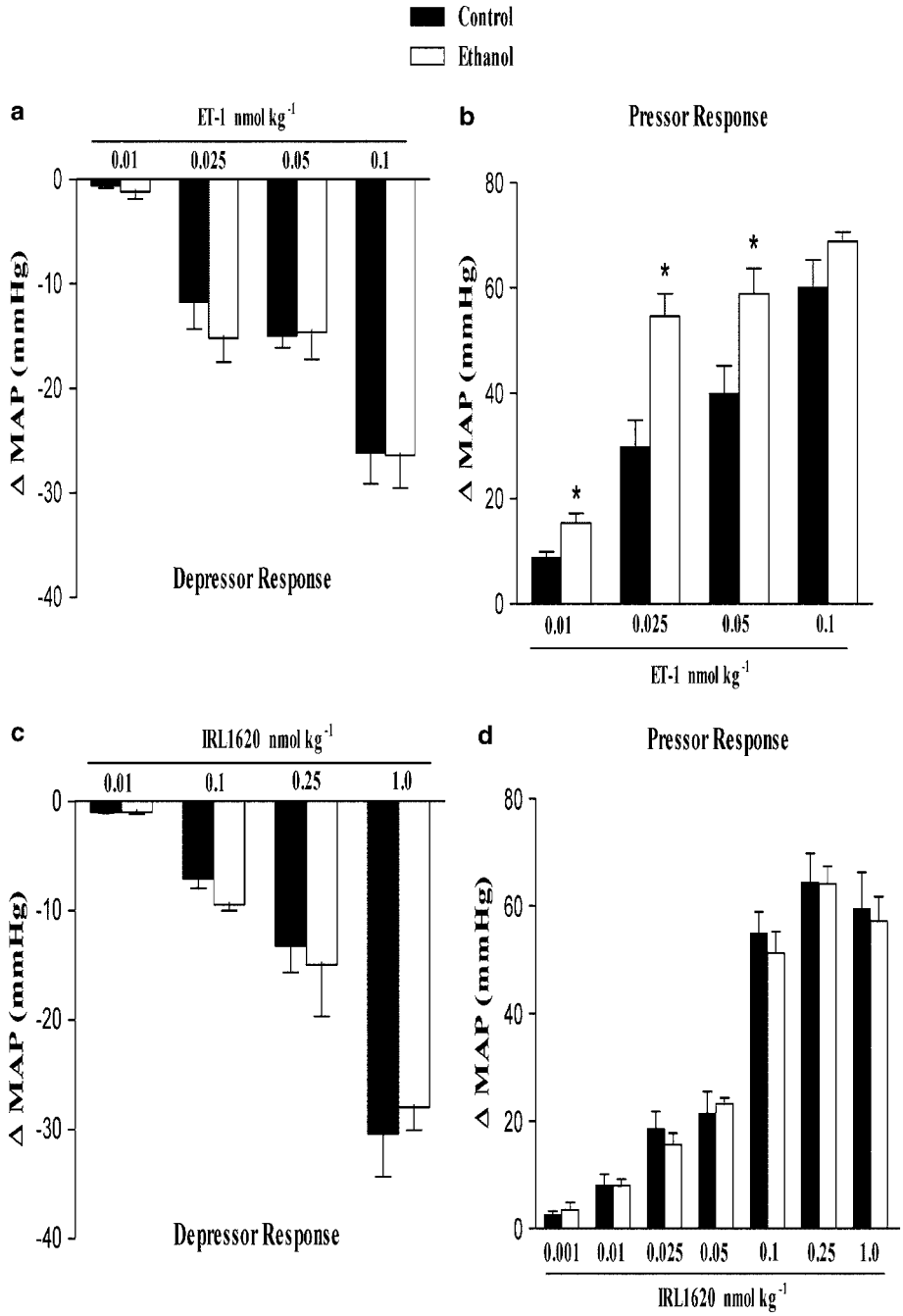

Figure 1 Effect of chronic ethanol consumption on the maximal variation in mean arterial blood pressure (MAP) induced by i.v. injection of ET-1 (0.01, 0.025, 0.05 or 0.1 nmol kg^{-1}) or IRL1620 (0.001, 0.01, 0.025, 0.05, 0.1, 0.25 or 1.0 nmol kg^{-1}) in the anaesthetized rat. Each point represents the mean ± s.e.mean of 6–11 independent experiments for the maximal depressor (**a**, **c**) and pressor (**b**, **d**) response induced by ET-1 or IRL1620. No depressor responses to IRL1620 were found in the dose range of 0.001–0.05 nmol kg^{-1} (only the doses of 0.01 nmol kg^{-1} and higher of IRL1620 are shown) (*$P<0.05$ compared with control group; Student's *t*-test).

differ between groups. However, after treatment for 2 weeks with ethanol, rats presented a higher increase in MAP induced by ET-1, at all doses except the highest (0.1 nmol kg^{-1}). On the other hand, IRL1620-induced increase in MAP did not differ between treatment groups. Note that as shown in Figure 2, chronic ethanol consumption did not alter either the increase in MAP induced by phenylephrine, or the decrease in MAP induced by acetylcholine (Figure 2). In addition, no differences were observed in the ET-1 or IRL1620-dependent increases in HR between control or ethanol-treated groups (Figures 5a and d).

Effect of BQ123 and BQ788 on the pressor or depressor response and changes in HR induced by ET-1 in control and ethanol-treated rats

To investigate further the role of ET receptors in cardiovascular responses to ET-1 after ethanol treatment, we

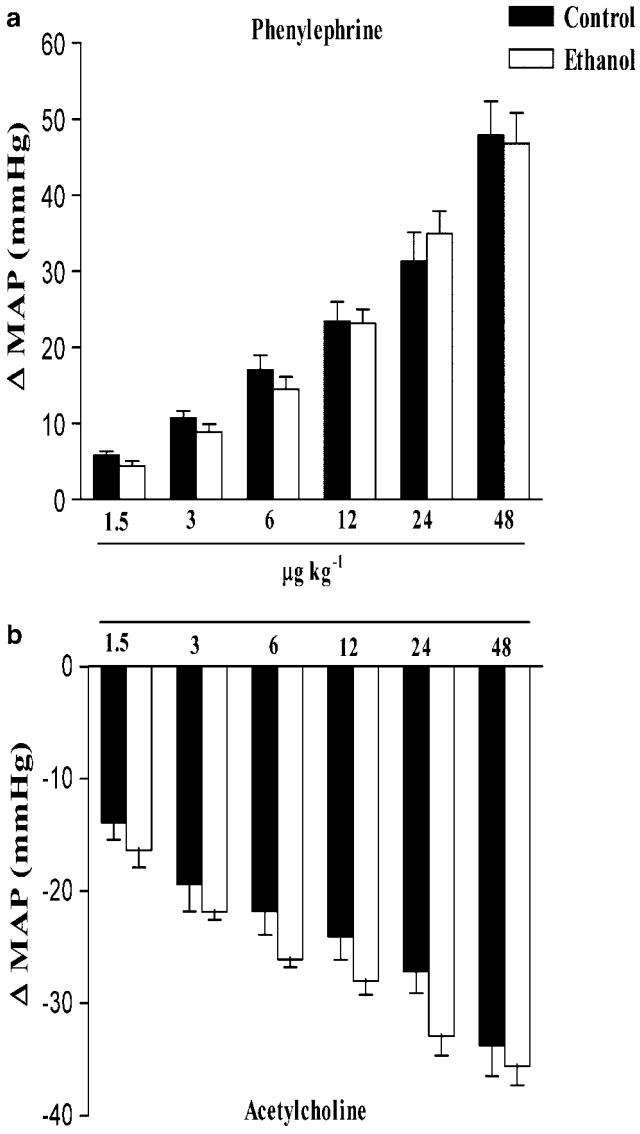

Figure 2 Effect of chronic ethanol consumption on the maximal variation in mean arterial blood pressure (MAP) induced by i.v injection of phenylephrine (1.5–48 $\mu g\,kg^{-1}$) or acetylcholine (1.5–48 $\mu g\,kg^{-1}$) in the anaesthetized rat. Each point represents the mean ± s.e.mean of 6–7 independent experiments for the maximal pressor (**a**) and depressor (**b**) response induced by phenylephrine or acetylcholine, respectively.

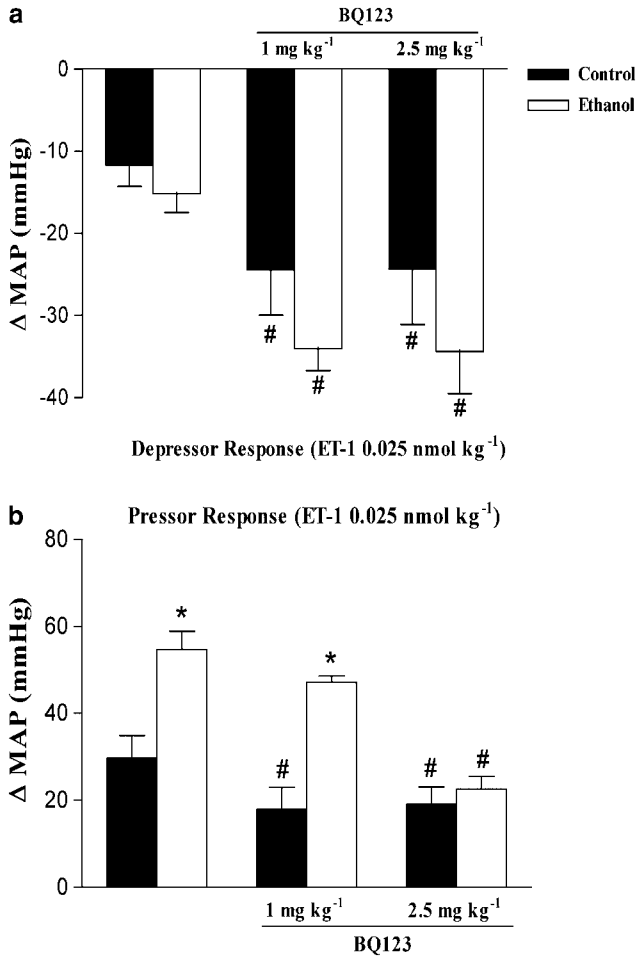

Figure 3 Effect of BQ123 (1 or 2.5 $mg\,kg^{-1}$) on the maximal variation in mean arterial blood pressure (MAP) induced by i.v. injection of endothelin (ET)-1 (0.025 $nmol\,kg^{-1}$) in anaesthetized control and ethanol-treated rats. Each point represents the mean ± s.e.mean of 5–6 independent experiments for the maximal depressor (**a**) and pressor (**b**) response induced by ET-1 (*$P<0.05$ compared with control group; # compared with the respective group in the absence of BQ123; ANOVA followed by Bonferroni's comparison test).

performed experiments with selective ET_A and ET_B receptor antagonists. The effects of BQ123 and BQ788 on the depressor and pressor responses to ET-1 are represented in Figures 3 and 4. At 1 $mg\,kg^{-1}$, BQ123, a selective ET_A antagonist, significantly reduced the increase in MAP induced by ET-1 (0.025 $nmol\,kg^{-1}$) in control rats, but failed to do so in ethanol-treated rats (Figure 3b). On the other hand, when administered at 2.5 $mg\,kg^{-1}$, BQ123 reduced ET-1-induced increase in MAP in both control and ethanol-treated rats (Figure 3b). The selective ET_A antagonist also potentiated the initial ET-1-induced hypotensive responses in control or ethanol-treated rats (Figure 3a). At the concentrations used in the present study, BQ123 did not alter basal MAP as previously observed (Honore *et al.*, 2002).

On the other hand, BQ788, a selective ET_B antagonist, abolished the ET-1-induced hypotensive responses (at 0.025 $nmol\,kg^{-1}$) in control and ethanol-treated rats (Figure 4b). Conversely, the increase in MAP induced by ET-1 (0.01 $nmol\,kg^{-1}$) was potentiated by BQ788 (0.25 mg kg^{-1}) in control and ethanol-treated rats (Figure 4c), whereas the pressor response to a higher dose of the same agonist (0.025 $nmol\,kg^{-1}$) was significantly enhanced by the ET_B antagonist only in control animals (Figures 4c and d). Administration of BQ788 produced the same increases in basal MAP in control (6.2 ± 1.0 mm Hg, $n=16$) as in ethanol-treated rats (8.5 ± 2.0 mm Hg, $n=16$).

Finally, the increase in HR induced by ET-1 (0.025 nmol kg^{-1}) was unaltered by BQ123 (1 or 2.5 $nmol\,kg^{-1}$) in control or ethanol-treated rats (Figure 5b). In contrast, increases in HR induced by ET-1, at 0.025 but not 0.01 $nmol\,kg^{-1}$, were potentiated by BQ788 (0.25 $mg\,kg^{-1}$) in ethanol-treated but not in control rats (Figure 5c).

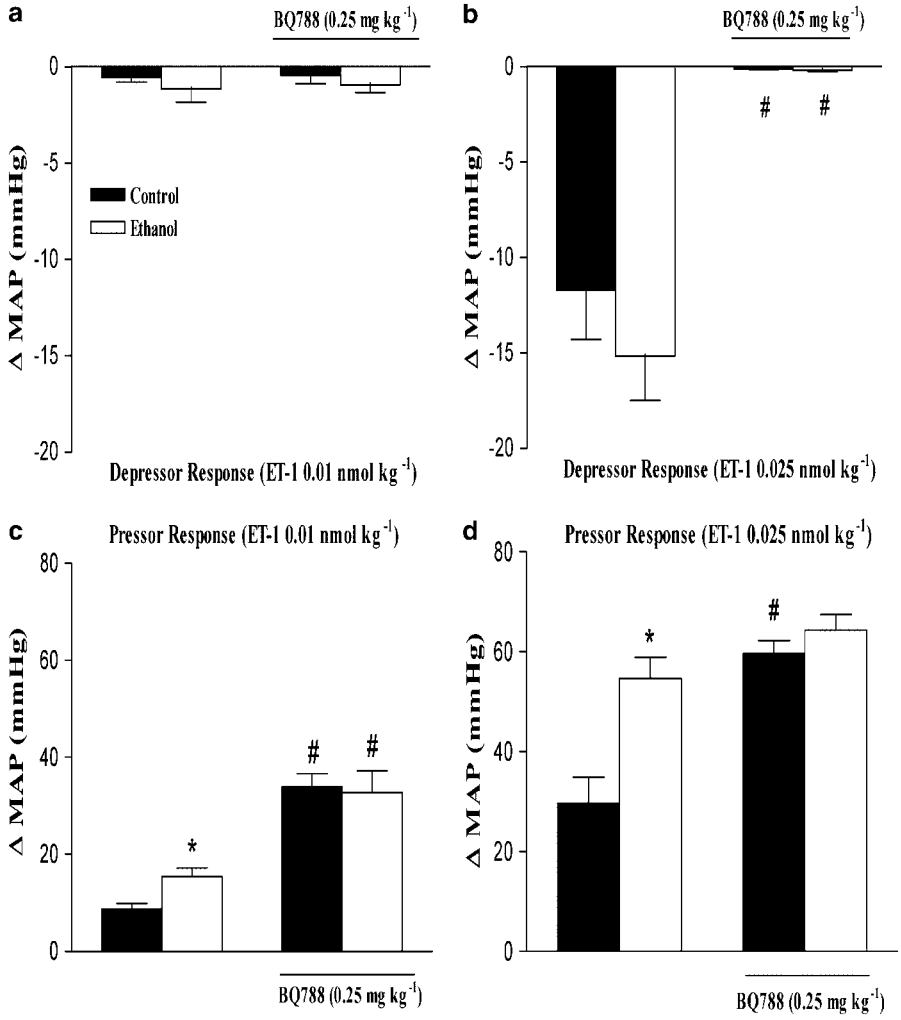

Figure 4 Effect of BQ788 (0.25 mg kg^{-1}) on the maximal variation in mean arterial blood pressure (MAP) induced by i.v. injection of endothelin (ET)-1 (0.01 or 0.025 nmol kg^{-1}) in anaesthetized control and ethanol-treated rats. Each point represents the mean ± s.e.mean of 6–11 independent experiments for the maximal depressor (**a**, **b**) and pressor (**c**, **d**) response induced by ET-1 (*$P<0.05$ compared with control group; # compared with the respective group in the absence of BQ788; ANOVA followed by Bonferroni's comparison test).

Effect of chronic ethanol consumption on protein levels of ET_A and ET_B receptors in the heart, mesenteric artery, kidney, aorta and liver

Western blot assays showed that protein levels of ET_A receptors were increased in the heart and mesenteric artery from ethanol-treated rats when compared with control (Figure 6). On the other hand, no alterations in protein levels of ET_A receptors were found in the kidney (Figure 6), aorta (control: 100.0 ± 7.7%, $n=4$; ethanol: 65.2 ± 16.0%, $n=4$) and liver (control: 100.0 ± 4.2%, $n=4$; ethanol: 157 ± 45.7%, $n=4$). The protein levels of ET_B receptors were decreased in the kidney (Figure 6) and aorta (control: 100.0 ± 9.2%, $n=4$; ethanol: 40.7 ± 6.7%, $n=4$) ($P<0.05$; Student's *t*-test), but not altered in the heart, mesenteric artery (Figure 6) and liver (control: 100 ± 26%, $n=4$; ethanol: 77 ± 18%, $n=4$) from ethanol chronically treated rats. As a final control, Figure 7 shows the specificity of the antibodies used in the present study in tissue homogenates derived from both control or ethanol-treated animals, as pretreatment of total protein containing membranes of the heart, kidney or mesentery artery incubated with the ET_A or ET_B antibodies pre-incubated with their respective specific antigenic peptide, markedly reduced the detection of ET_A and ET_B receptors. Similar results were obtained with antigenic peptides for ET_A and ET_B receptors incubated with tissue homogenates of aorta and liver (results not shown).

Discussion

We have shown in the present study that rats chronically treated with ethanol exhibit an enhanced, ET_A-dependent, pressor response to ET-1. In addition, a positive correlation was found between this enhanced response to the 21 amino-acid peptide and an increased ET_A receptor protein level in cardiac tissue and in a resistance vessel, whereas the ET_B receptor was reduced in aortic and renal tissue homogenates.

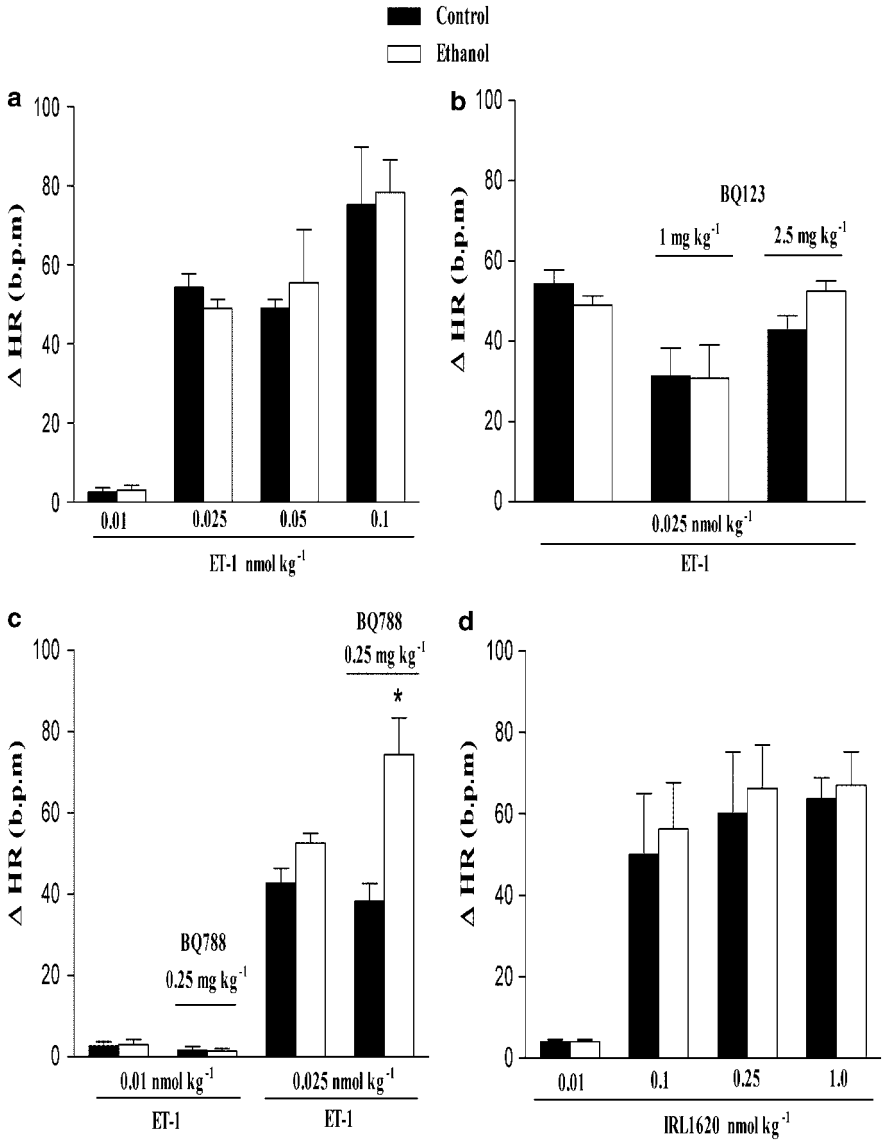

Figure 5 Maximal decrease in heart rate (HR) induced by i.v. injection of endothelin (ET)-1 (0.01, 0.025, 0.05 or 0.1 nmol kg^{-1}) (**a**), ET-1 (0.025 nmol kg^{-1}) in the presence of BQ123 (1 or 2.5 mg kg^{-1}) (**b**), ET-1 (0.01 or 0.025 nmol kg^{-1}) in the presence of or BQ788 (0.25 mg kg^{-1})(**c**), IRL1620 (0.01, 0.1, 0.25 or 1.0 nmol kg^{-1}) (**d**) in the anaesthetized rat. Each point represents the mean ± s.e.mean of 5–6 independent experiments (*$P<0.05$ compared with control group; Student's *t*-test).

Our data show that chronic ethanol consumption increases ET-1-induced pressor response but does not alter the initial hypotensive response to the peptide. Furthermore, chronic ethanol consumption did not alter the biphasic pressor response elicited by IRL1620, a selective ET_B receptor agonist. Thus, enhanced ET-1-induced pressor response may not be due to decreased endothelial-dependent dilatation or vasoconstriction following activation of ET_B receptors.

We also found that chronic ethanol consumption did not alter acetylcholine-induced hypotension or the pressor response induced by phenylephrine, a selective α_1-adrenoreceptor agonist. Thus, the two above-mentioned series of experiments further strengthen our hypothesis that ethanol treatment specifically affects the ET-1 pathway. It is of interest that Resstel *et al.* (2006) recently reported an enhanced pressor response to phenylephrine in non-anaesthetized (telemetry instrumented) rats, whereas we were unable, in the present study, to observe the same potentiation of the hypertensive effect of the α_1-adrenoreceptor agonist in anaesthetized animals. Worthy of mention, Resstel *et al.* (2006) also applied a continuous infusion protocol rather than the bolus injections of phenylephrine used in the present study.

Abdel-Rahman *et al.* (1985) suggested that anaesthetics depress the cardiovascular system to a greater extent in ethanol-fed than in control rats. In the present study however, no significant alterations in basal haemodynamic parameters (or HR) were noted in ethanol-treated rats under anaesthesia when compared with non-anaesthetized animals. These findings are in accordance with previous results in conscious rats using the same ethanol treatment protocol (Resstel *et al.*, 2006).

Interestingly, changes in MAP induced by ET-1 at 0.01 nmol kg^{-1} were higher in ethanol-treated rats after

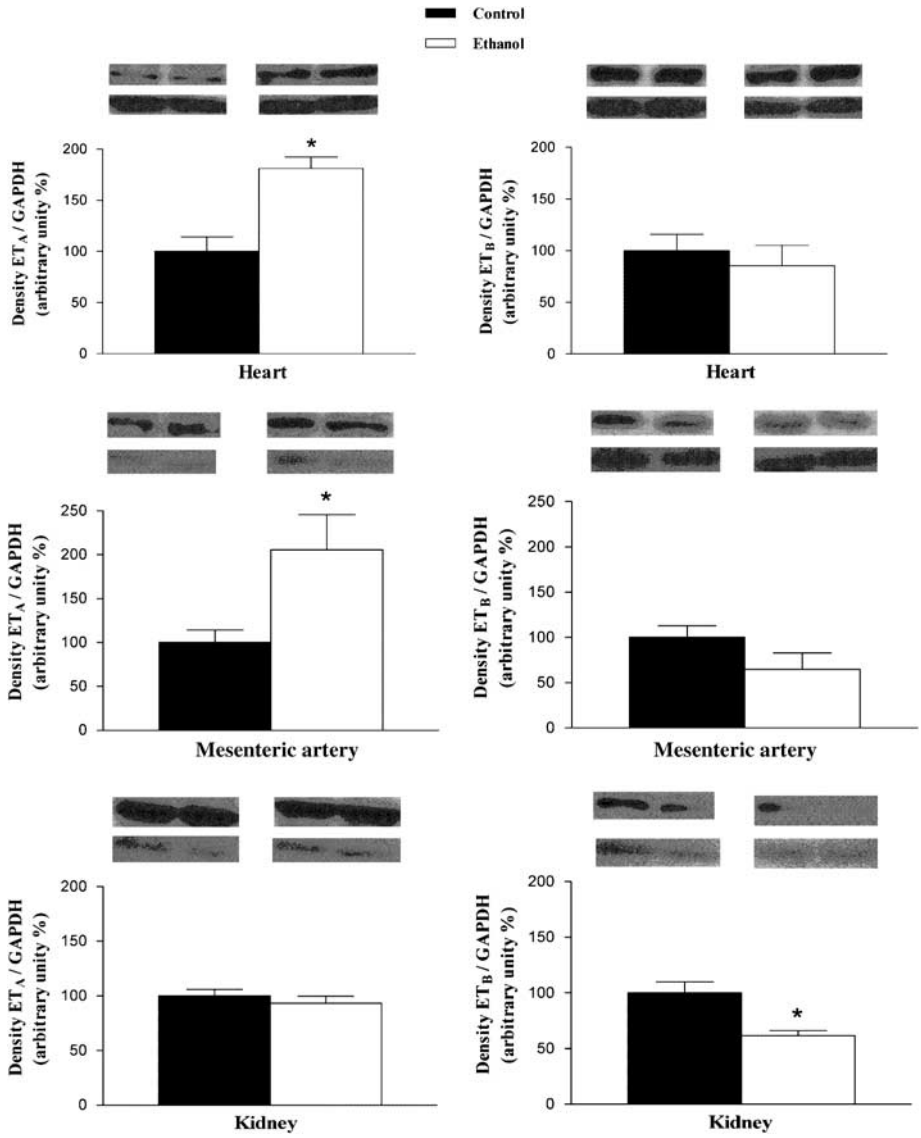

Figure 6 Representative western immunoblots of 50 μg total protein extracted from heart, mesenteric artery and kidney from control or ethanol-treated rats. The bar graphs show the relative absorbance values of ET_A and ET_B receptor bands. Values were normalized by the corresponding GAPDH bands (used as an internal standard). Results are reported as means ± s.e.mean and are representative of 4–5 experiments (*$P<0.05$ compared with control group; Student's *t*-test).

administration of BQ788. This result suggests that the enhancement of ET-1-induced pressor responses after ethanol treatment involves an increased response to the peptide mediated by ET_A but not ET_B receptors. However, it is noteworthy that the ET_A antagonist, BQ123 failed to reduce the hypertensive state induced by chronic ethanol in the present rat model. We attribute this lack of effect of BQ123 to the acute (that is, 5 min before injection of ET-1), rather than a chronic, administration protocol in the present study. It remains to be seen if a 4- to 5-day regimen with BQ123 i.v. or an orally available ET_A antagonist would correct the ethanol-induced hypertensive state in this rat model.

Patients with cirrhosis and experimental animal models of this disease show elevated serum and hepatic levels of ET-1 (Moore *et al*., 1992; Gandhi *et al*., 1996; Ikura *et al*., 2004). Metabolic parameters were also analysed in our study to assess whether alterations in hepatic function following ethanol consumption could be involved in the exacerbated cardiovascular responses to ET-1 in our model. Glucose, AST, ALT, alkaline phosphatase, total proteins and bilirubin, which are markers for hepatic dysfunction in cirrhotic stages, were unchanged by the 2-week ethanol treatment.

Chronic ethanol intake has been associated with increases in blood pressure attributed to several mechanisms, including the secretion of hormones and neurotransmitters, stimulation of the sympathetic nervous system, alteration of baroreceptor activity and volume overload (Altura and Altura, 1982; Chan and Sutter, 1982). The present results support the concept that enhanced vascular responsiveness to ET-1 may also be involved in the increases in systemic resistance induced by chronic ingestion of ethanol.

The results obtained with ET antagonists suggest that the enhancement of ET-1-induced pressor responses after ethanol treatment might involve an increased response to

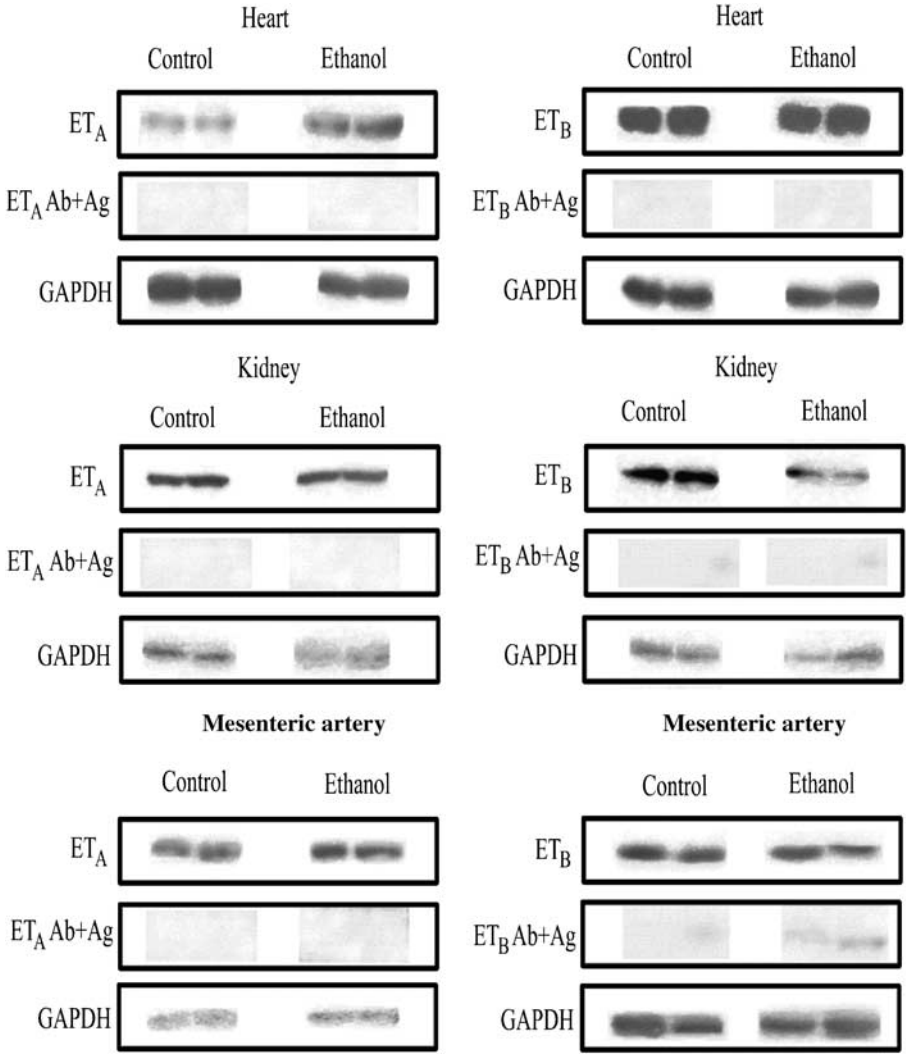

Figure 7 Representative western immunoblots of 50 μg total protein extracted from heart, kidney and mesenteric artery from control or ethanol-treated rats with ET_A or ET_B antibodies in absence (upper panels) or presence (mid-panels) of their respective antigenic peptides. Ab: antibody, Ag: antigenic peptide.

the peptide mediated by ET_A but not ET_B receptors. However, ethanol consumption for 2 weeks did not affect resting HR, suggesting that alterations in chronotropic cardiac parameters do not mediate the elevation of basal blood pressure observed in ethanol-treated rats. This postulate is in accordance with previous studies reporting a lack of alteration in resting HR after long-term ethanol consumption (Beilin *et al*., 1992; Resstel *et al*., 2006). Furthermore, increases in HR triggered by ET-1 and IRL1620 were not altered by chronic ethanol consumption. Moreover, the administration of BQ123 did not alter the changes in HR induced by ET-1. Interestingly, changes in HR induced by ET-1 were significantly higher in ethanol-treated rats after administration of BQ788. Taken together, these results suggest that chronic ethanol consumption alters the pattern of response mediated by cardiac ET_A receptors, which exerts positive chronotropic effect in the heart (Robu *et al*., 2003).

By western blots, we also demonstrated that ET_A protein levels in the heart and mesenteric artery were increased in rats chronically treated with ethanol. Thus, we suggest that changes in HR elicited by ET-1 in the ethanol-treated rats, under BQ788 treatment, were caused by increased cardiac ET_A receptor protein levels, reported to be involved in ET-1-induced positive chronotropic effects (Robu *et al*., 2003). Alternatively, based on our experiments with BQ788, we suggest that ET_B receptors play a protective role in ethanol-treated rats by counteracting ET_A-mediated positive chronotropic effects. Additionally, long-term ethanol consumption may be associated with remodelling as ET_A receptor activation mediates cardiac hypertrophy in rats (Hafiz *et al*., 2004). Furthermore, the overexpression of ET_A receptors, which mediate ET-1-induced contraction in the rat arterial mesenteric bed (D'Orleans-Juste *et al*., 1993), could also be involved in the increased pressor response induced by ET-1 in ethanol-treated rats. Finally, the reduced expression of ET_B protein levels in the aorta could also contribute to the increased responsiveness to ET-1 after treatment with ethanol, as these receptors are known to produce relaxation (Sudjarwo *et al*., 1992) in this tissue as previously suggested

by Tirapelli *et al*. (2006b) using rat carotid arteries under the same experimental conditions.

Previously, renal ET_B receptor deficiency has been associated with increased blood pressure (Tazawa *et al*., 2004), suggesting that the reduced expression of this receptor type observed in the present investigation in kidney homogenates could also contribute to the increased responsiveness to ET-1 as well as the overall hypertensive state of rats chronically treated with ethanol. Whether the altered expression of ET receptors is a cause or consequence of ethanol-induced enhancement in blood pressure remains to be determined.

Alterations in the ET pathway have been reported in some vascular diseases such as cerebral ischaemia (Salom *et al*., 2000), subarachnoid haemorrhage (Alabadi *et al*., 1997) and hypertension (Cardillo *et al*., 1999). The present study demonstrates for the first time that chronic ethanol potentiates pressor responses to ET-1 and alters ET_A and ET_B receptor proteins in the heart, kidney, aorta and mesenteric artery confirming that chronic ethanol consumption alters the ET-1 pathway. Treatment for 2 weeks with ethanol induced a twofold increase in plasma ET-1 levels in rats that presented blood ethanol levels within the range that we found in our experiments (Nanji *et al*., 1994). On the basis of previous findings from the literature as well as from the present study it is suggested that the ET pathway could play a role in the pathogenesis of cardiovascular complications associated with chronic ethanol consumption (Alabadi *et al*., 1997; Salom *et al*., 2000).

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Conflict of interest

The authors state no conflict of interest.

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