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Ethanol Consumption Enhances Endothelin-1-Induced Contraction in the Isolated Rat Carotid

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Abbreviations: L-NAME (N^G -nitro-L-arginine methyl ester), ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one) TEA (tetraethylammonium chloride), 4-AP (4-aminopyridine), IRL1620 (succinyl-[Glu⁹,Ala^{11,15}]-ET-1(8-210)), BQ788 ([N-cis-2,6-dimethyl-piperidinocarbonyl-L- γ -methylleucyl¹-D-1-methoxycarbonyltryptophanyl-D-norleucine]), BQ123 (c(DTrp – Dasp – Pro – Dval – Leu)).

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Abstract

We investigated the mechanisms involved in the enhancement of endothelin (ET)-1 vascular reactivity induced by ethanol consumption. Ethanol intake for 2-, 6- and 10-weeks enhanced ET-1- induced contractile response of endothelium-intact, but not endothelium-denuded, rat carotid rings independently of the treatment duration. Conversely, phenylephrine-induced contraction was not affected by ethanol intake. The contraction induced by IRL1620, a selective ET_B agonist, was increased after treatment with ethanol in endothelium-intact, but not in endothelium-denuded, carotids. Moreover, ET-1- and IRL1620-induced relaxation were reduced in endothelium-intact phenylephrine-pre-contracted rings from ethanol-treated rats. Acetylcholine-induced relaxation was not affected by ethanol treatment. N^G-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), indomethacin and tetraethylammonium (TEA) reduced the relaxation induced by IRL1620 in carotids from control but not ethanol-treated rats. The mRNA levels for ET_A and ET_B receptors were not altered by ethanol consumption. However, ethanol treatment reduced the protein expression of ET_B receptors. Furthermore, immunohistochemical assays showed reduced immunostaining for endothelial ET_B receptors after treatment with ethanol. We conclude that ethanol consumption enhances ET-1-induced contraction in the rat carotid and that this response is not different among the three periods of treatment employed in this study. Finally, the potentiation of ET-1 induced vascular reactivity is likely caused by a reduced expression of relaxing endothelial ET_B receptors.

Introduction

Epidemiological and clinical studies have established a positive relationship between long-term ingestion of ethanol and the development of hypertension (Resstel et al., 2006), brain ischemia and stroke-like events (Altura et al., 1983; Gill et al., 1991; Kitohara et al., 1995). Ethanol consumption results in atrophy of cerebral areas that may be related to reduction in cerebral blood flow (Melgaard et al., 1990; Oishi et al., 1999). Much of the research on the effects of ethanol on the cardiovascular system has dealt with vascular responsiveness to vasoconstrictor agents (Strickland and Wooles, 1988; Hatton et al., 1992). Several groups have reported enhanced vascular reactivity to vasoconstrictor agents (Pinard et al., 1992; Tirapelli et al., 2006) or impairment of the vascular relaxation (Kähönen et al., 1999) with regards to cardiovascular complications associated with ethanol consumption.

The positive correlation between the duration of ethanol intake and the development of cardiovascular abnormalities reported by previous studies (Abdel-Rahman and Wooles, 1987; Strickland and Wooles, 1988) suggests that the period of exposure to ethanol is a major factor in the development of cardiovascular complications. However, the time scale for ethanol treatment varies among most of the published studies (Abdel-Rahman et al. 1985; Chan et al., 1985; Utkan et al., 2001), albeit most reports related to changes in vascular reactivity use only single periods of treatment (Utkan et al., 2001; Brown et al., 2002).

Endothelin-1 (ET-1), the predominant isoform of the ET peptide family, has potent vasoconstrictor, mitogenic, and pro-inflammatory properties, and is implicated in numerous cardiovascular diseases (Yanagisawa et al. 1988; Tostes & Muscara, 2005). Interestingly, Nanji et al. (1994) observed increased plasma ET-1 levels in rats treated with ethanol, suggesting that the later peptide plays a role in the cardiovascular complications induced by ethanol consumption. However, to our knowledge, no studies have evaluated the vascular responses to ET-1 in ethanol-treated rats

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The aim of this study was therefore to determine whether there are any changes in vascular reactivity to ET-1 in carotid arteries from ethanol-treated rats. The carotid artery was chosen based on evidence of the importance of this vascular bed in the cerebral blood flow (Eugene et al., 1999). As we are not familiar with any reports in the literature, which deal with the time-course for the effect of ethanol intake on the vascular responsiveness to ET-1, we investigated the effect of ethanol intake for 2, 6 and 10 weeks.

Materials and Methods

Experimental design

Male Wistar rats were housed under standard laboratory conditions with free access to food and water. The housing conditions and experimental protocols are in accordance with the Ethical Animal Committee from the University of São Paulo.

The rats initially weighing 300-350 g (80-100 days old) were randomly divided into three groups: control, isocaloric and ethanol. Control rats received tap water *ad libitum*. Rats from the isocaloric group received a solution containing an isocaloric amount of sucrose (290.50 g/L) instead of ethanol. Rats in the ethanol group received 20% (v/v) ethanol in their drinking water (Tirapelli et al., 2006; Resstel et al., 2006). In order 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 in the first week, 10% in the second and 20% in the third week. At the end of the third week the experimental stage began. The same procedure was adopted for the isocaloric group. In these groups, the caloric content of the liquid diet was adjusted to match that of the ethanol-exposed groups. The isocaloric groups were included in the study protocol to evaluate whether alterations in caloric intake following ethanol consumption would explain the possible influences of ethanol on arterial responses. The rats were treated for 2, 6 and 10 weeks and weighted weekly.

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Blood ethanol and serum glucose measurements

Blood was collected from the aorta of anesthetized rats and ethanol analysis was carried out using a CG-17A gas chromatography (Shimadzu, Kyoto, Japan) as previously described (Tirapelli et al., 2005). For glucose measurements, the blood was centrifuged and the serum was analyzed for glucose content using available commercial kits (Labtest Diagnóstica, São Paulo, Brazil) and the auto-analyzer ABBOTT (model ABAA VP, USA).

Vessel ring preparation

The rats were anaesthetized and killed by aortic exsanguination. The carotid artery was quickly removed, cleaned of adherent connective tissues and cut into rings (5–6 mm in length) and placed in a 5-ml organ chamber (basal tension of 1.0 g) as previously described (Tirapelli et al., 2005). 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 μ M) in the presence of contractile tone induced by phenylephrine (0.1 μ M). 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.

Experimental Protocols

Concentration response curves for ET-1 and phenylephrine

Cumulative concentration–response curves for ET-1 (10^{-12} – 10^{-7} M) or phenylephrine (10^{-10} – 10^{-5} M) were performed in endothelium-intact and -denuded rings by a stepwise increase in the concentration of the agonists. Additions were made as soon as a steady response was obtained from the preceding concentration. The vascular responsiveness to these agonists was studied in carotid rings from control, isocaloric and ethanol-treated rats after 2, 6 and 10 weeks.

Effects of BQ123 and BQ788 on ET-1-induced contraction

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The antagonists were added 30 min prior to the construction of the concentration-response curves for ET-1. 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} – 3×10^{-7} M) were obtained. Six concentrations of BQ123 (0.001, 0.01, 0.3, 1, 3 and 5 μ M) and four concentrations of BQ788 (0.1, 0.3, 1 and 3 μ M) were tested in endothelium-intact rings. The curves for ET-1 in the presence of the antagonists were obtained in rings from control, isocaloric and 2-week ethanol-treated rats. Each ring was used for a single experiment since we observed tachyphylaxis for ET-1 in the rat carotid (Tirapelli et al., 2005)

IRL1620-induced contraction

Cumulative concentration-response curves for IRL1620 (10^{-10} – 3×10^{-7} M) were performed in endothelium-intact and -denuded rings from control, isocaloric and 2-week ethanol-treated rats.

ET-1, IRL1620 and acetylcholine-induced relaxation

Endothelium-intact rings were pre-contracted with phenylephrine (0.1 μ M). After reaching a stable and sustainable contraction, ET-1 (10^{-14} – 3×10^{-11} M), IRL1620 (10^{-10} – 3×10^{-8} M) or acetylcholine (10^{-10} – 10^{-5} M) were added cumulatively to the organ bath.

A possible influence of ethanol consumption on the mechanisms underlying the relaxant effect induced by IRL1620 was studied in endothelium-intact rings from control and ethanol-treated rats. These mechanisms were evaluated by experiments performed in the presence of N^G-nitro-L-arginine-methyl-ester (L-NAME, a non-selective NO synthase inhibitor, 100 μ M), indomethacin (a cyclooxygenase inhibitor, 10 μ M), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor, 1 μ M). Concentrations of the channel blockers tetraethylammonium chloride (TEA, a non-selective K⁺ channel blocker, 10 mM), apamin (selective blocker of low conductance Ca²⁺-activated channels, 1 μ M), glibenclamide

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(selective blocker of ATP-sensitive K⁺ channels, 3 μM), charybdotoxin (selective blocker of large conductance Ca²⁺-activated K⁺ channels, 0.1 μM) and 4-aminopyridine (selective blocker of voltage-dependent K⁺ channels, 4-AP, 1 mM) were used according to Nelson and Quayle, 1995. All drugs were incubated for 30 min before further experimental procedures. Relaxations were expressed as percentage change from the phenylephrine-contracted tissues. Since we noted that L-NAME and ODQ enhanced phenylephrine-induced contraction, the rings with intact endothelium exposed to these compounds were pre-contracted with phenylephrine 0.03 μM, to induce a magnitude of contraction similar to that found in the intact rings not exposed to the inhibitors.

Reverse transcriptase-polymerase chain reaction

Reverse transcriptase-polymerase chain reaction was performed as previously described (Tirapelli et al., 2005). 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 CTGTGCTGCTCGCCCTTGTA, sense primer GAAGTCGTCCGTGGGCATCA (216-bp fragment); ET_B, antisense primer CACGATGAGGACAATGAGAT, sense primer TTACAAGACAGCCAAAGACT (565-bp fragment); GAPDH, anti-sense primer CACCACCCTGTTGCTGTA, sense primer TATGATGACATCAAGAAGGTGG (219-bp fragment). The band intensities were measured using a software package (Kodak Digital Science, Eastman Kodak Company, New Haven, CT, USA) and the signals are reported relatively to the intensity of GAPDH amplification in each co-amplified sample.

Western immunoblotting

The Bradford assay was used to determine protein concentration. Total protein (20 μg) was separated by electrophoresis on 10% SDS polyacrylamide gel and transferred to methanol-activated PVDF membrane (Amersham). Membranes were blocked on TBST with 8

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% non fat 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 a second antibody, the donkey polyclonal antiserum against rabbit IgG coupled to Horse-Radish Peroxidase (NA9340V) (Amersham) was used. Densitometric analysis was performed with a densitometer (Gel Doc, Bio-Rad) to determine level of protein expression (Tirapelli et al., 2005).

Immunohistochemistry

Longitudinal sections (5 μm) of the rat carotid were incubated with 3% H₂O₂ and a Pierce solution to block endogenous peroxidase and biotin, respectively. Sections were subsequently incubated 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) and streptavidin-conjugated peroxidase (Vectastain ABC kit, Vector Laboratories Inc, Burlingame, CA). Color was developed by the addition of DAB (Sigma). To evaluate the background reaction, procedures were also performed in sections incubated only with the secondary antibody (indirect technique) or in the absence of antibodies (direct technique). The number of positive immunostaining for ET_A and ET_B receptors was measured by using a camera DXC-107A (Sony) and the program Image-Pro Plus (Media Cybernetics). The positive staining for each receptor was determined per unit area (positive staining/ μm^2).

Drugs

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, ODQ, glibenclamide, 4-AP, ET-1 (Sigma, St. Louis, MO, USA), L-NAME, TEA (Sigma/RBI, Natick, MA, USA), indomethacin (Calbiochem, USA), apamin, IRL1620 ({{succinyl-[Glu9,Ala11,15]-ET-1(8-210)}}, BQ788 ([N-cis-2,6-dimethyl-piperidinocarbonyl-

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L- γ -methylleucyl1-D-1methoxycarbonyltryptophanyl-D-norleucine]], BQ123 (c(DTrp – Dasp – Pro – Dval – Leu) (American Peptide Company, Sunnyvale, CA, USA), 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% and 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 expressed as changes in the displacement (grams) from baseline since no differences on tissue mass among the groups were observed. 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). Agonist potencies and maximal responses were expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximal response) and E_{max} (maximum effect elicited by the agonist), respectively. 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

Body weight, blood ethanol and serum glucose measurements

Body weight of the rats before beginning the treatment averaged 305 ± 10 g in the control, 312 ± 6 g in the isocaloric and 317 ± 11 g in the ethanol groups. The treatment for 2 weeks did not alter the body weight of the rats (control= 446 ± 14 g, isocaloric= 442 ± 13 g, ethanol= 429 ± 15 g). On the other hand, the treatment for 6 weeks reduced the body weight of the rats from the ethanol group (445 ± 11 g) and increased the body weight of the rats from

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the isocaloric group (570 ± 10 g) when compared to control group (521 ± 13 g). Similarly, animals receiving ethanol in the drinking water for 10 weeks showed reduced body weight (479 ± 12 g) in comparison to age matched control (544 ± 12 g) and isocaloric rats (587 ± 10 g) ($p < 0.05$; ANOVA).

Blood ethanol levels in the ethanol treated rats averaged 1.87 ± 0.20 ; 1.73 ± 0.22 and 1.72 ± 0.18 mg/mL in the second, sixth and tenth week, respectively ($n=8-9$) with no differences among the three different periods of treatment ($p < 0.05$; ANOVA). No ethanol was detectable in the blood of control and isocaloric animals.

In the 2-week treated rats, serum glucose levels (mg/dL) in the control ($n=10$), isocaloric ($n=6$) and ethanol ($n=9$) groups averaged, 100.81 ± 9.15 ; 102.30 ± 6.25 and 99.95 ± 6.05 , respectively. Similarly, no differences were found in serum glucose levels after six weeks of treatment (control: 100.50 ± 6.70 , $n=10$; isocaloric: 101.35 ± 11.79 , $n=8$; ethanol: 103.22 ± 6.60 , $n=9$). The treatment for 10 weeks did not modify the serum glucose levels in control (103.60 ± 7.42 , $n=8$), isocaloric (110.85 ± 6.82 , $n=6$) or ethanol-treated rats (105.42 ± 7.00 , $n=6$) ($p < 0.05$; ANOVA).

Concentration response curves for ET-1 and phenylephrine

The E_{max} for ET-1 was significantly higher in endothelium-intact, but not endothelium-denuded arteries from ethanol-treated rats than in arteries from control or isocaloric animals, without any significant differences among the pD_2 values (Fig.1, Table 1). Moreover, the magnitude of the enhancement observed after ethanol treatment on ET-1-induced contraction of endothelium-intact rings did not differ among the three different periods of treatment.

Phenylephrine-induced contraction in rat carotid rings was not altered at any time after treatment with ethanol in either endothelium-intact or denuded rings (Data not shown). Since the period of treatment did not influence the effect induced by ethanol on ET-1-induced

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contraction of endothelium-intact rings, the following experiments designed to investigate the mechanisms underlying this response were obtained in carotid rings from 2-week treated rats and their respective age-matched control and isocaloric animals.

Effects of antagonists on ET-1-induced contraction

The E_{\max} and pD_2 values for ET-1 concentration-response curves observed in the absence and presence of BQ123 and BQ788 are shown in Table 2. The incubation of carotid rings from control, isocaloric and ethanol-treated rats with BQ123 produced concentration-dependent rightward displacements of the ET-1 response curves with reduction of the maximum response. However, in the presence of BQ123, the E_{\max} values for ET-1 obtained in the rings from ethanol-treated rats were significantly higher with respect to the values obtained for arteries from control and isocaloric rats when exposed to the same concentration of the ET_A antagonist.

Effect of ethanol consumption on IRL1620-induced contraction

The E_{\max} of IRL-1620 was significantly higher in endothelium-intact but not in denuded rings from ethanol-treated rats when compared to control or isocaloric animals. No significant differences among pD_2 values were found in endothelium-intact or denuded rings (Fig 2, Table 3).

Effect of ethanol consumption on ET-1, IRL1620 and acetylcholine-induced relaxation

Figure 3 shows that ethanol consumption reduced ET-1-induced relaxation (E_{\max} : 27.87 ± 3.86 %; $n=13$) when compared to control (E_{\max} : 47.83 ± 3.91 %; $n=9$) or isocaloric arteries (E_{\max} : 49.35 ± 5.71 %; $n=9$) ($p<0.05$; ANOVA). The mean pD_2 values of ET-1 in rings derived from ethanol-treated rats (12.66 ± 0.15) were not significantly different from that found in the arteries from control (13.10 ± 0.16) or from isocaloric rats (13.01 ± 0.10). Similarly, the relaxation induced by IRL1620 in endothelium-intact rings from ethanol treated rats (E_{\max} : 21.61 ± 2.47 %; $n=13$) was significantly reduced when compared to control (E_{\max} :

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46.39 ± 2.71 %; n=10) or isocaloric arteries (E_{\max} : 48.28 ± 3.97 %; n=7) ($p < 0.05$; ANOVA). The mean pD_2 values of IRL1620 in rings from ethanol-treated rats (8.83 ± 0.21) were not significantly different from that found in arteries from control (8.92 ± 0.20) or isocaloric rats (9.01 ± 0.12). On the other hand, acetylcholine-induced relaxation in the rat carotid did not significantly differ among control (E_{\max} : 102.30 ± 9.30 %; pD_2 : 7.26 ± 0.11, n=8), isocaloric (E_{\max} : 109.56 ± 3.26 %; pD_2 : 7.27 ± 0.12, n=6) or ethanol rats (E_{\max} : 113.60 ± 3.49 %; pD_2 : 7.18 ± 0.10, n=6).

We noted no differences in the relaxation induced by ET-1 or IRL1620 between control and isocaloric arteries. Thus, the experiments designed to verify whether ethanol consumption affects the mechanisms underlying ET_B -induced relaxation were performed in arteries from control and ethanol-treated rats (Table 4).

When added alone, L-NAME, ODQ, indomethacin or TEA reduced IRL1620-induced relaxation of control arteries to a similar extent. On the other hand, these compounds did not affect IRL1620-induced relaxation of ethanol-treated arteries. The combination of L-NAME and indomethacin showed further suppression than that observed with either L-NAME or indomethacin alone in control arteries and reduced IRL1620-induced relaxation of ethanol-treated arteries. The combination of TEA, L-NAME and indomethacin strongly reduced IRL1620-induced relaxation of control and ethanol-treated arteries. The relaxant response induced by IRL1620 on control arteries was reduced by 4-AP, whereas apamin, glibenclamide or charybdotoxin had no effect in this response. On the other hand, neither of these compounds altered IRL1620-induced relaxation of ethanol-treated arteries. The mean pD_2 of IRL1620 concentration-dependent vasodilatory responses as well as its E_{\max} values, in absence or presence of the above treatments, are given in Table 4.

ET_A and ET_B receptor mRNA expression in the rat carotid artery

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The results obtained by RT-PCR show that there is no difference in the expression of mRNA for both ET_A (Control: 1.66 ± 0.10 , n=5; Isocaloric: 1.73 ± 0.07 , n=4; Ethanol: 1.66 ± 0.14 , n=6) and ET_B (Control: 1.59 ± 0.13 , n=5; Isocaloric: 1.60 ± 0.14 , n=4; Ethanol: 1.61 ± 0.20 , n=6) receptors among the three experimental groups. The band signals are reported relatively to the intensity of GAPDH amplification in each co-amplified sample.

Protein levels of ET_A and ET_B receptors in the rat carotid artery

Western immunoblotting assays showed that the treatment with ethanol reduced rat carotid ET_B receptors protein levels when compared to control or isocaloric tissues (Fig. 4). On the other hand, the protein levels of ET_A receptors in arteries from ethanol-treated rats were not significantly different in vessels derived from the same three above mentioned groups .

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

Immunohistochemical studies revealed intense staining for ET_A and ET_B-immunoreactivity in the rat carotid (Fig. 5). The positive staining for each receptor was determined per unit area (positive staining/ μm^2). Ethanol consumption did not modify the staining of either ET_A receptors (control: 0.45 ± 0.02 , n=5; isocaloric: 0.50 ± 0.02 , n=4; ethanol: 0.46 ± 0.02 , n=5) or ET_B receptors (control: 0.60 ± 0.06 , n=5; isocaloric: 0.57 ± 0.05 , n=4; ethanol: 0.54 ± 0.03 , n=4) located in the smooth muscle. Conversely, in endothelial cells the positive immunostaining for ET_B was reduced after ethanol consumption (control: 0.86 ± 0.09 , n=5; isocaloric: 0.81 ± 0.05 , n=4; ethanol: 0.41 ± 0.05 , n=4) ($p < 0.05$; ANOVA).

Discussion

The present study showed enhancement of the contractile response of isolated carotid artery to ET-1 in ethanol treated rats. Endothelium denudation of arterial rings from control, but not ethanol-treated rats, enhanced the contractile response of these arterial vessels to ET-

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1, indicating that the endothelium partially counteracts the ET-1-mediated vasoconstriction. This result suggests that ethanol consumption impairs the modulatory activity of the endothelium; this alteration could partly contribute to the hyperreactivity of the carotid artery to ET-1 observed in ethanol-treated animals. Interestingly, the hyperreactivity to ET-1 is not the result of a non-specific increase in the reactivity of the rat carotid artery induced by ethanol consumption, since the contractile response of these arteries to phenylephrine, a selective α_1 -adrenoreceptor agonist, did not differ among the three groups. Therefore, the enhanced reactivity of the carotid artery from ethanol-treated rats to ET-1 cannot be explained by a non-specific impairment of the modulatory action of the endothelium but rather by a selective alteration of the response to ET-1.

Elevated glucose levels have been reported to alter vascular responsiveness (Tesfamariam, 1991; Lloréns et al., 2004). In the present work the level of glucose did not differ among the isocaloric groups and their respective control and ethanol age-matched rats. Also, sucrose feeding did not alter the vascular reactivity to ET-1, suggesting that the caloric content of the ethanol diet did not play a significant role in the present findings.

Some reports suggest that the period of exposure to ethanol is the major factor in the development of cardiovascular abnormalities (Abdel-Rahman and Wooles, 1987; Strickland and Wooles, 1988). In the present work no relation between the period of treatment and the increment on ET-1-induced contraction was observed. However, our data does not rule out the possibility that ethanol displays a time-dependent effect at periods of treatment shorter or longer than that employed in the present study.

Previously we demonstrated the existence of both ET_A and ET_B vasoconstrictor receptors located on smooth muscle of rat carotid arteries and endothelial ET_B receptors responsible for ET-1 induced vasorelaxation via NO-cGMP pathway, vasodilator cyclooxygenase product(s) and the activation of voltage-dependent K^+ (K_V) channels

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(Tirapelli et al., 2005). We observed that in the presence of BQ123, but not BQ788, the E_{\max} values for ET-1 obtained in the rings from ethanol-treated rats were significantly higher with respect to values obtained for arteries from control and isocaloric rats when exposed to the same concentration of the antagonist. Accordingly, the hyperreactivity to ET-1 could be related to a greater participation of ET_A or ET_B receptors located on the vascular smooth muscle or a reduced relaxation mediated by endothelial ET_B receptors. Ethanol consumption enhanced the contraction induced by IRL1620, a selective agonist for ET_B receptors (Takai et al., 1992), in endothelium intact but not denuded arteries, indicating that the contraction mediated by ET_B receptors located on the smooth muscle was not altered by the treatment. Removal of the endothelium significantly enhanced IRL1620-induced contraction further suggesting that the endothelium counteracts the contraction induced by ET_B receptors. This observation corroborates our initial finding namely that the hyperreactivity to ET-1 is endothelium-dependent and also indicates that ethanol consumption impairs the modulatory activity of the endothelium by a mechanism that is selective to the endothelinergic pathway. Thus, it seems that the selective enhancement of the ET-1-induced contraction shown by isolated rat carotid from ethanol-treated rats is related to an altered function of ET receptors located on endothelial cells. Ethanol consumption reduced both ET-1 and IRL1620-induced vasodilatory responses, further supporting the concept of impaired endothelial ET_B -dependent responses. Furthermore, ethanol consumption did not alter acetylcholine-induced endothelium-dependent relaxation, further confirming that the treatment selectively affects the endothelinergic pathway.

Incubation of carotid arteries from ethanol-treated rats with L-NAME, indomethacin, TEA and 4-AP did not significantly modify the maximal relaxation induced by IRL1620, suggesting that, in these arteries, the role of NO, vasodilator prostanoid(s) and voltage-dependent K^+ (K_V) channels in response to ET_B activation was attenuated by the treatment.

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However, the association of L-NAME and indomethacin and the association of L-NAME, indomethacin and TEA reduced ET_B-mediated relaxation in the rings from ethanol-treated rats. This finding suggests that ethanol consumption attenuates but does not abolish the intracellular pathways involved on ET_B-mediated relaxation.

The mRNA expression of both ET_A and ET_B receptors was not altered by ethanol consumption. By using western immunoblotting we demonstrated that the protein levels of ET_B, but not ET_A receptors, were reduced by the treatment. Moreover, immunohistochemical assays showed reduced immunostaining for endothelial ET_B receptors after treatment, while ET_A and ET_B receptor levels in the vascular smooth muscle was not altered. These results show that ethanol consumption down-regulates endothelial ET_B receptors at the post-transcriptional level.

Increased vascular response to ET-1 has been reported in different pathophysiological conditions such as cerebral ischemia (Salom et al., 2000), subarachnoid hemorrhage (Alabadi et al., 1997) and hypertension (Cardillo et al., 1999). The vascular hyperreactivity to ET-1 described in this study, together with the increased plasma levels of this peptide in ethanol treated rats (Nanji et al., 1994), could play a role in the pathogenesis of cerebral ischemia associated to ethanol consumption.

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Legends for Figures

Fig. 1. Effect of chronic ethanol consumption on ET-1-induced contractile response in rat carotid rings. Concentration–response curves for ET-1 were determined in endothelium-intact or endothelium-denuded carotid rings from control, isocaloric and ethanol-treated rats.

Fig. 2. Concentration-response curves for IRL1620 obtained in endothelium-intact or denuded rat carotid rings from control, isocaloric or ethanol-treated rats. Values are means \pm SEM of 6 to 11 independent preparations.

Fig. 3. Relaxation responses induced by ET-1, IRL1620 and acetylcholine on rat carotid rings pre-contracted with phenylephrine. The concentration-response curves for both agonists were obtained in endothelium-intact rings from control, isocaloric or ethanol-treated rats. Sustained tension was evoked by phenylephrine and then the vasorelaxant agents were added cumulatively. Values are means \pm SEM of 6 to 8 independent preparations.

Fig. 4. Representative western immunoblotting products of 20 μ g total protein extracted from endothelium intact rat carotid arteries from control, isocaloric 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 COX-1 bands, used as internal standard. Results are reported as means \pm SEM and are representative of 4 to 5 experiments. (* $p < 0.05$ compared to control and isocaloric groups; ANOVA)

Fig. 5. Representative immunohistochemical photomicrographs of ET_A (up) and ET_B (down) receptors in rat carotid artery sections from control (A,D), isocaloric (B,E) or ethanol-treated

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rats (C,F). Arrows indicate expression of ET_A receptor in smooth muscle cells and ET_B in both endothelial and smooth muscle cells.

Table 1. Effect of chronic ethanol consumption on the E_{\max} (g) (A) and pD_2 values (B) for ET-1 in endothelium-intact (Endo+) or denuded (Endo-) carotid rings.

A	Control		Isocaloric		Ethanol	
	Endo+	Endo-	Endo+	Endo-	Endo+	Endo-
Period (Weeks)						
2	0.44 ± 0.04 (8)	0.66 ± 0.09 ^b (8)	0.46 ± 0.03 (9)	0.62 ± 0.08 ^b (7)	0.58 ± 0.04 ^a (12)	0.61 ± 0.05 (8)
6	0.43 ± 0.04 (10)	0.66 ± 0.04 ^b (7)	0.45 ± 0.04 (6)	0.66 ± 0.07 ^b (7)	0.56 ± 0.03 ^a (10)	0.61 ± 0.02 (8)
10	0.42 ± 0.05 (8)	0.66 ± 0.04 ^b (8)	0.41 ± 0.03 (9)	0.63 ± 0.03 ^b (10)	0.55 ± 0.04 ^a (7)	0.65 ± 0.08 (8)
B	Control		Isocaloric		Ethanol	
	Endo+	Endo-	Endo+	Endo-	Endo+	Endo-
Period (Weeks)						
2	8.75 ± 0.12 (8)	9.10 ± 0.17 (8)	8.81 ± 0.08 (9)	9.04 ± 0.13 (7)	8.79 ± 0.05 (12)	9.05 ± 0.09 (8)
6	8.77 ± 0.04 (10)	8.90 ± 0.05 (7)	8.87 ± 0.05 (6)	9.08 ± 0.10 (7)	8.82 ± 0.02 (10)	9.08 ± 0.05 (8)
10	8.83 ± 0.05 (8)	8.86 ± 0.08 (8)	8.66 ± 0.08 (9)	8.93 ± 0.09 (10)	8.85 ± 0.09 (7)	8.98 ± 0.13 (8)

Number between parentheses indicates the number of replicates. Values are means±S.E.M.

^a Compared to control and isocaloric groups with intact endothelium ($p < 0.05$; ANOVA). ^b

Compared to the respective groups with intact endothelium ($p < 0.05$; ANOVA).

Table 2. Effect of BQ123 (A) and BQ788 (B) on the E_{\max} (g) and pD_2 values for ET-1 in endothelium-intact carotid rings.

A	Control		Isocaloric		Ethanol	
	E_{\max}	pD_2	E_{\max}	pD_2	E_{\max}	pD_2
BQ123 [μ M]						
Absent	0.44 \pm 0.04 (8)	8.75 \pm 0.12	0.46 \pm 0.03 (9)	8.81 \pm 0.08	0.58 \pm 0.04 ^a (12)	8.79 \pm 0.05
0.001	0.45 \pm 0.04 (6)	8.75 \pm 0.06	0.39 \pm 0.03 (6)	8.69 \pm 0.03	0.55 \pm 0.02 ^a (6)	8.68 \pm 0.01
0.01	0.46 \pm 0.04 (6)	8.68 \pm 0.04	0.41 \pm 0.04 (6)	8.60 \pm 0.05	0.56 \pm 0.03 ^a (6)	8.64 \pm 0.03
0.3	0.35 \pm 0.05 (6)	8.22 \pm 0.11	0.34 \pm 0.03 (6)	8.15 \pm 0.09 ^b	0.51 \pm 0.04 ^a (6)	8.32 \pm 0.04 ^{b,e}
1.0	0.26 \pm 0.06 ^b (6)	7.93 \pm 0.10 ^{b,c,e}	0.23 \pm 0.08 ^{b,c,e} (6)	7.94 \pm 0.03 ^{b,c,d}	0.43 \pm 0.06 ^a (6)	7.92 \pm 0.05 ^{b,c,e}
3.0	0.13 \pm 0.03 ^{b,c} (6)	7.94 \pm 0.10 ^{b,c,e}	0.13 \pm 0.04 ^{b,c,e} (6)	8.12 \pm 0.20 ^b	0.22 \pm 0.06 ^{b,c} (6)	7.71 \pm 0.07 ^{b,c,e}
5.0	0.19 \pm 0.03 ^{b,c} (6)	7.78 \pm 0.06 ^{b,c,e}	0.13 \pm 0.06 ^{b,c,e} (6)	7.73 \pm 0.03 ^{b,c,e}	0.31 \pm 0.04 ^{a,b,c} (6)	7.76 \pm 0.10 ^{b,c,e}
B	Control		Isocaloric		Ethanol	
BQ788 [M]	E_{\max}	pD_2	E_{\max}	pD_2	E_{\max}	pD_2
Absent	0.44 \pm 0.04 (8)	8.75 \pm 0.12	0.46 \pm 0.03 (9)	8.81 \pm 0.08	0.58 \pm 0.04 ^a (12)	8.79 \pm 0.05
0.1	0.42 \pm 0.03 (6)	8.82 \pm 0.06	0.45 \pm 0.03 (5)	8.86 \pm 0.03	0.47 \pm 0.05 (7)	8.66 \pm 0.02
0.3	0.43 \pm 0.06 (6)	8.70 \pm 0.08	0.49 \pm 0.04 (5)	8.70 \pm 0.13	0.49 \pm 0.05 (7)	8.66 \pm 0.04

1.0	$0.26 \pm 0.02^{b,d}$ (6)	$8.45 \pm 0.02^{b,d}$	$0.29 \pm 0.02^{b,d}$ (5)	8.67 ± 0.09	$0.31 \pm 0.05^{b,d}$ (7)	$8.37 \pm 0.10^{b,d}$
3.0	$0.29 \pm 0.07^{b,d}$ (6)	$8.16 \pm 0.11^{b,d}$	$0.33 \pm 0.03^{b,d}$ (5)	$8.45 \pm 0.08^{b,d}$	$0.32 \pm 0.05^{b,d}$ (7)	$8.24 \pm 0.07^{b,d}$

Number between parentheses indicates the number of replicates. Values are means \pm S.E.M. ^a Compared to control and isocaloric groups. ^b Compared to the respective group in the absence of the antagonist (BQ123 or BQ788). ^c Compared to the respective group in the presence of BQ123 0.001, 0.01, 0.3 μ M. ^d Compared to the respective group in the presence of BQ788 0.1 and 0.3 μ M. ^e Compared to the respective group in the presence of BQ123 0.001 and 0.01 μ M.

Table 3. Effect of chronic ethanol consumption on the E_{\max} (g) (A) and pD_2 values (B) for IRL1620 in endothelium-intact (Endo+) or denuded (Endo-) carotid rings.

Groups	Intact endothelium			Denuded endothelium		
	E_{\max}	pD_2	n	E_{\max}	pD_2	n
Control	0.041 ± 0.007	7.48 ± 0.13	9	0.065 ± 0.008^a	7.42 ± 0.13	11
Isocaloric	0.035 ± 0.006	7.41 ± 0.11	6	0.060 ± 0.009^a	7.48 ± 0.22	6
Ethanol	0.066 ± 0.008^a	7.53 ± 0.11	9	0.068 ± 0.007^a	7.35 ± 0.10	10

Values are means \pm S.E.M of n experiments. ^a Compared to control and isocaloric groups with intact endothelium ($p < 0.05$; ANOVA).

Table 4. Effect of chronic ethanol consumption on the E_{\max} (% relaxation) and pD_2 values for IRL1620 in endothelium-intact phenylephrine-pre-contracted carotid rings in the absence or presence of different inhibitors.

Inhibitor	Control		Ethanol	
	E_{\max}	pD_2	E_{\max}	pD_2
Absent	46.39 ± 2.71 (10)	8.92 ± 0.20 (10)	21.61 ± 2.47 ^a (12)	9.43 ± 0.18 (12)
L-NAME	25.31 ± 3.84 ^a (9)	9.10 ± 0.14 (9)	24.30 ± 2.43 ^a (7)	9.14 ± 0.07 (7)
ODQ	28.60 ± 4.54 ^a (9)	9.18 ± 0.10 (9)	19.56 ± 4.61 ^a (11)	9.85 ± 0.25 (11)
Indomethacin	25.49 ± 4.98 ^a (8)	9.05 ± 0.20 (8)	26.38 ± 3.78 ^a (6)	9.26 ± 0.12 (6)
L-NAME + Indomethacin	13.81 ± 2.99 ^a (8)	8.85 ± 0.10 (8)	14.98 ± 3.18 ^{a,b} (7)	8.65 ± 0.24 (7)
TEA	25.04 ± 3.10 ^a (9)	8.79 ± 0.17 (9)	29.83 ± 4.40 ^a (8)	8.92 ± 0.12 (8)
L-NAME + Indomethacin + TEA	8.11 ± 3.60 ^a (7)	-	10.35 ± 3.16 ^{a,b} (8)	-
4-AP	17.21 ± 7.56 ^a (16)	8.70 ± 0.28 (16)	18.90 ± 3.81 (13)	9.53 ± 0.32 (13)
Apamin	40.33 ± 3.46 ^b (8)	9.03 ± 0.15 (8)	22.70 ± 3.60 (9)	9.72 ± 0.30 (9)
Glibenclamide	38.61 ± 3.03 ^b (8)	8.98 ± 0.20 (8)	23.60 ± 3.70 (10)	9.47 ± 0.33 (10)
Charybdotoxin	49.71 ± 6.12 ^b (8)	8.86 ± 0.12 (8)	20.72 ± 2.52 (7)	9.10 ± 0.13 (7)

Number between parentheses indicates the number (n) of replicates. Values are means ± S.E.M. ^a Compared to control group in the absence of the inhibitors ($p < 0.05$; ANOVA). ^b Compared to ethanol group in the absence of the inhibitors ($p < 0.05$; ANOVA). L-NAME: N^G-nitro-L-arginine methyl ester; ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; TEA: tetraethylammonium; 4-AP: 4-aminopyridine









