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

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Received February 16, 2006; accepted April 27, 2006

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 the 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 [succinyl-(Glu9,Ala11,15)-ET-1(8–21)], a selective ETB agonist, was increased after treatment with ethanol in endothelium-intact but not in endothelium-denuded carotid rings. Moreover, ET-1- and IRL1620-induced relaxation was reduced in endothelium-intact phenylephrine-precontracted rings from ethanol-treated rats. Acetylcholine-induced relaxation was not affected by ethanol treatment. N²-Nitro-L-arginine methyl ester, 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one, indomethacin, and tetraethylammonium reduced the relaxation induced by IRL1620 in carotid glands from control but not ethanol-treated rats. The mRNA levels for ETα and ETβ receptors were not altered by ethanol consumption. However, ethanol treatment reduced the protein expression of ETβ receptors. Furthermore, immunohistochemical assays showed reduced immunostaining for endothelial ETB 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 used in this study. Finally, the potentiation of ET-1-induced vascular reactivity is probably caused by reduced expression of relaxing endothelial ETβ receptors.

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 a reduction in cerebral blood flow (Melgaard et al., 1990; Oishi et al., 1991). Much of the research on the effects of ethanol on the cardiovascular system has dealt with vascular responsiveness to vasoconstrictor agents (Strickland and Woolfes, 1988; Hatton et al., 1992). Several groups have reported enhanced vascular reactivity to vasoconstrictor agents (Pinardi et al., 1992; Tirapelli et al., 2006) or impairment of the vascular relaxation (Kähönen et al., 1999) with regard 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 Woolles, 1987; Strickland and Woolles, 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 used only single periods of treatment (Utkan et al., 2001; Brown et al., 2002).

Endothelin (ET)-1, the predominant isoform of the ET peptide family, has potent vasoconstrictor, mitogenic, and proinflammatory properties and is implicated in numerous cardiovascular diseases (Yanagisawa et al., 1988; Tostes and Muscara, 2005). Interestingly, Nanji et al. (1994) observed increased plasma ET-1 levels in rats treated with ethanol, suggesting that the 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.

Therefore, the aim of this study was 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 that deal with the time course for the effect of ethanol intake on the vascular reactivity 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 were in accordance with guidelines of the Ethical Animal Committee from the University of Sao Paulo.

The rats, initially weighing 300 to 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 (290.5 g/l) instead of ethanol. Rats in the ethanol group received 20% (v/v) ethanol in their drinking water (Resstel et al., 2006; Tirapelli et al., 2005). These mechanisms were evaluated by experiments performed in endothelium-intact and -denuded rings from control, isocaloric, and ethanol-treated rats after 2, 6, and 10 weeks.

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 chromatograph (Shimadzu, Kyoto, Japan) as described previously (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, Sao Paulo, Brazil) and the AutoAnalyzer (model ABAA VP; Abbott Laboratories, Chicago, IL).

Vessel Ring Preparation

The rats were anesthetized 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), which were placed in a 5-ml organ chamber (basal tension of 1.0 g) as described previously (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 μM) in the presence of contractile tone induced by phenylephrine (0.1 μM). For studies of endothelium-intact vessels, the rings were discarded if relaxation with acetylcholine was not ≥80%. 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, 10−11 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 BQ-123 and BQ-788 on ET-1-Induced Contraction. The antagonists were added 30 min before the construction of the concentration-response curves for ET-1. Both the selective ETA (BQ-123, Ihara et al., 1992) and ETB (BQ-788, 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 BQ-123 (0.001, 0.01, 0.3, 1, 3, and 5 μM) and four concentrations of BQ-788 (0.1, 0.3, 1, and 3 μM) 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 because 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 precontracted with phenylephrine (0.1 μM). After they reached 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 Nω-nitro-l-arginine methyl ester (l-NNAME) (a nonselective NO synthase inhibitor, 100 μM), indomethacin (a cyclooxygenase inhibitor, 10 μM), or 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (a guanylyl cyclase inhibitor, 1 μM). Concentrations of the channel blockers tetroxybammonium chloride (TEA) (a nonspecific K+ channel blocker, 10 mM), ampin (selective blocker of low-conductance Ca2+-activated channels, 1 μM), glibenclamide (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 (4-AP) (selective blocker of voltage-dependent K+ channels, 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 percent change from the phenylephrine-contracted tissues. Because we noted that l-NNAME and ODQ enhanced phenylephrine-induced contraction, the rings with intact endothelium exposed to these compounds were precontracted with 0.03 μM phenylephrine 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 described previously (Tirapelli et al., 2005). Polymerase chain reaction primers were designed on the basis of published rat cDNA sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ETα/ETβ receptors and are as follows (5′–3′): ETα, antisense primer

```plaintext
5'-GTC CAT GCT CAC CAC CTT GAT T-3'
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CTGTGCTGCTGCCCTTGTA, sense primer GAAGTCGTCCGTCGGCATCA (216-bp fragment); ETB, antisense primer CACGGAGCAGCATCAGAT, sense primer TTACAAGCAGCAGAAAGCT (565-bp fragment); and GAPDH, antisense primer CACCAACCCTGTGCTGTA, sense primer TATGATGACATCAAGAAGGTGG (219-bp fragment). The band intensities were measured using a software package (Kodak Digital Science; Eastman Kodak, New Haven, CT), and the signals are reported relative to the intensity of GAPDH amplification in each coamplified 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 a methanol-activated polyvinylidene difluoride membrane (Amer sham Biosciences Inc., Piscataway, NJ). Membranes were blocked on Tris-buffered saline/Tween 20 with 8% nonfat dry milk and incubated with rabbit polyclonal antisera (1:200) raised against rat ETB receptor (AER-002) and (1:100) raised against rat ETA receptor (AER-001) (Alomone Labs, Jerusalem, Israel). Cyclooxygenase-1 was used as an internal control and detected with rabbit polyclonal antiserum (1:750) (160109; Cayman Chemical, Ann Arbor, MI). Densitometric analysis was performed with a densitometer (Gel Doc; Bio-Rad, Hercules, CA) to determine the 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 ETA and ETB receptors (1:10 dilution; Alomone Labs) 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.). Color was developed by the addition of DAB (Sigma Chemical, St. Louis, MO). 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 with positive immunostaining for ETA and ETB receptors was measured by using a camera (DXC-107A; Sony Corporation of America, New York, NY) and the program, Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD). Positive staining for each receptor was determined per unit area (positive staining per square micrometer).

Drugs. The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, ODQ, glibenclamide, 4-AP, and ET-1 (Sigma); L-NAME and TEA (Sigma/RBI, Natick, MA); indomethacin (Calbiochem, San Diego, CA); apamin, IRL1620, BQ-788, and BQ-123 (American Peptide Co., Inc., Sunnyvale, CA), and charybdotoxin (Alomone Labs). Glibenclamide and ODQ were prepared as stock solutions in ethanol and dimethyl sulfoxide, respec-
action, respectively. Statistically significant differences were calculated when the treatment averaged 305 ± 50% of the maximal response and the agonist potencies and maximal responses were expressed as pD2 values. Agonist concentrations 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 pD2 (negative logarithm of the agonist concentration 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 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, and 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 the isocaloric group (570 ± 10 g) compared with that of the control group (521 ± 13 g). Likewise, animals receiving ethanol in the drinking water for 10 weeks showed reduced body weight (479 ± 12 g) in comparison with 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 weeks 2, 6, and 10, 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 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 mg/dl, respectively. Likewise, no differences were found in serum glucose levels after 6 weeks of treatment (control: 100.50 ± 6.70 mg/dl, n = 10; isocaloric: 101.35 ± 11.79 mg/dl, n = 8; and ethanol: 103.22 ± 6.60 mg/dl, n = 9). The treatment for 10 weeks did not modify the serum glucose levels in control (103.60 ± 7.42 mg/dl, n = 8), isocaloric (110.85 ± 6.82 mg/dl, n = 6), or ethanol-treated rats (105.42 ± 7.00 mg/dl, n = 6) (p < 0.05; ANOVA).

**TABLE 1**

<table>
<thead>
<tr>
<th>Period (Weeks)</th>
<th>Control</th>
<th>Isocaloric</th>
<th>Ethanol</th>
</tr>
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<tr>
<td></td>
<td>E_max</td>
<td>pD2</td>
<td>E_max</td>
</tr>
<tr>
<td>2</td>
<td>0.44 ± 0.04 (8)</td>
<td>0.66 ± 0.09 (8)</td>
<td>0.46 ± 0.03 (9)</td>
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<tr>
<td>6</td>
<td>0.43 ± 0.04 (10)</td>
<td>0.66 ± 0.04 (7)</td>
<td>0.45 ± 0.04 (6)</td>
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<tr>
<td>10</td>
<td>0.42 ± 0.05 (8)</td>
<td>0.66 ± 0.04 (8)</td>
<td>0.41 ± 0.03 (9)</td>
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**TABLE 2**

<table>
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<tr>
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<th>Control</th>
<th>Isocaloric</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E_max</td>
<td>pD2</td>
<td>E_max</td>
</tr>
<tr>
<td>BQ-123</td>
<td>0.001 μM</td>
<td>0.45 ± 0.04 (6)</td>
<td>0.39 ± 0.03 (6)</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>0.46 ± 0.04 (6)</td>
<td>0.41 ± 0.04 (6)</td>
<td>0.80 ± 0.05</td>
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<tr>
<td>0.3 μM</td>
<td>0.35 ± 0.05 (6)</td>
<td>0.34 ± 0.03 (6)</td>
<td>0.31 ± 0.04 (5)</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>0.26 ± 0.06 (7)</td>
<td>0.23 ± 0.04 (8)</td>
<td>0.43 ± 0.06 (7)</td>
</tr>
<tr>
<td>3.0 μM</td>
<td>0.13 ± 0.03 (6)</td>
<td>0.13 ± 0.04 (6)</td>
<td>0.22 ± 0.06 (6)</td>
</tr>
<tr>
<td>5.0 μM</td>
<td>0.19 ± 0.03 (6)</td>
<td>0.13 ± 0.06 (6)</td>
<td>0.31 ± 0.04 (6)</td>
</tr>
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</table>

**TABLE 3**

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Isocaloric</th>
<th>Ethanol</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>E_max</td>
<td>pD2</td>
<td>E_max</td>
</tr>
<tr>
<td>BQ-788</td>
<td>Absent</td>
<td>0.44 ± 0.04 (8)</td>
<td>0.46 ± 0.03 (9)</td>
</tr>
<tr>
<td>0.1 M</td>
<td>0.42 ± 0.03 (6)</td>
<td>0.45 ± 0.03 (5)</td>
<td>0.47 ± 0.05 (7)</td>
</tr>
<tr>
<td>0.3 M</td>
<td>0.43 ± 0.06 (6)</td>
<td>0.49 ± 0.04 (5)</td>
<td>0.49 ± 0.05 (7)</td>
</tr>
<tr>
<td>1.0 M</td>
<td>0.26 ± 0.02 (6)</td>
<td>0.29 ± 0.02 (5)</td>
<td>0.31 ± 0.05 (7)</td>
</tr>
<tr>
<td>3.0 M</td>
<td>0.27 ± 0.01 (7)</td>
<td>0.33 ± 0.03 (5)</td>
<td>0.32 ± 0.05 (7)</td>
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</tbody>
</table>

*Compared with the respective groups with intact endothelium (p < 0.05; ANOVA).
**Compared with control and isocaloric groups with intact endothelium (p < 0.05; ANOVA).
***Compared with the respective group in the presence of BQ-123, 0.001 and 0.01 μM.
****Compared with the respective groups with intact endothelium.
*****Compared with the respective group in the absence of the antagonist (BQ-123 or BQ-788).
******Compared with the respective group in the presence of BQ-123, 0.001 and 0.01 μM.
*******Compared with the respective group in the presence of BQ-788, 0.1 and 0.3 μM.
********Compared with the respective group in the presence of the agonist (BQ-123 or BQ-788).
curves observed in the absence and presence of BQ-123 and BQ-788 are shown in Table 2. The incubation of carotid rings from control, isocaloric, and ethanol-treated rats with BQ-123 produced concentration-dependent rightward displacements of the ET-1 response curves with reduction of the maximum response. However, in the presence of BQ-123, the \( E_{\text{max}} \) 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 \( E_{\text{max}} \) of IRL-1620 was significantly higher in endothelium-intact but not in endothelium-denuded arteries compared with 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 \( (E_{\text{max}}: 27.87 \pm 3.86\%; n = 13) \) compared with control \( (E_{\text{max}}: 47.83 \pm 3.91\%; n = 9) \) or isocaloric arteries \( (E_{\text{max}}: 49.35 \pm 5.71\%; n = 9) \) \((p < 0.05; \text{ANOVA})\). The mean \( pD_2 \) values of ET-1 in rings derived from ethanol-treated rats \((12.66 \pm 0.15)\) were not significantly different from those found in the arteries from control \((13.10 \pm 0.16)\) or isocaloric rats \((13.01 \pm 0.10)\). Likewise, the relaxation induced by IRL1620 in endothelium-intact rings from ethanol-treated rats \( (E_{\text{max}}: 21.61 \pm 2.47\%; n = 13) \) was significantly reduced compared with control \( (E_{\text{max}}: 46.39 \pm 2.71\%; n = 10) \) or isocaloric arteries \( (E_{\text{max}}: 48.28 \pm 3.97\%; n = 7) \) \((p < 0.05; \text{ANOVA})\). The mean \( pD_2 \) values of IRL1620 in rings from ethanol-treated rats \((8.83 \pm 0.21)\) were not significantly different from those found in arteries from control \((8.92 \pm 0.20)\) or isocaloric rats \((9.01 \pm 0.12)\). On the other hand, acetylcholine-induced relaxation in the rat carotid did not significantly differ among control \( (E_{\text{max}}: 102.30 \pm 9.30\%; pD_2: 7.26 \pm 0.11, n = 8) \), isocaloric \( (E_{\text{max}}: 109.56 \pm 3.26\%; pD_2: 7.27 \pm 0.12, n = 6) \), or ethanol-treated rats \( (E_{\text{max}}: 113.60 \pm 3.49\%; pD_2: 7.18 \pm 0.10, n = 6) \).

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\( _A \)-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 arter-

### Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Intact Endothelium</th>
<th>Denuded Endothelium</th>
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<tbody>
<tr>
<td></td>
<td>( E_{\text{max}} )</td>
<td>( pD_2 )</td>
</tr>
<tr>
<td>Control</td>
<td>0.041 ± 0.007</td>
<td>7.48 ± 0.13</td>
</tr>
<tr>
<td>Isocaloric</td>
<td>0.035 ± 0.006</td>
<td>7.41 ± 0.11</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.066 ± 0.008( ^a )</td>
<td>7.53 ± 0.11</td>
</tr>
</tbody>
</table>

\( ^a \) Compared with control and isocaloric groups with intact endothelium \((p < 0.05; \text{ANOVA})\).
ies. 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 $\text{pD}_2$ of IRL1620 concentration-dependent vasodilatory responses as well as its $E_{\text{max}}$ values, in the absence or presence of the above treatments, are given in Table 4.

**ETA and ETB Receptor mRNA Expression in the Rat Carotid Artery.** The results obtained by reverse transcription-polymerase chain reaction show that there is no difference in the expression of mRNA for both ETA (control: 1.66 ± 0.10, $n = 5$; isocaloric: 1.73 ± 0.07, $n = 4$; ethanol: 1.66 ± 0.14, $n = 6$) and ETB (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 relative to the intensity of GAPDH amplification in each coamplified sample.

**Protein Levels of ETA and ETB Receptors in the Rat Carotid Artery.** Western immunoblotting assays showed that the treatment with ethanol reduced rat carotid ETB receptors protein levels compared with control or isocaloric tissues (Fig. 4). On the other hand, the protein levels of ETA receptors in arteries from ethanol-treated rats were not significantly different in vessels derived from the same three above-mentioned groups.

**Immunohistochemical Localization of ETA and ETB Receptors in the Rat Carotid Artery.** Immunohistochemical studies revealed intense staining for ETA and ETB immunoreactivity in the rat carotid (Fig. 5). The positive staining for each receptor was determined per unit area (positive staining per square micrometer). Ethanol consumption did not modify the staining of either ETA receptors (control: 0.45 ± 0.02, $n = 5$; isocaloric: 0.50 ± 0.02, $n = 4$; ethanol: 0.46 ± 0.02, $n = 5$) or ETB 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 ETB 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 the 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-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
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 et al., 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. In addition, 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 suggested 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 do not rule out the possibility that ethanol displays a time-dependent effect at periods of treatment shorter or longer than those used in the present study.

Previously, we demonstrated the existence of both ETA and ETB vasoconstrictor receptors located on 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+ (Kv) channels (Tirapelli et al., 2005). We observed that in the presence of BQ-123, but not BQ-788, the \( E_{\text{max}} \) 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 ETA or ETB receptors located on the vascular smooth muscle or a reduced relaxation mediated by endothelial ETB receptors. Ethanol consumption enhanced the contraction induced by IRL1620, a selective agonist for ETB receptors (Takai et al., 1992), in endothelium-intact but not -denuded arteries, indicating that the contraction mediated by ETB 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 ETB receptors. This observation corroborates our initial finding, namely that the hyperreactivity to ET-1 is

result of a nonspecific increase in the reactivity of the rat carotid artery induced by ethanol consumption, because the contractile response of these arteries to phenylephrine, a selective \( \alpha_1 \)-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 nonspecific impairment of the modulatory
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<sub>B</sub>-dependent responses. Furthermore, ethanol consumption did not alter acetylcholine-induced endothelium-dependent relaxation, further confirming the theory 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 K<sub>Ca</sub> channels in response to ET<sub>B</sub> activation was attenuated by the treatment. However, the association of L-NAME and indomethacin and the association of L-NAME, indomethacin, and TEA reduced ET<sub>B</sub>-mediated relaxation in the rings from ethanol-treated rats. This finding suggests that ethanol consumption attenuates but does not abolish the intracellular pathways involved in ET<sub>B</sub>-mediated relaxation.

The mRNA expression of both ET<sub>A</sub> and ET<sub>B</sub> receptors was not altered by ethanol consumption. By using Western immunoblotting we demonstrated that the protein levels of ET<sub>B</sub>, but not ET<sub>A</sub>, receptors were reduced by the treatment. Moreover, immunohistochemical assays showed reduced immunostaining for endothelial ET<sub>B</sub> receptors after treatment, whereas ET<sub>A</sub> and ET<sub>B</sub> receptor levels in the vascular smooth muscle was not altered. These results show that ethanol consumption down-regulates endothelial ET<sub>B</sub> receptors at the post-transcriptional level.

An 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 with ethanol consumption.

Acknowledgments

We thank Eleni Gomes, Mirian de Melo, Juliana Vercesi, Eduardo Tozatto, Sonia Dreossi, and Antonio Zanardo Filho for technical assistance.

References


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