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Ethanol Consumption Increases Endothelin-1 Expression and Reactivity in the Rat Cavernosal Smooth Muscle

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Abstract — Aims: We investigated the effects of chronic ethanol consumption on the cavernosal smooth muscle (CSM) reactivity to endothelin-1 (ET-1) and the expression of ET system components in this tissue. Methods: Male Wistar rats were treated with heavy dose of ethanol (20% v/v) for 6 weeks. Reactivity experiments were performed in the isolated rat CSM. Plasma and CSM nitrate generation and also superoxide anion generation in rat CSM were measured by chemiluminescence. Protein and mRNA levels of pre-pro-ET-1, endothelin-converting enzyme-1 (ECE-1), ETα and ETβ receptors, eNOS, nNOS and iNOS were assessed by western immunoblotting and quantitative real-time polymerase chain reaction, respectively. Results: Chronic ethanol consumption increased plasma ET-1 levels and the contractile response induced by this peptide in the isolated CSM. The relaxation induced by acetycholine, but not IRL1620, a selective ETβ receptor agonist, was reduced in CSM from ethanol-treated rats. BQ123, a selective ETα receptor antagonist, produced a rightward displacement of the ET-1 concentration–response curves in CSM from control, but not ethanol-treated rats. Reduced levels of nitrate were found in the plasma and CSM from ethanol-treated rats. Ethanol consumption increased superoxide anion generation in the rat CSM. The mRNA levels of pre-pro-ET-1, ECE-1, ETα and ETβ receptors, eNOS, nNOS and iNOS were not altered by ethanol consumption. Protein levels of ET-1, ETα receptor and iNOS were higher in the CSM from rats chronically treated with ethanol. Conclusion: The major findings of the present study are that heavy ethanol consumption increases plasma ET-1 levels and the contraction induced by the peptide in the CSM. Increased CSM reactivity to ET-1 and altered protein levels of ET-1 and ETα receptors could play a role in the pathogenesis of erectile dysfunction associated with chronic ethanol consumption.

INTRODUCTION

Endothelin-1 (ET-1) causes strong and sustained contraction of trabecular smooth muscle cells of the corpora cavernosa and contributes to the maintenance of cavernosal smooth muscle (CSM) tone (Andersson, 2001; Carneiro et al., 2008a). ET-1 is a 21 amino-acid peptide, belonging to a family of potent vasoconstrictors (Yanagisawa et al., 1988), which produces its effects via stimulation of two specific G-protein-coupled receptors, namely ETα and ETβ. Both receptors have been reported in the cavernosal tissue of humans (Holmquist et al., 1992; Granchi et al., 2002), rabbits (Holmquist et al., 1992) and rats (Bell et al., 1995; Dai et al., 2000). ET-1-induced cavernous vasoconstriction in vivo and in vitro is mainly mediated by ETα receptors (Dai et al., 2000; Carneiro et al., 2008a), whereas ETβ receptors induce relaxation of the CSM (Carneiro et al., 2008a,b). ET-1 as well as its converting enzyme (ECE-1) is expressed in both endothelial and smooth muscle cells of the human penis (Granchi et al., 2002), indicating that penile smooth muscle cells not only respond to, but also synthesize, ET-1.

ET-1 has potent vasoconstrictor, mitogenic and proinflammatory properties and is implicated in numerous cardiovascular diseases (Rodríguez-Pascual et al., 2011). In addition, there is evidence to suggest that ET-1 plays a pathophysiological role in erectile dysfunction (ED). In this line, increased levels of ET-1 have been described in hypercholesterolemic and diabetic patients with ED (Arendt et al., 1993; Francavilla et al., 1997). In rats, activation of the ET-1/ETα pathway contributes to mineralocorticoid hypertension-associated ED (Carneiro et al., 2008b). Chronic ethanol consumption is also considered as a risk factor for ED (O’Farrell et al., 1997; Wetterling et al., 1999). The prevalence of ED among alcoholic subjects varies from 59 to 72% and the most common sexual dysfunctions are diminished sexual interest, impotence and premature ejaculation (Van Thiel and Lester, 1979; Mandell and Miller, 1983; Arackal and Benegal, 2007). 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. 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. Moreover, chronic ethanol intake enhances ET-1-induced contraction in the isolated rat carotid artery, a consequence of a reduced expression of vasodilator endothelial ETβ receptors (Tirapelli et al., 2006). Finally, chronic ethanol consumption was described to potentiate the pressor responses to ET-1 and to alter ETα and ETβ receptor expression in the heart, kidney, aorta and mesenteric artery (Tirapelli et al., 2008a). Whereas ET-1 actions on cavernosal tissue have been studied, the pathophysiologial role of this peptide in ethanol-induced ED remains unclear.

In the present study, we aimed to determine whether and how chronic ethanol consumption affects the ET system in the CSM of rats. We compared the effect of ethanol intake for 6 weeks on the responses to ET-1 in isolated CSM and assessed putative alterations in the expression of ET-1, ECE-1 as well as ETα and ETβ receptors in this tissue. The influence of nitric oxide (NO) on the response to ET-1 was also evaluated.
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 approved by the Animal Ethics Committee of the University of São Paulo—Campus of Ribeirão Preto (Protocol number: 10.1.1084.53.6). The rats, initially weighing 250–300 g (50–70 days old), were randomly divided into two groups: control and ethanol. Control rats received water ad libitum. Rats from the ethanol group received 20% (v/v) ethanol in their drinking water (Tirapelli et al., 2006, 2008a). 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 week and 20% in the third week. At the end of the third week, the experimental stage began. The rats were treated for 6 weeks and weighed weekly.

Blood ethanol measurements
Blood was collected from the aorta of anaesthetized rats, and ethanol analysis was carried out using a CG-17A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame-ionization detector and an HSS-4A headspace sampler (Shimadzu, Kyoto, Japan). 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) and results were expressed as milligrams of ethanol per millilitre of blood (Tirapelli et al., 2008a).

Functional studies
The CSM was isolated as previously described (Lizarte et al., 2009). In brief, the penis was harvested by cutting the corporal body at the level of its attachment to the ischium bone and immersed in Krebs solution. The composition of the Krebs solution was as follows (mmol/l): NaCl 130, KCl 4.7, KH2PO4 1.18, MgSO4·7H2O 1.17, CaCl2·2H2O 1.6, NaHCO3 14.9 and glucose 5.5. The tunica albuginea was carefully opened from its proximal extremity towards the penile shaft, and the erectile tissue within the corpus cavernosum was surgically dissected free. The strips of CSM (1 × 1 × 10 mm) were mounted in a 5-ml organ chamber containing Krebs solution at 37°C and continuously bubbled with a gas mixture of 95% oxygen and 5% carbon dioxide (pH 7.4). One end of each corporal strip was attached to the bottom of the organ bath and the other end tied to a force transducer (TRI201, Panlab, Spain). The strips were stretched to a resting tension of 3 mN and allowed to equilibrate for 60 min. The responses were recorded on a computer system using Chart Pro 5 (PowerLab, ADInstruments, Australia).

After equilibration for 60 min, each strip was exposed to KCl (120 mmol/l) to assess its maximum contractility. The strips were sequentially washed and allowed to relax to base-line. Cumulative concentration–response curves for ET-1 (0.1–100 μmol/l) or phentolamine (0.1–100 μmol/l) were performed in CSM 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 contraction responses of ET-1 and phenylephrine were expressed as percentage of the contraction induced by KCl 120 mmol/l.

In another set of experiments, CSM strips were pre-contracted with phentolamine (10 μmol/l) and when the contraction reached a stable and sustainable plateau, concentration–response curves for succinyl-[Glu9,Ala11,15]-ET-1 (8-210) (IRL1620, a selective ETB receptor agonist, 0.001–10 nmol/l), adrenomedullin (10 pmol/l–0.1 μmol/l), acetylcholine (0.01 μmol/l–100 nmol/l) or sodium nitroprusside (SNP, 10 nmol/l–300 μmol/l) were performed. Relaxation was expressed as per cent change from phentolamine-contracted levels.

A possible influence of ethanol consumption on the mechanisms underlying the contractile effect induced by ET-1 was studied in CSM from control and ethanol-treated rats. These mechanisms were evaluated by experiments performed in the presence of c(DTIp—Dasp-Pro-Dval-Leu) (BQ123, a selective ET1 receptor antagonist, 1 or 10 μmol/l), N-cis,2-6-dimethyl-piperidinocarbonyl-1- J-methyleucyl1-n-1methoxy carbonyl tryptophanyl-n-norleucine) (BQ788, a selective ETB receptor antagonist, 3 μmol/l), NG-nitro-L-arginine-methyl-ester (l-NAME, a non-selective NOS inhibitor, 100 μmol/l), 7-nitroindazole (a selective nNOS inhibitor, 100 μmol/l) or N[(3-aminomethyl)phenyl]methyl]ethanimidamide dihydrochloride (1400W, a selective iNOS inhibitor, 0.1 μmol/l), BQ788 and 7-nitroindazole were dissolved in dimethyl sulfoxide (DMSO). The bath concentration of DMSO did not exceed 0.5%, which was shown to have no effect per se on the basal tone of the preparations. All drugs were incubated for 30 min before further experimental procedures. The agonist concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software, Inc., San Diego, CA, USA). Agonist potencies and maximal responses were expressed as pD2 (negative logarithm of the molar concentration of agonist producing 50% of the maximal response) and Emax (maximum effect elicited by the agonist, respectively).

Quantitative real-time polymerase chain reaction
Total cellular RNA was extracted using Trizol® Reagent (Invitrogen, Carlsbad, CA), and RNA was reverse-transcribed to double-stranded cDNA using a High Capacity Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s protocol. For the quantitative analysis of the genes of interest, which consisted of pre-pro-ET-1 (Rn00561129_m1), ET1 (Rn00561137_m1), ETB (Rn00569139_m1), ECE (Rn00585943_m1), eNOS (Rn.PT.47.6917365), nNOS (Rn00583793_m1) and iNOS (Rn.PT.47.13446725), we used the commercially available TaqMan Assay-on-Demand System, which consists of oligonucleotides and probes (Applied Biosystems). Reverse transcription was performed using 1 μg of total RNA for each sample in 20 μl of the total reaction mixture. The cDNA obtained was diluted 1:15, and 4.0 μl was used for each 10 μl of the Q-PCR mixture using the TaqMan Master Mix (Applied Biosystems). All reactions were carried out in duplicate and analyzed with the StepOnePlus Real-Time PCR system (Applied Biosystems). Data were analyzed using the StepOne software v.2.2.2. The relative quantification of the studied genes was performed using the 2–ΔΔCt method, with the GAPDH gene (Rn.PT.47.12624405) acting as the house-keeping gene and a group of six control animals as calibrators.

Western immunoblotting
CSM from control and ethanol-treated rats was used as follows. Frozen tissue was homogenized in lysis buffer [50

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mmol/l Tris–HCl (pH 7.4), NP-40 (1%), sodium deoxycholate (0.5%), SDS (0.1%). Homogenates were centrifuged at 5000 g for 10 min, the pellet discarded and the supernatant was stored at −80°C. Sixty micrograms of protein were separated by electrophoresis on a 7.5, 10 or 12% polyacrylamide gel, and transferred onto a nitrocellulose membrane. Nonspecific binding sites were blocked with 7% skim milk in Tris-buffered saline solution with Tween 20 for 1 h at 24°C. Membranes were then incubated with specific antibodies overnight at 4°C as follows: anti-ET-1 (1:500, Santa Cruz Biotechnology), anti-ET\(_A\) (1:250, Santa Cruz Biotechnology), anti-ET\(_B\) (1:500, Santa Cruz Biotechnology), anti-ECE-1 (1:500, Santa Cruz Biotechnology), anti-eNOS (1:1000, Sigma-Aldrich), anti-phospho-eNOS (ser1177) (1:1000, Santa Cruz Biotechnology), anti-nNOS (1:500, Cell Signaling Technology) and anti-iNOS (1:500, Sigma-Aldrich). After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified by densitometry. Beta-actin (1:5000, Santa Cruz Biotechnology) was used as an internal control.

**Plasma ET-1 determination**

Blood samples were collected in heparinized syringes. The samples were centrifuged (15 min, 4000 g) and the plasma was used for ET-1 measurement by enzyme-linked immunosorbent assay (ELISA) using commercially available kit (EDN1, USCN-LIFE™, China). Results are expressed as picograms per millilitre.

**Measurement of plasma and tissue nitrate**

The CSM was isolated and frozen in liquid nitrogen. Nitrate (NO\(_3\), a metabolite of NO) levels were measured in supernatants from CSM homogenates. Aliquots of 5 µl were injected into a Sievers Chemiluminescence Analyzer (Nitric Oxide Analyser, NOA™ 280, Sievers Instruments, Colorado, USA) and pelleted by centrifugation with VCl\(_3\) and HCl (at 95°C), which act as reductants for nitrate. Protein concentrations were determined with protein assay reagent (Bio-Rad Laboratories). Results were normalized for protein concentration and are expressed as micromoles per litre per milligram-protein. To evaluate plasma nitrate levels, blood was collected into chilled plastic tubes, containing heparin (200 U), centrifuged for 20 min at 2000 g at 4°C for plasma separation and stored at −70°C before dosage. On the day of the assay, plasma samples were thawed and deproteinized with 95% ethanol (at 4°C) for 30 min, subsequently centrifuged, and the supernatant was used for measurement of nitrate as described above. Results are expressed as micromoles per litre.

**Detection of superoxide anions in the rat CSM by lucigenin enhanced chemiluminescence**

The rat CSM was isolated and frozen in liquid nitrogen. The lucigenin-derived chemiluminescence assay was used to determine superoxide anion levels in CSM homogenates 10% (w/v) prepared in phosphate buffer (20 mmol/l of KH\(_2\)PO\(_4\), 1 mmol/l of EGTA and protease inhibitors [pH 7.4]) with a glass-to-glass homogenizer. The reaction was started by the addition of NAD(P)H (0.1 mmol/l) to the suspension (250 µl of final volume) containing sample (50 µl), lucigenin (5 µM) and assay buffer (50 mmol/l of KH\(_2\)PO\(_4\), 1 mmol/l of EGTA and 150 mmol/l of sucrose [pH 7.4]). Luminescence was measured every 1.8 s for 3 min in a luminometer (Orion II Luminometer, Berthold detection systems). Buffer blank was subtracted from each reading. Superoxide production was expressed as relative light unit (RLU) per milligram protein. Protein concentrations were determined with protein assay reagent (Bio-Rad Laboratories) (Yogi et al., 2012).

**Statistical analysis**

Data are presented as means ± standard error of the mean (SEM). Groups were compared using Student’s \(t\)-test or one-way analysis of variance (ANOVA). Bonferroni correction was used to compensate for multiple testing procedures. Results of statistical tests with \(P < 0.05\) were considered as significant.

**RESULTS**

**Body weight and blood ethanol levels**

The body weights of the rats prior to treatment averaged 272 ± 2 g \((n = 27)\) in the control group and 274 ± 6 g \((n = 27)\)
in the ethanol group. Animals receiving ethanol in the drinking water for 6 weeks showed reduced body weight (462 ± 16 g) when compared with age-matched control rats (591 ± 10 g) (P < 0.05; Student’s t-test). Blood ethanol levels in the ethanol-treated rats averaged 1.91 ± 0.21 mg/ml (~41 mmol/l, n = 11). No ethanol was detectable in the blood of control animals.

**Effect of ethanol consumption on CSM reactivity**

The contraction induced by KCl at 120 mmol/l was similar in both groups, with contractions of 1.8 ± 0.1 g (n = 27) in CSM from ethanol-treated rats and 1.9 ± 0.1 g (n = 27) in controls. No significant differences were found in the dry weight of CSM from control (21.9 ± 1.1 mg, n = 27) and ethanol-treated rats (21.5 ± 0.6 mg, n = 27). Chronic ethanol consumption increased ET-1-induced contraction (31.8 ± 2.7%, n = 7) in isolated CSM when compared with control group (21.5 ± 1.5%, n = 6). However, no differences were observed in the pD2 values between the experimental groups (control: 7.04 ± 0.03; ethanol: 7.23 ± 0.11) (Fig. 1). The E_max values for phenylephrine did not differ significantly between CSM from the control (127.6 ± 4.2%, n = 6) or ethanol-treated rats (142.7 ± 7.7%, n = 7). Similarly, no differences were observed in the pD2 values between the experimental groups (control: 5.14 ± 0.06; ethanol: 5.30 ± 0.08) (Fig. 1). The relaxation induced by acetylcholine in CSM was decreased after the treatment with ethanol when compared with the control group (Table 1). Finally, ethanol treatment did not alter the relaxation induced by IRL1620, SNP or adrenomedullin (Table 1). The pre-contraction levels induced by phenylephrine (10 µmol/l) were similar in CSM from control and ethanol-treated rats (data not shown).

**Effects of antagonists on ET-1-induced contraction**

BQ123 produced a rightward displacement of the ET-1 response curves in CSM from control but not ethanol-treated rats (Table 2). However, in the presence of BQ123, the E_max values for ET-1 obtained in CSM from ethanol-treated rats were significantly higher with respect to the values obtained for tissues from control rats (Table 2).

**Contribution of NOS isoforms in modulating the response to ET-1**

Pre-incubation with L-NAME or 1400W increased ET-1-induced contraction in CSM from both control and ethanol-treated rats. On the other hand, 7-nitroindazole induced a leftward displacement of the ET-1 response curves in CSM from control but not ethanol-treated rats (Fig. 2; Table 2).

**Effect of ethanol consumption on mRNA levels of pre-pro-ET-1, ECE-1, ETA and ETB receptors, eNOS, nNOS and iNOS in the rat CSM**

Chronic ethanol consumption did not alter mRNA levels of pre-pro-ET-1, ECE-1, ETA and ETB receptors, eNOS, nNOS and iNOS in the CSM (Table 3).

**Effect of ethanol consumption on protein levels of ET-1, ECE-1, ETA and ETB receptors, eNOS, nNOS and iNOS in the rat CSM**

Western immunoblotting assays showed that protein levels of ET-1, ETA receptors and iNOS were increased in the CSM from ethanol-treated rats when compared with control (Figs 3 and 4). On the other hand, no alterations in protein levels of ECE-1, ETB receptors, eNOS and nNOS were found in the CSM from ethanol chronically treated rats (Figs 3 and 4). Finally, ethanol consumption did not alter eNOS phosphorylation at ser1177 (Fig. 4).

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**Table 1. Effect of ethanol consumption on the E_max (% relaxation) and pD2 values for acetylcholine, IRL1620, sodium nitroprusside (SNP) and adrenomedullin in the rat CSM**

<table>
<thead>
<tr>
<th>E_max (%)</th>
<th>pD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>39.5 ± 1.5 (10)</td>
</tr>
<tr>
<td>IRL1620</td>
<td>51.4 ± 3.0 (5)</td>
</tr>
<tr>
<td>SNP</td>
<td>100.0 ± 3.1 (6)</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>40.2 ± 3.5 (7)</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number between parentheses indicates the number of isolated preparations.

*Compared with control (P < 0.05; Student’s t-test).

**Table 2. Effects of BQ123, BQ788, L-NAME, 7-nitroindazole and 1400W on the E_max (% contraction KCl 120 mmol/l) and pD2 values for ET-1 in the rat CSM from control and ethanol-treated rats**

<table>
<thead>
<tr>
<th>Antagonist/inhibitor</th>
<th>Control</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E_max</td>
<td>pD2</td>
</tr>
<tr>
<td>Absent</td>
<td>21.5 ± 1.5 (6)</td>
<td>7.04 ± 0.03</td>
</tr>
<tr>
<td>BQ123 (1 µmol/l)</td>
<td>19.3 ± 1.5 (6)</td>
<td>6.67 ± 0.06*</td>
</tr>
<tr>
<td>BQ123 (10 µmol/l)</td>
<td>12.6 ± 0.5 (4)*</td>
<td>5.9 ± 0.11*</td>
</tr>
<tr>
<td>BQ788 (3 µmol/l)</td>
<td>19.3 ± 0.8 (6)</td>
<td>7.35 ± 0.08</td>
</tr>
<tr>
<td>L-NAME (100 µmol/l)</td>
<td>34.8 ± 3.0 (6)*</td>
<td>7.35 ± 0.25</td>
</tr>
<tr>
<td>7-Nitroindazole (100 µmol/l)</td>
<td>26.8 ± 3.9 (4)</td>
<td>7.83 ± 0.10*</td>
</tr>
<tr>
<td>1400W (0.1 µmol/l)</td>
<td>48.6 ± 3.4 (4)*</td>
<td>7.82 ± 0.23*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number between parentheses indicates the number of isolated preparations.

*Compared with control group in the absence of the antagonist/inhibitor.

*Compared with ethanol group in the absence of the antagonist/inhibitor.

*Compared with respective control in the presence of the antagonist/inhibitor (P < 0.05; ANOVA followed by Bonferroni’s multiple comparison test).
Effect of ethanol consumption on plasma ET-1 levels and nitrate generation

Plasma ET-1 levels were significantly increased in ethanol-treated rats compared with control rats (Fig. 5A). Chronic ethanol consumption significantly decreased plasma nitrate levels (Fig. 5B). Nitrate content in CSM from ethanol-treated rats was also significantly lower when compared with control rats (Fig. 5C).

DISCUSSION

The major new findings of the present study are that ethanol consumption increased plasma ET-1 levels and that CSM from rats chronically treated with ethanol exhibits an enhanced, ETA-dependent, contractile response to ET-1. Importantly, these responses were observed in rats with blood concentrations of ethanol that are relevant (~41 mmol/l) since they are well within those found in humans after heavy consumption of ethanol (Urso et al., 1981). The blood ethanol concentration is an important variable to the development of ED since the chance of developing sexual dysfunctions increases with increasing quantity of ethanol consumed (Arackal and Benegal, 2007).

Our results are consistent with previous findings showing that chronic ethanol consumption increases plasma ET-1 levels (Nanji et al., 1994). ET-1 acts as a circulating hormone and locally in an autocrine/paracrine fashion. Endothelial cells and CSM cells express ET-1 and its converting enzyme, ECE-1 (Saenz de Tejada et al., 1991; Granchi et al., 2002). ET-1 produced in the rat CSM plays a role in the autocrine/paracrine regulation of penile erection due to its vasoconstrictor action (Saenz de Tejada et al., 1991; Holmquist et al., 1992; Granchi et al., 2002). In the present study, increased ET-1 production was observed in the CSM from chronically treated rats. Increased production of local and/or systemic ET-1 is relevant to the pathogenesis of ED since the chance of developing sexual dysfunctions increases with increasing quantity of ethanol consumed (Arackal and Benegal, 2007).

Effect of ethanol consumption on superoxide anion generation in the rat CSM

Lucigenin-derived luminescence was significantly higher in CSM from ethanol-treated rats when compared with control (Fig. 6).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-pro-ET-1</td>
<td>$1.05 \pm 0.11$ (9)</td>
<td>$0.99 \pm 0.11$ (9)</td>
</tr>
<tr>
<td>ECE-1</td>
<td>$1.03 \pm 0.10$ (9)</td>
<td>$1.02 \pm 0.12$ (9)</td>
</tr>
<tr>
<td>ETA receptor</td>
<td>$1.12 \pm 0.14$ (8)</td>
<td>$1.31 \pm 0.18$ (8)</td>
</tr>
<tr>
<td>ETB receptor</td>
<td>$1.04 \pm 0.11$ (9)</td>
<td>$1.18 \pm 0.16$ (9)</td>
</tr>
<tr>
<td>eNOS</td>
<td>$1.03 \pm 0.10$ (9)</td>
<td>$1.18 \pm 0.13$ (9)</td>
</tr>
<tr>
<td>nNOS</td>
<td>$1.01 \pm 0.05$ (9)</td>
<td>$1.07 \pm 0.09$ (9)</td>
</tr>
<tr>
<td>iNOS</td>
<td>$1.00 \pm 0.04$ (9)</td>
<td>$1.16 \pm 0.11$ (9)</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number between parentheses indicates the number of animals. GAPDH gene was used as housekeeping gene.
without effect on pre-pro-ET-1 mRNA expression, further suggesting that ethanol acts at a post-transcriptional level.

Chronic ethanol consumption not only increased ET-1 production but also affected the responsiveness of CSM to ET-1 itself. The increased response to ET-1 is probably not the result of a nonspecific increase in the reactivity of the CSM induced by ethanol consumption, as the contractile response of this tissue to phenylephrine, a selective $\alpha_1$-adrenoceptor agonist, did not differ between the groups. Our findings are consistent with a previous result demonstrating that ethanol consumption did not alter phenylephrine-induced contraction in the CSM (Lizarte et al., 2009). In the rat CSM, ET-1-induced contraction is mediated by the ET$_A$ receptors (Dai et al., 2000; Carneiro et al., 2008a, b), whereas ET$_B$ receptors induce relaxation (Carneiro et al., 2008a). Accordingly, the hyper-reactivity to ET-1 could be related to a greater participation of contractile ET$_A$ receptors or a reduced relaxation mediated by ET$_B$ receptors.

Ethanol consumption did not alter IRL1620-induced CSM relaxation, further discounting a role for ET$_B$ receptors in the hyper-reactivity to ET-1. Moreover, BQ788, a selective ET$_B$ receptor antagonist, did not affect ET-1-induced contraction in CSM from both control and ethanol-treated rats. Finally, no difference on ET$_B$ receptor expression was observed in our study. Thus, it seems that the selective increase of the ET-1-induced contraction shown by CSM from ethanol-treated rats is related to an altered function or expression of ET$_A$ receptors.

Fig. 3. Effect of chronic ethanol consumption on ET-1, ECE-1, ET$_A$ and ET$_B$ receptors in the rat CSM. Top panels: representative immunoblots for ET-1, ECE-1, ET$_A$ and ET$_B$ receptors protein expression. Bottom panels: corresponding bar graphs show densitometric data for ET-1, ECE-1, ET$_A$ receptor and ET$_B$ receptor expression. Results are presented as means ± SEM of 4–6 experiments. *Compared with control ($P < 0.05$, Student’s $t$-test).
BQ123 produced a rightward displacement of the ET-1 response curves in CSM from control but not ethanol-treated rats. Interestingly, increased expression of ETA receptors was detected in the CSM from ethanol-treated rats, suggesting a role for ETA receptors on the increased response to ET-1. It is important to note that the imbalance in ETA/ETB receptors due to increased expression of ETA receptors may be associated with ED in different conditions such as hypertension (Carneiro et al., 2008b) and diabetes mellitus (Bell et al., 1995). Thus, the increased ET_A-dependent contractile response to ET-1 observed in CSM from ethanol-treated rats may contribute to pathophysiology of ethanol-associated ED.

NO derived from both nerves and the vascular endothelium is the main chemical mediator of the vasodilatation and trabecular relaxation that leads to penile erection (Burnett et al., 1992). NO counteracts ET-1-induced contraction in the vasculature serving as a modulator of the contractile actions displayed by this peptide (de Nucci et al., 1988). Ethanol consumption has been described to affect NO synthase (NOS)-derived NO and NOS expression in different tissues such as the aorta, mesenteric arterial tissues and the CSM (El-Mas et al., 2006; Tirapelli et al., 2008b,c; Lizarte et al., 2009). Appreciating the importance of NO-mediated regulation of CSM and the modulatory action of NO on the ET-1 pathway, we investigated whether ethanol consumption would alter NO-mediated relaxation and its modulatory effect on ET-1-induced CSM contraction. Our results are consistent with previous findings in mouse (Aydinoglu et al., 2008) and

![Image of immunoblots and bar graphs for eNOS, phospho-eNOS, nNOS, and iNOS expression in control and ethanol-treated rats.](https://doi.org/10.1093/alcalc/aga/aar095)
rat CSM (Lizarte et al., 2009) showing that chronic ethanol consumption impaired the endothelium-dependent relaxation induced by acetylcholine. The impaired response to acetylcholine is not the result of a nonspecific impairment of the endothelial function but rather by a selective alteration in the response to this agonist, which is mediated by muscarinic type 3 receptors (Traish et al., 1995). Thus, ethanol consumption displays a selective effect on the endothelial cell receptor-stimulated production/release of NO.

Incubation of CSM from control rats with L-NAME significantly enhanced ET-1-induced contraction, indicating an inhibitory role for NO in the modulation of the contractile response of the CSM to this agonist. Similarly, incubation of CSM from ethanol-treated rats with L-NAME significantly enhanced the maximal contraction induced by ET-1, suggesting that the inhibitory role of NO in the response of CSM to ET-1 did not differ from that seen in control rats.

7-Nitroindazole displaced to the left the ET-1-induced contractions in CSM from control, but not ethanol-treated rats, suggesting that nNOS-derived NO counteracts the contractile effect induced by ET-1. Our results show that the lack of effect of 7-nitroindazole on CSM from ethanol-treated rats is not the consequence of a reduced expression of nNOS. This response could be the consequence of a reduced production/release of neuronal NO in CSM from ethanol-treated rats. However, whether ethanol alters neuronal NO production needs further investigation.

The selective iNOS inhibitor 1400W significantly enhanced the maximal contraction induced by ET-1 in CSM from both control and ethanol-treated rats. The protein levels of iNOS, but not its mRNA levels, were increased by the treatment, suggesting that ethanol consumption up-regulates iNOS at the post-transcriptional level. This finding is in accordance with earlier reports describing that ethanol increases iNOS expression in different tissues (El-Mas et al., 2006; Yuan et al., 2006; Tirapelli et al., 2008b, 2012; Lizarte et al., 2009). Interestingly, ET-1-induced contraction in CSM from control rats was increased in the presence of 1400W and mRNA as well as protein for iNOS was detected in this tissue. Therefore, the impaired reactivity of the CSM from ethanol-treated rats to acetylcholine cannot be explained by a nonspecific impairment of the endothelial function but rather by a selective alteration in the response to this agonist, which is mediated by muscarinic type 3 receptors (Traish et al., 1995). Thus, ethanol consumption displays a selective effect on the endothelial cell receptor-stimulated production/release of NO.

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the rat CSM, which is in accordance with previous findings (Lizarte et al., 2009). Taken together, our functional findings show that ethanol consumption does not impair the modulatory effect displayed by NO on ET-1-induced contraction.

Our results are consistent with previous findings showing that chronic ethanol consumption decreases plasma NO levels (Husain et al., 2004, 2005). Despite the fact that chronic ethanol treatment up-regulated iNOS protein expression, the overall levels of NO in the CSM were reduced. A decline in NO bioavailability is mainly caused by inactivation of NO by superoxide anion or the reduction of NO synthesis by eNOS (Griendling et al., 2000). eNOS activity is regulated by its phosphorylation at serine1177 (Luo et al., 2000). In our study, ethanol intake did not alter eNOS phosphorylation or expression in the rat CSM, suggesting that reduced stimulation of this enzyme is not one of the factors contributing to the reduced levels of NO in the CSM. Ethanol intake is described to increase superoxide anions generation in the vasculature (Husain et al., 2011). Our findings show that ethanol increased superoxide anion generation in the rat CSM. Thus, it is possible that ethanol-induced superoxide anion generation, which reacts with NO to form peroxynitrite, is most likely implicated in the diminished NO bioavailability here described. Although we observed an increase on the protein levels for iNOS, no alteration on eNOS or nNOS protein levels was detected. In fact, chronic ethanol consumption has been described to exert a differential effect on the NOS isoforms, this response being tissue-specific (Karaa et al., 2005; Krecsmarik et al., 2006; Husain et al., 2011; Tirapelli et al., 2012).

The implication of ET-1 in vascular dysfunction and ED has been reported in several conditions including diabetes mellitus and hypertension. Our results describe the first evidence of endothelin-1 in diabetic and nondiabetic men with erectile dysfunction. J Urol 158:1770–4.


Tirapelli CR, Legros E, Brochu I et al. (2008a) Chronic ethanol intake modulates vascular levels of endothelin-1 receptor and