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Ethanol induces vascular relaxation via redox-sensitive and nitric oxide-dependent pathways

Juliana T. Rocha [a], Ulisses V. Hipólito [b], Glaucia E. Callera [c], Alvaro Yogi [c], Mario dos Anjos Neto Filho [d], Lusiane M. Bendhack [d], Rhian M. Touyz [c], Carlos R. Tirapelli [a,*]

[a] Department of Psychiatric Nursing and Human Sciences, Laboratory of Pharmacology, College of Nursing of Ribeirão Preto, University of São Paulo (USP), Ribeirão Preto, SP, Brazil
[b] Department of Pharmacology, Faculty of Medicine of Ribeirão Preto, USP, Ribeirão Preto, SP, Brazil
[c] Kidney Research Centre, Ottawa Hospital Research Institute, University of Ottawa, Ontario, Canada
[d] Department of Physics and Chemistry, Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, USP, Ribeirão Preto, SP, Brazil

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ABSTRACT

We investigated the role of reactive oxygen species (ROS) and nitric oxide (NO) in ethanol-induced relaxation. Vascular reactivity experiments showed that ethanol (0.03–200 mmol/L) induced relaxation in endothelium-intact and denuded rat aortic rings isolated from male Wistar rats. Pre-incubation of intact or denuded rings with L-NAME (non selective NOS inhibitor, 100 μmol/L), 7-nitroindazole (selective nNOS inhibitor, 100 μmol/L), ODQ (selective inhibitor of guanylyl cyclase enzyme, 1 μmol/L), glibenclamide (selective blocker of ATP-sensitive K^+ channels, 3 μmol/L) and 4-aminopyridine (selective blocker of voltage-dependent K^+ channels, 4-AP, 1 mmol/L) reduced ethanol-induced relaxation. Similarly, tiron (superoxide anion (O_2^-) scavenger, 1 mmol/L) and catalase (hydrogen peroxide (H_2O_2) scavenger, 300 U/mL) reduced ethanol-induced relaxation to a similar extent in both endothelium-intact and denuded rings. Finally, prodifen (non-selective cytochrome P450 enzymes inhibitor, 10 μmol/L) and 4-methylpyrazole (selective alcohol dehydrogenase inhibitor, 10 μmol/L) reduced ethanol-induced relaxation. In cultured aortic vascular smooth muscle cells (VSMCs), ethanol stimulated generation of NO, which was significantly inhibited by L-NAME. In endothelial cells, flow cytometry studies showed that ethanol increased cytosolic Ca^{2+} concentration ([Ca^{2+}]c), O_2^- and cytosolic NO concentration ([NO]c). Tiron inhibited ethanol-induced increase in [Ca^{2+}]c and [NO]c. The major new finding of this work is that ethanol induces relaxation via redox-sensitive and NO–cGMP-dependent pathways through direct effects on ROS production and NO signaling. These findings identify putative molecular mechanisms whereby ethanol, at pharmacological concentrations, influences vascular reactivity.

1. Introduction

The relationship between ethanol consumption and blood pressure is complex. Chronic consumption of high doses of ethanol is associated with hypertension in animals and humans (Wakabayashi, 2008; Tirapelli et al., 2008). On the other hand, consumption of low to moderate amounts of ethanol has been described to exert beneficial effects on the cardiovascular system. For example, chronic consumption of low doses of ethanol is effective in decreasing blood pressure in rat models of hypertension (Vasdev et al., 2006). Ethanol consumption was also described to reduce the risk of myocardial infarction in hypertensive patients (Beulens et al., 2007). Moreover, the inverse correlation between moderate ethanol intake and coronary mortality has long been recognized (Albert et al., 1999; Klatsky, 2002).

Vascular relaxation might explain, at least in part, the beneficial effects of moderate ethanol consumption on the cardiovascular system. Arterial dilatation accompanied by hypotension is observed in the first hours after consumption of ethanol (Abe et al., 1994; Rosito et al., 1999; Bau et al., 2005). In vitro studies using isolated tissues show that ethanol induces direct relaxant responses in different blood vessels (Greenberg et al., 1993; Ru et al., 2008). Ethanol-induced vascular relaxation is related to the synthesis and action of endothelial factors, such as nitric oxide (NO) (Greenberg et al., 1993; Puddey et al., 2001), and prostaglandin (Greenberg et al., 1993). In fact, the beneficial effect of low ethanol doses on the

Abbreviations: NO, Nitric oxide; eNOS, endothelial NO synthase; nNOS, neuronal NO synthase; ROS, reactive oxygen species; ([Ca^{2+}]c), Cytosolic calcium concentration; ADH, alcohol dehydrogenase; ([NO]c), Cytosolic NO concentration; VSMCs, vascular smooth muscle cells; O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; L-NAME, *NG*-nitro-L-arginine methyl Ester; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; DHE, dihydroethidium.

* Corresponding author at: Universidade de São Paulo, Escola de Enfermagem de Ribeirão Preto, Laboratório de Farmacologia, Avenida Bandeirantes 3900, CEP 14040-902, Ribeirão Preto, SP, Brazil. Tel.: +55 16 36020532; fax: +55 16 36020518.
E-mail address: crtirapelli@eerp.usp.br (C.R. Tirapelli).

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cardiovascular system has been related to the release of NO (Puddey et al., 2001). Ethanol increases the expression of eNOS (Venkov et al., 1999) and stimulated Ca^{2+}-activated potassium channels increasing production of NO in cultured vascular endothelial cells (Kuhlmann et al., 2004). Moreover, ethanol relaxes bovine pulmonary arteries through enhancement of both basal and stimulated release of NO (Greenberg et al., 1993). Interestingly, the relaxation induced by ethanol is not completely abolished after endothelial removal, suggesting that this response is partially mediated by vascular smooth muscle cells (VSMC) (Greenberg et al., 1993; Ru et al., 2008).

The effect of ethanol on the vasculature is complex. Although ethanol is primarily metabolized in the liver, it is also metabolized in other tissues, including the vascular tissue. Ethanol-metabolizing enzymes alcohol dehydrogenase (ADH) and cytochrome P450-2E1 (CYP-2E1) are functionally active in the vasculature, and ethanol metabolism in this tissue leads to oxidative stress and the generation of reactive oxygen species (ROS) (Haorah et al., 2005). Superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) are two of the most important ROS in the vessel wall (Touyz, 2000; Touyz and Schiffrin, 2004). Studies in endothelial cells have shown that O_2^- induces a rapid rise in cytosolic concentration of Ca^{2+} ($[Ca^{2+}]c$) (Franceschi et al., 1990; Dreher and Junot, 1995; Hirosumi et al., 1988), which activates endothelial NO synthase (eNOS) with consequent vasorelaxation (Duarte et al., 2004). Superoxide anion is reduced by superoxide dismutase to H_2O_2 (Touyz and Schiffrin, 2004), which is implicated in the regulation of signaling pathways that leads to vascular relaxation (Barlow and White, 1998a, 1998b; Barlow et al., 2000). Moreover, H_2O_2 is known to activate phospholipase A_2 (PLA_2) (Rao et al., 1995; Boyer et al., 1995), resulting in the stimulation of arachidonic acid release (Sporn et al., 1992; Boyer et al., 1995) and increase in $[Ca^{2+}]c$ (Suzuki et al., 1997). Recently, we provided evidence that ethanol increases the generation of O_2^- and H_2O_2 in aortic smooth muscle cells and that ethanol-induced increase of $[Ca^{2+}]c$ in these cells is attenuated by ROS scavengers and cyclooxygenase inhibitors (Yogi et al., 2010). Our results show that ethanol stimulates ROS generation, which in turn may lead to production of prostanoids that induce increase of $[Ca^{2+}]c$. However, whether ethanol-induced NO generation and relaxation is modulated by ROS remains to be determined.

Since ethanol may have both protective and harmful effects in the cardiovascular system, the identification of biochemical mechanisms that could explain such paradoxical effects is warranted. Based on the above mentioned observations, we hypothesized that the relaxant effect of ethanol involves the generation of ROS and activation of NOS in the vasculature. To test this hypothesis the present study has attempted to investigate the role played by ROS in ethanol-induced relaxation and NO generation, thereby giving us some insight into the potential contribution of these cellular-signaling pathways to ethanol-induced vasorelaxation.

2. Material and methods

2.1. Vascular reactivity studies

Male Wistar rats weighting between 200 and 250 g (50–60 days old) were anesthetized and killed by aortic exsanguination in accordance to standards and policies of the University of São Paulo's Animal Care and Use Committee. The thoracic aorta was quickly removed, cleaned of adherent connective tissues and cut into rings (5–6 mm in length). Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer TRI201 (Panlab, Spain) to measure tension in the vessels. The rings were placed in a 5 mL organ chamber containing Krebs solution, pH 7.4, gassed with 95%O_2/5%CO_2, and maintained at 37 °C. The composition of Krebs solution was as follows (mmol/L): NaCl, 118.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 15.0; Glucose, 5.5; and $CaCl_2$, 2.5. The rings were stretched until they reached a basal tension of 1.5 g, as determined by length-tension relationship experiments and were then allowed to equilibrate for 60 min; during this time, the bath fluid was changed every 15–20 min. For some rings, the endothelium was removed mechanically by gently rolling the vessel lumen on a thin wire. Endothelial integrity was assessed qualitatively by the degree of relaxation induced by acetylcholine (1 μmol/L) in the presence of contractile tone induced by phenylephrine (0.1 μmol/L). For studies of endothelium-intact vessels, a ring was discarded if relaxation with acetylcholine was not 80% or greater. For studies of endothelium-denuded vessels, a ring was discarded if there was any degree of relaxation.

2.2. Effects of ethanol on vascular reactivity

Steady tension was evoked by phenylephrine (concentrations of 0.1 μmol/L for endothelium-intact rings and 0.03 μmol/L for endothelium-denuded rings were used to induce contractions of similar magnitude) and ethanol was then added in a stepwise fashion (0.03–200 mmol/L). Additions of ethanol were made as soon as a steady response was obtained at the preceding concentration. The concentration of ethanol used in our work was based on previous studies in different type of blood vessels (Greenberg et al., 1993; Hendrickson et al., 1999; Ru et al., 2008). In the present investigation we used concentrations of ethanol that are well within those described in the bloodstream of humans (Kalant, 1971) and rats (Husain et al., 2005; Tirapelli et al., 2008) after ethanol ingestion. For comparison, the effect of acetylcholine (10 nmol/L to10 μmol/L) and sodium nitroprusside (SNP, 10 nmol/L–0.1 μmol/L), were also evaluated in endothelium-intact and -denuded rings, respectively.

The mechanisms underlying the relaxant effect induced by ethanol were studied in endothelium-intact and endothelium-denuded rings pre-incubated for 30 min with the following drugs: N^G-nitro-L-arginine-methyl-ester (L-NAME, non-selective NO synthase inhibitor, 100 μmol/L), 7-nitroindazole (selective nNOS inhibitor, 100 μmol/L), wortmannin (PI3K inhibitor, 0.5 μmol/L), indomethacin (non-selective cyclooxygenase inhibitor, 10 μmol/L), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, selective guanylyl cyclase inhibitor, 1 μmol/L), tiron (O_2^- scavenger, 1 mmol/L), catalase (H_2O_2 scavenger, 300 U/mL), prodifen (non-selective cytochrome P450 enzymes inhibitor, 10 μmol/L), apocynin (NADPH inhibitor, 100 μmol/L) and 4-methylpyrazole (selective ADH inhibitor, 10 μmol/L). The participation of K^+ channels on ethanol-induced relaxation was investigated using the following inhibitors: apamin (selective blocker of low-conductance Ca^{2+}-activated channels, 1 μmol/L), glibenclamide (selective blocker of ATP-sensitive K^+ channels, 3 μmol/L), charybdotoxin (selective blocker of large-conductance Ca^{2+}-activated K^+ channels, 0.1 μmol/L) and 4-aminopyridine (selective blocker of voltage-dependent K^+ channels, 4-AP, 1 mmol/L), which were used as described by Nelson and Quayle (1995). In some protocols, combinations of these drugs were used. Relaxation was expressed as percentage change from the phenylephrine-contracted levels. Because L-NAME and ODQ enhanced phenylephrine-induced contraction, the rings with intact endothelium exposed to these compounds were pre-contracted with phenylephrine 0.03 μmol/L, to induce a magnitude of contraction similar to that found in the intact rings not exposed to the inhibitors. Concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA). The potency and maximum response are expressed as pD_2 (negative logarithm of the molar concentration of the drug producing 50% of the maximum response) and E_{max} (maximum effect), respectively.

2.3. Detection of NO in cultured VSMCs

VSMCs from aorta of Wistar–Kyoto rats were examined. Cells were maintained in DMEM containing 10% fetal bovine serum (FBS). Low