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

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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.

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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

passaged cells (passages 2–7) were studied. The study was approved by the Animal Ethics Committee of the University of Ottawa and performed according to recommendations of the Canadian Council for Animal Care. Adult male Wistar–Kyoto rats were euthanized by decapitation. VSMCs derived from aorta were isolated and characterized as described in detail previously (Callera et al., 2005). In brief, arteries were cleaned of adipose and connective tissue, and VSMCs were dissociated by digestion of vascular arcades with enzymatic solution (collagenase, elastase, soybean trypsin inhibitor, and bovine serum albumin type I; 60 min, 37 °C). The tissue was filtered and the cell suspension centrifuged and resuspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mmol/L glutamine, 20 mmol/L HEPES (pH 7.4), and antibiotics. At subconfluence, the culture medium was replaced with serum-free medium for 24 h to render the cells quiescent. Low-passage cells (passages 4 to 7), from at least 4 different primary cell cultures, were used in the experiments.

Production of NO was determined using the NO-specific fluorescent 4-Amino-5-methylamino-2′,7′-difluorescein, Diaminofluorescein-FM (DAF-2 FM, Sigma, St Louis, MO, USA). Cells were loaded with DAF-2 FM (final concentration 5 μmol/L, 30 min, 37 °C), kept in the dark, and maintained at 37 °C. Cells were then stimulated with ethanol (100 mmol/L) for 10 min. Because NOS generates O_2^- instead of NO in the absence of L-arginine (Xia et al., 1998), we added 100 μmol/L of L-arginine (Nakatsubo et al., 1998) to all solutions used for NO measurement, except for the experiment with L-NAME-treated cells, where L-NAME (100 μmol/L) was added 30 min before the stimulation with ethanol. Exposure to light was avoided as far as possible in all samples due to light sensitivity of the fluorescent probe. Subsequently the probe was washed out with PBS and cells were harvested with mild trypsinization. PBS (10 mL, 37 °C) was added to the cells, which were centrifuged for 3 min at 1200 rpm. The supernatants were discarded and the pellets were resuspended in PBS (200 μL), which were transferred to black microplates. DAF-FM nitrosation was assessed spectrofluorometrically at excitation/emission wavelengths of 495/515 nm (Cary Eclipse, Varian Canada, Inc). The fluorescence intensity was adjusted to the protein concentration and expressed as fluorescence emission per micrograms of protein. Protein concentrations were determined with protein assay reagent (Bio-Rad Laboratories).

2.4. Visualization of NO generation by intact VSMCs detected with fluorescent dye 4-Amino-5-methylamino-2′,7′-difluorescein, Diaminofluorescein-FM (DAF-2 FM)

Generation of intracellular NO in living cells was measured with the fluoroprobe DAF-2 FM (excitation at 495 nm and emission at 515 nm). Growth-arrested VSMCs from WKY rats were incubated in Hank's balanced salt solution (HBSS) containing 1.3 mmol/L $CaCl_2$ and 5.5 mmol/L glucose supplemented with 5 μmol/L DAF-2 FM in a light protected chamber at 37 °C, for 10 min. Cells were rinsed in HBSS and images were obtained before and after cells exposition to 100 mmol/L ethanol. Cells were imaged on a wide field epifluorescence microscope equipped with 40× oil immersion lens using the Stallion live-cell Digital Hi-Speed Multi-Channel Imaging System (Zeiss, Germany).

2.5. Flow cytometry for measurement of Ca^{2+}, NO and O_2^- in endothelial cells

Flow cytometry was performed as previously described (Bonaventura et al., 2008). The aortas were isolated and longitudinally opened. Endothelial cells were isolated from the vessels mechanically, by gently friction with plastic stem in plates containing Krebs solution. The cells suspension was centrifuged at 1000 rpm for 5 min. The cells pellet was suspended in 1.0 mL of Krebs solution and maintained in a humidified incubator (37 °C) until the use. Part of this cells suspension was used for viability test. Cells viability was determined by trypan blue staining (2%). The number of viable cells before the isolation was estimated for each animal by counting in a Neubauer chamber (Weber Scientific International, Germany). Cytofluorographic analysis was performed using a Becton–Dickinson FACScan (San Jose, CA, USA) with an argon ion laser tuned to 488 nm at 15 mW output. The cells were analyzed at the flow cytometer in the absence of the fluorescent dyes. Before the detection of $[Ca^{2+}]c$, cytoplasmatic concentration of NO ([NO]c) or O_2^-, the cells without the dyes were analyzed on the flow cytometer (Blank). After that, the cells were incubated with a selective fluorescent dye for Ca^{2+} (Fluo-3AM, 10 μmol/L), NO (4,5-diaminofluorescein-2 diacetate — DAF-2/DA, 10 μmol/L) or O_2^- (dihydroethidium — DHE, 2 μmol/L) for 20 min. Afterwards, ethanol (1, 10 or 100 mmol/L) was added to the cells loaded with one of the selective dyes. After the addition of ethanol, the samples were analyzed for 10 min in intervals of 1 min. Acquisition was set at 10,000 cells and the mean fluorescent intensity was measured in all the samples by using CellQuest 1.2 software (Becton–Dickinson, Franklin Lakes, NJ,

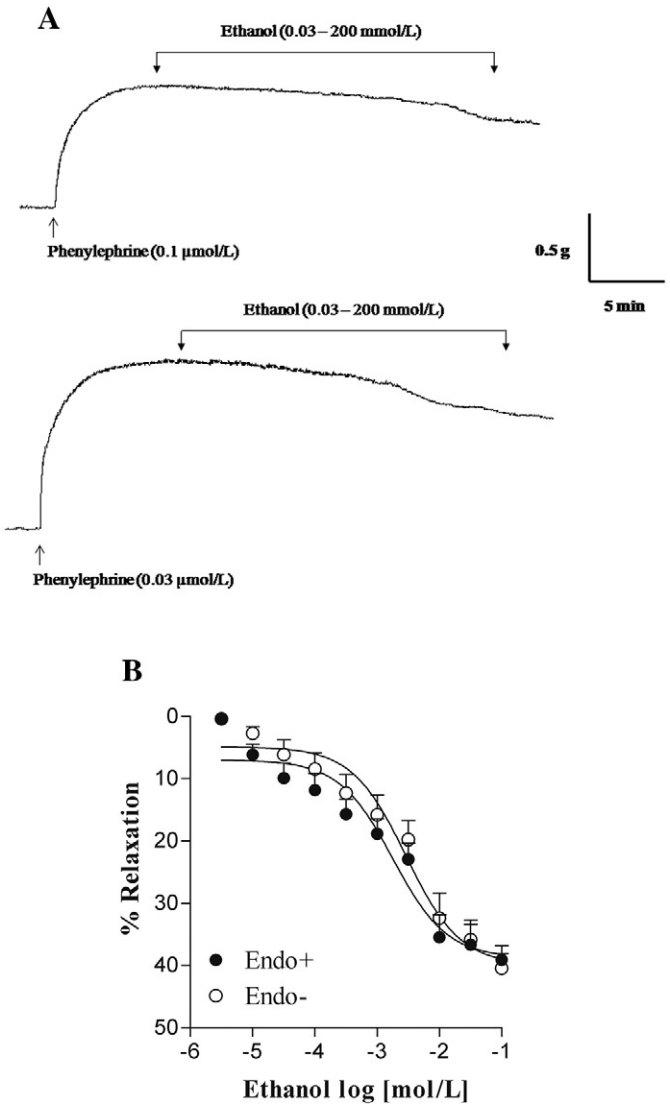

Fig. 1. (A) Original traces of the isometric tension recording of endothelium-intact (Endo+) or endothelium-denuded (Endo−) rat aortic rings in response to ethanol. (B) Concentration–response curves for ethanol in endothelium-intact and endothelium-denuded rat aortic rings. Endothelium-intact and -denuded rings were pre-contracted with phenylephrine (0.1 and 0.03 μmol/L, respectively). After reaching a stable and sustainable contraction, ethanol (0.03–200 mmol/L) was added cumulatively to the organ bath.

USA). The initial fluorescence intensity value was designated F_0 and the final fluorescence intensity value obtained after stimulation for 10 min with ethanol was designated *F*. In this way, the percentage of the difference in fluorescence intensity (%ΔFI), which reflects the increase of $[Ca^{2+}]c$, [NO]c or O_2^-, was obtained for each protocol in relation to F (100%). It was calculated by the following formula: $\%\Delta FI = (F - F_0/F) \times 100$. Control responses were obtained in experiments where ethanol was replaced by the vehicle (Hank's solution) (Rodrigues et al., 2010).

In order to investigate the mechanisms underlying ethanol-induced increase in $[Ca^{2+}]c$, endothelial cells were incubated for 30 min with one of the following inhibitors: tiron (1 mmol/L), indomethacin (10 μmol/L) or verapamil (Ca^{2+} channel blocker, 10 μmol/L). The cells were loaded with Fluo-3AM and exposed to ethanol (100 mmol/L). In order to investigate the mechanisms underlying ethanol-induced increase in [NO]c, endothelial cells were incubated for 30 min with one of the following inhibitors: tiron (1 mmol/L), indomethacin (10 μmol/L), verapamil (10 μmol/L) or L-NAME (100 μmol/L). The cells were loaded with DAF-2/DA and exposed to ethanol (100 mmol/L).

2.6. Drugs

ODQ, indomethacin and wortmannin were prepared as stock solutions in dimethyl sulfoxide (DMSO). The other drugs were dissolved in distilled water. The concentration of DMSO did not exceed 0.5%, which was shown to have no effects per se on the flow cytometry experiments or on the functional assays.

2.7. Statistics

Statistically significant differences were calculated by Student's *t* test or one-way analysis of variance (ANOVA) followed by Newman–

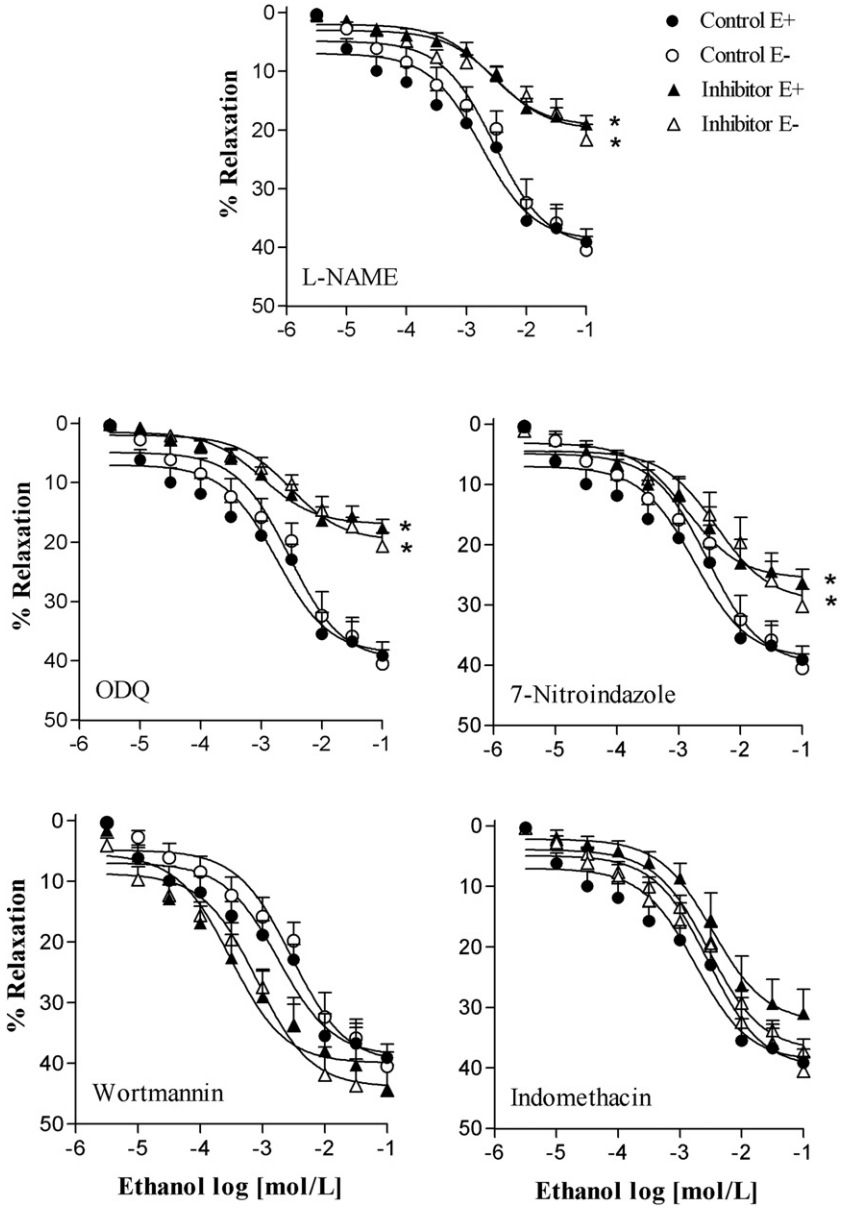

Fig. 2. Effect of L-NAME, ODQ, 7-nitroindazole, wortmannin and indomethacin on ethanol-induced relaxation of endothelium-intact (E+) and denuded (E−) aortic rings. Concentration–response curves for ethanol were determined in endothelium-intact or denuded aortic rings in the absence or after 30-min incubation with L-NAME (100 μmol/L), ODQ (1 μmol/L), 7-nitroindazole (100 μmol/L), wortmannin (0.5 μmol/L) and indomethacin (10 μmol/L). *Compared to the respective control group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

Table 1
Effect of L-NAME, ODQ, 7-nitroindazole, wortmannin, indomethacin, apamin, glibenclamide, charybdotoxin and 4-aminopyridine on ethanol-induced relaxation in endothelium-intact or endothelium-denuded rat aortic rings.

Group	Endothelium-intact		Endothelium-denuded	
	E_{max} (% relaxation)	pD_2	E_{max} (% relaxation)	pD_2
Control	39.0 ± 2.2 (8)	3.1 ± 0.2	40.5 ± 2.4 (8)	2.8 ± 0.3
L-NAME 100 μmol/L	19.0 ± 1.5 (6)*	2.9 ± 0.2	21.7 ± 2.7 (7)*	2.8 ± 0.2
ODQ 1 μmol/L	17.6 ± 1.4 (8)*	3.0 ± 0.2	20.7 ± 2.2 (6)*	2.7 ± 0.2
7-nitroindazole 100 μmol/L	26.4 ± 2.3 (7)*	2.9 ± 0.2	30.2 ± 2.9 (7)*	2.7 ± 0.3
Wortmannin 0.5 μmol/L	43.8 ± 5.0 (8)	3.3 ± 0.3	44.5 ± 4.5 (6)	3.4 ± 0.5
Indomethacin 10 μmol/L	32.0 ± 4.0 (6)	2.6 ± 0.2	37.3 ± 2.1 (8)	2.7 ± 0.1
Apamin 1 μmol/L	45.4 ± 3.9 (7)	2.9 ± 0.2	38.9 ± 5.6 (6)	2.6 ± 0.1
Glibenclamide 3 μmol/L	27.8 ± 4.3 (4)*	3.2 ± 0.2	21.7 ± 3.3 (6)*	3.0 ± 0.1
Charybdotoxin 0.1 μmol/L	49.2 ± 1.5 (5)	2.7 ± 0.1	42.1 ± 2.7 (6)	2.7 ± 0.2
4-aminopyridine 1 mmol/L	23.6 ± 4.9 (6)*	3.4 ± 0.2	25.4 ± 2.1 (5)*	2.9 ± 0.3

Data are represented as mean ± SEM. Number of experiments is indicated between parentheses.
* Compared to respective control group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

Keuls multiple comparison test. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Effects of ethanol on vascular reactivity

In endothelium-intact or endothelium-denuded rat aortic rings, phenylephrine induced sustained contraction, which lasted at least 90 min without any predominant decrease in tension (data not shown). The addition of ethanol in concentrations ranging from 0.03 to 200 mmol/L caused a concentration-dependent relaxation of endothelium-intact (E_{max}: 39.0 ± 2.2%; pD_2: 3.1 ± 0.2, n = 8) and denuded (E_{max}: 40.5 ± 2.4%, pD_2: 2.8 ± 0.3, n = 8) aortic rings (Fig. 1). The maximum relaxant effect was obtained with ethanol 200 mmol/L. When the subsequent concentration of ethanol (300 mmol/L) was added no relaxation was observed. Instead, at this concentration ethanol induced contraction of endothelium-intact or denuded aortas (data not shown). No differences were found between the pre-contractile levels induced by phenylephrine in endothelium-intact (1.0 ± 0.09 g, n = 8) and endothelium-denuded rings (1.1 ± 0.13 g, n = 8).

The relaxation induced by acetylcholine in endothelium-intact rings was significantly different (E_{max}: 97.8 ± 1.3%; pD_2: 7.0 ± 0.2, n = 5) from that found for ethanol (E_{max}: 39.0 ± 2.2%; pD_2: 3.1 ± 0.2, n = 8) ($P<0.05$, Student's *t* test). Similarly, SNP-induced relaxation of endothelium-denuded rings (E_{max}: 100.7 ± 4.2%; pD_2: 8.3 ± 0.1, n = 5) was significantly different from that found for ethanol (E_{max}: 40.5 ± 2.4%, pD_2: 2.8 ± 0.3, n = 8) ($P<0.05$, Student's *t* test).

To investigate the mechanisms underlying ethanol-induced relaxation, endothelium-intact or endothelium-denuded rings were exposed to NO–cGMP, cyclooxygenase pathway inhibitors and O_2^-/H_2O_2 scavengers. L-NAME, 7-nitroindazole and ODQ reduced the relaxation induced by ethanol in endothelium-intact and denuded rings. On the other hand, wortmannin and indomethacin did not affect ethanol-induced relaxation in both endothelium-intact and -denuded rings (Fig. 2, Table 1). The participation of K^+ channels on ethanol-induced relaxation was evaluated using selective K^+ channels blockers. Glibenclamide and 4-aminopyridine, but not apamin or charybdotoxin, reduced ethanol-induced relaxation (Fig. 3, Table 1). Tiron and catalase reduced ethanol-induced relaxation to a similar extent. Combination of these two compounds showed no further suppression on ethanol-induced relaxation (Fig. 4, Table 2). Combination of L-NAME and tiron or L-NAME and catalase showed no further suppression on ethanol-induced

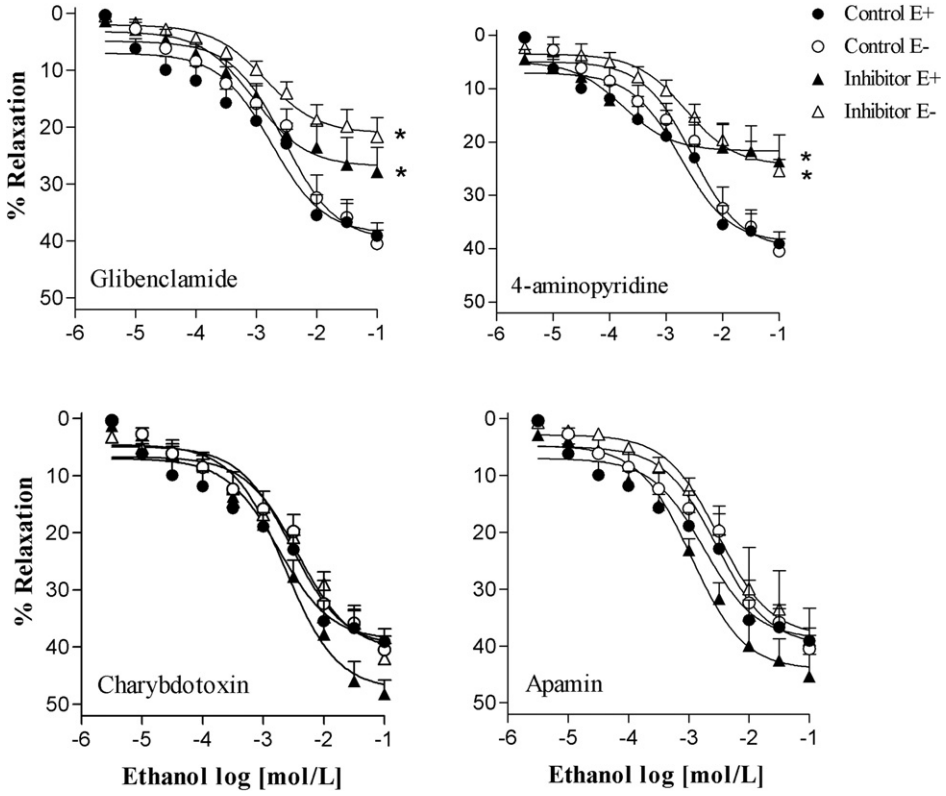

Fig. 3. Effect of apamin, glibenclamide, charybdotoxin and 4-aminopyridine on ethanol-induced relaxation of endothelium-intact (E+) and denuded (E−) aortic rings. Concentration–response curves for ethanol were determined in endothelium-intact or denuded aortic rings in the absence or after 30-min incubation with apamin (1 μmol/L), glibenclamide (3 μmol/L), charybdotoxin (0.1 μmol/L) and 4-aminopyridine (1 mmol/L). *Compared to the respective control group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

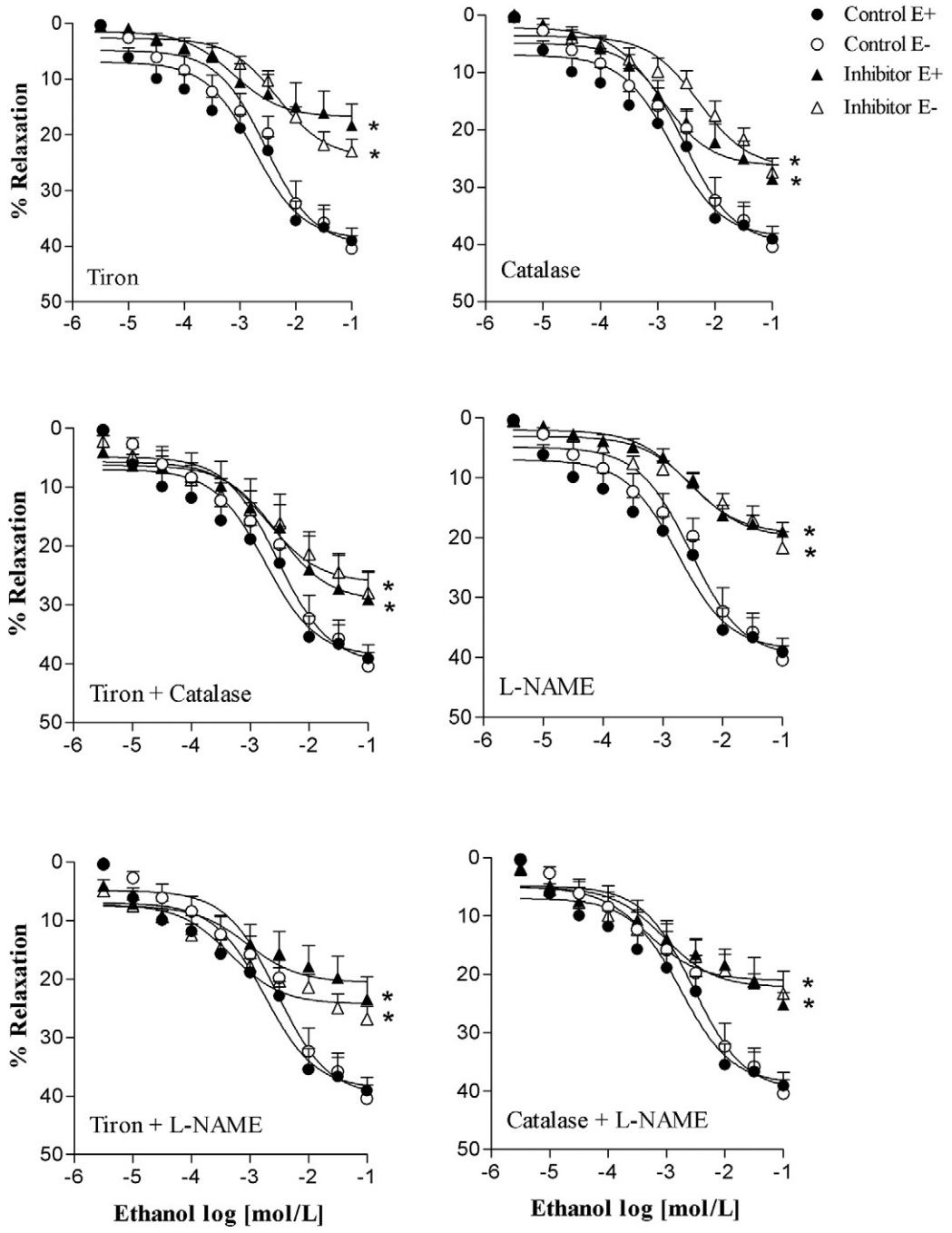

Fig. 4. Effect of tiron, catalase, L-NAME and the combination of these compounds on ethanol-induced relaxation of endothelium-intact (E+) and denuded (E−) rat aortic rings. Concentration–response curves for ethanol were determined in endothelium-intact or denuded aortic rings in the absence or after 30-min incubation with tiron (1 mmol/L), catalase (300 U/mL), L-NAME (100 μmol/L) or the combination of these compounds. *Compared to the respective control group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

relaxation when compared to the effects of these drugs when added alone (Fig. 4, Table 2). Finally, 4-methylpyrazole and prodifen, but not apocynin, reduced the relaxation induced by ethanol (Fig. 5, Table 3).

3.2. Detection of NO in cultured VSMCs

Fig. 6 shows that ethanol induced NO generation in VSMCs. L-NAME inhibited ethanol-induced NO generation.

3.3. Detection of $[Ca^{2+}]c$, [NO]c and O_2^- in endothelial cells

Fig. 7 shows that ethanol induced an increase in [NO]c and $[Ca^{2+}]$c in endothelial cells in a concentration-dependent manner. Similarly, ethanol induces O_2^- generation in endothelial cells. Our results show that the NO production represented as %ΔFI was lower in endothelial cells pre-treated with tiron, indomethacin, verapamil and L-NAME (Table 4). Finally, tiron, indomethacin and verapamil attenuated the increase in $[Ca^{2+}]c$ induced by ethanol in endothelial cells (Table 4). Ethanol (at the concentrations used), had no significant effect on endothelial cells viability as assessed by trypan blue exclusion (data not shown).

4. Discussion

Major findings from the present study demonstrate that ROS scavenging or suppression of ethanol metabolism in the aorta prevented

Table 2
Effect of tiron, catalase, L-NAME and the combination of these inhibitors on ethanol-induced relaxation in endothelium-intact or endothelium-denuded rat aortic rings.

Group	Endothelium-intact		Endothelium-denuded	
	E_{max} (% relaxation)	pD_2	E_{max} (% relaxation)	pD_2
Control	39.0 ± 2.2 (8)	3.1 ± 0.2	40.5 ± 2.4 (8)	2.8 ± 0.3
Tiron 1 mmol/L	18.4 ± 3.8 (6)*	2.8 ± 0.4	23.1 ± 2.2 (8)*	2.5 ± 0.2
Catalase 300 units/mL	28.6 ± 3.6 (6)*	3.2 ± 0.2	27.5 ± 1.4 (7)*	2.6 ± 0.2
Tiron + Catalase	29.1 ± 4.8 (6)*	3.3 ± 0.5	27.9 ± 3.4(6)*	3.1 ± 0.3
L-NAME 100 μmol/L	19.0 ± 1.5 (6)*	2.9 ± 0.2	21.7 ± 2.7 (7)*	2.8 ± 0.2
Tiron + L-NAME	23.5 ± 3.7 (6)*	3.6 ± 0.3	26.8 ± 2.1 (5)*	3.4 ± 0.2
Catalase + L-NAME	25.2 ± 2.1 (6)*	3.2 ± 0.2	23.3 ± 3.8 (4)*	3.6 ± 0.3

Data are represented as mean ± SEM. Number of experiments is indicated between parentheses.
* Compared to respective control group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

ethanol-induced relaxation, suggesting that this response is mediated, in part, by oxidative stress. Moreover, the present data suggest that ROS generation triggers the activation of the NO–cGMP pathway, which in turn increases NO generation and relaxation. These findings identify redox-sensitive and NO-dependent signaling as novel mechanisms underlying ethanol-induced vascular relaxation. It is important to note that the concentrations of ethanol used in our study to evaluate vascular relaxation are relevant since they are within those found in the bloodstream of humans after moderate ethanol consumption (approximately 5–25 mmol/L) (Klatsky et al., 1992; Thun et al., 1997; Mukamal et al., 2003). Moreover, in alcoholics, blood ethanol level can reach 100 mmol/L.

Ethanol treatment of rat aortic rings resulted in an endothelium-independent relaxation, which is in accordance with previous findings showing that the relaxation induced by ethanol in vascular tissues is not solely mediated by the endothelium (Greenberg et al., 1993; Ru et al., 2008). The relaxation induced by ethanol was approximately 40% of the relaxant response induced by acetylcholine and SNP in endothelium-intact and denuded rings, respectively. L-NAME and ODQ attenuated ethanol-induced relaxation in both endothelium-intact and denuded rings, supporting a role for the NO–cGMP pathway in this response. Moreover, ATP-sensitive and voltage-dependent K^+ channels are also involved in the vasorelaxant response induced by ethanol. On the other hand, vasodilator prostanoids do not participate on ethanol induced relaxation since indomethacin did not alter this response. Our functional results support previous observation demonstrating that VSMCs produce NO (Kleinert et al., 2004; Carrillo-Sepúlveda et al., 2010). This finding, associated with the fact that ethanol increased NO generation in cultured VSMCs, suggest that relaxation of rat aorta in response to ethanol is mediated, at least in part, by NO generated in VSMCs. In fact, this is the first study showing a direct effect of ethanol in the generation of NO in VSMCs, since currently available data show that only endothelial cells were previously identified as targets for ethanol-induced NO production (Greenberg et al., 1993; Hendrickson et al., 1999; Kuhlmann et al., 2004). The presence of eNOS and nNOS in VSMCs was previously described in the cytoplasm of these cells under basal conditions (Carrillo-Sepúlveda et al., 2010). Our data showed that inhibition of nNOS significantly reduced the relaxation induced by ethanol, showing that nNOS play a significant role in ethanol-induced NO production by VSMCs. This finding is consistent with the study of El-Mas et al. (2009), which demonstrated that ethanol increases NO production in the myocardium by a mechanism that involves nNOS activation via the PI3K/Akt/nNOS signaling pathway. Interestingly, our data rule out the participation of the PI3K pathway in ethanol-induced relaxation since this response was not attenuated by wortmannin.

Our functional results showed that antioxidant treatment of endothelium-intact or denuded aortic rings prevented ethanol-induced

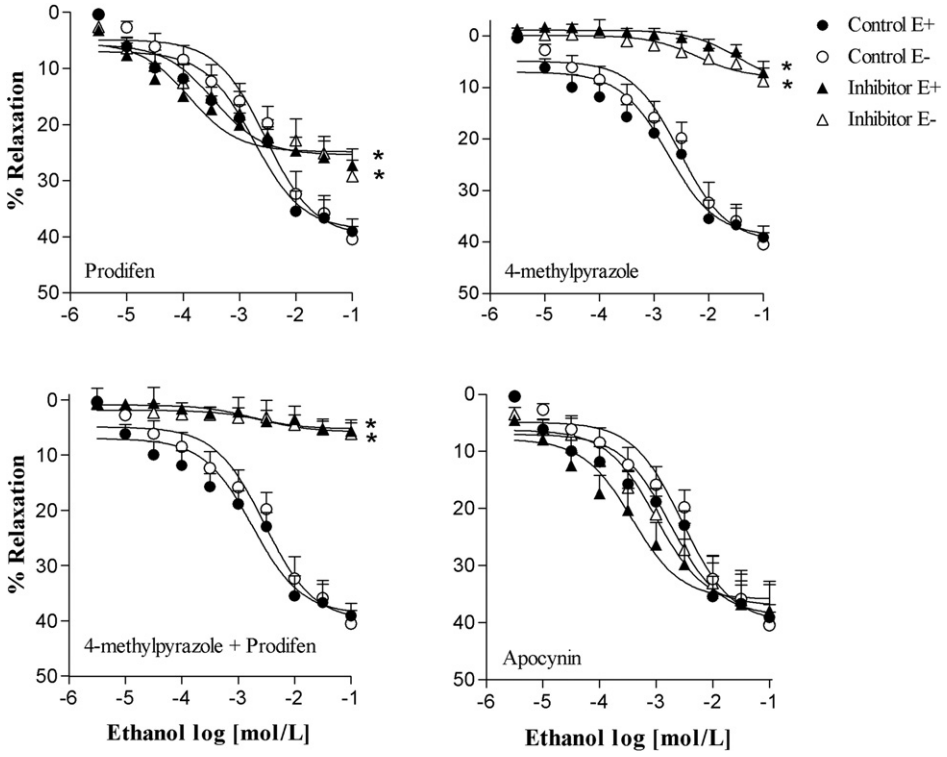

Fig. 5. Effect of 4-methylpyrazole, prodifen and apocynin on ethanol-induced relaxation of endothelium-intact (E+) and denuded (E−) aortic rings. Concentration–response curves for ethanol were determined in endothelium-intact or denuded aortic rings in the absence or after 30-min incubation with 4-methylpyrazole (100 μmol/L), prodifen (10 μmol/L), apocynin (100 μmol/L) or the combination of these compounds. *Compared to the respective control group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

Table 3
Effect of 4-methylpyrazole, prodifen, apocynin or the combination of these inhibitors on ethanol-induced relaxation in endothelium-intact or endothelium-denuded rat aortic rings.

Group	Endothelium-intact		Endothelium-denuded	
	E_{max} (% relaxation)	pD_2	E_{max} (% relaxation)	pD_2
Control	39.0 ± 2.2 (8)	3.1 ± 0.2	40.5 ± 2.4 (8)	2.8 ± 0.3
4-methylpyrazole 10 μmol/L	7.2 ± 2.1 (4)*	_	8.8 ± 2.5 (4)*	–
Prodifen 10 μmol/L	27.3 ± 2.9 (8)*	3.6 ± 0.2	29.2 ± 2.8 (6)*	3.1 ± 0.3
4-methylpyrazole + Prodifen	5.2 ± 1.3 (6)*	_	6.0 ± 2.1 (5)*	–
Apocynin 100 μmol/L	37.9 ± 5.1 (6)	3.6 ± 0.2	39.2 ± 5.8 (6)	3.2 ± 0.2

Data are represented as mean ± SEM. Number of experiments is indicated between parentheses.
* Compared to respective control group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

relaxation, suggesting that this response is mediated, in part, by oxidative stress. In fact, low levels of O_2^- contribute to vasodilatation particularly after dismutation to H_2O_2 (Wei et al., 1996). We have previously demonstrated that ethanol at 100 mmol/L increases the generation of O_2^- and H_2O_2 in cultured aortic smooth muscle cells (Yogi et al., 2010). Thus, the ethanol-induced relaxation here described is dependent on ROS generation by the vasculature. Interestingly, the association of ROS scavengers with L-NAME showed no additional inhibitory effect on the relaxation induced by ethanol, suggesting that ROS generation is the trigger for NO production. This result is consistent with those reported in the literature showing that O_2^- enhances Ca^{2+}/NO signaling (Graier et al., 1996) and induces vasorelaxation (Duarte et al., 2004). Moreover, H_2O_2 was also described to play a positive role in regulating NO signaling. In endothelial cells, H_2O_2 activates eNOS (Thomas et al., 2001) and the generation of H_2O_2 has been implicated as a mediator of flow-induced vasodilatation in coronary and cerebral vessels (Barlow and White, 1998a, 1998b; Miura et al., 2003). More recently, Kumar et al. (2010) demonstrated that H_2O_2 increases eNOS phosphorylation and NO generation in pulmonary arterial endothelial cells. It should also be noted that ROS scavengers and L-NAME combined did not completely abolish ethanol-induced relaxation, indicating that this response may involve other factors and/or activation of pathways that are not associated with ROS generation or NO–cGMP pathway.

In order to determine the sources of O_2^- implicated in the vascular action of ethanol, the effect of ethanol-metabolizing enzyme inhibitors was tested. ADH and CYP-2E1 are ethanol-metabolizing enzymes that are functionally active in the vasculature, and ethanol metabolism in this tissue leads to oxidative stress and generation of ROS (Haorah et al., 2005). We found that inhibition of ethanol metabolism by ADH strongly inhibited ethanol-induced relaxation while CYP-2E1 inhibition by prodifen attenuated this response. The lack of effect of apocynin on the vascular relaxation induced by ethanol. ruled out

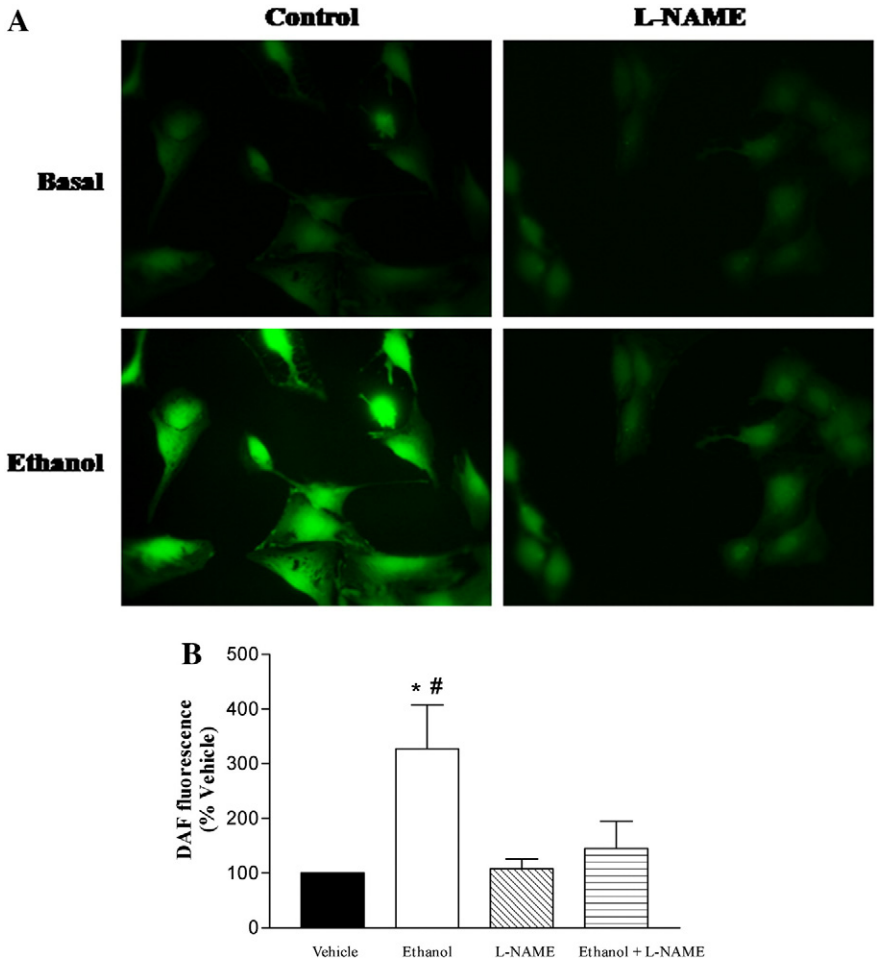

Fig. 6. Effect of ethanol on NO generation in VSMCs. VSMCs were loaded with DAF-2 FM and exposed to ethanol (100 mmol/L), in the absence or presence of L-NAME (100 μmol/L). A: representative fluorescence images of VSMCs in basal conditions and in cells stimulated with ethanol in the absence and presence of L-NAME. B: bar graphs demonstrate NO generation in VSMCs. Data are presented as DAF-2 FM fluorescence (excitation/emission — 495/515 nm). Data are means ± SEM of 4 experiments. *Compared to basal ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

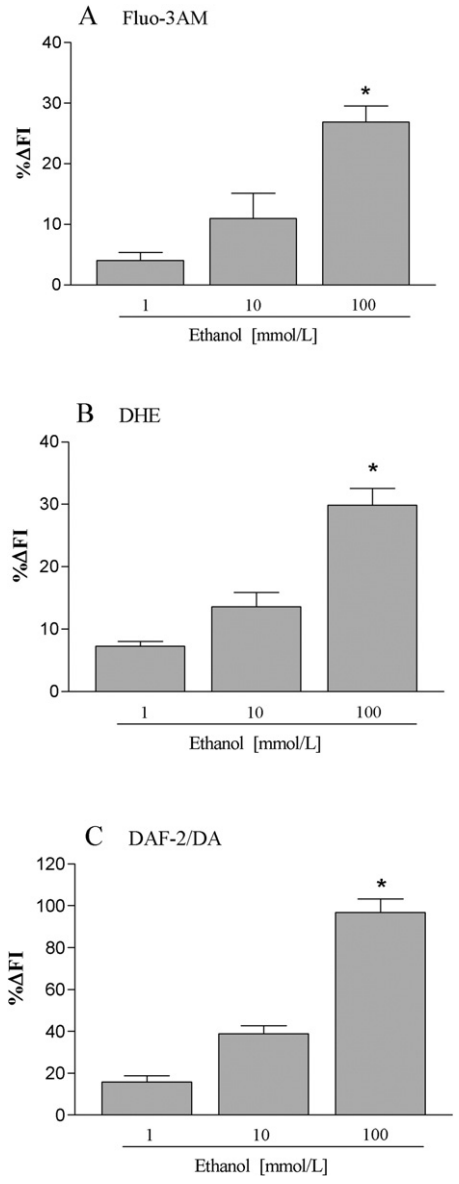

Fig. 7. Effect of ethanol on $[Ca^{2+}]c$ (A), O_2^- levels (B) and [NO]c (C) in aortic endothelial cells. Endothelial cells were preloaded with DAF-2/DA (10 μmol/L), Fluo-3 AM (10 μmol/L) or dihydroethidium (DHE, 2 μmol/L) and analyzed by flow cytometry. The initial fluorescence intensity value was designated F_0, and the final fluorescence intensity value obtained after stimulation with ethanol (100 mmol/L) was designated *F*. The percentage of the difference in fluorescence intensity (%ΔFI), which reflects the increase on $[Ca^{2+}]c$, [NO]c or O_2^- generation was obtained for each protocol in relation to *F* (100%). The bars represent the mean ± SEM of n = 6–9 experiments. *Compared to ethanol at 1 and 10 mmol/L ($P<0.05$; ANOVA followed by Newman − Keuls multiple comparison test).

the involvement of NADPH oxidases as a source of O_2^-. Thus, we present evidence that ethanol metabolism by ADH and CYP2E1 in the vasculature is a source for ROS generation, which acts as a signaling molecule to induce relaxation.

The fact that ethanol-induced relaxation of isolated aorta is endothelium-independent does not rule out a possible effect of ethanol on endothelial cells. Endothelial NO synthesis was directly measured with DAF-2/DA fluorescence. Our data demonstrate a dose-dependent effect of ethanol on endothelial NO production. This data are consistent with the findings of Acevedo et al. (2001), which demonstrated an ethanol-induced dose-dependent increase of NO production in endothelial cells from human umbilical vein. In cultured bovine aortic endothelial cells ethanol (0.8–160 mmol/L) was

Table 4
Effect of different inhibitors on ethanol-induced increase in [NO]c (%ΔFI DAF-2/DA) and $[Ca^{2+}]c$ (%ΔFI Fluo-3AM) in aortic endothelial cells.

Groups	%ΔFI DAF-2/DA	%ΔFI Fluo-3AM
Ethanol (100 mmol/L)	96.8 ± 6.5 (6)	27.0 ± 2.6 (6)
+ tiron (1 mmol/L)	53.2 ± 1.1 (6)*	7.8 ± 1.3 (9)*
+ indomethacin (10 μmol/L)	41.2 ± 2.5 (5)*	7.0 ± 1.5 (7)*
+ tiron plus indomethacin	32.0 ± 1.3 (5)*	4.9 ± 1.9 (7)*
+ verapamil (10 μmol/L)	29.8 ± 5.1 (5)*	1.8 ± 0.6 (5)*
+ L-NAME (100 μmol/L)	18.1 ± 1.9 (5)*	–

The initial fluorescence intensity value was designated F_0, and the final fluorescence intensity value obtained after stimulation with ethanol (100 mmol/L) was designated *F*. The percentage of the difference in fluorescence intensity (%ΔFI) was obtained for each protocol in relation to *F* (100%). Data are represented as mean ± SEM. Number of experiments is indicated between parentheses.

* Compared to respective ethanol group ($P<0.05$; ANOVA followed by Newman–Keuls multiple comparison test).

shown to increase eNOS activity (Hendrickson et al., 1999). In our study, endothelial NO production stimulated by ethanol was sensitive to L-NAME further implicating the activation of eNOS in this response. These findings indicate a possible beneficial effect of low-dose ethanol on endothelial function since NO plays a crucial role in vasodilatation, regional blood flow increase, hypotension and atherosclerosis prevention (Moncada and Higgs, 1991). However, we must be aware of the fact that ethanol-induced generation of NO was reduced in the presence of tiron, further suggesting a role for O_2^- in this response. Moreover we found that ethanol induced O_2^- generation in endothelial cells, which is involved in the ethanol-induced increase in $[Ca^{2+}]c$. Studies in endothelial cells have shown that O_2^- induces a rapid rise in $[Ca^{2+}]c$ (Franceschi et al., 1990; Dreher and Junot, 1995; Hirosumi et al., 1988), which might activate eNOS with consequent vasorelaxation (Duarte et al., 2004). Ca^{2+} is required for the activation of eNOS. Our data show that verapamil partially inhibited ethanol-induced NO generation in endothelial cells. Recently, we provided evidence that ethanol induced an increase of $[Ca^{2+}]c$ in aortic smooth muscle cells and that this response is attenuated by ROS scavengers and cyclooxygenase inhibitors (Yogi et al., 2010). Similar results are described in the present investigation in endothelial cells where ethanol stimulated O_2^- generation. The latter, may lead to production of prostanoids that induce increase on $[Ca^{2+}]c$. However, it is important to note that although indomethacin reduced NO generation in endothelial cells, this cyclooxygenase inhibitor did not affect the relaxation induced by ethanol in the isolated aorta. A possible explanation for such observation is that the relaxation induced by ethanol is partially, but not totally, dependent on the NO generated in endothelial cells. As mentioned before, relaxation of rat aorta in response to ethanol is also mediated by NO generated in VSMCs. Taken together our findings support a key role for O_2^- in ethanol-induced NO generation in endothelial cells.

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. At pharmacologically relevant concentrations, ethanol induces vascular relaxation by acting in both endothelial cells and VSMCs. The vasodilator effect is mediated by increased production of NO, which occurs rapidly by the activation of constitutive NOS. Ethanol metabolism in the vasculature is possibly the main source of ROS generation, which acts as a signaling molecule that triggers the vascular production of NO with consequent relaxation.

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