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Ethanol-induced erectile dysfunction and increased expression of pro-inflammatory proteins in the rat cavernosal smooth muscle are mediated by NADPH oxidase-derived reactive oxygen species

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ABSTRACT

Ethanol consumption is associated with an increased risk of erectile dysfunction (ED), but the molecular mechanisms through which ethanol causes ED remain elusive. Reactive oxygen species are described as mediators of ethanol-induced cell toxicity/damage in distinctive tissues. The enzyme NADPH oxidase is the main source of reactive oxygen species in the endothelium and vascular smooth muscle cells and ethanol is described to increase NADPH oxidase activation and reactive oxygen species generation. This study evaluated the contribution of NADPH oxidase-derived reactive oxygen species to ethanol-induced ED, endothelial dysfunction and production of pro-inflammatory and redox-sensitive proteins in the rat cavernosal smooth muscle (CSM). Male Wistar rats were treated with ethanol (20% v/v) or ethanol plus apocynin (30 mg/kg/day; p.o. gavage) for six weeks. Apocynin prevented both the decreased in acetylcholine-induced relaxation and intracavernosal pressure induced by ethanol. Ethanol increased superoxide anion (O_2^-) generation and catalase activity in CSM, and treatment with apocynin prevented these responses. Similarly, apocynin prevented the ethanol-induced decreased of nitrate/nitrite (NOx), hydrogen peroxide (H_2O_2) and SOD activity. Treatment with ethanol increased p47phox translocation to the membrane as well as the expression of Nox2, COX-1, catalase, iNOS, ICAM-1 and p65. Apocynin prevented the effects of ethanol on protein expression and p47phox translocation. Finally, treatment with ethanol increased both TNF-α production and neutrophil migration in CSM. The major new finding of this study is that NADPH oxidase-derived reactive oxygen species play a role on chronic ethanol consumption-induced ED and endothelial dysfunction in the rat CSM.

1. Introduction

Chronic consumption of high doses of ethanol has been associated with increased reactive oxygen species generation, altered vascular contractility/relaxation, endothelial dysfunction and hypertension (Passaglia et al., 2015; Marchi et al., 2016). Some studies have demonstrated that chronic ethanol consumption is also associated with an increased risk of erectile dysfunction (ED) (Wetterling et al., 1999; Peugh et al., 2001). However, the molecular mechanisms and possible mediators through which ethanol causes ED remain elusive. In this context, previous studies from our laboratory have linked ethanol-induced ED to decreased acetylcholine-induced relaxation and increased reactive oxygen species production in the cavernosal smooth muscle (CSM) (Leite et al., 2013; Muniz et al., 2015). Reactive oxygen species are described as mediators of ethanol-induced cell toxicity/damage in distinctive tissues as they induce lipid peroxidation, activation of mitogen-activated protein kinases (MAPK) and production of inflammatory mediators. Moreover, ethanol consumption increases the expression of the catalytic subunits of the enzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is an important source of reactive oxygen species (Yeligar et al., 2012; Simplicio et al., 2017). Thus, it is possible that reactive oxygen species-induced production of inflammatory cytokines and activation of MAPK may contribute to ethanol-induced ED.

The enzyme NADPH oxidase is the main source of reactive oxygen species in the endothelium and vascular smooth muscle cells

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(Montezano and Touyz, 2014). The catalytic subunit of NADPH oxidase catalyzes the production of superoxide anion (O_2^-) using an electron derived from NADPH or NADH. Seven catalytic subunits termed Nox1-5, Duox1 and Duox2 have been identified. The homologues Nox1, Nox2 and Nox4 are expressed in endothelial and smooth muscle cells. The NADPH oxidase family is also composed of regulatory subunits p22phox, p47phox, Noxo1, p67phox, Noxa1, p40phox, and the major binding partner Rac (Rodiño-Janeiro et al., 2013). Importantly, up-regulation of NADPH oxidase subunits in the corpus cavernosum has been associated with ED in different conditions such as hypercholesterolemia and diabetes (Li et al., 2012; Fraga-Silva et al., 2013, 2015; Musicki et al., 2016).

Reactive oxygen species generated by NADPH oxidase are important in the regulation of physiologic functions but they also contribute to the development of vascular diseases, such as hypertension, diabetes and ED (Li et al., 2012; Montezano and Touyz, 2014; Murat et al., 2016). Recently, we showed that chronic ethanol consumption increases NADPH oxidase activity and reactive oxygen species production in the aorta, mesenteric arteries and corpus cavernosum. The effects of ethanol included decreased nitric oxide (NO) bioavailability, vasoconstriction, endothelial dysfunction, and increased production of systemic and vascular inflammatory cytokines (Muniz et al., 2015; Passaglia et al., 2015; Simplicio et al., 2017). However, the role of NADPH oxidase-derived reactive oxygen species on ethanol-induced ED and inflammation of the CSM is unclear.

We hypothesized that ethanol consumption increases NADPH oxidase-derived reactive oxygen species with further reduction on NO bioavailability, endothelial dysfunction and activation of redox-sensitive signaling pathways that will lead to an increased production of pro-inflammatory proteins in the CSM. Altogether, these responses may be responsible for ethanol-induced ED. Therefore, we aimed to evaluate the contribution of NADPH oxidase-derived reactive oxygen species to ethanol-induced ED, endothelial dysfunction and production of pro-inflammatory and redox-sensitive proteins in the rat CSM. With this purpose, animals were treated with apocynin, which was shown to inhibit NADPH oxidase activation and to prevent ED (Stolk et al., 1994; La Favor et al., 2013; Silva et al., 2013).

2. Material and methods

2.1. Animals and grouping

Male Wistar rats were housed in a room with controlled temperature (25 ± 1 °C) and on a 12 h light/dark cycle with free access to rat chow and filtered water. The rats, initially weighing 250–300 g (60–70 days old), were assigned into four groups: control, ethanol, apocynin and ethanol+ apocynin. Rats from the control and apocynin groups had free access to water. Animals from the ethanol-treated groups were submitted to a two-week period of adaptation in which they received ethanol 5% and 10% (vol./vol.), respectively (Leite et al., 2013; Simplicio et al., 2017). From the 3rd to the 9th week, rats had free access to ethanol 20%. Apocynin (30 mg/kg/day) was administered by gavage (Simplicio et al., 2017). Rats not treated with apocynin received water (vehicle) daily. The described protocol is previously reported to produce ED (Muniz et al., 2015), therefore is used in the present study. The animal experiment was conducted as per the guidelines of Conselho Nacional de Controle de Experimentação Animal (CONCEA, Brazil) after the necessary approval of the Ethics Committee on Animal Use of the University of São Paulo, *Campus* of Ribeirão Preto (#13.1.471.53.9).

2.2. Blood ethanol levels

Blood samples were collected from vena cava in heparinized syringes and transferred to tubes containing sodium fluoride (1 mg/ml). Blood ethanol concentration was measured using a Varian CP3380 gas chromatograph (Varian, CA, USA), equipped with a flame ionization detector as described previously (Gonzaga et al., 2015). The levels of ethanol in blood are expressed as mg/dl.

2.3. In vivo measurements of intracavernosal pressure (ICP)/mean arterial pressure (MAP)

Intracavernosal pressure in response to electrical stimulation of the major pelvic ganglion was assessed as described previously (Carneiro et al., 2010). In brief, rats were anaesthetized with 4% isoflurane in 10% oxygen and the left femoral artery and the right crura were cannulated for continuous monitoring of MAP and ICP, respectively. The major pelvic ganglion was stimulated with bipolar silver electrode and ICP changes were monitored in response to frequency curves (0.2–20 Hz; 1 ms pulses at 6 V). The erectile response was quantified as the ratio between ICP and MAP. The area under the curve (AUC) ratio of the ICP/MAP was recorded performing stimulation at 12 Hz. Three measurements were analyzed for each animal. The erectile response was calculated using the maximum ICP response normalized to MAP at the time of maximum ICP.

2.4. Cavernosal smooth muscle function test

The CSM was isolated and placed in 5-ml organ chamber containing Krebs solution at 37 °C as previously described (Lizarte et al., 2009). The strips of CSM were stretched to a resting tension of 3 mN and allowed to equilibrate for 60 min. CSM strips were pre-contracted with phenylephrine (10 μmol/l) and when the contraction reached a plateau, concentration-response curves for acetylcholine (0.01 nmol/l to 100 nmol/l) or sodium nitroprusside (SNP, 10 nmol/l to 300 μmol/l) were performed. Agonist concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 5.0; GraphPad Software Inc., San Diego, CA, USA). Agonist potencies are expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximal response). Maximum effect elicited by the agonist (E_{max}) values is represented as a percentage change from the phenylephrine-contracted levels for relaxation responses. The amplitude of contraction induced by phenylephrine was not different among the experimental groups.

2.5. Detection of O_2^- generation and hydrogen peroxide (H_2O_2) concentration in the rat CSM

Superoxide anion production in CSM homogenates was measured by lucigenin chemiluminescence using the method described by Leite et al. (2013). Superoxide anion levels are expressed as relative light units (RLU)/mg protein·H_2O_2 concentration (μmol/l/mg protein) was measured using the Amplex Red Hydrogen Peroxide Assay Kit (#A22188, Invitrogen, Carlsbad, CA, USA). Protein concentration in all experiments was quantified by the Lowry protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Measurement of nitrate/nitrite (NOx) concentration in the CSM

The level of stable metabolites of NO, the nitrate/nitrite were measured (nmol/mg protein) using a commercial kit (#780001, Cayman Chemical, Ann Arbor, MI, USA) following manufacturer's protocol.

2.7. Determination of superoxide dismutase (SOD) and catalase activities in the CSM

SOD activity was determined by inhibition of pyrogallol autoxidation as previously described (Gonzaga et al., 2014) and SOD activity is expressed as inhibition %/mg protein. catalase activity was determined as previously described by Gonzaga et al. (2014). One catalase unit (U)