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Ethanol withdrawal increases oxidative stress and reduces nitric oxide bioavailability in the vasculature of rats

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ABSTRACT

We analyzed the effects of ethanol withdrawal on the vascular and systemic renin-angiotensin system (RAS) and vascular oxidative stress. Male Wistar rats were treated with ethanol 3–9% (v/v) for a period of 21 days. Ethanol withdrawal was induced by abrupt discontinuation of the treatment. Experiments were performed 48 h after ethanol discontinuation. Rats from the ethanol withdrawal group showed decreased exploration of the open arms of the elevated-plus maze (EPM) and increased plasma corticosterone levels. Ethanol withdrawal significantly increased systolic blood pressure and plasma angiotensin II (ANG II) levels without an effect on plasma renin activity (PRA), angiotensin converting enzyme (ACE) activity, or plasma angiotensin I (ANG I) levels. No differences in vascular ANG I, ANG II levels, and ACE activity/expression and AT_1 and AT_2 receptor expression were detected among the experimental groups. Plasma osmolality, as well as plasma sodium, potassium, and glucose levels were not affected by ethanol withdrawal. Ethanol withdrawal induced systemic and vascular oxidative stress, as evidenced by increased plasma thiobarbituric acid-reacting substances (TBARS) levels and the vascular generation of superoxide anion. Ethanol withdrawal significantly decreased plasma and vascular nitrate/nitrite levels. Major new findings of the present study are that ethanol withdrawal induces vascular oxidative stress and reduces nitric oxide (NO) levels in the vasculature. Additionally, our study provides novel evidence that ethanol withdrawal does not affect the vascular ANG II generating system while stimulating systemic RAS. These responses could predispose individuals to the development of cardiovascular diseases.

Introduction

Abrupt cessation of regular intake of ethanol in a dependent person causes a withdrawal syndrome (McKeon, Frye, & Delanty, 2008). Ethanol withdrawal syndrome generally begins 6–24 h after the last intake of ethanol, and the signs and symptoms include agitation, tremors, sweating, nausea, vomiting, seizures, hallucinations, insomnia, delirium, anxiety, tachycardia, and hypertension (McKeon et al., 2008). Some studies show that arterial blood pressure is elevated during ethanol withdrawal (Clark & Friedman, 1985; King, Errico, Parsons, & Lovallo, 1991). Despite extensive investigation of withdrawal syndrome-induced hypertension, a clear mechanism for the pressor effects of ethanol withdrawal has not yet been established.

Previous studies have linked the pressor effects of ethanol withdrawal to increased levels of catecholamines, and activation of the hypothalamic-pituitary-adrenal axis and the renin-angiotensin system (RAS) (Bezzegh, Nyuli, & Kovács, 1991; Clark & Friedman, 1985; Potter, Bannan, Saunders, Ingram, & Beevers, 1983). The latter is critically involved in the control of blood pressure and hydromineral homeostasis. Angiotensin II (ANG II), the major bioactive peptide of the RAS, is produced systemically and locally within the vascular wall (Nguyen Dinh Cat & Touyz, 2011). ANG II exerts its diverse actions via two G-protein-coupled receptors named AT_1 and AT_2. The AT_1 receptor mediates most of the main actions of ANG II including sodium retention, aldosterone secretion, and vasoconstriction (Touyz & Schiffrin, 2000). The AT_2 receptor

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tends to counteract AT_1 effects and its activation is associated with antiproliferative, proapoptotic, and vasodilator actions of ANG II (Touyz & Schiffrin, 2000).

Most components of the RAS are expressed in the vasculature, and the vascular system may act independently from the systemic RAS to generate ANG II. The vascular ANG II generating system may be activated even when the systemic RAS is suppressed or normal (Nguyen Dinh Cat & Touyz, 2011). The exact function of the vascular RAS remains elusive, but it may amplify the effects of the systemic RAS, particularly in pathological conditions, such as in hypertension (Nguyen Dinh Cat & Touyz, 2011). Ethanol withdrawal was found to activate the systemic RAS (Bezzegh et al., 1991; Clark & Friedman, 1985; King et al., 1991; Potter et al., 1983), but no information on the effects of ethanol withdrawal in the vascular RAS is available.

ANG II induces reactive oxygen species (ROS) generation in the vasculature via NAD(P)H oxidase activation (Touyz & Schiffrin, 2000). NAD(P)H oxidase is the major source of ROS in the cardiovascular system, and increased ROS generation or bioavailability has been implicated in the progression and/or onset of cardiovascular diseases, including hypertension (Touyz & Schiffrin, 2000). It has been previously described that ethanol withdrawal increases ROS generation and lipid peroxidation in the rat hippocampus and cerebellum (Jung, Rewal, Perez, Wen, & Simpkins, 2004; Jung, Simpkins, Wilson, Downey, & Mallet, 1985). However, whether ethanol withdrawal increases vascular production of ROS remains elusive.

Another aspect that has been related to ethanol withdrawal is the development of symptoms of anxiety (Doremus, Brunell, Varlinskaya, & Spear, 2003). The elevated-plus maze (EPM) is a test of anxiety-related behavior in rats, and it has been employed as a test to evaluate ethanol withdrawal-induced anxiety in rodents (Doremus et al., 2003). Ethanol withdrawal generally decreases the exploratory activity in the EPM, indicating the occurrence of an anxiety-like effect of ethanol withdrawal in rodents (Cabral et al., 2006). Since vascular RAS contributes to the control of blood pressure and vascular oxidative stress (Nguyen Dinh Cat & Touyz, 2011), the aim of the present study was to assess the effects of ethanol withdrawal on vascular oxidative stress and vascular RAS. In the present study, the effectiveness of the model for ethanol withdrawal was assessed by the EPM test and the measurement of corticosterone levels.

Materials and methods

Ethanol administration

Experimental protocols were approved by the animal ethics committee of the University of São Paulo (#11.1.1212.53.55). Male Wistar rats weighting between 200 and 230 g (50 days old) were divided into three groups: Control ($n = 25$): animals received water *ad libitum* for 23 days; Ethanol ($n = 25$): treatment with ethanol was started with a solution of 3% ethanol (v/v) being gradually increased every 3 days to 6% (4th to 6th day) and 9% (7th to 21st day); Ethanol withdrawal ($n = 25$): the animals were treated as described for the ethanol group until the 20th day. Then the ethanol solution (9%) was removed and returned the next day (21st day) for 2 h. After that, the animals received water until the 23rd day, thereby ensuring an abstinence period of 48 h (Padovan, Batistela, Queiroz, & Tirapelli, 2010). A period of 48 h was chosen based on previous studies showing anxiety-like behavior after this period of abstinence to ethanol (Bonassoli, Milani, & de Oliveira, 2011; Cabral et al., 2006). A short period of treatment using a low dose of ethanol was used to avoid any systemic or vascular effects of ethanol. The choice of treatment for 21 days with ethanol (3–9%) was based on pilot experiments conducted in our laboratory.

Determination of plasma ethanol levels

The rats were anesthetized with urethane (1.25 g/kg, intraperitoneally [i.p.], Sigma–Aldrich, St. Louis, MO, USA) and the blood (1 mL) was collected from inferior vena cava using heparinized syringes. Blood samples (100 μL) were transferred into a 20-mL headspace vial containing 500 μL of a 10% aqueous $(NH_4)_2SO_4$ solution, to increase the recovery by salting out effects. Isobutanol (100 mg/dL) was added as an internal standard. The vials were sealed with silicon/PTFE septa and steel caps. Headspace vials containing the samples were placed into the vial rack of the automatic sampler, operating in the headspace mode, and incubated for 15 min at 80 °C under stirring (400 rpm, agitator on-time: 3 s, agitator off-time: 10 s). Ethanol analyses were carried out on a Varian CP3380 gas chromatographer (Varian, CA, USA) equipped with a flame ionization detector and a Carbowax fused silica capillary column (30 m × 0.25 mm internal diameter, film thickness 0.25 μm) (Chrompack, São Paulo, SP, Brazil). The injection mode was splitless for 18 sec. The column temperature was set for 40 °C (4 min) to 220 °C (2 min) at 15 °C/min. The temperatures of the injector and detector were set for 220 and 300 °C, respectively. Nitrogen was used as a carrier gas with linear velocity of 33 cm/s. Calibrations standards were prepared in the same headspace vials (0.02–2.0 mg/mL) using the blood of control animals. Plasma ethanol levels were expressed as mg/dL.

Elevated-plus maze (EPM)

Rats from the three experimental groups were individually placed in the center of the EPM facing a closed arm. The number of entries with the four paws, and time spent in the open or enclosed arms of the EPM, were recorded for 5 min. The percent of open-arm entries (100 × open/total entries) and of time spent in the open arms (100 × open/[open + enclosed]) were calculated for each rat as standard anxiety indices. The total closed arm entries were calculated as a relative pure index of locomotor activity.

Systolic blood pressure measurements

Systolic blood pressure was measured by plethysmography (Plethysmograph EFF-306, Insight, Ribeirão Preto, SP, Brazil). The rats were maintained for 5–10 min in a warm chamber, and three consecutive recordings (~1 min apart) were performed. Results are expressed as mm Hg.

Determination of hormone levels

For measurements of plasma and tissue hormones, the animals were decapitated and blood, aorta, and mesenteric arterial bed (MAB) were collected as previously described (Yogi et al., 2012). For measurements of tissue ANG I and ANG II concentrations, aorta and MAB were homogenized and peptides were extracted onto a bond Elut SPE-Column (Peninsula Laboratories, Inc., Belmont, CA, USA) as described previously (Yogi et al., 2012). Plasma levels of atrial natriuretic peptide (ANP), vasopressin (AVP), ANG I, and ANG II were measured by radioimmunoassay (RIA). The antibodies for AVP, ANG I, and ANG II were obtained from Peninsula Laboratories (AVP: T4561; ANG I: T4166; ANG II: T4007) and the ANP antibody was kindly donated by Jolanta Gutkowska (University of Montreal, Canada). Plasma protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, CA, USA). For measurements of plasma corticosterone, a specific RIA was used after extraction with ethanol as described previously (Mecawi et al., 2013).

The levels of AVP, ANP, corticosterone, and ANG I and II were determined by RIA (Botelho, Block, Khosla, & Santos, 1994;