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Acute treatment with alcohol affects calcium signaling and contraction associated with apoptosis in vas deferens of periadolescent rats

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ABSTRACT

Our purpose was to verify if alcohol causes alterations on translocation of Ca^{2+} and tension induced by KCl or noradrenaline in vas deferens of periadolescent Wistar rats. A single dose of alcohol (i.p. 3.0 g/kg) or saline as control, was given 4 h before sacrifice. Longitudinal strips of prostatic portion were mounted *in vitro* for simultaneous measurements of intracellular Ca^{2+} and contractions. Fluorescence and tension were measured in strips loaded with the fluorescent dye fura-2. The mean values (±S.E.M.) of fluorescence ratios ($F_{340/380}$) evoked by KCl were significantly lower by about 70% after alcohol, in relation to control. It was about 50% lower when evoked by noradrenaline. In relation to tension, the respective mean values (±S.E.M.) were lower by about 60% in organs treated with KCl or by about 80% after noradrenaline. In some experiments, before noradrenaline contraction, the vas deferens was incubated with verapamil 10^{-6} M for 30 min. In these experiments, contractions by noradrenaline in the presence of verapamil were decreased by about 70% by alcohol. Alcohol decreases cytosolic calcium and contractility after KCl and noradrenaline, as compared with controls. In addition, alcohol promoted damage of lumen structures. Prostatic portion showed no striking morphometric change after treatment, but the number of TUNEL positive cells in muscular layer, basal lamina and lumen were increased by alcohol, indicating apoptosis, compared with controls. This investigation shows that alcohol treatment alters signaling of calcium which in turn compromises the contraction associated with a process of apoptosis of periadolescent rats.

1. Introduction

In the periphery, evidences show that acute or chronic treatment with ethanol alters the function of peripheral noradrenergic system in various tissues (Pohorecky, 1982). Historically, ethanol has been considered exerting effects through effects on biological membranes on its fluidity, but the mechanism of action of this agent is incompletely understood (Altura and Altura, 1982; Rubin, 1982). It was suggested that ethanol is involved in attenuation of smooth muscle contraction (Boselli and Govoni, 2000; DeTurcky and Pohorecky, 1986; Keshavarzian et al.,1994; Kesim et al., 2004; Sahna et al., 2000; Turlapaty et al.,1979). In this context, Ca^{2+} is a major determinant of smooth muscle contractility (Jurkiewicz et al., 1975, 1994; Lopes et al., 2006). Since calcium is essential for the contractile process, a possible mechanism for this depression is an inhibition of calcium entry into cell. In addition, the source for this ion can be the various intracellular Ca^{2+} storage compartments, or the extracellular medium, by Ca^{2+} entrance through different channel types as receptor-operated channels (ROCs), or voltage-operated channels (VOCs) (Jurkiewicz et al., 1975; Lopes et al., 2006).

Periadolescence animals (postnatal day 20–60; Spear, 2000) show vulnerability of acute effects of alcohol on spatial working memory (Markwiese et al., 1998). In humans, these periods are important, during the time in which youngsters first experiment with alcohol (Spear, 2000). While adults typically drink more frequently, adolescents consume more than adults, engaging in heavy episodic or "binge" drinking (Substance Abuse and Mental Health Services Administration, 2003), defined as consuming 5 or more drinks. Given the use of alcohol during periadolescence, it is of great interest to study rats injected by only high doses of alcohol. For this, we used vas deferens that has been extensively used for diverse studies of different aspects of autonomic neurotransmission (Jurkiewicz et al., 1977; Burnstock and Verkhratsky, 2010).

Studies have shown that ethanol (2–3 g/kg) affects the contractility of prostatic or epididimal portions of rat vas deferens (Boselli and Govoni, 2000, 2001; DeTurcky and Pohorecky, 1986) and seems to cause dysfunction of male reproductive tract (Boselli

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and Govoni, 2000; Dixit et al.,1976; Freeman and Coffey, 1973; Johnson, 1972).

We have shown in our laboratory that ethanol 1.2 g/kg (four h before gavage experiments) in 30–45 days old rats depressed neurogenic and adrenergic contractile responses in rat vas deferens (Silva-Júnior et al., 2012). Now, we investigated the influence of acute treatment with alcohol in $[Ca^{2+}]_i$ signaling, and contraction in vas deferens of periadolescent rats, as well as apoptosis and other morphological parameters after using alcohol.

2. Material and methods

2.1. Animals and treatment

Male wistar rats (40–45 day old) from our BAW-2 colony, were treated with a single dose of alcohol (i.p. 3.0 g/kg) 4 h before sacrifice. Absolute ethanol was diluted to 30% *w/v* solutions in physiological saline (0.9%) and given i.p. in a volume of 10 ml/kg. Controls received intraperitoneal injection of saline. The animals were sacrificed with an overdose of ethyl ether and vasa deferentia were removed for simultaneous measurements of fluorescence and contraction, for morphometric analysis or TUNEL experiments.

All protocols were approved by the Ethical committee of the Federal University of São Paulo.

2.2. Simultaneous measurements of fluorescence and contraction

Fluorescence and tension experiments were made according to Lopes et al., 2006 and modificated for the vas deferens. The system containing a transducer and a fluorimeter coupled to a computer system allowed simultaneous measurements of dual wavelength fluorimetry and tension. Recordings of isometric contractions were obtained with a force-displacement transducer.

Strips (about 1.0 × 0.1 cm) of prostatic segments of vasa deferencia were mounted vertically in quartz cuvettes and fixed in isometric force transducers. The resting tension was adjusted to 1 g and the tissue perfused with 2.5 ml of Tyrode composed of (mM): 138.0 NaCl; 5.7 KCl; 1.8 $CaCl_2$; 15.0 $NaHCO_3$; 0.36 NaH_2PO_4 and 5.5 glucose, in distilled water (Verde et al., 2002) (pH=7.4), at 37 °C, bubbled with a mixture of 95% O_2 and CO_2, with addition In some experiments of a calcium-free solution with 1 μM EGTA.

Simultaneous registration of isometric tension and fluorescence were recorded. Fura-2 was excited ratiometrically at wavelengths of 340 and 380 nm and detection was measured at 510 nm. After 15 min washing, the buffer was replaced with a Tyrode solution containing fura-2/AM (10 μM) and non-cytotoxic detergent Pluoronic F-127 (0.08%) for 3 h at room temperature (Grynkiewicz et al., 1985). After loading with Fura-2, the muscle strip was washed with fresh Tyrode solution at 37 °C. Noradrenaline 10^{-4} M (submaximal dose) or KCl-induced fluorescence and contractions were recorded for 300 s. At the end of experiments, the Fura-2—Ca^{2+} signal was calibrated, for all experiments and measurements of the tissue autofluorescence were made. The maximal ratio (R_{max}) was measured in Ca^{2+} saturating medium by addition of digitonin (0.1 mM). To be sure that the registered signals came really from the fluorescence of the complex Fura-2—Ca^{2+}, 2 mM $MnCl_2$ was added and fura-2 fluorescent signal was quenched. Finally, 4 mM EGTA was added and the minimum ratio (R_{min}) was obtained. The changes in fluorescence (ΔF) were calculated by subtracting basal fluorescence before stimulation (F_0) from the intensity of fluorescence recorded at a given time (F) after stimulation [$\Delta F=F-F_0$]. Changes of tension values were expressed in $\Delta T=(T-T_0)$. Fluorescence and tension intensities were plotted in relation to the average of baseline values and measured after the initial 100 min of the stimulation with drug [$\Delta F=F-F_0$ or $\Delta T=T-T_0$, respectively]. The results were presented as the mean ± S.E.M.

To investigate the involvement of influx of Ca^{2+} through calcium channels, some experiments were performed in the presence of verapamil (10^{-6} M), a VOCs antagonist that was added to incubation medium 30 min before the beginning of the experiments in controls and alcohol-treated group. Verapamil was diluted in distillated water.

2.3. Histological preparation of vas deferens

Prostatic segment of vas deferens was fixed in Bouin liquid for 24 h, dehydrated in ethanol, cleared in Xilol, infiltrated and embedded in paraffin-wax. Microtome-cut of transversal sections (4 μm) were placed onto glass slides and silane- treated glass slides and stained with haemotoxylin and eosin (H&E) and Picrosirius red stains or analyzed by the terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) experiments (see details bellow).

2.4. Morphometric analysis

Prostatic sections (4 μm) were stained with H&E and Picrosirius red. The images of histological sections of prostatic portion of controls and alcohol—treated were captured with a video camera Olympus Q-Color 3, America, inc., coupled with research binocular microscope Olympus BX-40 under 100 × magnification. Photomicrographs were obtained from Q Capture Pro 6.0 software and transmitted to a microcomputer for measurements through morphometry software (Image Tool for Windows VS. 3.0-University of Texas). The thickness of muscular layer, diameters of lumen and organ, were calculated by the average of four measurements on four separate quadrants for each parameter assessed. The mean of muscular area was obtained through the difference between external and inner areas (longitudinal external–internal longitudinal muscle area). To measure the total area, we used the outer circumference in micrometers.

The sections stained with Picrosirius were submitted to polarized collagen to quantify the collagen in digitalized images (prostatic portion). The collagen was analyzed by software image (Image Tool for Windows, Texas, USA) and values were expressed as mean gray levels as pixels image. Digital images translated into numerical values, describe staining intensity as a numeric variable, which is more precise than visual qualitative observation. The images were captured in the same microscopic system and enlargement illumination, by the same individual groups (blinded analysis at the same time). The image was converted into gray levels and the darkest signal (black) was expressed as 256 and the lightest (white) was expressed as 0 in the 256 Gy levels scale. This tool compares the gray level intensity expressing a strongly stained structure with a high numerical gray level. Seven slides of prostatic portion of vas deferens of each group (controls and alcohol treated group) were analyzed.

2.5. TUNEL method

Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) experiments, based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3′-OH ends of DNA (Gavrieli et al., 1992), were performed with the Apop Tag plus Kit (Intergen Discovery products, USA). Prostatic sections were fixed in 4% formaldehyde buffered at pH 7.2 with 0.1 M sodium phosphate at room temperature. After dehydration, the specimens were embedded in paraffin and 4-Am sections were attached to silanized slides. Deparaffinized sections were washed in PBS (50 mM sodium phosphate, pH 7.4, 200 mM NaCl) and pre treated with 20 μg/ml proteinase K (Sigma-Protein Digesting Enzyme) for 15 min at room temperature. Sections were washed in distilled

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