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Ethanol modulates the synthesis and catabolism of retinoic acid in the rat prostate

Beatriz Aparecida Fioruci-Fontanelli[a,b], Luiz Gustavo A. Chuffa[a,*], Leonardo O. Mendes[a,b], Patricia Fernanda F. Pinheiro[a], Luis Antônio Justulin Jr.[c], Sérgio Luis Felisbino[c], Francisco Eduardo Martinez[a]

[a] Department of Anatomy, Institute of Biosciences, UNESP—Univ Estadual Paulista, Botucatu, SP, Brazil
[b] Structural and Cell Biology Program, UNICAMP, Campinas, SP, Brazil
[c] Department of Morphology, Institute of Biosciences, UNESP—Univ Estadual Paulista, Botucatu, SP, Brazil

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ABSTRACT

All-trans retinoic acid (atRA) maintains physiological stability of the prostate, and we reported that ethanol intake increases atRA in the rat prostate; however the mechanisms underlying these changes are unknown. We evaluated the impact of a low- and high-dose ethanol intake (UChA and UChB strains) on atRA metabolism in the dorsal and lateral prostate. Aldehyde dehydrogenase (ALDH) subtype 1A3 was increased in the dorsal prostate of UChA animals while ALDH1A1 and ALDH1A2 decreased in the lateral prostate. In UChB animals, ALDH1A1, ALDH1A2, and ALDH1A3 increased in the dorsal prostate, and ALDH1A3 decreased in the lateral prostate. atRA levels increased with the low activity of CYP2E1 and decreased with high CYP26 activity in the UChB dorsal prostate. Conversely, atRA was found to decrease when the activity of total CYP was increased in the UChA lateral prostate. Ethanol modulates the synthesis and catabolism of atRA in the prostate in a concentration-dependent manner.

1. Introduction

Retinoic acid (RA), an active metabolite of retinol (vitamin A), is responsible for the maintenance of several biological functions in vertebrates, including reproduction [1]. RA acts through four distinct isomers: *all-trans*-RA (*at*RA), 9-*cis*-RA, 13-*cis*-RA, and 9,13-*dicis*-RA. Among these, *at*RA is the most abundant and is considered to be the biologically active isomer [2]. Because abnormal *at*RA shares pathological conditions with alcoholism, retinol activation into *at*RA has been focused widely as a mechanism of ethanol toxicity [3].

In the prostate, *at*RA controls development of prostatic buds, differentiation of epithelial cells, and contributes to the maintenance of tissue homeostasis [4,5]. In conjunction with members of the steroid hormone receptor superfamily, *at*RA acts as a transcription factor, consequently regulating cell differentiation, apoptosis, and proliferation [6]. Recently, *atRA* has been pointed out to induce proteins that sensitize tumors to drug combinations [7]. Although mechanistic studies of retinoid signaling continue to suggest novel drug targets for therapies, only few reports on *at*RA regulation in the prostate are available.

The concentration of *at*RA is regulated by enzymes responsible for its synthesis and catabolism [8]. The synthesis of *at*RA occurs by the irreversible oxidation of retinal, a derivative of retinol oxidation, by aldehyde dehydrogenase (ALDH), also known as retinaldehyde dehydrogenase [9]. The proteins of ALDH1A family (ALDH1A1, ALDH1A2, and ALDH1A3) are responsible for *at*RA synthesis [10,11]. On the other hand, cytochrome P450 enzyme, family 26, breaks down RA in the tissues and reduces its levels [9]. The CYP26 family comprises three isoforms: CYP26A1, CYP26B1, and CYP26C1 [12]. CYP26A1 and CYP26B1 enzymes metabolize the *at*RA isomer [13], whereas CYP26C1 appears to prefer 9-*cis*-RA as a substrate [14]. Additionally, CYP2E1 enzyme is involved mainly in the catabolism of ethanol, but also participates in the excretion of RA upon induction by ethanol [15,16].

Ethanol has been shown to interfere with RA metabolism and change its concentration in tissues differentially [17,18]. In the liver, ethanol decreases the *at*RA levels [19], while increasing it in the hippocampus, testis, and serum [3]. In the prostate, only a high dose of chronic ethanol ingestion increases *at*RA, while a lower dose of ethanol does not [20]. Importantly, changes in the *at*RA pathway and *at*RA levels may contribute to the development of

* Corresponding author at: Department of Anatomy, Institute of Bioscience, UNESP—Univ Estadual Paulista, P.O. Box 510, Postal Code: 18618-970, Rubião Júnior, s/n, Botucatu, SP, Brazil. Tel.: +55 14 3880 0027; fax: +55 14 3811 6361.
E-mail address: chuffa@ibb.unesp.br (L.G.A. Chuffa).

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benign prostatic hyperplasia (BPH) and prostate cancer in humans [21,22]. Therefore, the knowledge of how ethanol modulates the ALDHs and CYPs related to *at*RA metabolism in the prostate is important and can yield new insights into damage caused by ethanol and prostate disease.

The present study was aimed to evaluate the effects of both high and low doses of ethanol intake upon the proteins responsible for synthesis and catabolism of *at*RA in the dorsal and lateral lobes of the UCh rat prostate.

2. Materials and methods

2.1. Animals, groups, and diet

Forty UCh (University of Chile) male rats, weighing between 240 and 280 g (~60 days old), were obtained from the Department of Anatomy, Bioscience Institute, Campus of Botucatu, UNESP—Universidade Estadual Paulista (IBB/UNESP). The UCh strain is an ethanol-preferring rat that voluntarily consume ethanol [23]. There are two UCh rat strains, UChA and UChB, displaying low ethanol consumption (0–2 g/kg/day) and high ethanol consumption (>2 g/kg/day), respectively [24]. When the animals reached 60 days of age, they were given, a choice between two bottles containing either water *ad libitum* or 10% (v/v) ethanol solution [25,26]. In this study, the animals were divided into four sub-groups (n = 10/group): UChA: low-dose ethanol; UChAC: control group; UChB: high-dose ethanol; UChBC: control group.

The ethanol ingestion was measured weekly throughout the experimental period (60 days) using a marked test tube. All rats were housed in individual cages in a temperature- and humidity-controlled room under a 12-h light:12-h dark cycle, had free access to filtered tap water, and were fed with standard rodent chow Nuvital® (Nuvilab CR-1). At 120 days old, the animals were euthanized in CO_2 chamber. The experimental protocol was approved by the Ethical Committee of the Institute of Bioscience, Campus of Botucatu, SP, Brazil (IBB/UNESP, Protocol no. 340/2011).

2.2. Protein extraction and Western blot analysis

After 60 days of ethanol consumption, the dorsal and lateral lobes of the prostate of UChA and UChB rats were collected and immediately frozen in liquid nitrogen and stored at −80 °C. Extraction of proteins and Western blot were performed using methods described previously [27,28]. Antibodies against ALDH1A1 (1:2000), ALDH1A2 (1:250), and ALDH1A3 (1:250) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-CYP2E1 (1:250), anti-CYP26A1 (1:500), and anti-CYP26B1 (1:500) were purchased from Abcam (Inc., Cambridge, MA, USA). After incubation with primary antibody, the membranes were incubated for 2 h at RT with rabbit or mouse HRP-conjugated secondary antibodies (diluted 1:1000 in 1% BSA; Sigma, St. Louis, MO, USA). Blots were revealed using ECL Western Blotting system (Amersham) and analyzed with a densitometer (G-BOX calibrated imaging densitometer, Syngen). β-Actin was used as an endogenous control and all results are expressed as mean ± SEM. Immunoblotting concentrations were represented as optical density (IOD) values (bands intensity mean/β-actin ratio).

2.3. Immunohistochemistry

Dorsal and lateral prostate tissue sections (4-μm thick), paraffin embedded, of the 5 samples/group were deparaffinized and treated in a water bath for 30 min, at 95 °C, in Tris/EDTA + Tween 20 (pH 9.0) for antigen recovery. After cooling, the sections were incubated with monoclonal antibodies: anti-ALDH1A1 (1:100), anti-CYP26A1 (1:100), anti-CYP26B1 (1:100), and anti-CYP2E1 (1:70) (Abcam, Inc., Cambridge, MA, USA), at 4 °C overnight, subsequently rinsed in PBS and incubated with anti-rabbit HRP-conjugated secondary antibody (Sigma, St. Louis, MO, USA) for 1 h. The color reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co.). The reaction was stopped in distilled water, and sections were counterstained with hematoxylin. The sections were analyzed and photographed using an Olympus (BX-41) microscope, equipped with a DP-12 digital camera (Olympus, Inc., Japan).

2.4. Immunofluorescence

Frozen sections of dorsal and lateral lobes of rat prostate were mounted on precooled chucks (−20 °C) in a Leica cryostat, and 8-μm sections were thaw-mounted on silane-coated glass slides. For fixing and permeabilizing the tissue, the sections were placed in an ice-cold acetone/methanol solution (2:1, v/v), for 10 min. After the sections were washed with TBS buffer (pH 7.4) 3 times (5 min per wash), and blocked using 5% nonfat milk for 1 h. The sections were incubated with monoclonal antibodies anti-ALDH1A2 and anti-ALDH1A3 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and were placed in a moist chamber at 4 °C overnight. The fluorescein isothiocyanate (FITC)-labeled anti-goat secondary antibody (Sigma, St. Louis, MO, USA) was used against the ALDH1A2 and ALDH1A3 antibodies. The slides were mounted with Vectashield containing 4′,6-diamido-2-phenylindole hydrochloride (DAPI, Vector Laboratories, Inc.), to visualize nuclei. Photographs of slides were taken using a laser scanning confocal microscope (Leica, Inc., Wetzlar, Germany).

2.5. Subcellular fractions from prostate tissue

Initially, dorsal and lateral prostates were homogenized in 500 μl of buffer A (Tris–HCl (10 mM), sacarose 10% (w/w), EDTA (1 mM), DTT (1.5 mM), pH 7.4), using a tissue homogenizer (Ika, T 10 basic Ultra-Turrax, Staufen, Germany). The homogenates were centrifuged (4 °C, 14,000 rpm, 20 min), and the supernatants were pipetted, and subjected to differential centrifugation to isolate the microsomal fraction, according to the method described by Kane et al. [3]. After differential centrifugation (4 °C, 100,000 × g, 1 h), supernatants were removed (cytosol) and the pellet (microsomes) was resuspended and hand-homogenized in buffer A. The total protein concentration was determined using the Bradford assay.

2.6. Microsomal activity

Assessment of activity of P450 enzymes of the microsomal fraction (CYP26A1, CYP26B1 and CYP2E1), was performed in triplicate with or without CYP enzyme inhibitors. Initially, the buffer (50 mM Tris–HCl, 150 mM KCl, 5 mM $MgCl_2$, pH 7.4), with NADPH included, was placed in glass tubes at a total volume of 0.5 ml and agitated at 65 rpm in a water bath at 37 °C for 3 min. The reaction was started by adding 5 μl of substrate (2 nmol of *at*RA dissolved in DMSO) in samples containing prostatic microsomes (200 μg protein) and incubated at 37 °C in a shaking water bath (65 rpm) for 15 min. Before incubation with the substrate (*at*RA), to evaluate the effects of ethanol consumption on the catabolism of the CYP (CYP26A1, CYP26B1 and CYP2E1), the samples were preincubated for 10 min with specific inhibitors of CYP, with some adjustments to the method described in Liu et al. [16]. The specific inhibitors of CYP used were chlormethiazole (specific inhibitor of CYP2E1), α-naphthoflavone (of CYP1A2), troleandomycin (of the CYP3A family), and liarozole (of the CYP26 family), followed by incubation with *at*RA for 15 min [16].

After incubation for 15 min, *at*RA was extracted from the samples, quantified by LC-tandem mass spectrometry (MS/MS) and analyzed in the Laboratory for analysis of retinoids at the Department of Nutritional Science and Toxicology, University of California-Berkeley, USA.