Life Sciences 88 (2011) 719–724

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Age-dependent effect of ouabain on renal Na^+,K^+-ATPase

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ARTICLE INFO

Article history:
Received 25 June 2010
Accepted 2 February 2011

Keywords:
Na^+,K^+-ATPase
Hypertension
Cardiotonic steroids
Ouabain
Kidney

ABSTRACT

Aims: This study examines the effect of chronic ouabain-treatment on renal Na^+ handling in 12-week and 52-week old rats.
Main methods: Wistar Kyoto rats aged 5 weeks or 45 weeks were treated with ouabain or vehicle during 7 weeks. Blood pressure was measured in conscious animals throughout the study. After 7 weeks of treatment urinary electrolyte concentration, Na^+,K^+-ATPase activity and α_1-subunit expression were determined in 12-week and 52-week old rats.
Key findings: In 12-week and 52-week old rats ouabain produced a significant increase in systolic blood pressure. Although no differences were observed in Na^+ excretion in these animals, 12-week old ouabain-treated rats had lower Na^+,K^+-ATPase activity in proximal tubules. However, 12-week old ouabain-treated rats had decreased fractional excretion of Na^+. In proximal tubules of 52-week old rats Na^+,K^+-ATPase activity did not differ between vehicle and ouabain-treated groups.
Significance: Our results show that in Wistar Kyoto rats renal response to ouabain treatment may be age-dependent and that the hypertensive effect of ouabain is independent of the effect on renal Na^+,K^+-ATPase.

Introduction

Na^+,K^+-ATPase is an oligomeric transmembrane protein responsible for the active transport of Na^+ and K^+ across the plasma membrane. In the kidney it is located in the basolateral membrane, providing the driving force for water and Na^+ transport (Kaplan, 2002; Lingrel and Kuntzweiler, 1994; Skou, 1957). The α-subunit of Na^+,K^+-ATPase is the catalytic domain of the enzyme and contains the binding site for Na^+, K^+, ATP and cardiotonic steroids, which are specific inhibitors of the enzyme. Several endogenous cardiotonic steroids such as ouabain, digoxin and marinobufagenin, have been identified (Nesher, et al., 2007; Schoner and Scheiner-Bobis, 2007a,b). Endogenous ouabain and marinobufagenin seem to play a role in the elevation of blood pressure in contrast to digoxin which counteracts the hypertensinogenic effect of ouabain (Hamlyn, et al., 1982; Nesher, et al., 2007; Schoner and Scheiner-Bobis, 2007b).

When chronically administered to normotensive rats, exogenous ouabain induces moderate but sustained hypertension (Huang, et al., 1994; Kimura, et al., 2000; Manunta, et al., 1994; Rossoni, et al., 2002b; Xavier, et al., 2004). In this model of induced hypertension increased sympathetic tone, activation of the brain renin–angiotensin and endothelin systems has been demonstrated (Huang, et al., 2001; Zhang and Leenen, 2001). Although much work has been done in an attempt to clarify the network of pathogenic mechanisms activated by ouabain that are responsible for the development of hypertension not much is known at the kidney level.

Studies performed in vivo suggest a natriuretic role for endogenous Na^+,K^+-ATPase inhibitors. Natriuretic response to an acute salt load was augmented in mice in which the α_1-subunit of Na^+,K^+-ATPase was conferred ouabain sensitivity (Loreaux, et al., 2008). Moreover, treatment with anti-digoxin antibody fragment lowered Na^+ excretion in these mice. However, it has been reported that Na^+,K^+-ATPase activity is enhanced in renal cell lines chronically treated with ouabain (Ferrari, et al., 1998; Zhang, et al., 2010). Previous work from our group demonstrated that renal cells chronically treated with ouabain had increased Na^+,K^+-ATPase activity only in cells with increased oxidative stress levels and alterations in the cytoskeleton (Silva and Soares-da-Silva, 2009).

In view of this and in line with our previous findings it is possible that the mechanisms activated by ouabain treatment might be altered with age. Thus, in the present work we evaluated the role of ouabain in the regulation of renal Na^+ transport in an in vivo model using for this purpose 5-week and 45-week old Wistar Kyoto rats (WKY) chronically treated with ouabain.

Materials and methods

Animal preparation and experimental design

Three-week old male WKY rats were obtained from Harlan-Interfauna Ibérica (Barcelona, Spain). This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National

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0024-3205/$ – see front matter
doi:10.1016/j.lfs.2011.02.012

Institutes of Health (NIH Publication no. 85-23, revised 1996) and the experiments were performed according to the Portuguese law on animal welfare and according to the guidelines issued by Federation of European Laboratory Animal Science Associations (FELASA). Rats were kept under controlled conditions (12-hour light/dark cycle and room temperature at 22±2 °C) and had free access to tap water and standard rat chow (PANLAB, Barcelona, Spain). At 5-weeks of age rats were sub-divided into two groups and one controlled time release pellet (Innovative Research of America, Saracota, FL, USA) containing ouabain (0.5 mg/pellet) or vehicle was implanted subcutaneously, as previously described (Rossoni, et al., 2002b). At 45-weeks of age rats were subdivided into two groups and as they weighed the double of 5-week old rats two controlled time release pellets (Innovative Research of America) containing ouabain (0.5 mg/pellet) or vehicle were implanted subcutaneously. These pellets were designed to release a constant amount of ouabain (8.3 μg/day) or vehicle per pellet for a 60-day period. The length of treatment was 7 weeks. Blood pressure was measured in conscious animals using a photoelectric tail-cuff detector (LE 5000, Letica, Barcelona, Spain). A minimum of 5 consecutive measurements were made each time and the mean value was used for further calculations.

Metabolic study

Fourty-eight hours before the experiments, all groups were placed in metabolic cages (Tecniplast, Buguggiate-VA, Italy) for 24 hour urine collection and monitoring of food and water intake. After completion of this protocol, rats were anesthetized with sodium pentobarbital (60 mg kg^{-1}) and the kidneys were rapidly removed through an abdominal midline incision, rinsed free of blood with normal saline (0.9% NaCl), weighed, decapsuled and cut in half. One kidney was placed in ice-cold saline for measurement of Na^+,K^+-ATPase and the other was stored at −80 °C for further experiments.

Urinary Na^+ and K^+

The quantification of Na^+ and K^+ in urine was performed by an ion-selective electrode in a Cobas Mira Plus analyzer (ABX Diagnostics, Geneva, Switzerland).

Isolation of epithelial cells

The isolation of proximal tubules was performed as previously described (Soares-da-Silva, et al., 1994). In brief, the outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste. The cortical paste was filtered sequentially through a series of Nybolt nylon sieves, first 180 μm and then 75 μm. Undissociated cortex remained on the upper (180 μm) sieve, while the lower one (75 μm) retained predominantly proximal nephron segments. The sieves were continuously rinsed with cold Hanks' solution. The retained tubules were then washed with cold Hanks' solution and collected into a pellet by centrifugation at 200 *g*, 5 min at 4 °C; renal tubules used in incubation experiments were suspended in Hanks' solution.

Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity in isolated renal proximal tubules was measured as previously described (Quigley and Gotterer, 1969) and adapted in our laboratory with slight modifications. Briefly, isolated renal proximal tubules obtained as described above were pre-incubated for 20 min at 37 °C. After de pre-incubation period the cells were permeabilized by rapid freezing in dry ice-acetone and thawing. The reaction mixture (pH 7.4) contained (in mM) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium-EDTA, 5 $MgCl_2$, 6 NaN_3, 75 tris(hydroxylmethyl) aminomethane(tris) hydrochloride and 100 μl cell suspension (100 μg protein). The reaction was initiated by the addition of 4 mM ATP (25 μl). For determination of ouabain-sensitive ATPase, NaCl and KCl were omitted, and 100 μl of ouabain (final concentration 1 mM) or vehicle (water) was added to the assay. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 50 μl of ice-cold trichloroacetic acid. Samples were centrifuged (1500 *g*), and liberated Pi (free phosphorus) in the supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nanomoles Pi per mg protein per minute and determined as the difference between total and ouabain-insensitive ATPase. The protein content was determined using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with human serum albumin as standard.

Western blot

Isolated renal cortex was sliced very thinly and lysed by the addition of RIPA buffer [150 mM NaCl, 50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1% NP-40 (IGEPAL), and 0.25% sodium deoxycholate] containing protease inhibitors (1 mM PMSF, 1 μg/ml leupeptine and 1 μg/ml aprotinin) and phosphatase inhibitors (1 mM Na_3VO_4 and 1 mM NaF). Lysed tissue was briefly sonicated, incubated on ice for 1 h and centrifuged (13,000 *g* for 45 min). Proteins were mixed with 6× Laemmeli buffer (0.35 M Tris–HCl, 4% SDS, 30% glycerol, 9.3% DTT, pH 6.8, and 0.01% bromophenol blue) and warmed at 37 °C for 15 min. Equal amounts of total protein were separated on a 7.5% SDS-polyacrylamide gel and electrotransfered to a nitrocellulose membrane in Tris–Glycine transfer buffer containing 20% methanol. Membranes were blocked in 3% non-fat dry milk in phosphate buffered saline (PBS) for 1 h and then incubated overnight at 4 °C with mouse monoclonal anti-α_1-Na^+,K^+-ATPase subunit (Santa Cruz Biotechnology, Inc., CA, USA). For the assay of β-actin abundance, membranes were blocked in 3% non-fat dry milk in PBS overnight and then incubated for 1 h with anti-β-actin primary antibody (Santa Cruz Biotechnology, Inc.). The immunoblots were subsequently washed, incubated with 0.5 μg/ml of fluorescently labeled goat anti-mouse secondary antibody (AlexaFlour 680, Molecular Probes, Inc., Eugene, OR, USA) for 1 h at room temperature and protected from light. Membranes were washed and imaged by scanning at 700 nm with Odyssey Infrared System (LI-COR Biosciences, Lincoln, NE, USA).

α_1-Na^+,K^+-ATPase immunoprecipitation

Isolated renal cortex was sliced very thinly and homogenized with a Polytron® (Kinematica AG, Littau-Lucerne, Switzerland) in RIPA buffer. Lysed tissue was incubated on ice for 1 h and centrifuged (13,000 *g* for 45 min). An aliquot of the supernatant (1 mg/ml of total cellular protein) was incubated with 2 μg of mouse monoclonal anti-α_1-Na^+,K^+-ATPase subunit (Santa Cruz Biotechnology, Inc.) for 1 h at 4 °C. Antigen–antibody complex was precipitated using 30 μl of Protein A/G-PLUS-Agarose (Santa Cruz Biotechnology, Inc.) overnight at 4 °C under rotary agitation. Immunoprecipitates were collected by centrifugation (1000 *g* for 5 min) at 4 °C and pellet was washed 4 times with 1 ml RIPA buffer. The antigen–antibody complex was dissociated with 30 μl of 2× Laemmeli buffer (125 mM Tris–HCl, 4% SDS, 5% β-mercaptoethanol, and 20% glycerol) by incubating at 37 °C for 1 h. The immunoprecipitated samples (15 μl) were detected by western blot following the same procedure described above except that the mouse monoclonal phosphoserine antibody (Sigma-Aldrich, St. Louis, MO, USA) was used as the primary antibody and PBS was replaced by tris-buffered saline.

Data analysis

Arithmetic means are given with standard error mean (SEM). Statistical analysis was performed by Student's *t*-test or by 2-way ANOVA followed by Bonferroni post-test. A *P*-value less than 0.05 was assumed to denote a significant difference.