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Spironolactone Attenuates Experimental Uremic Cardiomyopathy by Antagonizing Marinobufagenin

by

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Short Title: Spironolactone attenuates signaling via Na pump.

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Expanded Methods:

Animals: Male Sprague-Dawley rats were used for all of the studies. All of the animal experimentation described in the manuscript was conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals using protocols approved by the University of Toledo, Medical Health Campus, Institutional Animal Use and Care Committee (IACUC).

Experimental Groups: Rats weighing between 250 and 300 grams were divided into 6 groups. Pentobarbital (50 mg/kg IP) was used for surgeries or cardiac physiological measurements (vida infra). The first group was subjected to sham surgery (SS) while the second group was subjected to 5/6th nephrectomy as previously described [1]. In the third group, SS was performed and a minipump (model 2004, Alzet, Palo Alto, CA) infusing spironolactone (Sigma-Aldrich, St. Louis, MO) at 20 mg/kg per day was inserted subcutaneously through a flank incision [2]. The fourth group was subjected to a partial nephrectomy (PNx) with a minipump infusing spironolactone at 20 mg/kg per day. The fifth group was subjected to SS with a minipump infusing MBG at 10 μg/kg per day whereas the sixth group was subjected to SS with minipumps infusing MBG at 10 μg/kg per day and spironolactone at 20 mg/kg per day.

Both before and after surgeries, animals were fed a standard rat chow (Rodent Laboratory Chow 5001, PMI Nutrition International Inc., Brentwood, MO) which contained sodium (amount), potassium (amount). The rat chow also was (amount) protein, (amount) carbohydrate and fat.

Blood Pressure: After surgery, the rats were allowed to recover for 4 weeks with easy access to chow mix food (Rodent Laboratory Chow 5001, PMI Nutrition International Inc., Brentwood, MO) and water. Conscious blood pressure (BP) was measured by the tail cuff method with equipment made by IITC, Inc. (Amplifier model 229, Monitor model 31, Test chamber Model 306; IITC Life Science, Woodland Hills, CA) as previously described [1, 3]. At the end of 4 weeks following surgery, animals were euthanized, their hearts were weighed, and cardiac tissue was prepared for histology and biochemistry as previously described [1, 3, 4].

Cardiac Physiological Measurements: Some anesthetized rats were instrumented with a Millar 2.0-Fr catheter placed into the carotid artery. Left ventricular pressure and volume were recorded under steady state conditions as well as during inferior vena cava constriction. These data were analyzed using the PVAN 3.5 software as previously reported [3].

MBG Purity: MBG was isolated from Bufo Marinus venom as described previously [5]. The isolated MBG was >99% pure based on high-performance liquid chromatography and mass spectroscopy analysis.

MBG Measurement: Plasma MBG concentration was determined using a competitive enzyme-linked immunosorbent assay (ELISA) as described before [1, 6]. Briefly, the plasma samples (0.5 ml) were extracted on Sep-Pak C-18 columns. The combination of 20% and 80% acetonitril elutes was lyophilized and re-suspended in TBS buffer (Tris 50 mM, NaCl 150 mM, NaN_3 7.7 mM, pH 7.4). 100 μl of MBG standards or samples were mixed with 100 μl anti-MBG monoclonal antibody. The mixture was then added to MBG-thyroglobulin-coated and 1% BSA-blocked ELISA plate. After 1 h incubation, plates were washed and secondary anti-mouse antibody was added. A fluorescent signal

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amplifier, FDP Alkaline Phosphatase, from ANASpec (San Jose, CA) was used to detect the signals after washing out the secondary antibody.

Aldosterone Measurement: Plasma samples were first diluted 10 times and aldosterone concentrations were measured using an EIA kit from Cayman Chemical (Ann Arbor, MI Cat# 10004377). Samples or standards were mixed with equal volume of anti-aldosterone antibody and aldosterone-AChE tracer in a plate coated with anti-mouse antibody. After overnight incubation at 4°C, plates were washed and 200 μl Ellman's reagent was added to develop the signals. OD value was measured after 90 minutes at 410 nm [1].

Creatinine Measurement: Plasma creatinine was measured with colorimetric method using a commercial kit from Teco Diagnostics (Anaheim, CA, Cat# C515-490). Creatinine standards or plasma samples were mixed with the picric acid reagent and creatinine buffer reagent provided with the kit. The OD value at 510 nm was measured immediately after and at 15 min. The differences between the two time points were used to calculate the creatinine concentrations.

Isolation of Cardiac Fibroblasts: Isolation of cardiac fibroblasts was carried out as previously described by Brilla and coworkers [7] with modifications as previously reported [3]. Briefly, male Sprague Dawley rats weighing 250-300 grams were used to obtain fibroblast from the hearts. The rats were anesthetized with pentobarbital (50 mg/kg), and their hearts were removed and perfused under sterile condition via the ascending aorta with Joklik's medium (Sigma-Aldrich, St. Louis, MO) in a modified Langendorff apparatus. After 5 min of perfusion, the perfusate was placed in Joklik's medium containing 0.1% collagenase type 2 (Worthington Biochemical, Lakewood, NJ) and 0.1% BSA which was circulated for 15-25 min until the heart became flaccid. Ventricles were excised and finely cut, and shaken in Joklik's modified medium with 0.1% collagenase and 0.1%BSA for15 min. Cells /tissue suspension was allowed to settle for 15 min and was centrifugated at 500 rpm for 10 min. The supernatant then was centrifugated at 1500 rpm for 15 min. The resulting pellet was suspended in DMEM supplemented with antibiotics (penicillin/streptomycin/fungizone) plus 15% FBS (Hyclone, Logan, UT) and seeded onto plates and incubated for 1hr. Unattached cells were removed, and the attached fibroblasts cells were allowed to grow until confluence and then trypsinized and passaged once at 1:3 dilution. Cells were allowed to grow confluent prior to use for experimental purposes. All cells used in these experiments were from passage one unless otherwise specified.

Western Blot Analysis: Western Blot analysis was performed on proteins isolated from cell lysates or from tissue homogenates as previously reported [1, 3, 4, 8]. For the cell lysates, the cells were grown to confluence and starved for 18 h in DMEM with 1% FBS. The cells then were treated with MBG or spironolactone for 24 h when looking for procollagen expression and for 15 minutes when looking for phosphorylated proteins expression such as ERK1/2. The cells were washed with phosphate buffered saline (BPS) twice and exposed to lysis buffer. As for tissue analysis, the left ventricles from the heart were homogenized in ice-cold buffer (pH 7.0) containing 25mM imidazole and protease inhibitors. The homogenate was centrifugated at 12,000 *G* for 10 min at 4° Celsius. The protein was quantified in the supernatant and the proteins were solubilized in sample buffer (2% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.005% bromophenol blue and 50mM Tris-HCl pH 7.0). The proteins, obtained from cell lysates or tissue

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homogenates, were resolved on an SDS-PAGE using Precast Ready Gels 4-15 % Tris-HCl, purchased from Bio-Rad (Hercules,CA). The proteins from the gel were electro-transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.5, 150 mM NaCl, and 0.1% Tween 20). Goat anti-type1 collagen antibody (Southern Biotech, Birmingham, AL) was used to probe for procollagen-1 (Invitrogen/ Biosource, Carlsbad, CA. Anti-ERK1/2 polyclonal antibody, anti-phospho-ERK1/2 monoclonal antibody, and secondary anti-goat and anti rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For detection, we used ECL and ECL plus purchased from Amersham Biosciences (Piscataway, NJ). Loading conditions were controlled for using either tubulin or actin (mouse monoclonal, Santa Cruz).

Determination of protein kinase C isoform delta (PKCδ) in the nucleus and cytosol: Cardiac fibroblasts were grown to confluence and starved for 18-24hrs. Cells were treated with MBG (1 nM) and/or canrenone (100 nM) added to the media. After 15min, cells were washed with ice-cold PBS and both cytosolic and nuclear extracts were prepared from treated and untreated cells. PKCδ in the cytosol and nucleus was determined by performing Western blot as described previously [9].

Histology: Left ventricle sections were immediately fixed in 4% formalin buffer solution (pH 7.2) for 18 h, dehydrated in 70% ethanol, and then embedded in paraffin and cut with a microtome. Trichrome staining was then performed and fibrosis quantified as we have previously reported using ImageJ software (ImageJ 1.36b, National Institutes of Health, USA) [1]. For confirmation of the histological findings, quantitative determination of Collagen-1 in left ventricular homogenates was performed using Western blot (as described above).

Collagen Synthesis: (^{3}H)Proline incorporation study by cardiac fibroblasts was done to investigate the rate of collagen synthesis. Cardiac fibroblasts were isolated from Wistar rats and the cells were grown to confluence in DMEM with 15% FBS. The cells then were incubated in DMEM supplemented with 1 % FBS for 18 h before treatment, then the cells were treated for 24 h in 1 % FBS fresh medium. (^{3}H)proline (1µCi/ml) was added 12 h prior to the termination of the treatment. The experiments were terminated by washing the cells twice with PBS and twice with ice-cold trichloroacetic acid (10%). The precipitate was solubilized in 100 µL of 0.5 N NaOH and 0.1% SDS then aliquoted from each well with 5 mL scintillation fluid were later counted in a liquid scintillation counter [3, 7].

(^{3}H)Ouabain binding: Ouabain binding studies were performed in a porcine cell line (LLC-PK1 cells) as previously described [10]. Briefly, cells were seeded into 12-well plates, cultured to 90% confluence and then serum starved overnight. Afterward, cells were rinsed, incubated in K+ free Krebs solution in the presence of 20 µM monensin to clamp intracellular Na+ and prevent recycling of the Na/K-ATPase. At the end of 15 minutes of incubation, cells were washed 4 times with ice-cold K+ free Kreb's solution, soulubilized in 0.1N NaOH/0-.2% SDS and measured with a scintillation counter.

Statistical analysis: Data presented are mean± standard error of the mean. Data obtained were first tested for normality. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney Rank Sum test were used to compare the data. If the data did pass the normality test, parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was

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performed prior to comparison of individual groups with the unpaired Student's t-test with Bonferroni's correction for multiple comparisons. If only two groups of normal data were compared, the Student's t-test was used without correction [11]. Statistical analysis was performed using Matlab™ software.

Expanded Results:

Spironolactone and canrenone inhibit ouabain binding: To further examine the interactions between spironolactone, canrenone and cardiotonic steroids, we performed experiments in LLC-PK1 cells as well as with the Na/K-ATPase isolated from porcine kidney. The reason we moved to porcine samples was because the alpha1 isoform from the pig binds ouabain quite tightly allowing for easy measurement of ouabain binding. As no radio-labeled form of MBG was available to us, we purchased (^{3}H)ouabain and performed binding studies as described in the methods. We found that both canrenone and spironolactone (at high concentrations) significantly shifted binding of ouabain to LLC-PK1 cells (representative data in figure 3a, quantitative curve fitting data shown in table S1). Based on analysis of these data, both canrenone and spironolactone increased the apparent Kd for ouabain without significantly affecting the Bmax (table S1). This was confirmed for canrenone using the purified Na/K-ATPase isolated from porcine kidney and porcine heart (Table S1). A Scatchard plot of these data also demonstrates that the slope of the lines fit to data were shifted dramatically by addition of canrenone whereas the intercept with the X axis was not significantly affected (Figure 3b), further illustrating the competitive nature of the interaction between canrenone and cardiotonic steroids.

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Table S1: Effect of Spironolactone and Canrenone on (^{3}H)Ouabain Binding

Group (N)	Bmax (Fraction Control)	Kd (nM)
LLCPK1 Cells		
Control (24)	1.00 +/- 0.07	165 +/- 12
Canrenone 10 uM (3)	1.18 +/- 0.12	220 +/- 13†
Canrenone 50 uM (3)	1.02 +/- 0.08	297+/- 34†
Canrenone 100 uM (6)	1.11 +/- 0.05	452 +/- 52 †
Spironolactone 1 uM (3)	1.07 +/- 0.09	169 +/- 14
Spironolactone 10 uM (3)	1.04 +/- 0.12	175 +/- 35
Spironolactone 100 uM (6)	1.07 +/- 0.11	233 +/- 65†
Isolated Porcine Na/K-ATPase from Kidney Tissue		
Control (21)	1.00+/- 0.02	66+/-3
Canrenone 10 uM (3)	1.03+/-0.04	58+/-4
Canrenone 50 uM (3)	0.98+/-0.05	78+/-5*
Canrenone 100 uM (6)	0.97+/-0.07	151+/-4†
Spironolactone 10 uM (3)	0.95+/-0.06	61+/-4
Spironolactone 100 uM (3)	0.96+/-0.06	95+/-5†
Isolated Porcine Na/K-ATPase from Heart Tissue		
Control (15)	1.00+/-0.03	55+/-4
Canrenone 10 uM (3)	1.05+/-0.04	52+/-5
Canrenone 100 uM (6)	1.04+/-0.06	148+/-14†
Spironolactone 10 uM (3)	0.98+/-0.02	68+/-5
Spironolactone 100 uM (3)	1.06+/-0.04	95+/-4†

Controls were performed with each dose of canrenone or spironolactone. Because the LLCPK1 cell density varied from experiment to experiment or the precise amount of enzyme was also variable, Bmax data is expressed as a fraction of control. Kd and Bmax measurements were performed fitting experimental data to the function Y= Bmax * (X)/(Kd + X) where Y is (^{3}H) ouabain binding and X is the concentration of ouabain. * $p<0.05$ vs Control, † $p< 0.01$ vs Control.

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