Supplementary Material

High salt intake augments the activity of the RhoA/ROCK pathway and reduces intracellular calcium in arteries from rats

Running head: *Na^+ overconsumption and Ca^{2+} homeostasis*

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Supplementary Methods and Experimental Protocols

Regular and high-salt diets

This study was performed at both Universidade Federal do Paraná (Curitiba, PR, Brazil) and Augusta University (Augusta, GA, USA). For the experiments performed in Brazil (all included in Figure 2), the animals received a regular chow containing 0.7% NaCl (~0.27% of Na^+) purchased from Quimtia Feed (Colombo, PR, Brazil; manufacturer code NUVILAB CR-1; macronutrients in approximated values: 22% crude protein, 4% fat by ether extract, 55% carbohydrates, and gross energy of 3.91 kcal/g). The chow used for animals subjected to 2%, 4%, and 8% NaCl diets was prepared in-house using powdered regular chow supplied by the same manufacturer and added by NaCl until reach the desired concentrations. The animals used in the experiments conducted in USA (all from Figures 3 and 4) received the Teklad 4% Mouse/Rat Diet as regular chow (manufacturer code 7001; macronutrients in approximated values: 25.2% crude protein, 4.4% fat by ether extract, 39.5% carbohydrates, and gross energy of 3.0 kcal/g), containing 1% NaCl (~0.4% of Na^+), or the Teklad Custom Diet 4% NaCl (manufacturer code TD.92034; macronutrients in approximated values: 19.1% crude protein, 5.3% fat by ether extract, 47.5% carbohydrates, and gross energy of 3.1 kcal/g), both from Envigo (Madison, WI, USA). Regarding the mineral and vitamin amounts, all diets used in our control groups met the recommendation specified in AIN-93.[1]

Isolation of aorta and small mesenteric arteries for measurement of contractile force generation

After 6 weeks receiving high sodium or regular food the animals were killed by anesthetic overdose, and their thoracic aorta or second order small mesenteric artery (external

diameter: ~250 μm) were removed, cleaned, and cut into ring-like segments, as previously detailed,[2,3] with minor modifications. The aortas and second order small mesenteric arteries were mounted in organ baths containing physiological saline solution (PSS; composition in mM: 130.3 NaCl, 4.7 KCl, 1.6 $CaCl_2 \cdot 2H_2O$, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 5.5 D-glucose, 14.9 $NaHCO_3$), warmed at 37 °C and continuously aerated with 95% O_2/95% CO_2. Both endothelium–intact and –denuded arteries (confirmed by the absence of acetylcholine-induced relaxation under contraction elicited by phenylephrine) were used in our experiments. The preparations were subjected to a basal tension of 0.25 g (small mesenteric arteries), or 3 g (aorta), and were allowed to equilibrate for 60 min prior to the addition of any drug. Isometric tension was recorded using digital polygraphs coupled to a computer running the software LabChart v. 7.1 (both from AD Instruments, Castle Hill, Australia). Experiments with isolated small mesenteric arteries were conducted in wire myographs (Danysh Myo Technology, Aarhus, Denmark).

***In vitro* determination of vascular reactivity**

For all protocols described in this study, the responses obtained in aortic or mesenteric rings from rats belonging to high-salt groups were compared with those obtained in vessels isolated from control rats (fed with regular chow). In addition, when the preparations were subjected to incubation with any drug, time-matched experiments using the vehicle were performed to confirm whether the effects obtained were induced by the drug used or were consequences of the time under experimentation.

Since the major aim of the protocols using small mesenteric arteries was to check the applicability of our findings for resistance arteries, only groups of animals fed with regular chow (control group) or 4% NaCl diet were used in these experiments.

Evaluation of the vascular reactivity to high KCl, phenylephrine, acetylcholine and Y-27632

After the stabilization, the vessels were exposed to a modified PSS solution containing 120 mM KCl (composition in mM: 14.4 NaCl, 119.9 KCl, 1.6 $CaCl_2 \cdot 2H_2O$, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 5.5 D-glucose, 14.9 $NaHCO_3$), and the contractile force generated was recorded during 15 min, followed by a new stabilization period of 30 min.

In our first set of experiments we evaluated the ability of endothelium-intact aortic or mesenteric rings obtained from high-sodium groups to relax in response to acetylcholine or compound Y-27632, a selective inhibitor of ROCK. For this, the rings were contracted by phenylephrine (PE; 1 μM for aorta; 10 μM for mesenteric arteries), and in the tonic phase of the contraction the vessels were exposed to cumulative concentrations of acetylcholine (0.1 nM–30 μM), or Y-27632 (0.1 nM–100 μM). Importantly, each vessel was exposed to only one vasodilator.

Measurement of phenylephrine-induced contraction in the presence of Y-27632

To investigate the functionality of the RhoA/ROCK pathway, aortic rings from control and 2%, 4% and 8% NaCl groups were incubated for 15 min with the ROCK inhibitor Y-27632 (1, 3, or 10 μM), or vehicle (PSS; control), and were subjected to cumulative concentration response curves to PE (1 nM–100 μM). The effects of the Y-27632 (3, 30, or 50 μM) on PE-induced contraction were also evaluated in small mesenteric arteries from control and 4% NaCl groups. These experiments were performed in endothelium-intact and endothelium-denuded preparations.

Assessment of calcium-induced contractile force generation

In our next set of experiments, we investigated whether the high-salt diet (2%, 4% and 8% NaCl) could impair the influx and role of extracellular calcium in the contractile responses of aortic rings to PE. For this, experiments using a Ca^{2+}-free depolarizing PSS (containing 60 mM KCl and free of calcium; composition in mM: 71.4 NaCl, 59.8 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 5.5 D-glucose, 14.9 $NaHCO_3$, 0.04 EDTA) were carried out. In this approach, after the initial stabilization period with regular PSS and verification of the absence of functional endothelium, the regular PSS was replaced by Ca^{2+}-free depolarizing PSS for an additional 60 min, and the preparations were subjected to cumulative concentrations of $CaCl_2$ (10 μM–100 mM). The same protocol was carried out in small mesenteric arteries from both control and 4% NaCl groups.

Analysis of phenylephrine- and caffeine-induced contraction in calcium-free medium

We also investigated whether a high-salt diet could change the intracellular mobilization of Ca^{2+}. For this, after initial stabilization with regular PSS, endothelium-denuded aortic rings from control (regular chow) and 2%, 4% and 8% NaCl groups were maintained for 45 min in regular PSS followed by an additional period of 15 min in Ca^{2+}-free PSS (composition in mM: 130.3 NaCl, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 5.5 D-glucose, 14.9 $NaHCO_3$, 0.04 EDTA). Under these conditions, the aortic rings were exposed to a single concentration of caffeine (1 mM), or PE (1 μM), and the contractions evoked were compared among the groups. Using the same experimental protocol, the ability of caffeine (20 mM) and PE (10 μM) to induce contractile responses in small mesenteric arteries from control and 4% NaCl groups was also evaluated. In addition, PE-induced contraction was evaluated in both aortic rings and mesenteric arteries previously incubated for 15 min with the selective SERCA inhibitor thapsigargin (1 μM).

In a different set of experiments, the contractile effect of calcium alone was also checked in small mesenteric arteries maintained in calcium-free solution (not depolarizing), which were exposed to cumulative concentrations of $CaCl_2$ (10 μM–100 mM) before and after incubation with 1 μM thapsigargin for 15 min.

Fluorimetric detection of intracellular calcium

These experiments were performed in smooth muscle cells that were freshly isolated from thoracic aortas. Animals from control or 4% NaCl groups were anesthetized with oxygen-isoflurane (3%) inhalation and their thoracic aorta were removed, cleaned and cut into rings. For the separation of smooth muscle cells, the tissue was immersed in enzymatic solution containing collagenase type 1 (0.2 mg/mL), elastase type 1 (0.075 mg/mL), bovine serum albumin (BSA; 1 mg/mL), chicken trypsin inhibitor (0.2 mg/mL), and DTT (0.2 mg/mL), dissolved in low calcium solution (0.16 mM). The preparations were maintained at 37 °C, continuously aerated with oxygen, and gently shaken for 40 min. The samples were then transferred to a new solution containing papain (1.2 mg/mL), DTT (0.54 mg/mL), and BSA (4 mg/mL), and were kept at 37 °C for 30 min. After this, the samples were washed 5 times with low-calcium solution, moved to tubes with 2 ml of culture medium (DEMEM) containing 10% BSA, and gently shaken. A 500 μL volume of this solution was incubated with 25 μL of fura-2-acetoxymethyl-ester (Fura-2 AM, 200 μg/mL), and kept protected from light at room temperature for 1 h, after which time the reaction was stopped on ice. Aliquots of cells (50 μL) were transferred to a perfusion chamber (RC-20H, Warner Instruments, Hamden, CT, USA), and allowed to rest for 5 min. The perfusion rate was adjusted to 25 μL/min during calcium measurements. The system was coupled to an inverted microscope (Olympus, 1X50, Hicksville, NY, USA) and a fluorometer for detection of calcium (excitation and emission wavelengths 340/380 and 512 nm, respectively). The fluorometric

determination of calcium was carried out in both resting and stimulated (ATP, 100 μM) cells. For this, cells were perfused with low-calcium PSS solution followed by a first exposure to ATP (which generates a peak of calcium released from intracellular stores). After that, the cells were perfused with low-calcium PSS plus EGTA (1 mM) to reload the sarcoplasmic reticulum, followed by a second stimulation with ATP. The data obtained were recorded and analyzed by the software Felix for Windows® (version 1.41, Photon Technology International, Birmingham, NJ, USA). These experiments were performed in cells obtained from control and 4% NaCl groups (n = 3 different rats per group).

Western blotting

The expression of proteins was measured in samples of thoracic aortas obtained from control and 4% NaCl groups (*n* = 3-5). Excepting the experiment used to explore the translocation of RhoA (which is detailed in the sequence), the entire thoracic aorta was immediately frozen in liquid nitrogen after removal from the animal and maintained at -80 °C until it was processed for protein purification and quantification, as previously detailed.[4] Electrophoretic separation was carried out using 40 μg of protein per well in 10% polyacrylamide gels in a Mini-PROTEAN® Tetra cell apparatus coupled to a PowerPac TM HC Power Supply (both from Bio-Rad, CA, USA). The proteins were transferred onto nitrocellulose membranes (Hybond; Amersham Biosciences, NJ, USA), blocked in 5% non-fat dry milk (prepared in TBS-T buffer, pH 7.4; concentration in mM: 20 Tris-HCl, 137 NaCl, and 0.1% Tween 20), and subjected to incubation (overnight, at 4 °C) with polyclonal primary antibodies against RhoA, ROCKI, ROCKII, SERCA2, SERCA3, IP_3R, MYPT1, p-MYPT1, ryanodine receptor, STIM1 or Orai1. Peroxidase-conjugated monoclonal antibody against β-actin was used as a loading control for all samples tested. After incubation with primary antibodies, the membranes were washed 3 times (10 min each) with nonfat dry milk

in TBS-T and incubated with the specific secondary antibody conjugated to horseradish peroxidase (HPR) at room temperature for 1 h. The membranes were washed again 3 times (10 min each) with nonfat dry milk in TBS-T, and exposed to HPR substrate (Pierce Biotechnology, Rockford, IL, USA) for chemiluminescent detection of proteins using a FluorChem® HD2 Imaging System (Alpha Innotech Corp., CA, USA). The bands were quantified by densitometry using the UN-SCAN-IT gel software (Silk Scientific, Inc., Orem, UT, USA).

To evaluate the translocation of RhoA to the plasma membrane, thoracic aortas were collected from animals, cleaned from adjacent tissues, and incubated in organ baths under standard conditions (PSS at 37 °C, aerated with 5% CO_2/95% O_2). After 1 h of stabilization the vessels were incubated with phenylephrine (1 μM) or vehicle (PSS) for 15 min, after which time the tissues were removed from the baths and immediately frozen in liquid nitrogen. The samples were kept at -80 °C until the Western blotting assay. For this, the aortas were pulverized and transferred to tubes containing a modified lysis buffer (concentration in mM: 100 Tris-HCl, 1 EDTA, 1 EGTA, 0.5 PMSF, 150 NaCl, 2 Na_3VO_4, 104 AEBSF, 0.08 aprotinin, 4 bestatin, 1.4 E-64, 2 leupeptin, 1.5 pepstatin A; pH 7.5). After sonication and a period of 30 min immersed in ice, the samples were centrifuged at 3000 g for 30 min to remove nuclei and intact cells. The supernatant was then centrifuged at 60,000 g for 45 min to generate membrane and cytosolic fractions, as previously detailed.[5,6] The supernatant, containing the cytosolic fraction, was collected and resuspended in PBS. The pellet containing the membrane fraction was resuspended in RIPA buffer (Millipore Corporation, Billerica, MA, USA) containing PMSF, Na_3VO_4 and protease inhibitors. Protein concentration was quantified and samples were subjected to electrophoretic separation as previously described [4]. Nitrocellulose membranes to which were bound either cytosolic or membrane proteins were incubated with primary antibody against RhoA.

Drugs, reagents and antibodies

Phenylephrine hydrochloride (PE), acetylcholine chloride (ACh), caffeine, papain, 1,4 dithiothreitol (DTT), sodium orthovanadate, phenylmethylsulfonyl fluoride (PMSF), protease cocktail inhibitor, collagenase type 1, elastase type 1, bovine serum albumin (BSA; 1 mg/mL), chicken trypsin inhibitor, adenosine triphosphate (ATP), and thapsigargin were purchased from Sigma (St Louis, MO, USA). Fura-2-acetoxymethyl-ester (Fura 2-AM) was obtained from Invitrogen (Grand Island, NY, USA). (*R*)-(+)-*trans*-4-(1-Aminoethyl)-*N*-(4-yridyl) cyclohexanecarboxamide dihydrochloride monohydrate (Y-27632) was acquired from Tocris (Ellisville, MO, USA). Tissue protein extraction reagent was purchased from Thermo Fischer Scientific (Rockford, IL, USA). Tris-HCl buffer, Tris-glycine buffer, glycine, ammonium persulfate (APS), N,N,N′,N′-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), bovine serum albumin, milk and acrylamide were acquired from Bio-Rad Laboratories (Hercules, CA, USA). All salts used to prepare the physiological salt solution (PSS) were from Merck (Darmstdat, Germany). Stock solutions of drugs were prepared in fresh distilled water, excepting thapsigargin, which was dissolved in dimethyl sulfoxide. All drugs were diluted in PSS prior to use. Primary antibodies against the following proteins were used in this study: RhoA, ROCKI, ROCKII, inositol trisphosphate receptor (IP_3R) (all from Cell Signaling Technology; Danvers, MA, USA), ryanodine receptor (from Abcam; Cambridge, MA, USA), STIM1 and ORAI1 (both from ProSci Incorporated; Poway, CA, USA), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) 2 and 3 (from Thermo Fischer Scientific; Rockford, IL, USA), and β-actin (St Louis, MO, USA). All secondary antibodies were from GE Healthcare Life Sciences (Piscataway, NJ, USA).

Supplementary Results

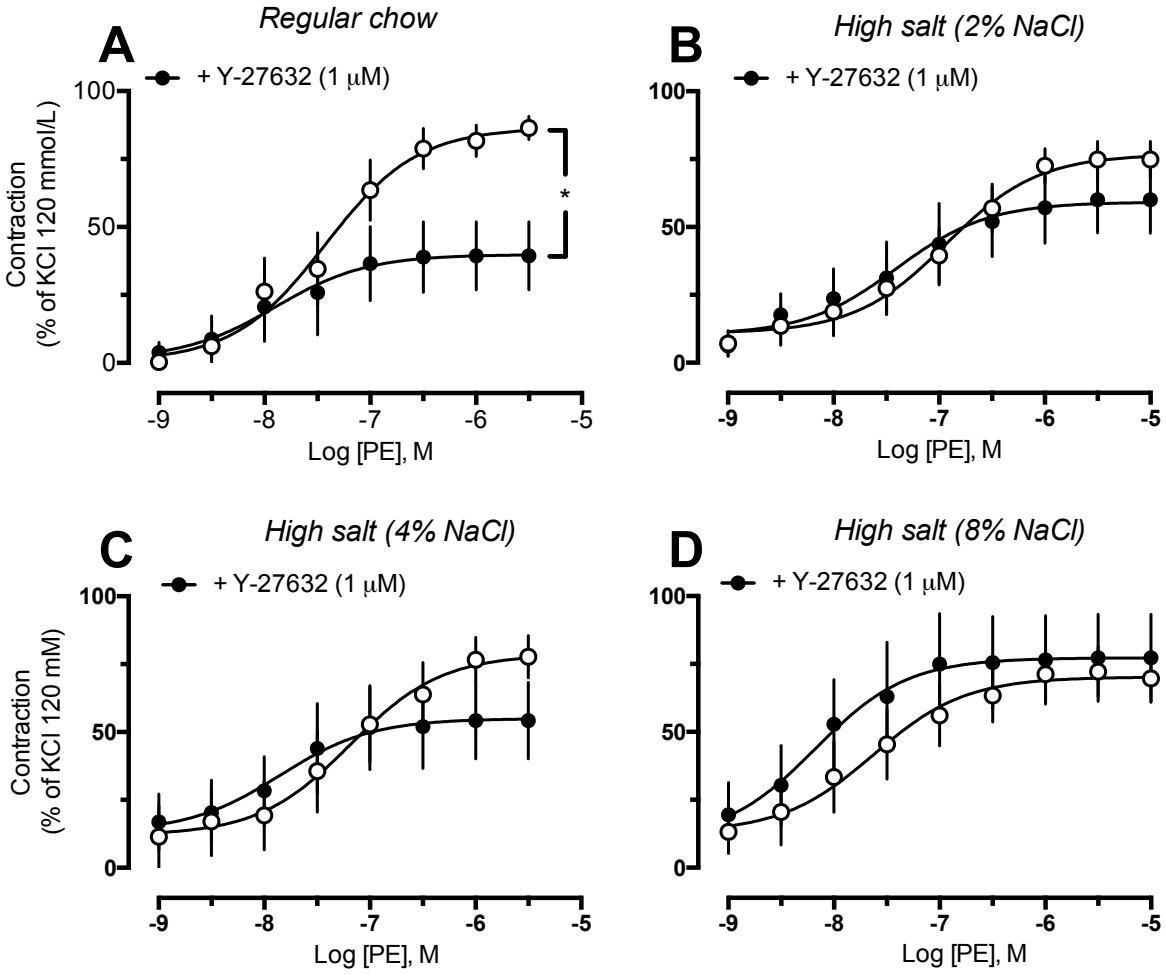

Figure S1
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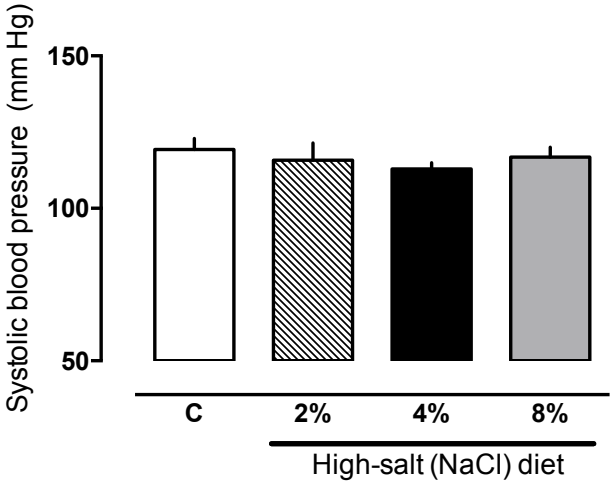

Figure S2
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Supplementary Figure Legends

Figure S1. Phenylephrine-induced contraction is less sensitive to inhibition of ROCK in endothelium-intact aortic rings. Preparations obtained from rats fed regular chow (A) or 2% NaCl (B), 4% NaCl (C), or 8% NaCl (D) diets, were incubated with the ROCK inhibitor Y-27632 (1 μM) for 30 min, followed by cumulative concentrations of phenylephrine (PE; 1 nM-10 μM). Results show the mean ± SEM of 5-6 preparations from different animals from each group. * $P < 0.05$ compared with control preparations.

Figure S2. Lack of changes in the systemic arterial pressure of rats subjected to a high-salt diet. The systolic blood pressure was measured by the tail cuff method in animals fed regular chow or 2% NaCl, 4% NaCl, or 8% NaCl diets for six weeks. Results show the mean ± SEM of eight animals per group. No statistical differences were found.

Supplementary References

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