

SUPPLEMENTAL MATERIAL

Methods

Animal Studies

Mice were housed in groups of two to five per cage on a 12:12 h light/dark cycle; with lights on at 6 a.m. Tissues, blood and urine samples were collected between 8 and 10 am. Male mice were studied. Food and water were available ad libitum.

Metabolic Cage Studies

Renal function was evaluated in metabolic cage clearance studies. After acclimation (3 days) in metabolic cages (Nalgene (Thermo Scientific), #NALGE650-0322), food and water consumption was assessed and urine samples were collected. Urine samples were collected several times a day to prevent contamination from food, water, and fecal matter. Urine samples were collected in tubes containing mineral oil to prevent evaporation.

Protein Preparation

Tissues were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) using microtube pestles (USA Scientific, Ocala, FL), and protein was extracted according to manufacturer's recommendations. An aliquot was used for total protein quantification using a BCA assay and the remainder was stored frozen at -80°C until use.

Antibodies

Affinity-purified antibodies to Rhbg and Rhcg generated in our laboratory that have been previously characterized¹⁻⁴. H. Moo Kwon (Ulsan National Institute of Science and Technology, Ulsan, South Korea) graciously provided antibodies to NKCC2. Dr. Fiona Karet Frankl (Cambridge Institute for Medical Research, Cambridge, UK) graciously provided antibodies to a4 subunit of H^{+} -ATPase. Antibodies to phosphoenolpyruvate carboxykinase (PEPCK) were

obtained from Cayman Chemical (Ann Arbor, MI; Cat 10004943), antibodies to glutamine synthetase (GS) were obtained from Chemicon (Temecula, CA; Cat Ab73593), antibodies to NHE3 were obtained from StressMarq Biosciences (Victoria BC, Canada; Cat SPC-400D), and antibodies to NBCe1 (Cat 885-1-AP) and to the KGA isoform of phosphate-dependent glutaminase (PDG) (Cat 20170-1-AP) were obtained from Proteintech (Rosemont, IL).

Immunoblot Procedure

Fifteen micrograms of renal protein were electrophoresed on 10% PAGE ReadyGel (Bio-Rad, Hercules, CA). Gels were then transferred electrophoretically to nitrocellulose membranes, blocked with 5 g/dl nonfat dry milk diluted in Blotto buffer (50mM Tris, 150 mM NaCl, 5mM Na₂EDTA, and 0.05% Tween 20; pH 7.6), and incubated at 4°C overnight with the primary antibody in nonfat dry milk. Loading and transfer equivalence were assessed with Ponceau S staining. After being washed, membranes were exposed to secondary antibody (goat anti-rabbit IgG, Millipore, Billerica, MA) conjugated to horseradish peroxidase at a dilution of 1:5,000. Sites of antibody-antigen reaction were visualized using enhanced chemiluminescence (SuperSignal West Pico Substrate, Pierce) and a Kodak Image Station 440CF digital imaging system. In selected experiments, blots were stripped with a second antibody directed against a second protein of different molecular weight. Band density was quantified using Kodak ID (version 5.0) software (Kodak Scientific Imaging, New Haven, CT). Band density normalized such that mean density in the same region (cortex or medulla) in wild type tissue was 100. The absence of saturation was confirmed by examining pixel intensity distribution in all immunoblots.

Immunohistochemistry

Anesthetized mice were fixed by perfusion with 2% paraformaldehyde in PBS via the left ventricle for 5 minutes at room temperature. The kidneys were then removed and fixed for an

additional 24 h at 4°C, rinsed in PBS, embedded in paraffin and 3 µm- thick sections were cut and mounted on gelatin-coated glass slides.

Immunolocalization for GS, NBCe1, NHE3, α4 subunit of H⁺-ATPase, Rhbg and Rhcg was accomplished using previously described immunoperoxidase procedures⁵⁻⁷. Briefly, sections were deparaffinized in xylene, rehydrated, heated in Trilogy (Cell Marque, Rocklin CA) to 88°C for 30 minutes and then to 96°C for 30 minute, cooled for 30 minutes, and rinsed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in 3% H₂O₂ in distilled water for 45 minutes. Sections were blocked for 15 minutes with Serum-Free Protein Block (Dako Cytomation) and then incubated overnight at 4°C overnight with the primary antibody. Sections were washed in PBS and incubated for 30 minutes with polymer-linked, peroxidase-conjugated horse anti-rabbit IgG (ImmPRESS, Vector Laboratories, Burlingame, CA) washed again with PBS, and then exposed to diaminobenzidine (DAB) for 5 minutes. Sections were washed in distilled water, dehydrated with xylene, mounted and observed by light microscopy. Comparisons of labeling were made between sections of the same thickness from the same immunohistochemistry experiment. Sections were examined on a Nikon E600 microscope equipped with DIC optics and photographed using a DXM1200F digital camera and ACT-1 software (Nikon).

Immunolocalization for PDG and PEPCK was accomplished using slightly modified immunoperoxidase procedures. Briefly, sections were deparaffinized in xylene, rehydrated, heated in Trilogy (Cell Marque, Rocklin CA) to 96°C for 60 minute, cooled for 30 minutes, and rinsed in PBS. Endogenous peroxidase activity was blocked by incubation of the sections in 3% H₂O₂ in methanol for 45 minutes. The sections were treated with 0.5% Triton X-100 in PBS for 15 minutes. The sections then underwent several washes in PBS containing 1% bovine serum

albumin (BSA), 0.05% saponin, and 0.2% gelatin, followed by blocking for 15 minutes with Serum-Free Protein Block (Dako Cytomation) and then incubated overnight at 4°C overnight with the primary antibody. Sections were washed in PBS containing 0.1 % BSA, 0.05% saponin and 0.2% gelatin, followed by PBS and incubated for 60 minutes with polymer-linked, peroxidase-conjugated goat anti-rabbit IgG (MACH2, Biocare Medical, Concord, CA) washed again with PBS, and then exposed to diaminobenzidine (DAB) for 5 minutes. Sections were washed in distilled water, dehydrated with xylene, mounted and observed by light microscopy. Comparisons of labeling were made between sections of the same thickness from the same immunohistochemistry experiment. Sections were examined on a Nikon E600 microscope equipped with DIC optics and photographed using a DXM1200F digital camera and ACT-1 software (Nikon).

References

1. Bishop JM, Verlander JW, Lee HW, Nelson RD, Weiner AJ, Handlogten ME, Weiner ID: Role of the Rhesus glycoprotein, Rh B Glycoprotein, in renal ammonia excretion. *Am J Physiol Renal Physiol* 299: F1065-F1077, 2010.
2. Lee HW, Verlander JW, Bishop JM, Igarashi P, Handlogten ME, Weiner ID: Collecting duct-specific Rh C Glycoprotein deletion alters basal and acidosis-stimulated renal ammonia excretion. *Am J Physiol Renal Physiol* 296: F1364-F1375, 2009.
3. Mak DO, Dang B, Weiner ID, Foskett JK, Westhoff CM: Characterization of transport by the kidney Rh glycoproteins, RhBG and RhCG. *Am J Physiol Renal Physiol* 290: F297-F305, 2006.
4. Verlander JW, Miller RT, Frank AE, Royaux IE, Kim YH, Weiner ID: Localization of the ammonium transporter proteins, Rh B Glycoprotein and Rh C Glycoprotein, in the mouse kidney. *Am J Physiol Renal Physiol* 284: F323-F337, 2003.
5. Lee HW, Osis G, Handlogten ME, Verlander JW, Weiner ID: Proximal Tubule Glutamine Synthetase Expression is Necessary for the Normal Response to Dietary Protein Restriction. *American Journal of Physiology - Renal Physiology* 313: F116-F125, 2017.
6. Lee HW, Osis G, Handlogten ME, Lamers WH, Chaudhry FA, Verlander JW, Weiner ID: Proximal tubule-specific glutamine synthetase deletion alters basal and acidosis-stimulated ammonia metabolism. *American Journal of Physiology - Renal Physiology* 310: F1229-F1242, 2016.
7. Lee HW, Osis G, Handlogten ME, Guo H, Verlander JW, Weiner ID: Effect of dietary protein restriction on renal ammonia metabolism. *American Journal of Physiology - Renal Physiology* 308: F1463-F1473, 2015.