THE ROLE OF RH B GLYCOPROTEIN IN RENAL AMMONIA METABOLISM

By

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To my family, friends, teachers, mentors, and team mates. And to all those that stood beside me during my time in the United States Army, as 1st Ranger Battalion was my first step towards a higher education. To all those that never came home to pursue their higher education, your sacrifices have made milestones like this possible for countless individuals like myself.

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I thank the chair and members of my supervisory committee for their devotion and commitment to my education over the years. I thank my wife and daughter for their support in every facet of my life, and for the encouragement to complete my studies.

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LIST OF ABBREVIATIONS

Amt	Ammonia transporters
B1-Cre	Transgenic mice expressing Cre-recombinase under control of the H+ATPase B1 subunit promoter
CCD	Cortical collecting duct
CD-Rhbg-KO	Collecting Duct Rh B Glycoprotein Knockout
CNT	Connecting segment
DCT	Distal convoluted tubule
IC-Rhbg-KO	Intercalated Cell Rh B Glycoprotein Knockout
ICT	Initial collecting tubule
Mep	Methylammonium permeases
NAE	Net acid excretion
NEAP	Net endogenous acid production
OMCD	Outer medullary collecting duct
PBS	Phosphate buffered saline
PDG	Phosphate dependent glutaminase
PEPCK	Phosphoenolpyruvate carboxykinase
PLP	Periodate-lysine-2% paraformaldehyde
Rhbg	Rh B Glycoprotein
Rhcg	Rh C Glycoprotein

Abstract of Thesis Presented to the Graduate School
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By

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Renal ammonia metabolism is a critical mechanism involved in the maintenance of acid-base homeostasis. Renal epithelial cell transport, along with renal ammoniagenesis are two major facets of renal ammonia metabolism. Recent studies have identified members of a family of ammonia specific transporters that play a critical role in renal epithelial transport.

Rh B glycoprotein (Rhbg) is one member of the large family of ammonia specific transporters. Rhbg is expressed in the kidney, liver, lung, gastrointestinal tract and skin. In the kidney, Rhbg is expressed in the basolateral plasma membrane in cells of the distal convoluted tubule (DCT), connecting segment (CNT), initial collecting tubule (ICT) and throughout the collecting duct.

The current study investigates the role of Rhbg in response to conditions that involve altered renal ammonia metabolism. This includes an investigation into Rhbg's role in hypokalemia and a preliminary investigation into the renal response to dietary protein restriction. This study included the use of intercalated cell Rhbg knockout mice (IC-Rhbg-KO) and collecting duct Rhbg knockout mice (CD-Rhbg-KO) and control mice that had normal Rhbg expression.

Rhbg protein expression increased in both the cortex and outer medulla in response to hypokalemia. In control mice, hypokalemia increased total urinary ammonia excretion, absolute ammonia concentration and urine pH, whereas in IC-Rhbg-KO mice, urine ammonia concentration did not change significantly from baseline and total urinary ammonia excretion was significantly less than in C mice. Urine pH, Na+, K+ and volume were unaltered by IC-Rhbg-KO.

CHAPTER 1
INTRODUCTION

Acid-base homeostasis is essential for many facets of normal human health. Acid-base disorders lead to stunted growth and skeletal disorders in children and in adults it leads to muscle wasting, nephrolithiasis, and circulatory disorders (2, 3, 29, 43). Net endogenous acid production is the amount of net acid produced by the metabolic system. For an individual consuming a typical western diet the endogenous acid production is estimated to be 50 mEq/d (20) . Systemic acid-base homeostasis is maintained when endogenous acid production is equivalent to net acid excretion. Net Acid excretion is composed urinary ammonia, titrateable acids and bicarbonate, with renal ammonia metabolism making up 50-70% of the total net acid excretion (32).

Renal ammonia metabolism is a critical mechanism involved in the maintenance of acid-base homeostasis, it involves integrated ammoniagenesis and renal epithelial (8, 10, 16, 42) cell transport. Recent studies have identified novel mechanisms of renal epithelial cell ammonia transport by members of a family of closely related ammonia specific transporters (4, 5, 14, 17, 19, 41, 42). Members of this ammonia transporter family include Mep proteins in yeast, Amt proteins in a wide variety of single cell organisms and plants, and Rh glycoproteins in amphibians, fish and mammals. In mammals, Rh glycoproteins mediate a central role in the regulation of renal ammonia excretion (4, 14, 22, 23, 41, 42). These proteins transport ammonia, but not Na+, K+ or H2O (21, 25-28) and they exhibit polarized expression in the distal convoluted tubule through the inner medullary collecting duct.

Two members of this family of ammonia specific transporters are Rh B glycoprotein (Rhbg) and Rh C glycoprotein (Rhcg). Both Rhbg and Rhcg are present in

collecting duct cells (both intercalated and principal cells), with Rhbg being expressed in the basolateral plasma membrane and Rhcg in the apical and basolateral membranes, in addition to cytoplasmic vesicles (9, 11, 15, 30, 33, 38). The coordinated action of these proteins enables regulated transcellular ammonia transport (40-42).

Recent studies in the mouse have shown that Rhbg mediates a critical role in ammonia metabolism under basal conditions and in response to metabolic acidosis. Metabolic acidosis increases Rhbg expression in both the cortex and the outer medulla (5). Moreover, intercalated cell-specific Rhbg deletion impaired the normal increase in renal ammonia excretion in response to metabolic acidosis, indicating a key role for Rhbg in renal ammonia transport and the maintenance of acid-base homeostasis in response to metabolic acidosis (5). Under basal conditions, intercalated cell-specific Rhbg deletion did not alter either serum $HCO3^-$ or urinary ammonia excretion, but did induce adaptive changes in glutamine synthetase expression which appear to compensate for the absence of Rhbg-mediated ammonia transport when ammonia excretion is not stimulated (5).

CHAPTER 2
THE ROLE OF RH B GLYCOPROTEIN IN THE RENAL RESPONSE TO HYPOKALEMIA

Hypokalemia is a common condition that increases urinary ammonia excretion and can lead to another acid-base disorder, metabolic alkalosis (35, 36). Hypokalemia results in altered ammoniagenesis (36), ammonia transport (6, 7, 39) and increased collecting duct ammonia secretion (7). The purpose of the current study was to determine Rhbg's role in the renal response to hypokalemia.

Methods

Animals

The generation of mice with loxP sites flanking critical exons of the murine Rhbg gene were described previously (5). Transgenic mice expressing Cre-recombinase under control of a 6.5-kb portion of the H+-ATPase B1 subunit promoter (B1-Cre) have been described previously (24). IC-Rhbg-KO mice were bred using floxed Rhbg mice and mice expressing Cre-recombinase under control of the B1 subunit of H+-ATPase promoter (B1-Cre) as described previously (5). Animal breeding was performed in the University of Florida College of Medicine Cancer and Genetics Transgenic Animal Core Facility by trained personnel. Mice were genotyped using tail-clip samples as described previously (15, 18). All mice used in this project were either floxed Rhbg, B1-Cre-positive or floxed Rhbg, B1-Cre-negative; we have shown previously that floxed Rhbg, B1-Cre-positive mice have intercalated cell-specific Rhbg deletion and that floxed Rhbg, B1-Cre-negative mice have normal Rhbg expression (5). All animal studies were approved by the Institutional Animal Care and Use Committees of the Gainesville VA Medical Center and of the University of Florida College of Medicine.

Antibodies

Affinity-purified antibodies to Rhcg and Rhbg were generated in our laboratory and have been previously characterized (15, 17, 21, 38). In particular, we have shown specificity of both the Rhbg and the Rhcg antibodies in studies using heterologous expression in Xenopus oocytes (21) and in studies using genetic deletion of Rhbg and Rhcg (15, 17). Norman Curthoys, Ph.D. (Colorado State University) provided antibodies to phosphate-dependent glutaminase (PDG) and Fiona Karet, Ph.D. (Cambridge Institute for Medical Research, Cambridge, UK) provided antibodies to the a4 subunit of H+-ATPase. Antibodies to glutamine synthetase were obtained from Millipore, Billerica, MA, and antibodies to phosphenolpyruvate carboxykinase (PEPCK) were obtained from Cayman Chemical Co., Ann Arbor, MI.

Induction of Hypokalemia

Hypokalemia was induced by feeding mice a K^+-free diet (TD.88239, Harlan Teklad, Madison, WI) for three days. Powdered food was mixed it with H_2O in a ratio of 6 gm food to 1 ml H_2O to form a semi-solid diet. Adult mice, greater than 8 weeks age, were placed into metabolic cages (Tecniplast diuresis metabolic cage, Fisher Scientific) and allowed to acclimate for three days while receiving control diet. They then received K^+-free or control diet and daily food intake was measured. At all times animals were allowed free access to water. Daily urine excretion was collected under mineral oil;, urine pH was measured and urine volume calculated. Urine samples were stored at -80°C until analyzed.

Electrolyte Measurements

Urine ammonia was measured using a commercially available kit (A7553, Pointe Scientific, Inc., Canton, MI) modified for use in 96 well plates. Serum bicarbonate was

measured as total CO_2 using a commercially available kit (C750-120, Pointe Scientific, Inc) modified for use with microliter quantities of serum. Urine pH was measured using a micro-pH electrode (Thermo Scientific, ROSS semi-micro pH, ORION 8115BN). Serum Na+ and K+ concentrations were measured using a flame photometer (Instrumentation Laboratory, Lexington, MA) as described previously (17). Serum and urine creatinine was measured by capillary electrophoresis using techniques described previously (17).

Tissue Preparation for Immunolocalization

Mice were anesthetized with inhalant isoflurane. The kidneys were preserved by *in vivo* cardiac perfusion with PBS (pH 7.4) followed by periodate-lysine-2% paraformaldehyde (PLP) and then cut transversely into several 2- to 4-mm-thick slices and immersed 24 to 30 hours at 4°C in the same fixative. For light microscopy, samples of kidney from each animal were embedded in polyester wax (polyethylene glycol 400 distearate (Polysciences, Warrington, PA) with 10% 1-hexadecanol), and 3-µm-thick sections were cut and mounted on gelatin-coated glass slides.

Immunohistochemistry

Immunolocalization was accomplished using immunoperoxidase procedures detailed previously (15, 17). Tissue sections were dewaxed in ethanol, rehydrated and then rinsed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in Peroxidase Blocking Reagent (DakoCytomation, Carpinteria, CA) for 45 min. The sections were blocked for 15 min with Serum-Free Protein Block (DakoCytomation), then incubated at 4°C overnight with primary antibody diluted in Dako Antibody Diluent. The sections were washed in PBS and incubated for 30 min with polymer-linked peroxidase conjugated goat anti-rabbit IgG (MACH2, Biocare

Medical), again washed with PBS, then exposed to diaminobenzidine for 5 minutes. The sections were washed in distilled water, then dehydrated in a graded series of ethanols and xylene, mounted and observed by light microscopy. Comparisons of labeling were made only 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). Color and contrast adjustment was performed using Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA).

Double Immunolabeling Procedure

Double immunolabeling was accomplished using sequential immunoperoxidase procedures described in detail previously (15). Briefly, tissue sections were labeled with the first primary antibody following the procedure described above, using Vector SG (Vector Laboratories) as the chromogen to produce a blue label. After the Vector SG reaction, sections were washed in PBS then blocked using the Peroxidase Blocking Reagent and Serum-Free Protein Block as described in the single label procedure. The above procedure was repeated with the substitution of a second primary antibody and the substitution of DAB for Vector SG. The sections were then washed with glass distilled water, dehydrated with xylene, mounted with Permount, and observed by light microscopy.

Protein Preparation

Animals were anesthetized with inhalant isoflurane and the kidneys were rinsed by *in vivo* cardiac perfusion with PBS (pH 7.4), rapidly removed, and stored frozen at -80°C until used. In some experiments, the right renal vasculature was clamped after in vivo cardiac perfusion with PBS, the right kidney removed, and then the left kidney was

perfused with PLP fixative for immunohistochemistry. Tissues were homogenized using microtube pestles (USA Scientific, Ocala, FL) and proteins were extracted using T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's recommended procedures. For membrane protein preparation for Rhcg, tissues were homogenized in buffer A (in mM: 50 sucrose, 10 Tris buffer, 1 EDTA, pH 7.4) and then diluted in buffer B (in mM: 250 sucrose, 10 Tris buffer, and 1 EDTA, pH 7.4). The sample was then centrifuged at 1,000 g for 5 min at 4°C. The pellet was resuspended in buffer B and again centrifuged at 21,000 g for 30 min at 4°C. The 21,000 g pellet was finally resuspended in buffer B. An aliquot was used for protein determination with a BCA assay, and the remainder was stored frozen at -80°C until used.

Immunoblotting Procedure

Five to twenty 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, and incubated for 2 h with primary antibody diluted in Blotto buffer (50 mM Tris, 150 mM NaCl, 5 mM Na2EDTA and 0.05% Tween-20, pH 7.5) with 5 g/dl nonfat dry milk. Loading and transfer equivalence was assessed with Ponceau S staining. After washing, membranes were exposed to secondary antibody (goat anti-rabbit IgG; Promega, Madison, WI or goat anti-mouse IgG; Upstate, Temecula, CA, conjugated to horseradish peroxidase) at a dilution of 1:5000. Sites of antibody-antigen reaction were visualized by using enhanced chemiluminescence (SuperSignal West Pico Substrate, Pierce, Rockford, IL) and a Kodak Image Station 440CF digital imaging system. Band density was quantified using Kodak 1D, version 3.5.4, software, Kodak Scientific Imaging, New Haven CT.

Band density was normalized such that mean density in the same region (cortex or outer medulla) in C kidneys was 100.0.

Statistical Analysis

Results are presented as mean ± SEM. N reflects number of mice examined. Tests of significance were performed using Student's t-test and ANOVA as appropriate. $P<0.05$ was required for evidence of statistical significance.

Results

Serum Chemistries

In order to study hypokalemia, mice were fed either normal diet or a K^+-free diet. Food intake was determined daily, and did not differ significantly between mice with intact Rhbg expression and IC-Rhbg-KO mice (data not shown). While on a normal diet, intercalated cell-specific Rhbg deletion did not significantly alter serum potassium concentration in mice on normal diet; serum potassium averaged 4.1 ± 0.2 mmol/L in mice with intact Rhbg expression and 4.1 ± 0.1 mmol/L in IC-Rhbg-KO mice (P=NS, N=8 in each group). Following three days of dietary potassium depletion, serum potassium averaged 3.2 ± 0.1 mmol/L in mice with intact Rhbg expression and 3.7 ± 0.2 mmol/L in IC-Rhbg-KO mice. Thus, three days of a K^+-free diet induced hypokalemia in mice with intact Rhbg expression and in mice with IC-Rhbg-KO ($P < 0.001$, N=8 in each group); the degree of hypokalemia did not differ significantly between mice with intact Rhbg expression versus those with IC-Rhbg-KO (P=NS, N=8 in each group).

Serum bicarbonate averaged 19.1 ± 0.9 and 19.1 ± 0.7 mmol L^{-1} in control and IC-Rhbg-KO mice on regular diet, respectively (N = 8 in each group) and 19.8 ± 0.8 and 20.2 ± 0.8 mmol L^{-1} in control and IC-Rhbg-KO mice on a K^+-free diet, respectively (N = 8 in each group). Serum bicarbonate did not change significantly in response to

hypokalemia, either in mice with intact or with intercalated cell-specific Rhbg deletion (P=NS by ANOVA). Also, intercalated cell-specific Rhbg deletion did not alter serum bicarbonate significantly, either while on regular diet or on 0 K+ diet (P = NS by ANOVA, N = 8 in each group).

A K^+-free diet induced mild polyuria, but neither baseline urine volume nor hypokalemia induced-increases in urine volume differed significantly between mice with intact Rhbg expression and intercalated cell-specific Rhbg deletion (data not shown). Similarly, there was no significant difference in serum sodium concentration, either under baseline conditions or with dietary potassium depletion, in either control or IC-Rhbg-KO mice (data not shown).

GFR was estimated using measurement of creatinine clearance. While on regular diet, creatinine clearance did not differ significantly between mice with intact Rhbg expression and those with IC-Rhbg-KO, respectively (intact, 363 ± 40 μl min^{-1}; IC-Rhbg-KO, 397 ± 78 μl min^{-1}, n=4 in each group, P=NS). After three days of a K+-free diet, creatinine clearance did not differ significantly between mice with intact Rhbg expression and those with IC-Rhbg-KO, respectively (intact, 466 ± 37 μl min^{-1}; IC-Rhbg-KO, 375 ± 30 μl min^{-1}, n=8 in each group, P=NS). In addition, hypokalemia did not change creatinine clearance significantly, either in mice with intact or with intercalated cell-specific Rhbg deletion (P=NS by ANOVA). Thus, IC-Rhbg-KO did not significantly alter GFR, either under basal conditions or in response to a K+-free diet for three days.

Urinary Ammonia Excretion

Basal ammonia excretion rates did not differ significantly between mice with intact Rhbg expression and with IC-Rhbg-KO (P=NS, N=8 in each group; Figure 2-1A), as we

reported previously (5). Hypokalemia increased total urinary ammonia excretion significantly both in mice with intact Rhbg expression and in mice with IC-Rhbg-KO. However, both total urinary ammonia excretion (Figure 2-1A) and the increase above the basal excretion rate in response to a K+-free diet (Figure 2-1B), a direct measure of an individual animal's response to hypokalemia, were significantly less in IC-Rhbg-KO mice than in mice with intact Rhbg expression ($P<0.05$ on each day, $n=8$ in each group at each time point). IC-Rhbg-KO decreased hypokalemia-induced changes in urinary ammonia excretion by 68 ± 9%, 52 ± 18% and 48 ± 15% on days 1, 2 and 3, respectively ($P<0.05$ for each comparison, $N=8$ in each group at each time point).

Changes in urinary pH often correlate with changes in urinary ammonia excretion, with, in general, more acidic urine pH associated with greater rates of ammonia excretion and more alkaline urine associated with less ammonia excretion. The most common exception to this correlation is hypokalemia, which typically increases urine pH. We observed in these studies that hypokalemia increased urine pH in both control and IC-Rhbg-KO mice. In contrast to the effects of IC-Rhbg-KO on ammonia excretion, IC-Rhbg-KO did not alter urinary pH in hypokalemic mice significantly (Figure 2-1C). Thus, differences in urinary pH do not mediate the differences in urinary ammonia excretion in response to hypokalemia.

Changes in total urinary ammonia excretion require either increases in ammonia concentration or increases in urine volume. As noted previously, Rhbg deletion did not alter hypokalemia-induced changes in urine volume. However, there was a significant difference in the effect of hypokalemia on urinary ammonia concentration. In mice with intact Rhbg expression, hypokalemia increased urinary ammonia concentration

significantly (Figure 2-1D). In contrast, in mice with intercalated cell-specific Rhbg deletion hypokalemia did not alter significantly urinary ammonia concentration (P=NS on each day of hypokalemia, N=8 on each day). Thus, intercalated cell-specific Rhbg deletion significantly impairs the expected increase in total urinary ammonia excretion and completely blocks changes in urinary ammonia concentration, and these changes are independent of changes in urine pH.

Effect of Hypokalemia on Rhbg Expression

The observation that intercalated cell-specific Rhbg deletion impairs the response to hypokalemia, but does not alter basal urinary ammonia excretion, suggests that Rhbg's role in ammonia excretion increases in response to hypokalemia. To examine this further, we determined the effect of hypokalemia on renal Rhbg expression using both immunoblot analysis and immunohistochemistry.

Immunoblot analysis showed that hypokalemia increased Rhbg protein expression significantly in both the cortex and the outer medulla in mice with intact Rhbg expression (Figure 2-2). Immunohistochemistry was used to determine whether hypokalemia increased Rhbg expression in intercalated cells, non-intercalated cells, or both. Hypokalemia increased basolateral Rhbg immunolabel in intercalated cells, but not in principal cells, in the cortical collecting duct (CCD) and in the outer medullary collecting duct (OMCD). Figure 2-3 shows representative micrographs demonstrating these findings. Thus, hypokalemia-induced increases in urinary ammonia excretion and urinary ammonia concentration is associated with increased Rhbg expression in intercalated cells in the CCD and OMCD.

Adaptive Responses to Intercalated Cell-specific Rhbg Deletion in Response to Hypokalemia

In a variety of conditions, adaptive responses in other proteins involved in renal ammonia metabolism can often compensate, either partially or completely, for the absence of the Rh glycoproteins, Rhbg and Rhcg (5, 17, 18). In preliminary studies, we confirmed that hypokalemia increased phosphate-dependent glutaminase, phosphoenolpyruvate carboxykinase and Rhcg expression and decreased glutamine synthetase expression in mice with intact Rhbg expression (data not shown). We then assessed in hypokalemic mice whether intercalated cell-specific Rhbg deletion induced alterations in the expression of these critical proteins in renal ammonia metabolism. Figure 2-4 summarizes these results. In hypokalemic mice, IC-Rhbg-KO did not alter expression of PEPCK, PDG, glutamine synthetase or Rhcg. Adaptive changes in the expression of these proteins does not appear to compensate for the absence of Rhbg-mediated ammonia transport in hypokalemic mice.

Correlation of Urinary K^+ and Ammonia Excretion

The well-known association of increased ammonia excretion and decreased urinary K^+ excretion in response to hypokalemia has sometimes suggested that there may be either direct or indirect NH_4^+ for K^+ exchange mechanism that regulates urinary K^+ excretion. To begin examining whether hypokalemia-induced changes in ammonia and K^+ excretion are directly related, we examined the effect of IC-Rhbg-KO, and its effect on urinary ammonia excretion, on urinary K+ excretion. Hypokalemia decreased urinary K^+ excretion rapidly in both control and IC-Rhbg-KO mice, and there was no significant difference between control mice and IC-Rhbg-KO mice (Figure 2-5A). Thus, inhibiting ammonia excretion, by IC-Rhbg-KO, did not alter net urinary K+ excretion.

Figure 2-5B shows summary data correlating changes in urinary K^+ and ammonia excretion in response to hypokalemia in control and IC-Rhbg-KO mice. IC-Rhbg-KO altered the slope of the correlation between changes in urinary K^+ and ammonia excretion significantly ($P<0.05$ by ANOVA).

Discussion

These studies provide the first detailed assessment of the effect of Rhbg deletion on the renal response to hypokalemia. In mice with intact Rhbg expression, hypokalemia increased total urinary ammonia excretion, urinary ammonia concentration, and renal Rhbg expression in intercalated cells. In mice with IC-Rhbg-KO, the expected increase in total urinary ammonia excretion was significantly blunted and the expected increase in urinary ammonia concentration changes was prevented. These changes occurred despite no significant effect of IC-Rhbg-KO on urinary pH. Thus, increased Rhbg expression in intercalated cells mediates a significant role in the increased urinary ammonia excretion observed in response to hypokalemia. Although IC-Rhbg-KO altered ammonia excretion significantly, it did not alter urinary K+ excretion, indicating that there is not a direct correlation between ammonia and K+ excretion in response to hypokalemia.

The primary finding of this study is that intercalated cell-specific Rhbg expression is necessary for the normal hypokalemia-induced changes in urinary ammonia excretion. Dietary potassium restriction in mice with intact Rhbg expression increased Rhbg protein expression in intercalated cells in both the CCD and OMCD and this was associated with increased renal ammonia excretion. In mice lacking Rhbg in intercalated cells the increase in total urinary ammonia excretion was significantly impaired, and there was no significant change in urinary ammonia concentration. Thus,

intercalated cell Rhbg expression is critical for the normal increases in ammonia excretion in response to hypokalemia.

This study of Rhbg's role in hypokalemia, along with the previous study in the metabolic acidosis model, show a clear role for Rhbg in renal ammonia metabolism. Mouse studies involving metabolic acidosis and hypokalemia have shown increases of ~ 300% in urinary ammonia excretion for metabolic acidosis and ~ 200% for hypokalemia, when Rhbg is knocked out in intercalated cells, these increase are only ~ 150% and ~ 75%. Because other disease states do not result in changes in urinary ammonia excretion as metabolic acidosis and hypokalemia, a more complete knockout of Rhbg will be required in order to study adaptive responses.

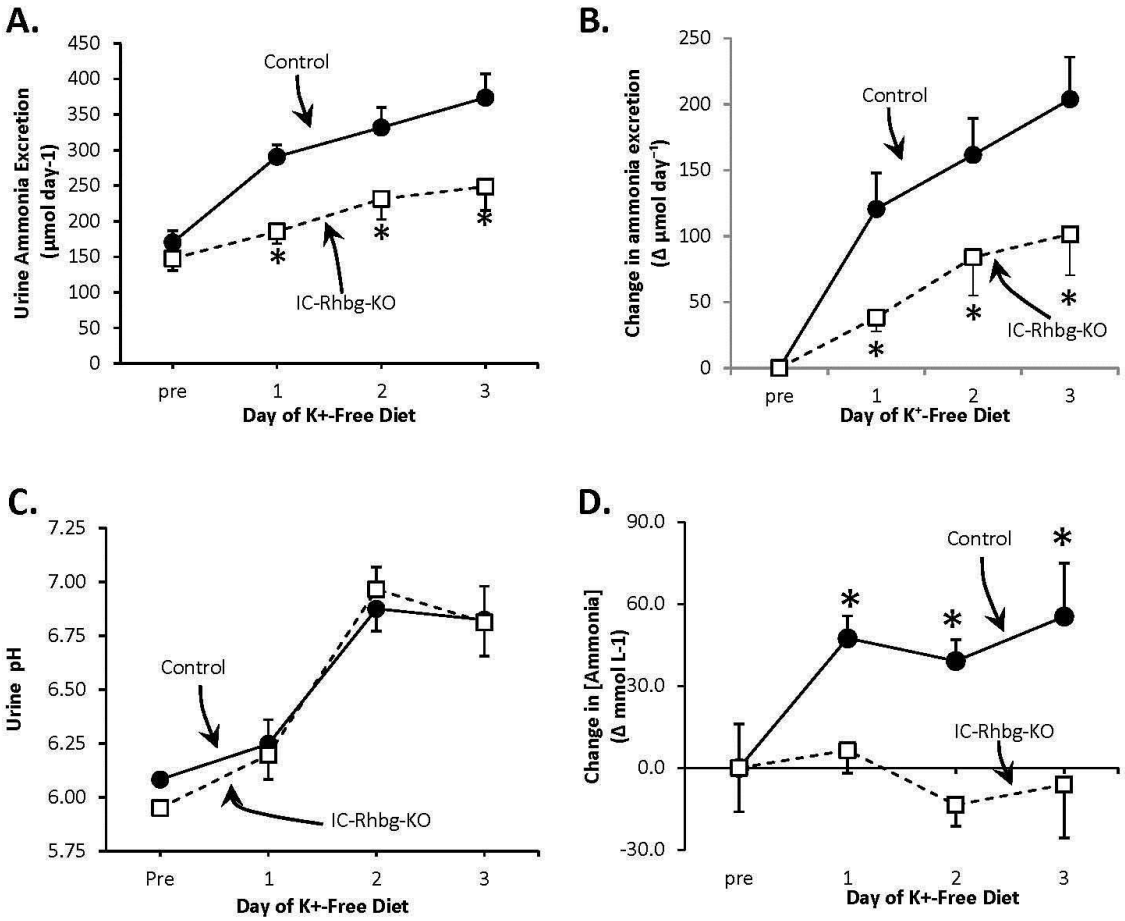

Figure 2-1 Effect of hypokalemia on urinary ammonia excretion. Panel A shows baseline urinary ammonia excretion ("Pre") and urine ammonia excretion on each day of a K^+-free diet. Urinary ammonia excretion increased each day in both control and IC-Rhbg-KO mice, but ammonia excretion was significantly less in IC-Rhbg-KO mice on each day of hypokalemia than in control mice (*, $P < 0.05$, N = 8 in each group). Panel B shows the change in urinary ammonia excretion from baseline rate in response to a K^+-free diet in control and IC-Rhbg-KO mice. The increase in urinary ammonia excretion was significantly less in IC-Rhbg-KO mice (N = 8 in each group on each day). Panel C shows urinary pH. Urine pH did not differ between control and IC-Rhbg-KO mice on any day of the K^+-free diet (N = 8 in each group on each day). Panel D shows changes in the concentration of ammonia in the urine in response to hypokalemia. Urinary ammonia concentration increased significantly on each day of the K^+-free diet in control mice, and did not change significantly from baseline urinary ammonia concentration in IC-Rhbg-KO mice (*, $P < 0.05$ vs baseline, N = 8 in each group).

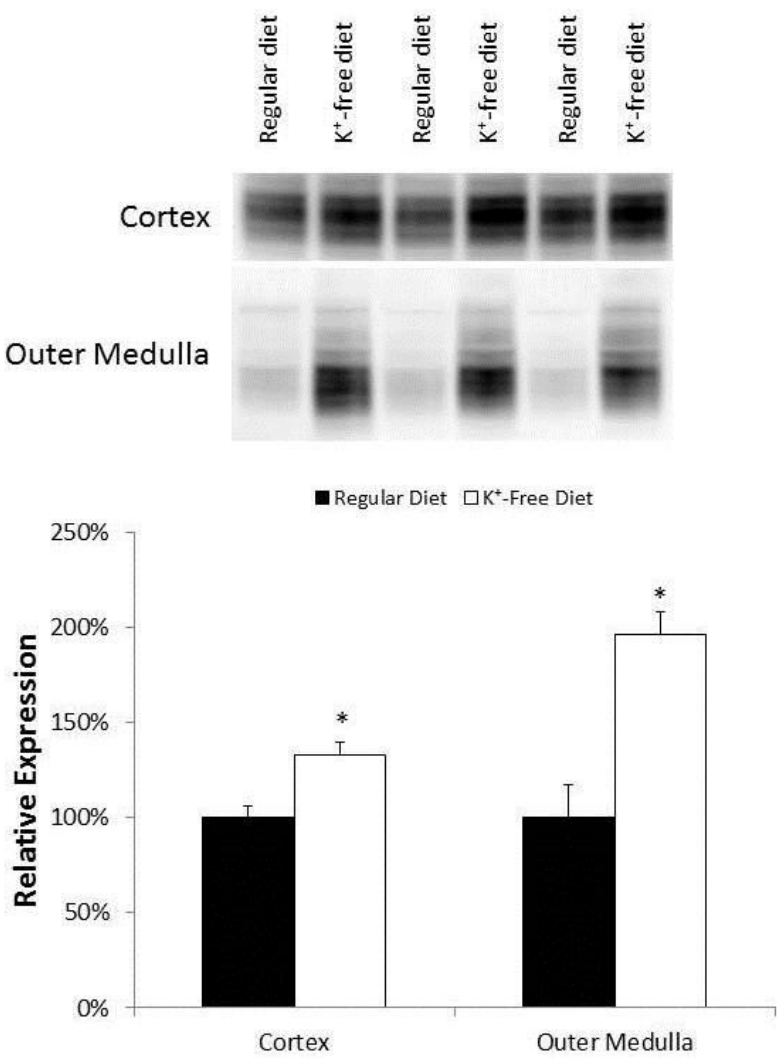

Figure 2-2 Effect of hypokalemia on renal Rhbg expression. Top Panel shows representative immunoblot analyses and bottom panel shows summary quantitative analyses of Rhbg protein expression in the cortex and outer medulla in response to K^+-free diet for three days. Rhbg protein expression increased significantly in both the cortex and the outer medulla (*, $P < 0.05$; N = 8 in each group).

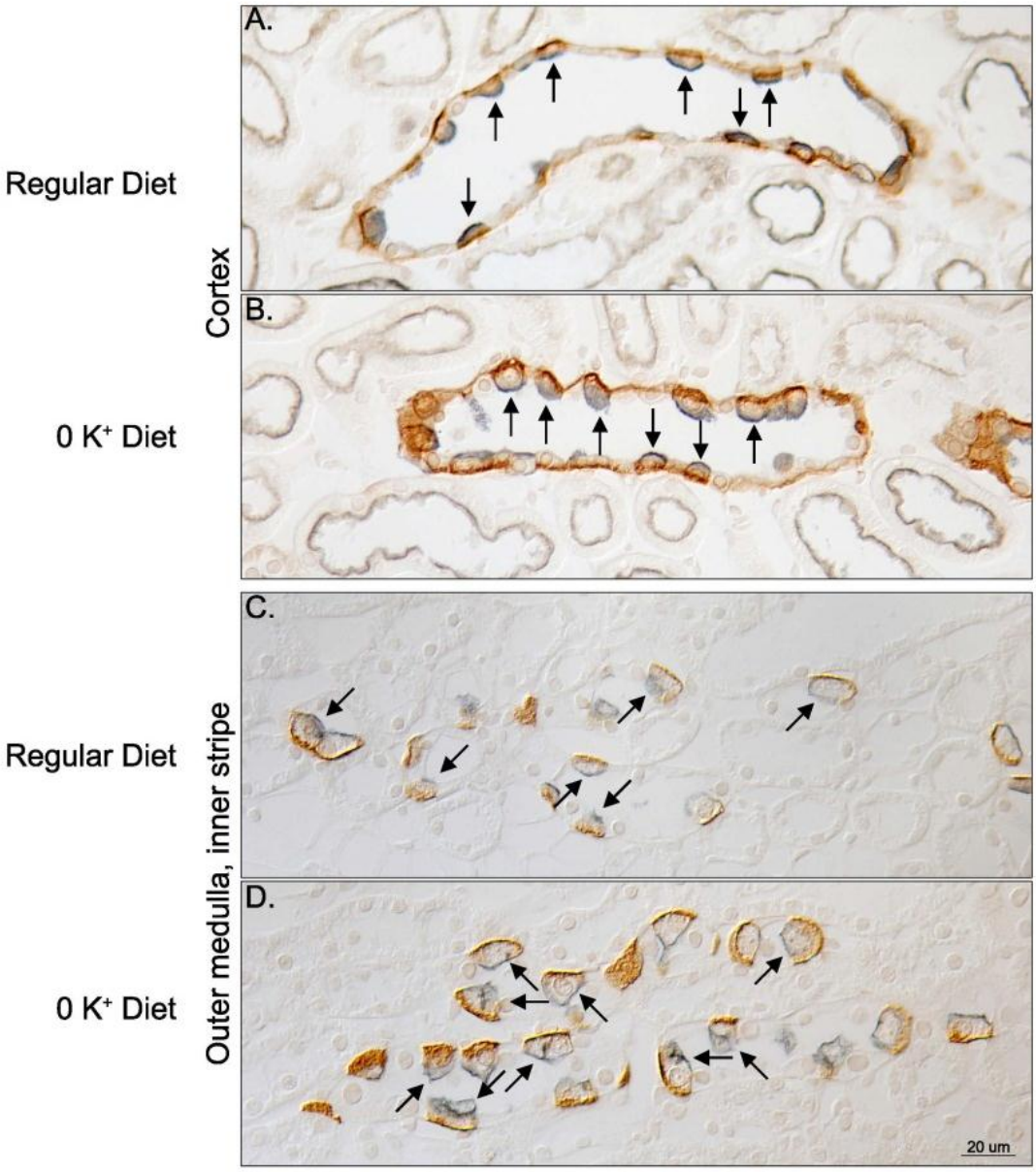

Figure 2-3 Rhbg expression by immunohistochemistry in response to hypokalemia.Rhbg immunolabel is shown in brown, and H^+-ATPase immunolabel in blue, used to differentiate between principal cells (H^+-ATPase-negative collecting duct cells) and intercalated cells (apical H^+-ATPase-positive collecting duct cells). In the CCD (Panels A and B), basolateral Rhbg immunolabel intensity increased in response to K^+-free diet in intercalated cells (arrows). There was no detectable change in principal cell basolateral Rhbg immunolabel in the CCD. In the OMCD (Panels C and D), Rhbg immunolabel increased in intercalated cells (arrows), but not in principal cells in response to the K^+-free diet. Images are representative of findings in 6 mice in each group.

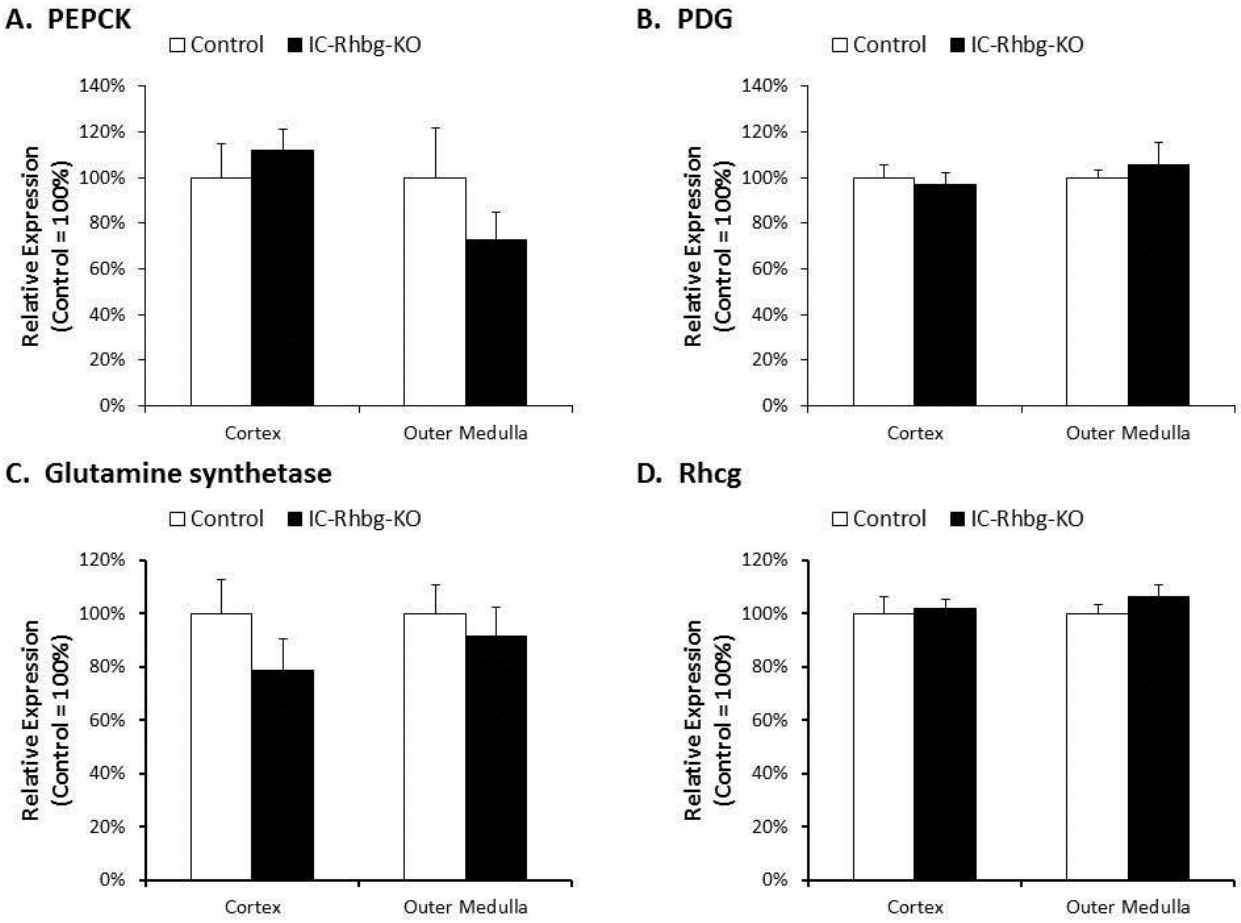

Figure 2-4 Adaptive responses of proteins involved in renal ammonia metabolism in response to IC-Rhbg-KO in hypokalemic mice. This figure summarizes experiments performed to determine whether adaptive changes in the expression of other proteins involved in renal ammonia metabolism compensate partially for the lack of Rhbg expression in intercalated cells. This shows a comparison of expression in hypokalemic mice with intact Rhbg expression to hypokalemic mice with IC-Rhbg-KO. Panels A-D show PEPCK, PDG, glutamine synthetase, and Rhcg protein expression, respectively. There were no significant differences in the expression of any of these proteins, either in cortex or outer medulla, between hypokalemic mice with intact or intercalated cell-specific Rhbg deletion (N = 8 in each group).

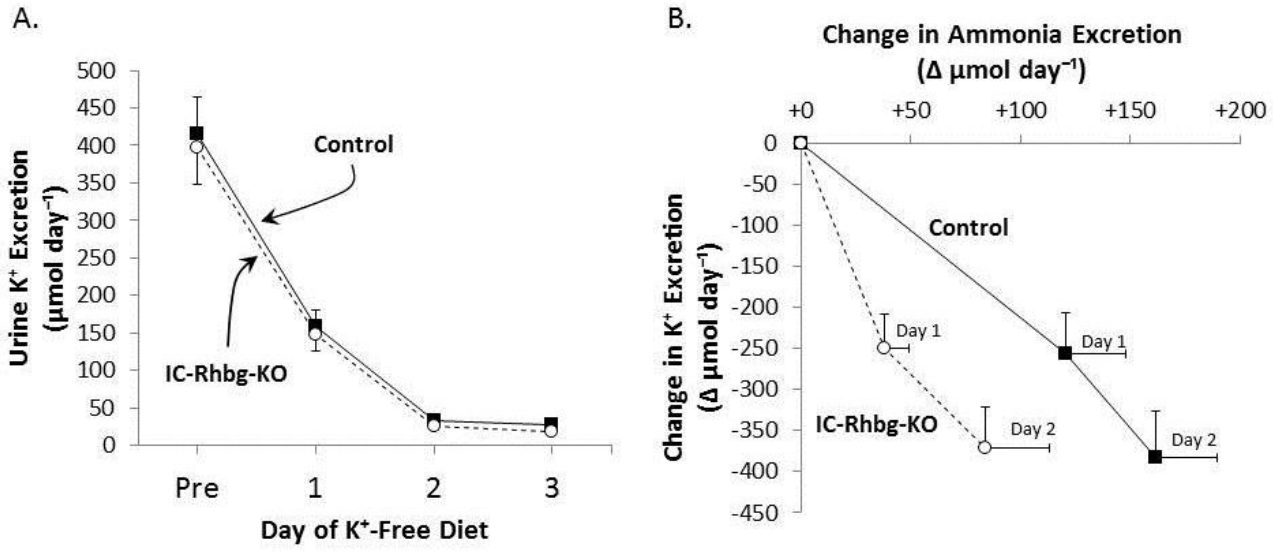

Figure 2-5 Correlation of changes in urinary K^+ and ammonia excretion in control and IC-Rhbg-KO mice in response to K^+-free diet. Panel A shows urinary K^+ excretion in response to K^+-free diet in control and IC-Rhbg-KO mice. Urinary K^+ excretion decreased rapidly in response to the K^+-free diet, and did not differ significantly between control and IC-Rhbg-KO mice on any day of the K^+-free diet (P = NS, N = 8 in each group). Panel B correlates the change in urinary ammonia with the change in K^+ excretion in response to K^+-free diet in control and IC-Rhbg-KO mice. The correlation differed significantly in control and IC-Rhbg-KO mice.

CHAPTER 3
CHARACTERIZATION OF COLLECTING DUCT KNOCKOUT RHBG MICE

INTRODUCTION

In order to better study Rhbg's role in renal ammonia metabolism, an additional animal model has been developed. Knocking out Rhbg in collecting duct intercalated cells has shown that Rhbg is important in overall renal ammonia metabolism, and also specifically that principal cell-mediated ammonia excretion is important to acid-base homeostasis. Here, a collecting duct knockout of Rhbg (CD-Rhbg-KO) is described. Whereas in the IC-Rhbg-KO animal model, Rhbg expression remained intact in principal cells, the goal of the CD-Rhbg-KO animal model is to study the effect of knocking out Rhbg in the entire collecting duct, and not just in principal cells. Future studies dealing with changes in urinary ammonia excretion of a lower magnitude will be made possible by CD-Rhbg-KO mice.

Methods

Animals

Mice used in this project were the result of mating mice homozygous for floxed Rhbg alleles and expressing Ksp-cadherin-Cre with mice homozygous for floxed Rhbg alleles but not expressing Ksp-cadherin-Cre. The generation of mice with loxP sites flanking critical exons of the murine Rhbg gene have been described previously (5). Transgenic mice expressing Cre-recombinase under control of the 1329 bp of the Ksp-cadherin promoter have been described previously (13). Animal breeding was performed in the University of Florida College of Medicine Cancer and Genetics Transgenic Animal Core Facility by trained personnel. Mice were genotyped using tail-clip samples as described previously (15, 18). All mice used in this project were either

floxed Rhbg, Ksp-Cre-positive or floxed Rhbg, Ksp-Cre-negative. All animal studies were approved by the Institutional Animal Care and Use Committees of the Gainesville VA Medical Center and of the University of Florida College of Medicine.

Antibodies

Affinity-purified antibodies to Rhbg have been previously characterized (15, 17, 21, 38). In particular, studies have shown the specificity of the Rhbg antibodies in studies using heterologous expression in Xenopus oocytes (21) and in studies using genetic deletion of Rhbg and Rhcg (15, 17).

Tissue Preparation for Immunolocalization

Mice were anesthetized with inhalant isoflurane. The kidneys were preserved by in vivo cardiac perfusion with PBS (pH 7.4) followed by periodate-lysine-2% paraformaldehyde (PLP) and then cut transversely into several 2- to 4-mm-thick slices and immersed 24 to 30 hours at 4°C in the same fixative. For light microscopy, samples of kidney from each animal were embedded in polyester wax (polyethylene glycol 400 distearate (Polysciences, Warrington, PA) with 10% 1-hexadecanol), and 3-μm-thick sections were cut and mounted on gelatin-coated glass slides.

Immunohistochemistry

Immunolocalization was accomplished using immunoperoxidase procedures detailed previously (15, 17). Tissue sections were dewaxed in ethanol, rehydrated and then rinsed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in Peroxidase Blocking Reagent (DakoCytomation, Carpinteria, CA) for 45 min. The sections were blocked for 15 min with Serum-Free Protein Block (DakoCytomation), then incubated at 4°C overnight with primary antibody diluted in Dako Antibody Diluent. The sections were washed in PBS and incubated for 30 min

with polymer-linked peroxidase conjugated goat anti-rabbit IgG (MACH2, Biocare Medical), again washed with PBS, then exposed to diaminobenzidine for 5 minutes. The sections were washed in distilled water, then dehydrated in a graded series of ethanols and xylene, mounted and observed by light microscopy. Comparisons of labeling were made only 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). Color and contrast adjustment was performed using Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA).

Results

Recombination in CD-Rhbg-KO mice

Transgenic mice with LoxP sites in introns flanking critical exons in the mouse Rhbg gene were bred with transgenic mice expressing Cre-recombinase under control of the 1,329-bp Ksp-cadherin promoter (13). Mice homozygous for floxed Rhbg alleles and expressing Ksp-cadherin-Cre were identified. Littermates homozygous for floxed Rhbg alleles but not expressing Ksp-cadherin-Cre were used as control mice. PCR amplification of tail clip DNA was used to confirm recombination in Ksp-cadherin-Cre mice. There was no recombination in Ksp-cadherin-Cre negative mice (data not shown).

Rhbg Expression in CD-Rhbg-KO Mice

The Ksp-cadherin-cre negative mice (control) showed typical Rhbg immunolabel in the kidney with basolateral expression in the distal convoluted tubule (DCT), CNT, initial collecting tubule, and collecting duct (Figure 3-1 A and C). There was nearly complete loss of Rhbg immunolabel in the CD-Rhbg-KO mice (Figure 3-1B and D). The only

Rhbg immunolabel found in the CD-Rhbg-KO mice was found in the CNT and DCT. These results were similar to those published in reports studying collecting duct Rhcg knockout mice using the same Ksp-Cadherin-cre promoter (15, 17).

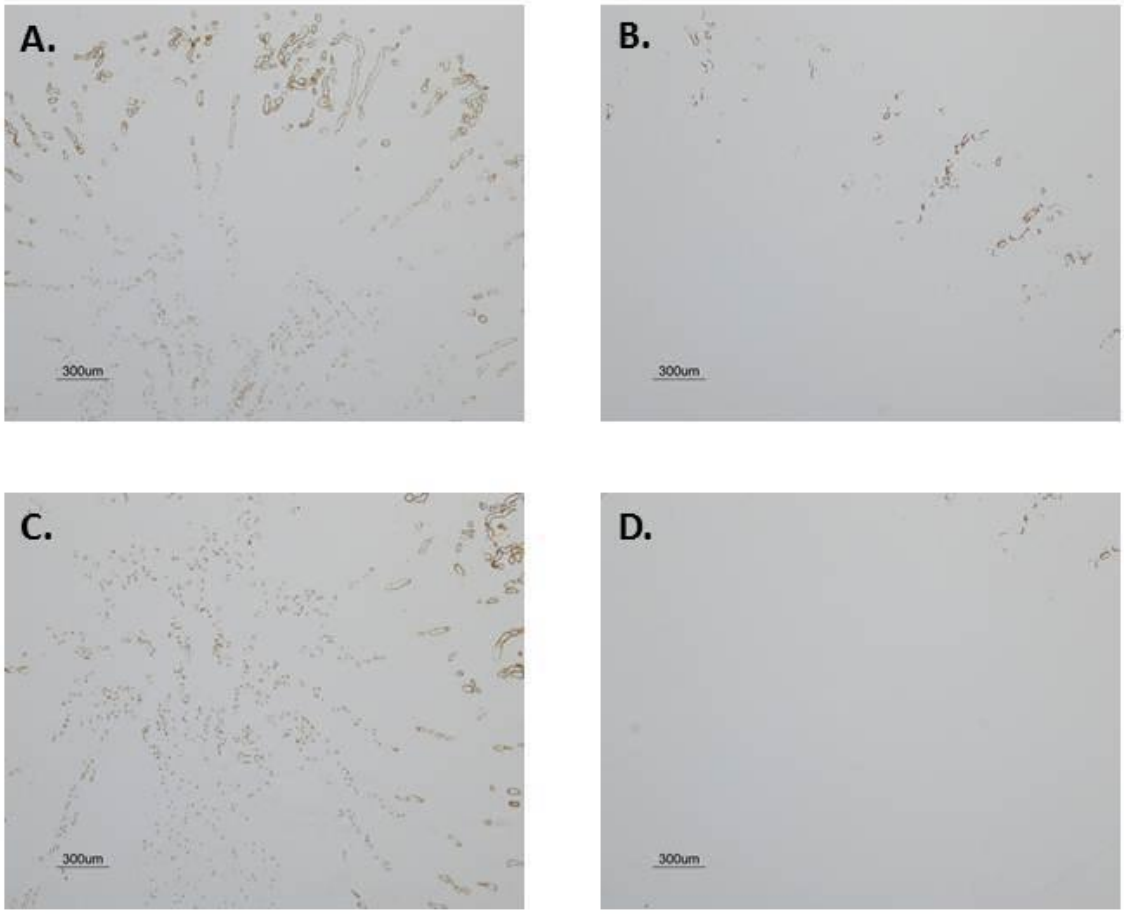

Figure 3-1 Rhbg immunolabel in CD-Rhbg-KO and control mice. Panel A and C show typical Rhbg expression in the cortex, outer medulla, and inner medulla of control mice. Panel B shows that the majority of Rhbg immunolabel has been knocked out, with only some left in the CNT and DCT. Panel D shows that in the outer medulla and inner medulla there is no Rhbg immunolabel in KD-Rhbg-KO mice.

CHAPTER 4
WORKS IN PROGRESS AND FUTURE DIRECTIONS

The primary goal of developing CD-Rhbg-KO mice was to study the role of Rhbg in response to a low protein diet. Is It has been well documented that the typical western diet contains a very high protein content that generates an acid load that over time, can lead to a mild metabolic acidosis (1, 37). The increased intake of protein leads to increased net acid excretion, the predominant component of that being urine ammonia excretion (12, 31), and in studies in both humans and rats, a low protein diet results in decreased urinary ammonia excretion (34). The low grade metabolic acidosis brought on by high protein diets leads to low urine pH, hyocitraturia and hypercalciuria which can also lead to kidney stone disease (1).

The purpose of the current study is to further investigate the renal response to changes in dietary protein intake. To do this, mice were placed on a low protein diet or a calorie matched control diet. Collecting duct knock out Rhbg mice were bred to study the effect of knocking out an ammonia transport protein in response to the low protein diet.

Methods

Animals

Mice used in this project were the result of mating mice homozygous for floxed Rhbg alleles and expressing Ksp-cadherin-Cre with mice homozygous for floxed Rhbg alleles but not expressing Ksp-cadherin-Cre. The generation of mice with loxP sites flanking critical exons of the murine Rhbg gene have been described previously (5). Transgenic mice expressing Cre-recombinase under control of the 1329 bp of the Ksp-cadherin promoter have been described previously (13). Animal breeding was

performed in the University of Florida College of Medicine Cancer and Genetics Transgenic Animal Core Facility by trained personnel. Mice were genotyped using tail-clip samples as described previously (15, 18). All mice used in this project were either floxed Rhbg, Ksp-Cre-positive or floxed Rhbg, Ksp-Cre-negative. All animal studies were approved by the Institutional Animal Care and Use Committees of the Gainesville VA Medical Center and of the University of Florida College of Medicine.

Antibodies

Affinity-purified antibodies to Rhbg have been previously characterized (15, 17, 21, 38). In particular, studies have shown the specificity of the Rhbg antibodies in studies using heterologous expression in Xenopus oocytes (21) and in studies using genetic deletion of Rhbg and Rhcg (15, 17).

Dietary Protein Restriction

Dietary protein intake was controlled through the use of Harlan-Teklad custom research diets. A 20% protein diet (TD.91532 Harlan Teklad, Madison, WI) was used as the control diet, or normal protein diet and a 6% protein diet (TD.90016 Harlan Teklad, Madison, WI) was used as the low protein diet. Powdered food was mixed with H_2O in a ratio of 6 gram food to 1 ml H_2O to form a semi-solid diet. Adult mice, greater than 8 weeks age, were placed into metabolic cages (Tecniplast diuresis metabolic cage, Fisher Scientific) and allowed to acclimate for three days while receiving control diet. They received low protein or control diet and daily food intake was measured. At all times animals were allowed free access to water. Daily urine excretion was collected under mineral oil;, urine pH was measured and urine volume calculated. Urine samples were stored at -80°C until analyzed.

Tissue Preparation for Immunolocalization

Mice were anesthetized with inhalant isoflurane. The kidneys were preserved by in vivo cardiac perfusion with PBS (pH 7.4) followed by periodate-lysine-2% paraformaldehyde (PLP) and then cut transversely into several 2- to 4-mm-thick slices and immersed 24 to 30 hours at 4°C in the same fixative. For light microscopy, samples of kidney from each animal were embedded in polyester wax (polyethylene glycol 400 distearate (Polysciences, Warrington, PA) with 10% 1-hexadecanol), and 3-µm-thick sections were cut and mounted on gelatin-coated glass slides.

Immunohistochemistry

Immunolocalization was accomplished using immunoperoxidase procedures detailed previously (15, 17). Tissue sections were dewaxed in ethanol, rehydrated and then rinsed in PBS. Endogenous peroxidase activity was blocked by incubating the sections in Peroxidase Blocking Reagent (DakoCytomation, Carpinteria, CA) for 45 min. The sections were blocked for 15 min with Serum-Free Protein Block (DakoCytomation), then incubated at 4°C overnight with primary antibody diluted in Dako Antibody Diluent. The sections were washed in PBS and incubated for 30 min with polymer-linked peroxidase conjugated goat anti-rabbit IgG (MACH2, Biocare Medical), again washed with PBS, then exposed to diaminobenzidine for 5 minutes. The sections were washed in distilled water, then dehydrated in a graded series of ethanols and xylene, mounted and observed by light microscopy. Comparisons of labeling were made only 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). Color and contrast adjustment was performed using Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA).

Results

Effect of Low Protein Diet on Rhbg Expression

Immunohistochemistry was used to determine whether low protein diet increased Rhbg expression. Preliminary results showed increased Rhbg expression in the cortex and outer medulla (Figure 4-1). Further immunohistochemistry and immunoblot studies will be necessary to confirm these results and further identify what cell types these increases occur in.

Urine Volume and Urine pH

Urine volume remained constant for all four experimental groups throughout the study (Figure 4-2).

Urine pH was increased in both groups of animals that were placed on the low protein diet, while the animals placed on control protein diet remained constant throughout the study.

Future Directions

Future studies will be required to identify other mechanisms involved in the renal response to a protein restricted diet. These experiments will include measurement of renal ammonia excretion in response to the low protein diet to determine if this animal model results in the expected decreases in urine ammonia excretion that have been seen in other studies (34). Previous studies have shown that Rhbg expression increases along with increased urinary ammonia excretion. If the preliminary data is accurate and Rhbg is increased in the low protein diet, then there may be other

mechanisms involved in the regulation of Rhbg in this model that have not been seen previously.

It will be necessary to determine if there are altered mechanism of glutamate transport, or changes in renal ammoniagenesis in response to the low protein diet. To study this, glutamate transporter, and glutamate dehydrogenase expression will be quantified by immunohistochemistry and western blotting.

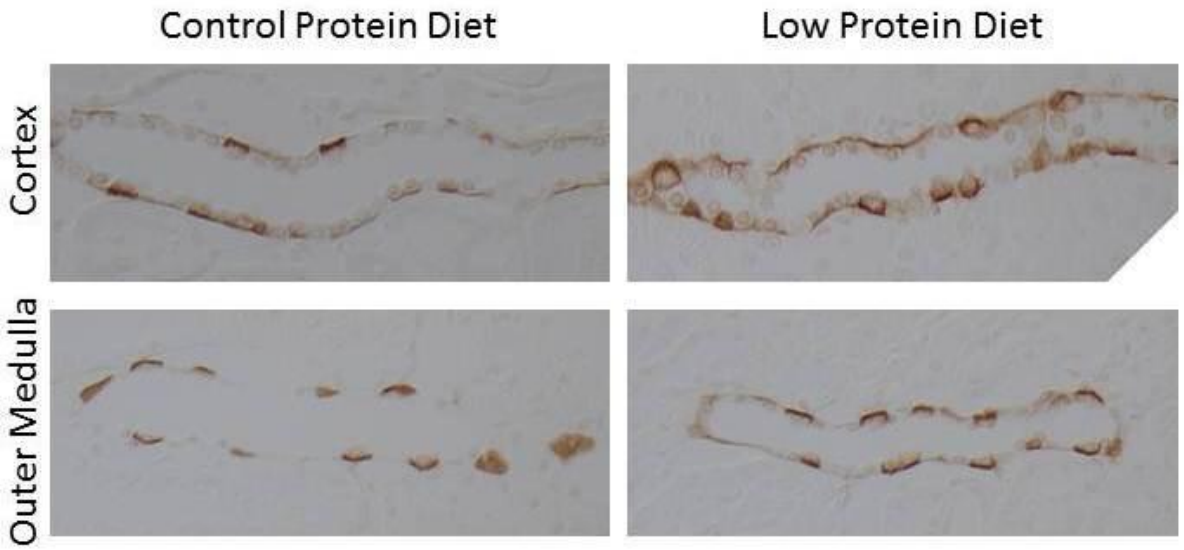

Figure 4-1 Rhbg in the cortex and outer medulla of control and low protein diet mice.The top two panels show Rhbg expression in the cortex in control protein diet and low protein diet. Bottom panels show Rhbg in the outer medulla. From this first set of preliminary studies Rhbg immunolabel is increased in both the cortex and outer medulla in response to the low protein diet.

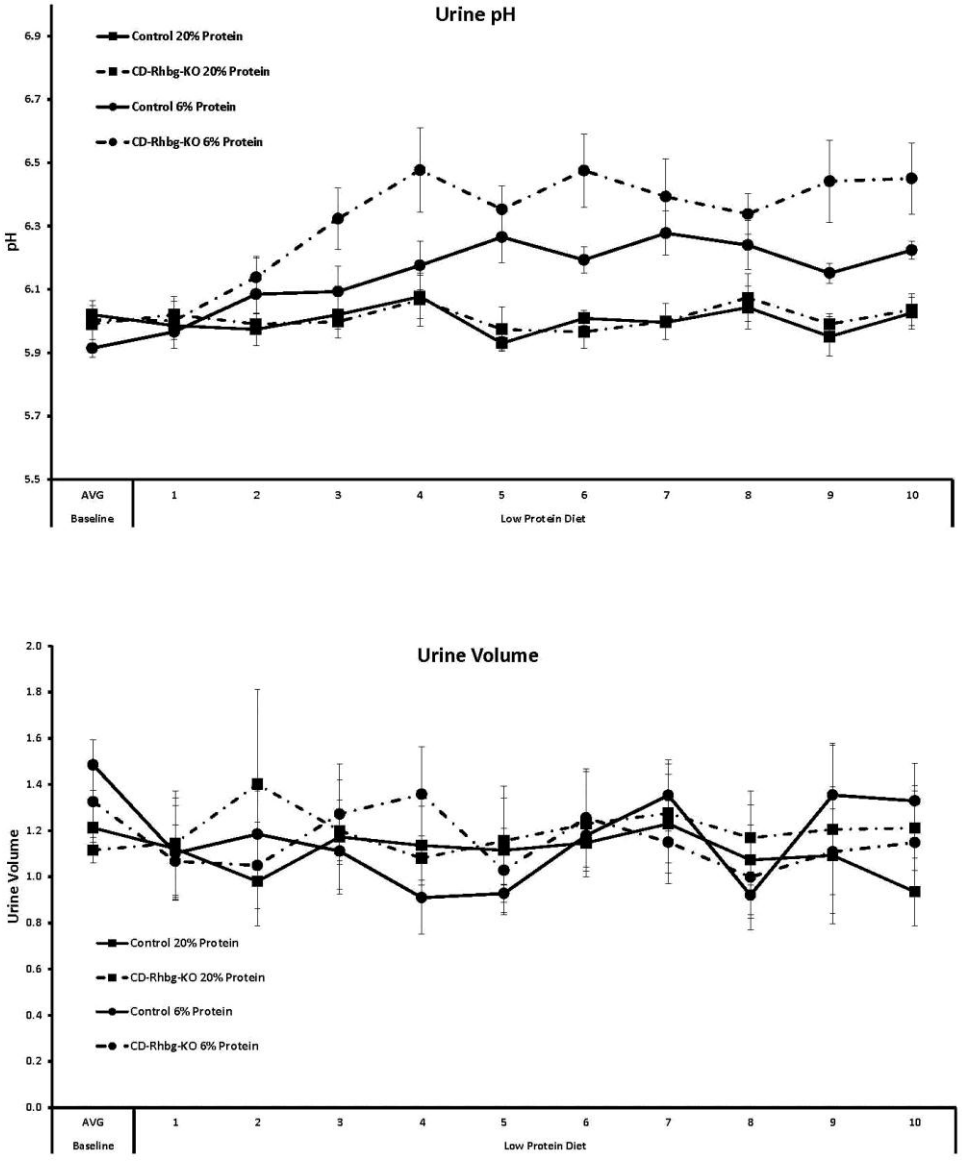

Figure 4-2 Urine Volume and Urine pH in Response to Low Protein Diet. The top panel shows changes in urine pH during the course of the study. Both groups of control protein animals maintained constant urine pH throughout the protocol. Low protein diet resulted in increased urine pH for both the control and CD-Rhbg-KO mice. There was no statistical difference at any time point. Urine volume remained constant for all four experimental groups.

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BIOGRAPHICAL SKETCH

Jesse Mitchell Bishop was born in 1980 in Cheshire, Connecticut. He attended elementary and middle school in Cheshire, prior to moving to Killingworth, Connecticut where he attended and eventually graduated from high school in 1998.

Upon graduation from high school Jesse enlisted in the United States Army. He completed Basic Combat Training and Airborne School in Fort Benning, Georgia prior to attending The Ranger Indoctrination Program. After successful completion of the Ranger Indoctrination Program Jesse was stationed with The 1st Battalion of the 75th Ranger Regiment at Hunter Army Airfield Georgia.

Jesse spent over four years with 1st Ranger Battalion, climbing through the ranks from Private to Sergeant, eventually serving as a team leader while deployed for combat operations in Afghanistan in support of Operation Enduring Freedom in 2002 and 2003.

After leaving the Army Jesse attended college at both Santa Fe College and the University of Florida for his undergraduate course work, eventually earning a degree in Human Nutrition from the University of Florida in 2007. After graduation Jesse went to work in the Lab of Dr. David Weiner in the Nephrology Division at the University of Florida. He continued doing research in Dr. Weiner's Lab after being accepted into the Master's Program in Translational Biotechnology.

Jesse has been married to Crystal Bishop for 1 year. They have a daughter Kathleen, who is currently 2 months old.