Downregulation of claudin-2 expression in renal epithelial cells by metabolic acidosis

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Departments of 1Medicine and 2Cell Biology, University of Alabama at Birmingham, and 3Birmingham Veterans Affairs Medical Center, Birmingham, Alabama; and 4Department of Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio

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Balkovetz DF, Chumley P, Amlal H. Downregulation of claudin-2 expression in renal epithelial cells by metabolic acidosis. Am J Physiol Renal Physiol 297: F604–F611, 2009. First published July 8, 2009; doi:10.1152/ajprenal.00043.2009—Chronic metabolic acidosis (CMA) is associated with an inhibition of fluid reabsorption in the renal proximal tubule. The effects of CMA on paracellular transport across the renal epithelial tight junction (TJ) is unknown. Claudin-2 is a transmembrane TJ-associated protein which confers TJ paracellular permeability to Na+. We examined the effects of CMA on the expression of TJ transport proteins using both in vivo and in vitro models of CMA. The results showed downregulation of claudin-2 mRNA and protein expression in the cortex of rats subjected to the NH4Cl loading model of CMA. Madin-Darby canine kidney (MDCK) and HK-2 cells are models of renal epithelial cells and express claudin-2 protein in their TJ. We examined the effects of acidic pH exposure on the expression of claudin-2 in MDCK and HK-2 renal epithelial cells. Exposure of MDCK cells to pH 6.96 medium caused a significant and reversible decrease in claudin-2 protein abundance. A dose-response analysis of acidic medium exposure of MDCK and HK-2 cells demonstrated a downregulation of claudin-2 protein. The downregulation effect of acidic pH is specific to claudin-2 expression as the expression of other TJ-associated proteins (i.e., claudin-1, -3, -4, and -7, occludin, and zonula occludens-1) remained unchanged compared with control pH (7.40). Collectively, these data demonstrate that CMA downregulates the expression of claudin-2 likely through a direct effect of acidic pH. Potential physiological significance of these changes is discussed.

paracellular transport

CHRONIC METABOLIC ACIDOSIS (CMA) is a clinical condition that is characterized by a decrease in blood pH and bicarbonate concentration and is caused by overproduction of an acid or excessive loss of base. The kidney plays a major role in the maintenance of systemic acid-base balance; thus the most common cause of CMA lasting more than a few weeks is chronic kidney disease (CKD) (33). CMA has been shown to have negative effects on several organ systems including bone loss, muscle wasting, and progression of CKD (19). Thus CKD and CMA appear to each contribute to the progression and development of the other. However, the mechanism by which CMA advances CKD is not known.

In CMA, the kidney undergoes numerous adaptive changes to increase net acid excretion and restore systemic acid-base balance. Renal proximal tubule ammonia synthesis and excretion increase during CMA (27). Other renal adaptive changes that occur in the proximal tubule in response to metabolic acidosis include an increase in active H+ secretion/bicarbonate reabsorption (2, 20), bicarbonate generation (39), and a reduction in the reabsorption of inorganic phosphate and sulfate (major determinants of titratable acidity in the collecting duct) (3). In addition to these adaptive and appropriate responses, several studies have shown that the proximal tubule exhibits a significant reduction in Na+ transport in response to CMA (14, 21, 43). Recent studies in our laboratory demonstrated that CMA is associated with a dual effect on Na+ excretion, with an early natriuresis (within 24 h) mediated through the downregulation of the epithelial Na+ channel (ENaC) in the collecting duct (14). This effect is followed by an increase in both Na+ retention and increased water conservation (4) by day 5 of acidosis. These studies indicate that normal kidneys can ultimately compensate for the decreased fluid reabsorption in the proximal tubule by increasing Na+ and water reabsorption in the collecting duct during chronic metabolic acidosis.

The reabsorption of Na+ in the proximal tubule occurs through both the paracellular [across the tight junction (TJ) between epithelial cells] and transcellular (through the cells via membrane channels and transporters). The paracellular transport of Na+ in the proximal tubule is a passive process and accounts for the reabsorption of ~50% of the filtered Na+ load (29). Presently, very little is known about the effect of CMA on proximal tubule paracellular transport of Na+ across the TJ.

The recent discovery of the claudin family of transmembrane TJ proteins has provided new information on the determinants of paracellular transport in the nephron (6). In particular, claudin-2 is a major determinant of the paracellular transport of Na+ in the proximal tubule (1). Because claudin-2 plays a role in paracellular reabsorption of Na+ in the proximal tubule, we have examined the effects of acid exposure on the expression of claudin-2 both in vitro in renal epithelial cell culture systems as well as in vivo rat model of CMA. In both model systems, the expression of claudin-2 is downregulated in the presence of acidic conditions. These data provide evidence explaining, in part, how proximal tubule Na+ reabsorption is inhibited during CMA.

MATERIALS AND METHODS

Cell culture and transfection. Low-passage Madin-Darby canine kidney (MDCK) strain II cells were used between passages 3 and 10 in these studies. HK-2 cells were kindly provided by Dr. Paul Sanders and cultured as previously described (32, 36). HK-2 cells are a primary proximal tubule cell culture from normal adult renal cortex immortalized by exposure to a recombinant retrovirus containing the HPV 16 E6/E7 genes. The HK-2 cell line is widely used to study human renal proximal tubule epithelial cell biology (36). These cells retain a phenotype indicative of well-differentiated proximal tubule cells (positive for alkaline phosphatase, γ-glutamyltranspeptidase,
leucine aminopeptidase, acid phosphatase, cytokeratin, α-βγ- integrin) (36). In addition, these cells exhibit functional characteristics of renal proximal tubule cells such as Na+-dependent sugar transport, adenylate cyclase responsiveness to parathyroid hormone, and lack of response to antidiuretic hormone (ADH) (36). The rationale for using MDCK strain II cells includes the fact that they are the most widely used cell line for the study of TJ biology in epithelia as well as the observation that this cell line exhibits features also found in proximal tubule epithelial cells. Two strains of MDCK cells have been established: MDCK I and II (35). Several studies report that MDCK II cells exhibit properties of proximal tubule epithelium while MDCK I displays properties of distal tubule epithelium. MDCK II cells possess measurable activities of proximal tubule markers alkaline phosphatase and γ-glutamyl transpeptidase whereas MDCK I cells do not (35). MDCK I cells form epithelial monolayers displaying a high transepithelial resistance (TER) (∼4,400 Ω·cm²) and respond to ADH. MDCK II cells form epithelial monolayers of low TER (∼200 Ω·cm²) and are insensitive to ADH (35). Na+-K+-ATPase activity is twofold greater in MDCK II than MDCK I cells (35), similar to the disparity of Na+-K+-ATPase in vivo where proximal tubules have higher activity than that of the collecting tubule (17). Phospho-ERK 1/2 (active) is observed only in the distal nephron of human kidneys (25), and MDCK I cells express high basal levels of phospho-ERK 1/2 while MDCK II cells have undetectable basal phospho-ERK 1/2 levels (24). The proximal tubule TJ-associated protein Claudin-2 is expressed in MDCK II cells under routine culture conditions but not in MDCK I cells (15). In vivo, Claudin-2 expression is restricted to the “leaky” nephron segments, including the proximal tubule, and is absent in the distal nephron (a “tight” epithelium) (12, 18, 34). MDCK strain II cells were cultured as previously described in modified Eagle’s MEM containing Earle’s balanced salt solution and glutamine supplemented with 5% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (7). In experiments with acidic medium, the pH was adjusted by reducing the concentration of NaHCO₃ in the medium and substituting an equimolar concentration of NaCl in its place.

Rat CMA model. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Sprague-Dawley rats (225-250 g) were allowed free access to regular rat chow and distilled water. After at least 3 days, the rats were divided into two groups. In the first group of rats, CMA was induced using the classic protocol of NH₄Cl (280 mM) added to the drinking water (14). The second group of animals remained on distilled water (control). Another set of rats was divided into two groups: One group was subjected to KCl loading (280 mM) added to the drinking water, and the other group remained on distilled water (control). The access of both control and KCl-loaded rats to water (control). Measurements of TER in MDCK and HK-2 cell monolayers cultured on 0.4-µm pore-size filters at various time points were done using an EVOM electrical resistance system (World Precision Instruments, New Haven, CT) (16). All conditions were established in triplicate for each experiment. TER results are expressed as the percentage of control of the measured resistance (in Ω) multiplied by the area of the filter (1 cm²).

Statistical analysis. Semiquantification of Northern hybridization and immunoblotting band densities was determined by densitometric analysis using appropriate scanners. The data are presented as means ± SD compared with control. Statistical significance between control and experimental groups was determined by one-way ANOVA or Student’s unpaired t-test as needed. P < 0.05 was considered significant.
RESULTS

Acid loading downregulates the expression of claudin-2 mRNA and protein in rat renal cortex. We examined the mRNA expression levels of claudin-2 in the kidney cortex of control and acid-loaded rats. Northern hybridization experiments shown in Fig. 1A indicate that the mRNA expression levels of claudin-2 are decreased 56% \((P < 0.002)\) within 24 h of NH4Cl loading and remained low for the 5-day duration of the experiment compared with control (Fig. 1A). Interestingly, the expression of claudin-2 mRNA was not significantly altered in KCl-loaded animals (Fig. 1B). We also examined the expression of claudin-2 mRNA in the kidney cortex of rats subjected to NaCl loading vs. control (water). In this protocol, we have also limited the access of the animals to both water and NaCl (280 mM) solution to the same amount of fluid consumed by NH4Cl group. The results clearly indicate that restricted NaCl loading did not decrease the expression of claudin-2. If any, there was a slight increase in its expression, but it was not significant. These data are shown in Fig. 1C.

Effect of acid loading on claudin-2 protein expression in the rat renal cortex. Figure 1 demonstrates that 5 days of acid loading result in downregulation of claudin-2 mRNA in renal cortex. To extend this finding, we performed Western blot and immunofluorescent analysis of renal cortex tissue for claudin-2 protein expression from control rats given NH4Cl (280 mM) in the drinking water for 5 days. Mixed blood-gas analysis from the control and 5-day CMA rats demonstrated a statistically significant metabolic acidosis in the rats given NH4Cl in the drinking water. Control rats had a mean arterial pH of 7.406 ± 0.03 while the 5-day CMA rats had a mean arterial pH of 7.25 ± 0.08. The differences in these two arterial pH values were significant \((P = 0.0019)\).

Renal cortex protein lysates were prepared in RIPA buffer. Western blot analysis was performed on equal amounts of lysate protein for claudin-2 and E-cadherin. Figure 2A shows that acidic rat renal cortex expresses less claudin-2 than control rat renal cortex. Figure 2B demonstrates a reduction of punctate claudin-2 in cortical tubules of acidic rats compared with control rats. These data are consistent with the RNA data in control and acidic rats.

Exposure of cultured renal epithelial cells to acidic medium decreases claudin-2 protein. We next examined the effect of exposure of cultured renal epithelial cells to acidic medium on claudin-2 expression. Monolayers of polarized MDCK strain II cells grown on permeable supports, which normally express claudin-2, were exposed to pH 6.96 medium on both the apical and basolateral surface for 0.25, 1, 6, 24, and 48 h. Equal amounts of cell protein lysates were analyzed for claudin-2 expression by Western blot analysis (Fig. 3, A and B). Protein levels of claudin-2 decreased after 6 h of pH 6.96 medium

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**Fig. 1.** mRNA expression of claudin-2 in the kidney cortex. The blots are Northern hybridizations of claudin-2 in the kidney cortex of rats subjected to 280 mM NH4Cl (A), 280 mM KCl (B), or 280 mM NaCl (C) loading and their respective controls. 28S rRNA was used as a constitutive gene for the control of the equity of RNA loading into Northern gels. The bars are the corresponding densitometric analysis showing the mean of claudin-2 mRNA-to-28S rRNA ratio. Thirty micrograms of total RNA from different rats were loaded per lane.
exposure and continued to decrease following 24 and 48 h of exposure. Levels of the cell-cell adhesion of protein E-cadherin were not affected by exposure to pH 6.96 medium. Exposure of cells to pH 6.96 medium for 48 h did not affect cell viability, as determined by reversibility experiments (see below), protein/RNA concentrations, and visual inspection (data not shown).

We also examined the dose-response of MDCK cells to acidic medium exposure on the apical and basolateral surface for a period of 48 h. Tissue culture medium with pH values of 7.40, 7.32, 7.23, 7.17, 7.11, and 6.96 were placed on the apical and basolateral surface of filter-grown MDCK cells for 48 h, cell lysates were prepared, and equal amounts of cell protein lysates were analyzed for claudin-2 expression by Western blot analysis (Fig. 3C). Decreased levels of claudin-2 were observed with decreasing pH values. The lowest levels of claudin-2 expression were observed following 48-h exposure to pH 6.96 medium. Levels of the housekeeping protein α-actin were not affected by acidic medium exposure (Fig. 3C). We also examined the effect of exposure to acidic medium in the immortalized human proximal tubule epithelial cell line HK-2 (36). Following exposure to increasingly acidic medium for a period of 48 h, levels of claudin-2 are reduced in HK-2 cells (Fig. 3D). α-Actin levels (Fig. 3D) and claudin-1 levels (data not shown) remained constant. These data demonstrate that exposure to acidic medium also inhibits expression of claudin-2 in a renal proximal tubule cell line.

Attempts to examine cell surface sensitivity (apical vs. basolateral cell surface exposure) were not successful due to the dissipation of the pH gradient across the MDCK cell monolayer within 1 h (data not shown). For example, placing pH 6.96 tissue culture medium in the basolateral surface compartment and pH 7.4 tissue culture medium in the apical surface compartment resulted in both compartments having an equilibrated pH of ~7.17 and a resulting intermediate decrease in claudin-2 expression similar to that seen in Fig. 3B at pH 7.17 compared with control. The rapid dissipation of the pH gradient across the MDCK cell monolayers is likely due to the
expression of claudin-2, which confers epithelial TJ paracellular permeability to monovalent cations including H⁺.

Specificity of acid exposure on decreasing TJ-associated claudin-2 protein. MDCK cell protein lysates of control and 48 h pH 6.96 medium-exposed MDCK cells were subjected to Western blot analysis for known TJ-associated proteins in MDCK cells (Fig. 4). The data confirm that pH 6.96 exposure of the cells inhibits the expression of claudin-2. However, other TJ-associated proteins (claudins-1–4, -7, occludin, and ZO-1) were not affected by exposure to acidic medium. These data indicate that the inhibition of claudin-2 expression in MDCK strain II cells by pH 6.96 medium exposure is specific with regard to other TJ-associated proteins found in MDCK strain II cells.

Reversibility of decreased claudin-2 expression following acid exposure. We next examined whether correction of the pH 6.96 medium to pH 7.4 restored levels of claudin-2 protein levels in MDCK cells (Fig. 5). In this experiment, MDCK cell monolayers were cultured in pH 7.40 or pH 6.96 medium for 48 h. Then medium was changed to create the following conditions:

- pH 7.4 medium 48 h → pH 7.4 medium 48 h (control)
- pH 7.4 medium 48 h → pH 6.96 medium 48 h (48 h pH 6.96)
- pH 6.96 medium 48 h → pH 7.4 medium 48 h (48 h pH 6.96 → 48 h pH 7.4)
- pH 6.96 medium 48 h → pH 6.96 medium 48 h (96 h pH 6.96)

Cell protein lysates were prepared, and equal amounts of protein were analyzed for claudin-2 and E-cadherin expression. In cells exposed to acid medium for 48 and 96 h, claudin-2 levels were decreased compared with controls. In cells exposed to acid medium for 48 h and then returned to control pH medium for 48 h, the claudin-2 levels returned to control levels. Levels of E-cadherin were not affected by acid exposure. These data indicate that the inhibition of claudin-2 protein expression in MDCK cells by exposure to acidic medium is a reversible process when the pH is returned to normal values.

Effect of acidic medium on TER of cultured renal epithelial cells. The relative permeability of the epithelial TJ barrier is commonly assessed by measuring transepithelial resistance (TER). The TER is a surrogate marker for TJ permeability to small ions in an experimentally applied electrical field in the cell medium (13). Therefore, the measurement of the TER reflects the integrity of the TJ across the epithelial cell monolayer. Claudin-2 is known to reduce the TER of cultured renal epithelial cells (15). To examine the functional significance of acid-induced downregulation of claudin-2, we examined the effect of pH 6.96 medium exposure on the TER of cultured MDCK cells. The results are shown in Fig. 6. Compared with control cells, pH 6.96 exposure resulted in a significant increase in TER after 24 and 48 h. We also observed a similar increase in TER of HK-2 renal epithelial cell monolayers following exposure to acidic medium (data not shown). Because claudin-2 is a major determinant of TER (15), this observation correlates well with the decrease in claudin-2 protein expression following exposure to pH 6.96 medium and provides functional consequences of acid-induced downregulation of claudin-2 in cultured renal epithelial cells.

DISCUSSION

The present studies demonstrate for the first time that metabolic acidosis is associated with a significant downregulation of claudin-2 expression in the proximal tubule cells of rat kidney at both the RNA and protein levels. In the kidney, claudin-2 expression is restricted to the proximal tubule (12, 18). Because claudin-2 expression induces cation-selective channels in TJ of epithelial cells (1) and gene deletion of claudin-2 expression in mice leads to renal Na⁺ wasting (26), we predict the CMA-induced loss of proximal tubule claudin-2 results in increased delivery of Na⁺ to the

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**Fig. 3. Claudin-2 expression in culture renal epithelial cells exposed to pH 6.96 medium.**

*A.* representative Western blots showing the amount of claudin-2, E-cadherin, and α-actin in equal amounts of RIPA lysate protein from control and pH 6.96-exposed Madin-Darby canine kidney (MDCK) cells (0.25, 1, 6, 24, and 48 h). *B.* average of the densitometric analysis of claudin-2 protein/E-cadherin protein ratio obtained from 3 separate blots. *P* < 0.001 for 24 and 48 h samples vs. control samples. Also shown is dose-response of cultured renal epithelial cells to decreasing pH on levels of claudin-2 in MDCK II (*C*) cells and HK-2 cells (*D*).
distal nephron segments. Future studies in this area should include analysis of proximal tubule transport of Na\(^+\)/H\(^+\) using microtubule perfusion. The potential physiological significance of decreased renal claudin-2 expression during CMA is discussed below.

This effect is independent of chloride/salt loading and is likely secondary to changes in systemic pH (acidic pH). Indeed, our studies clearly indicate that lowering the pH of cell culture medium inhibits the expression of the TJ-associated protein claudin-2 in MDCK strain II and HK-2 cells. The expression of other TJ-associated proteins including claudin-1, -3, -4, and -7, occludin, and ZO-1 were not altered by exposure to pH 6.96 medium. While MDCK cells are not recognized as a model of proximal tubule epithelium, they are known for being an unparalleled model to study TJ biology and epithelial cell polarity (1, 5, 15, 23, 38, 42). HK-2 cells are derived from adult renal cortex and recognized as a model to study human renal proximal tubule epithelial cell biology (22, 30, 36). Because both MDCK strain II and HK-2 cells express lower levels of claudin-2 following exposure to acidic medium (similar to in vivo inhibition of renal claudin-2 during CMA), these cell lines will be useful for studying mechanisms of acid-induced inhibition of claudin-2 on the cellular level. However, future study in this area should also include analysis of the effect of acidic medium exposure on claudin-2 in primary proximal tubule epithelial cells.

Several studies have demonstrated that CMA is associated with a significant decrease in reabsorption of Na\(^+\) in the proximal tubule (37, 43). The reabsorption of Na\(^+\) in the proximal tubule occurs through both paracellular and transcellular pathways. The paracellular reabsorption of Na\(^+\) in the proximal tubule is a passive process and accounts for the reabsorption of \(\sim 50\%\) of the filtered Na\(^+\) load (29). Claudin-2 expression in the kidney is restricted to the proximal tubule and the thin descending limb of Henle, both of which are considered to be leaky segments of the nephron. Claudin-2 confers TJ permeability to monovalent cations including Na\(^+\) (1). Thus the downregulation of claudin-2 in the renal cortex would result in diminution of paracellular Na\(^+\) reabsorption and would account for, in part, the reduction of...
Na\(^+\) reabsorption in the proximal tubule. To further characterize the role of CMA-induced downregulation of claudin-2 in the proximal tubule, future studies using perfused, isolated proximal tubules of control and CMA animals to study proximal tubule paracellular permeability should be informative.

CMA is a clinical condition frequently encountered in patients with CKD. CMA is associated with many adverse effects, including progression of CKD (19). The mechanism through which CMA contributes to the progression of CKD is not known. One potential mechanism by which CMA accelerates the progression of CKD is by increasing renal oxygen consumption and metabolic demand. Conditions of increased oxygen consumption and relative kidney hypoxia are thought to contribute to the progression of CKD (28). One of the major contributors to the oxygen demands of the kidney are the active, transcellular, Na\(^+\) dependent, transport processes in the kidney, many of which are critical for acid-base balance. Recent research has shown a linear relationship between kidney oxygen consumption and tubular Na\(^+\) reabsorption (11, 40). The reabsorption of Na\(^+\) in the proximal tubule occurs by both paracellular (between the cells) and transcellular (through the cells) pathways. The paracellular reabsorption of Na\(^+\) in the proximal tubule is a passive process and accounts for the reabsorption of ~50% of the filtered Na\(^+\) load (29). The inhibition of claudin-2 in the proximal tubule would decrease the relative amount of Na\(^+\) reabsorption by the paracellular route. Many of the adaptive mechanisms in proximal tubular transport involve transcellular pathways that are active (requiring energy) and are mediated by transport proteins of the apical and basolateral membrane. We are presently working on testing the hypothesis that during CMA there is a decrease in passive tubular Na\(^+\) reabsorption via claudin-2, an increase in active tubular Na\(^+\) reabsorption, and an increase in kidney oxygen utilization. Collectively, these changes may contribute to the long-term progression of CKD during CMA.

The ultimate renal adaptation to CMA is to eliminate the excess H\(^+\) into the urine. How would downregulation of proximal tubule claudin-2 contribute to this goal? Knockout of claudin-2 in mice causes relative renal Na\(^+\) wasting. The Na\(^+\) wasting is due to loss of reabsorption Na\(^+\) in the proximal tubule (26). One of the major mechanisms for H\(^+\) elimination and HCO\(_3^-\) generation is the Na\(^+\)/H\(^+\) exchanger isoform NHE3 in the proximal tubule. By reducing proximal tubule paracellular reabsorption of Na\(^+\) with downregulation of claudin-2, more Na\(^+\) would be available for NHE3 to facilitate both H\(^+\) secretion/bicarbonate reabsorption and NH\(_4^+\) secretion. In addition, loss of claudin-2 in the proximal tubule would increase delivery of Na\(^+\) to the distal nephron segments. In the collecting duct, the reabsorption of delivered Na\(^+\) is mediated through the ENaC, which generates a negative voltage in the lumen of collecting tubule and thus stimulates the electrogenic secretion of H\(^+\) through the apical H\(^+\)-ATPase of type A intercalated cells. Hence, downregulation of proximal tubule claudin-2 would increase the availability of Na\(^+\) for both proximal and distal tubule H\(^+\)-secreting mechanisms and thus contribute to the correction of CMA. Genetic deletion of claudin-2 in the proximal tubule results in Na\(^+\) wasting (26). Unfortunately, studies reporting the phenotype in mice lacking claudin-2 did not include analysis of acid-base status under conditions of metabolic acid/base stressors. Once these mice become available, this issue can be carefully studied to better clarify the role of renal claudin-2 in acid-base homeostasis.

Which pH sensor initiates the changes in renal claudin-2 expression? Future work will focus on the signaling cascade mediating changes in claudin-2 levels following acid exposure. This determination will provide important clues regarding potential signaling pathways that may be involved with this process. For example, members of the focal adhesion kinase family of tyrosine kinases called PYK2 pH sensors have been shown to activate NHE3 as an adaptation to acidosis (31). Another potential candidate to mediate pH-mediated cell signaling are the G protein pH sensors, such as GPR4 that senses extracellular pH changes in the kidney, leading to cAMP production (41).

In conclusion, metabolic acidosis causes a significant decrease in the expression and activity of TJ-associated protein claudin-2 in the proximal tubule. This effect is specific to claudin-2, independent of chloride loading, and is likely mediated via systemic acidic pH, as indicated by in vitro HK-2 and MDCK cell studies. We postulate that the downregulation of claudin-2 in CMA will promote the increase in transcellular Na\(^+\) reabsorption via NHE3 in the proximal tubule and ENaC in the collecting duct, which in turn will contribute to H\(^+\) secretion and correction of metabolic acido-sis.

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