Hypertonicity Compromises Renal Mineralocorticoid Receptor Signaling through Tis11b-Mediated Post-Transcriptional Control

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ABSTRACT

The mineralocorticoid receptor (MR) mediates the Na⁺-retaining action of aldosterone. MR is highly expressed in the distal nephron, which is submitted to intense variations in extracellular fluid tonicity generated by the corticopapillary gradient. We previously showed that post-transcriptional events control renal MR abundance. Here, we report that hypertonicity increases expression of the mRNA destabilizing protein Tis11b, a member of the tristetraprolin/ZFP36 family, and thereby, decreases MR expression in renal KC3AC1 cells. The 3′-untranslated regions (3′-UTRs) of human and mouse MR mRNA, containing several highly conserved adenylate/uridylate-rich elements (AREs), were cloned downstream of a reporter gene. Luciferase activities of full-length or truncated MR Luc-3′-UTR mutants decreased drastically when cotransfected with Tis11b plasmid, correlating with an approximately 50% shorter half-life of ARE-containing transcripts. Using site-directed mutagenesis and RNA immunoprecipitation, we identified a crucial ARE motif within the MR 3′-UTR, to which Tis11b must bind for destabilizing activity. Coimmunoprecipitation experiments suggested that endogenous Tis11b physically interacts with MR mRNA in KC3AC1 cells, and Tis11b knockdown prevented hypertonicity-elicited repression of MR. Moreover, hypertonicity blunted aldosterone-stimulated expression of glucocorticoid-induced leucine-zipper protein and the α-subunit of the epithelial Na⁺ channel, supporting impaired MR signaling. Challenging the renal osmotic gradient by submitting mice to water deprivation, diuretic administration, or high-Na⁺ diet increased renal Tis11b and decreased MR expression, particularly in the cortex, thus establishing a mechanistic pathway for osmotic regulation of MR expression in vivo. Altogether, we uncovered a mechanism by which renal MR expression is regulated through mRNA turnover, a post-transcriptional control that seems physiologically relevant.


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Na⁺ transport is tightly regulated by aldosterone, a steroid hormone exerting its effects through activation of the mineralocorticoid receptor (MR), a transcription factor (NR3C2) highly expressed in the distal nephron. Renal MR stimulates transcription of genes encoding ionic transporters involved in the reabsorption of Na⁺, such as the epithelial Na⁺ channel (ENaC) and the Na⁺/K⁺-ATPase. Other target genes have been identified, including the serum- and glucocorticoid-regulated kinase 1, the with no lysine 1 kinase, and the glucocorticoid-induced leucine zipper protein (GILZ), which all participate to the control of Na⁺ reabsorption in tight epithelium. Accumulating evidence underscores the major role played by MR and aldosterone in the pathogenesis of several human disorders, such as hypertension, heart failure, CKD, or mineralocorticoid resistance. Thus, MR signaling constitutes a central regulatory pathway given that activation of the mineralocorticoid pathway leads to marked Na⁺ retention, hypertension, high BP, and its subsequent end organ damages. It has been shown that renal MR expression positively correlates with the degree of renal scarring, the elevation of inflammatory markers, and the amount of proteinuria in patients. Importantly, pathogenesis of salt-sensitive hypertension and kidney injury were recently associated with activation of MR by Ras-related C3 botulinum substrate 1 (Rac1), a Rho-family small GTP binding protein, which emerged as a novel modulator of MR activity. Conversely, a low renal MR expression at birth accounts for the physiologic partial aldosterone resistance of human newborns, reminiscent of the MR haploinsufficiency reported in autosomal dominant pseudohypoaldosteronism. Lowering MR renal expression by RNA interference strategy both prevents progression of hypertension and attenuates renal damage. Thus, modulation of renal MR expression greatly affects kidney function. However, the mechanisms controlling renal MR expression remain poorly understood. MR gene transcription is directed by two functional promoters, whereas posttranslational modifications profoundly affect MR function. Nevertheless, post-transcriptional changes in mRNA processing in response to stress might also modify MR abundance in target cells.

Indeed, renal tubular cells are submitted to intense variations of the extracellular fluid tonicity generated by the corticopapillary gradient, which is pivotal for the regulation of ion and water transport. Of interest, MR expression level is high in the renal cortex, where tubular lumen is rather hypotonic (50 mOsm/kg). Protective mechanisms against hypertonic stress in mammalian cells involve the tonicity-responsive enhancer binding protein (TonEBP), a transcription factor belonging to the Rel family, with activity that is upregulated by both enhanced TonEBP transcription and increased TonEBP nuclear localization. We have previously shown that hypertonicity drastically reduced MR transcript and protein levels and concomitantly increased zfp36l1 gene expression in renal KC3AC1 cells. zfp36l1 encodes the protein tetradecanoyl phorbol acetate inducible sequence 11b (Tis11b), a member of the Tristetraprolin (TTP) RNA binding protein family, which regulates short-lived mRNA stability through its binding to adenylate-uridylate–rich elements (AREs) located in the 3’-untranslated region (3’-UTR) of target transcripts. We hypothesized that Tis11b may play a determinant role in the control of MR mRNA decay under hypertonic conditions. Here, we describe the molecular mechanisms by which hypertonicity-induced Tis11b expression in the kidney affects MR expression level and subsequently, MR signaling in cellular and mouse models. Modulation of such post-transcriptional mechanisms seems particularly relevant for the control of Na⁺ handling and BP.

RESULTS

Tis11b Decreases MR 3’-UTR-Driven Luciferase Activity through ARE

Several AREs were identified in mouse and human MR 3’-UTRs. These seven to nine regulatory motifs consist of AUUUA pentamers or UUAUUUA(U/A) nonamers, of which ARE3, -4, -5, and -6 and ARE8 and -9 are highly conserved among species. Human (2791 bp) and mouse (2781 bp) MR 3’-UTRs were cloned downstream of the luciferase reporter gene (pMIR-Luc report plasmid) and cotransfected in human embryonic kidney 293T (HEK293T) cells with pTarget-Tis11b–encoding plasmid. Tis11b expression resulted in a dose-dependent reduction of human and mouse 3’-UTR-driven luciferase activity but did not affect pMIR-Luc control activity, indicating that MR 3’-UTR is crucial for repression by Tis11b (Supplemental Figure 1). In addition, Tis11b accelerated Luc-3’-UTR mRNA degradation, because its half-life (t1/2) decreased from 7.2 to 4 hours in the presence of Tis11b (Supplemental Figure 2). The Tis11b mutant (RR), which is unable to bind ARE, did not efficiently reduce luciferase activity compared with wild-type Tis11b, indicating that specific Tis11b interaction with ARE is required for Luc-3’-UTR mRNA repression (Supplemental Figure 3). To identify Tis11b binding sites within MR 3’-UTR, truncated mutants (Δ1 to Δ44) were generated. Human Δ2 (hΔ2) and hΔ4 mutants, which harbor proximal ARE3, -4, -5, and -6 and distal ARE8, -9, and -10, respectively, led to a significant decrease in luciferase activity in the presence of Tis11b (68% and 35%, respectively) compared with the 60% reduction of luciferase activity observed with the entire MR 3’-UTR (Figure 1B). Of interest, hΔ1 and hΔ3 mutants, which lack ARE motifs, were ineffective in reducing reporter activity. Similar results were obtained with mouse MR 3’-UTR mutants (Supplemental Figure 4). To assess direct binding of Tis11b to the ARE-containing sequences, ribonucleoprotein immunoprecipitation (RNP-IP) was performed in HEK293T cells transfected with Tis11b and Luc-3’-UTR plasmids (full-length or truncated MR 3’-UTR) (Figure 1C). Although cross-linked immunoprecipitated RNP complexes were detected with the
entire MR 3’-UTR as well as hΔ2 and hΔ4 mutants, no specific signal was found with transcripts lacking AREs, such as Luc, hΔ1, or hΔ3, suggesting that Tis11b physically targets ARE-containing sequences. Given that Tis11b repressed Luc-hΔ2 3’-UTR and strongly interacted with hΔ2 region, we mutated ARE3, -4, -5, and -6 individually or in combination to determine which AREs were pivotal within hΔ2 sequence. Luciferase activities of single mutants ARE3 and ARE5 were still significantly reduced by Tis11b, whereas the mutant ARE4 lost its ability to be repressed by Tis11b (Figure 1D). Mutations of both ARE3 and ARE4 abrogated Tis11b-mediated repression of luciferase activity as observed for mutations of ARE4 alone, whereas mutations of both ARE5 and ARE6 had no significant effect. Altogether, these results indicate that ARE4 is the crucial core element in hMR 3’-UTR for the repression by Tis11b. However, Tis11b-induced decrease in mutant ARE3 luciferase activity was significantly impaired compared with the one driven by the wild-type 3’-UTR ($P<0.01$), suggesting that ARE3 may somehow also contribute to Tis11b inhibitory action.

**Hypertonicity Induces Tis11b-Mediated Decrease of MR mRNA Half-Life in Renal Cells**

In the renal Na⁺-transporting KC3AC1 cells, endogenous Tis11b is expressed at low level under isotonic conditions. A 6-fold increase in Tis11b protein expression was observed in rafinose-treated KC3AC1 cells (Figure 2A, hyper). Detection of several bands for Tis11b under hypertonic stress were reduced or remained unchanged at both mRNA and protein levels, respectively.
It is, therefore, very unlikely that Tis11d or Tis11 is involved in the downregulation of renal MR mRNA. To examine Tis11b functionality, pMIR-Luc and pMIR-Luc-3'-UTR plasmids were transfected in KC3AC1 cells submitted or not to hypertonic stress in the presence of scrambled small interfering RNA (siRNA) or Tis11b siRNA. As shown in Figure 2B, hypertonicity markedly decreased pMIR-Luc-3'-UTR activity (approximately 80% repression) given that MR 3'-UTR bears several AREs. Importantly, knockdown of Tis11b increased luciferase activity in hypertonicity-treated cells to the levels observed in the absence of MR 3'-UTR, whereas scrambled siRNA had no effect (Figure 2B, right panel). Of note, pMIR-Luc activity was also decreased; however, it was to a lesser extent (by approximately 40% under hypertonicity) whether Tis11b expression was knocked down or not (Figure 2B, left panel), suggesting that luciferase expression was likely affected at the transcriptional level.

Figure 2. Hypertonicity-induced Tis11b mediates MR transcript repression in renal cells. (A) Hypertonicity induces endogenous Tis11b expression in KC3AC1 cells. KC3AC1 cells were cultured under isotonic (iso) or hypertonic (hyper) conditions for 18 hours, and then, protein extracts were analyzed by Western blot. Quantified signals are means±SEMs (n=6). WB, Western blot analysis of Tis11b expression at indicated time points. **Significantly different from Tis11b expression under isotonicity with P, 0.01. (B) Hypertonicity-induced Tis11b is involved in the repression of MR 3'-UTR-driven luciferase activity. (Upper panel) KC3AC1 cells were transfected with pMIR-Luc or pMIR-Luc-3'-UTR vectors (50 ng per 12-well plate) under isotonic (white bars) or hypertonic (black bars) conditions in the presence of scrambled siRNA (hatched bars) or scrambled siRNA (gray bars). Under hypertonicity, Luc activity of pMIR-Luc-3'-UTR was significantly lower than the one of pMIR-Luc in the absence or presence of scrambled siRNA (**P<0.001). Luc activity of pMIR-Luc-3'-UTR measured under hypertonicity was also significantly lower than the one measured in the presence of Tis11b siRNA (**P<0.01). Values are means±SEMs from three independent experiments performed in duplicate. (C) Silencing Tis11b expression prevents hypertonicity-induced decrease of endogenous MR mRNA in renal cells. KC3AC1 cells were transfected with scrambled or Tis11b siRNA and then exposed to hypertonicity. MR mRNA steady state levels are quantified at time (t=0) prior to DRB treatment. Data are means±SEMs (n=4; ***P<0.001, Hyper versus Iso and Tis11b siRNA versus scrambled siRNA). RT-qPCR, quantitative RT-PCR. (D and E) MR transcript t1/2 in KC3AC1 cells exposed to (D) isotonic or hypertonic conditions or (E) hypertonic conditions in the presence of scrambled or Tis11b siRNA. DRB (10 μg/ml) was added to block transcription. MR mRNA values were plotted as the percentage of the initial value against time. Data are means±SEMs (n=4–8). (D) ***P<0.001 indicates significant difference from the corresponding time point under isotonicity. (E) *P<0.05 and **P<0.01 indicate significant difference from the corresponding time point under hypertonicity in the presence of scrambled siRNA.
independent from Tis11b and MR 3'-UTR. These results indicate that hypertonicity-induced Tis11b is involved in the destabilization of MR ARE-containing luciferase transcript. We next showed that hypertonicity induced a 60% decrease of endogenous MR transcript levels in KC3AC1 cells (Figure 2C). This effect was prevented by Tis11b knockdown with specific siRNA, indicating that MR mRNA was tightly controlled by hypertonicity-induced Tis11b expression. We further investigated the role of Tis11b in MR mRNA stability. Under isotonicity, KC3AC1 cells displayed a low Tis11b expression and a t1/2 for MR mRNA of 5 hours. This half-life was markedly decreased to 2.1 hours when Tis11b expression was induced by hypertonicity (Figure 2D). Likewise, transfection of KC3AC1 cells with scrambled siRNA did not alter hypertonicity-elicited MR mRNA half-life (t1/2 of 3.9 hours). In contrast, Tis11b knockdown both significantly reduced hypertonicity-stimulated Tis11b expression (Figure 2E, upper panel) and slowed down MR transcript turnover as revealed by a longer t1/2 of 7.4 hours (Figure 2E, lower panel).

Endogenous Tis11b Physically Interacts with MR Transcript

To examine whether MR transcript was a direct target of Tis11b, we performed RNP-IP assays in KC3AC1 cells exposed to hypertonicity (Figure 3). Figure 3, upper panel shows amplification of MR transcript in input samples. Anti-Tis11b antibody immunoprecipitated RNP complexes containing endogenous MR 3'-UTR as early as 4 hours after rafinose exposure (Figure 3, lower panel), whereas no specific MR amplification was observed under basal conditions (0 hours) or with preimmune serum. This result showed that hypertonicity-induced Tis11b protein physically interacts with MR mRNA expressed in KC3AC1 cells.

Hypertonicity Compromises MR Signaling

To examine the functional consequences of Tis11b-mediated destabilization of MR mRNA, KC3AC1 cells were cultivated on
filters to mimic vectorial hydroelectrolytic transports across polarized epithelial cells. Apical surface of cells was exposed to raffinose to mimic an increase of the luminal tonicity, whereas their basolateral compartment was bathed with isotonic medium. Hypertonicity induced a 1.5-fold increase in TonEBP expression, leading to a 2-fold increase in Aldose Reductase target gene expression (Figure 4A) and a 1.5-fold increase in Tis11b mRNA levels and resulting in a significant reduction of MR transcript levels. Likewise, hypertonicity stimulated Tis11b protein expression, which was accompanied by a slight but significant 20% reduction of MR protein (Figure 4B). Finally, aldosterone treatment of KC3AC1 cells for 1 hour induced a significant 1.5-fold increase in αENaC and Gilz expression under isotonic conditions (Figure 4C), but such stimulatory effects were abolished after apical exposure to raffinose. Similar results were obtained with sgk1 expression, but its mRNA levels dramatically increased on hypertonic stress (eight times) (Supplemental Figure 6) as already reported.20 Collectively, our findings indicate that hypertonicity compromised MR signaling in renal cells.

Challenging the Osmotic Corticopapillary Gradient Modulates Tis11b and MR Expression In Vivo

To address the physiologic relevance of our in vitro findings, the osmotic corticopapillary gradient was altered by submitting mice to 18 hours of water deprivation, 3 hours of diuretic (furosemide or indapamide) treatment, or high Na+ diet for 2 weeks; such conditions are known to be associated with increased Na+ fluxes in the distal nephron. Under basal conditions, a weak but positive cytoplasmic Tis11b labeling was observed in proximal and distal tubules but not glomeruli in the renal cortex (Figure 5A), whereas strong MR nuclear staining was detected in the distal nephron (Figure 5C). After high Na+ diet, Tis11b was dramatically increased in renal tubules (Figure 5B), with a concomitant reduction of MR in the nuclei of cortical collecting cells (Figure 5D). To quantify Tis11b and MR expression, Western blot analysis was performed on whole-kidney samples using multiplex detection of MR and Tis11b (Figure 5E, upper panel). Tis11b expression was slightly increased, although not significantly, by water deprivation, whereas furosemide, indapamide, and high Na+ diet (Na+) dramatically stimulated Tis11b expression by four- to six-fold. In contrast, a strong decrease in MR expression was observed (Figure 5E, lower panels). Altogether, we showed that, in vivo, an altered renal osmotic corticopapillary gradient associated with increased extracellular tonicity enhanced renal Tis11b expression, which negatively correlated with a concomitant repression of MR expression.

DISCUSSION

Accumulating evidence reveals that, other than transcription, post-transcriptional mechanisms constitute a second regulatory level of gene expression. Notably, transcript stability and translation efficiency are highly regulated steps, allowing the expression level of key proteins to be rapidly adjusted to cellular environment modifications. Here, we characterized a novel hypertonicity-triggered mechanism by which the RNA binding protein Tis11b destabilizes MR mRNA, leading to decreased MR expression. Tis11b is a member of the TTP family comprising Tis11 and Tis11d, which all regulate short-lived mRNA fate by their abilities to target ARE-containing mRNA to rapid degradation.18 Tis11b was shown to regulate steroidogenic acute regulatory protein mRNA stability19 and vascular endothelial growth factor in adrenocortical cells.21 Here, we identified seven to nine AREs located in MR 3’-UTR that were highly conserved.
between species. We showed that, by using site-directed mutagenesis, ARE3 (UUAUUUAAU) contributes to Tis11b-mediated repression of MR transcript, whereas ARE4 (UAAUUUA) is the crucial cis-acting element in this repression. AUUUA motifs have been also identified in the 3′-UTR of other steroid receptors, such as glucocorticoid receptor α and β,22 and estrogen receptor α,23 accounting for the destabilization of their transcripts. Interestingly, we were also able to show that endogenous glucocorticoid receptor expression in renal KC3AC1 cells was also slightly decreased by hypertonic stress (data not shown). Other RNA binding proteins might bind these AREs, which is exemplified by human antigen R (HuR) that acts as a positive regulator antagonizing Tis11b function.24 Given that MR mRNA levels increased on hypertonic stress in KC3AC1 cells, future analysis of HuR binding on MR 3′-UTR and subsequent consequences on MR transcript stability and signaling are warranted.

In cortical collecting KC3AC1 cells, binding of Tis11b to MR 3′-UTR accelerated MR transcript decay. Our study, thus, constitutes the first example of a steroid receptor mRNA turnover tightly controlled by hypertonicity-stimulated expression of Tis11b. Functional consequences of Tis11b-mediated destabilization of MR transcript were assessed in renal cells cultivated on filters under asymmetric culture conditions to be closer to the in vivo situation. Because basolateral osmolality may not change physiologically for cells in the cortex, we modified apical extracellular hypertonicity and showed that an increased TonEBP expression enhanced Tis11b expression, which in turn, reduced MR mRNA levels. Note that, as a result of accelerating MR degradation with halving the MR mRNA f1/2, a statistically significant difference in MR mRNA and protein abundance occurs, leading to a compromised MR signaling with blunted responses in terms of aldosterone-regulated gene expression (Gilz and eNAC). Because eNAC channel constitutes the rate-limiting step for Na+ reabsorption, Na+ transport might be rapidly affected when luminal extracellular toxicity increased in the distal tubules.

Although high Na+ diet condition and diuretic treatments are not likely to increase luminal osmolality to near 500 mOsM/kg, we interestingly showed in vivo that increased sodium fluxes in the distal part of the nephron and/or altered dilution capacities were associated with an increased renal Tis11b expression and a parallel decreased MR expression in the distal nephron.

Our results might be reminiscent of pathophysiological situations, in which the renal corticopapillary gradient is altered during osmotic diuresis, such as diabetes mellitus or tubular dysfunction. This in vivo mechanism at the organismal level may prevent accentuation of hypernatremia and rise in extracellular toxicity.

Altogether, our study provides new insights for better comprehension of the regulatory mechanisms controlling renal MR expression. We believe that our findings may have major physiologic impact, notably during renal development. Indeed, we have previously shown a physiologic renal aldosterone resistance in the neonatal period associated with a low MR expression in the kidney of both mice and humans at birth.7 Because the composition of the amniotic fluid, notably Na+ concentration, changes along gestation, a similar mechanism involving hypertonic stress and Tis11b may account for the control of renal MR expression in the fetus. Investigation on renal Tis11b ontogenesis during development and its expression pattern along nephronic segments may be particularly relevant.

We thus propose that Tis11b represents a physiologic regulator of renal MR abundance. It will be critical to establish whether osmotic stress may modulate MR abundance in other mineralocorticoid-sensitive tissues, not only in ionic transporting epithelia (colon and inner ear) but also cardiomyocytes, endothelial cells, adipocytes, or neurons, in which MR controls major biologic processes through rapid signaling. Osmotic stress and possibly, other stresses, including oxidative stress, oxygen depletion, and energy shortage are likely contributing to the rapid regulation of MR expression. MR abundance does constitute a key molecular determinant for mineralocorticoid signaling pathway and seems to be profoundly affected in various pathophysiological situations. Additional elucidation of the molecular mechanisms impacting MR-mediated signaling may open new therapeutic opportunities.

**CONCISE METHODS**

**Cell Culture**

HEK293T cells were cultured in DMEM with 10% FCS (BioWest), 2 mM glutamine, 20 mM Hepes (pH 7.4), 100 unit/ml penicillin, and 100 μg/ml streptomycin. All reagents were from PAA. KC3AC1 cells were cultured at 300 mOsM/kg (Isotonicity) as previously described.17 Hypertonic conditions (500 mOsM/kg) were achieved by adding 0.2 M raffinose (Sigma-Aldrich). Minimal medium lacking dexamethasone and serum was used to study aldosterone action (Acros Organics).

**Plasmid Constructs**

Murine and human MR 3′-UTRs (full-length and truncated sequences) were amplified by PCR from genomic DNA using specific primers (Supplemental Table 1), in which SpeI and SacI restriction sites were introduced to facilitate cloning into the pMIR-Report plasmid (Life Technologies). For ARE mutants, AUUUA was mutated to AggUA using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) (Supplemental Table 1). Tis11b-encoding pTarget vector was described previously.17 For pTarget-Tis11b-RK plasmid, the cytosine residues at positions 120 and 158 (TGC) were changed to cysteine residues using QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies) (Supplemental Table 1). Tis11b-encoding constructs and either empty or Tis11b-encoding pTarget vector using Lipofectamine 2000 (Life Technologies). Luciferase activities, nor-
malyzed to β-galactosidase or renilla luciferase activities, were performed 24 hours post-transfection as described.25

**RNP-IP**

RNP-IP was performed as previously described25 in either HEK293T cells transfected with Luc-MR 3′-UTR constructs or KC3AC1 cells exposed to hypertonicity. Luc-MR 3′-UTR, MR, or hypoxanthine-guanine phosphoribosyltransferase mRNA were amplified using specific primers (Supplemental Table 1).

**RNA Interference**

KC3AC1 cells were transfected two times (days 6 and 7 of culture) with 50 nM Tis1ib siRNA or scrambled siRNA (s2090 and 4390843; Life Technologies) using RNAImax reagent (Life Technologies). Cells were exposed overnight to hypertonicity, and 10 μg/ml 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole was added to determine MR mRNA half-life.

**Quantitative RT-PCR**

Quantitative RT-PCR was performed as described previously.17 After DNase treatment (Biolabs), RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit. Samples were analyzed by quantitative RT-PCR using the SYBR Green PCR Master Mix (Life Technologies) with the primers indicated (Supplemental Table 1) and a StepOne Real-Time PCR System (Life Technologies). Relative expression in a given sample was calculated as a ratio (attomoles specific gene per femtomole 18S or hypoxanthine-guanine phosphoribosyltransferase).

**Western Blot and Immunohistochemistry**

Protein extracts were processed for multiplex detection of Tis1ib or MR protein together with α-tubulin or β-actin protein for loading normalization; 5-μm sections of paraffin-embedded kidneys were deparaffinized in xylene and rehydrated. Antigen retrieval was done by microwaving (3 × 5 minutes) in citrate buffer (pH 6). Sections were subsequently treated as described previously.26 Antibody sources and dilutions are detailed in Supplemental Table 1.

**Investigations in Mice**

Animals had free access to water except for the water deprivation group, which did not receive any water for 18 hours. Mice were either subjected to a high Na+ diet (Genestil) for 2 weeks or received an intraperitoneal injection of furosemide (40 mg/kg; Renaudin) or in-dapamide (3 mg/kg; Sigma-Aldrich) for 3 hours. Animals (n=6–7 per group) were euthanized, and kidneys were collected and snap-frozen in liquid nitrogen for subsequent analyses. Mice were bred according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The animal facility was granted approval (C94–043–12) by the Ministère de l’Agriculture, France. All procedures were approved by the local ethics committee Consor tium des Animaleries Paris Sud (2012–2021).

**Statistical Analyses**

Data are means ± SEMs. One-way ANOVA or Mann–Whitney U test was used when appropriate to determine significant differences (GraphPad Prism software). A P value of 0.05 was considered as statistically significant (*P<0.05; **P<0.01; ***P<0.001).

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**DISCLOSURES**

None.

**REFERENCES**


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