Decreased ENaC expression compensates the increased NCC activity following inactivation of the kidney-specific isoform of WNK1 and prevents hypertension

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Mutations in WNK1 and WNK4 lead to familial hyperkalemic hypertension (FHHt). Because FHHt associates net positive Na+ balance with K+ and H+ renal retention, the identification of WNK1 and WNK4 led to a new paradigm to explain how aldosterone can promote either Na+ reabsorption or K+ secretion in a hypovolemic or hyperkalemic state, respectively. WNK1 gives rise to L-WNK1, an ubiquitous kinase, and KS-WNK1, a kinase-defective isoform expressed in the distal convoluted tubule. By inactivating KS-WNK1 in mice, we show here that this isoform is an important regulator of sodium transport. KS-WNK1+− mice display an increased activity of the NaCl cotransporter NCC, expressed specifically in the distal convoluted tubule, where it participates in the fine tuning of sodium reabsorption. Moreover, the expression of the ROMK and BKCa potassium channels was modified in KS-WNK1+− mice, indicating that KS-WNK1 is also a regulator of potassium transport in the distal nephron. Finally, we provide an alternative model for FHHt. Previous studies suggested that the activation of NCC plays a central role in the development of hypertension and hyperkalemia. Even though the increase in NCC activity in KS-WNK1+− mice was less pronounced than in mice overexpressing a mutant form of WNK4, our study suggests that the activation of NaCl cotransporter is not sufficient by itself to induce a hyperkalemic hypertension and that the deregulation of other channels, such as the Epithelial Na+ channel (ENaC), is probably required.

Results

Generation of KS-WNK1+− KO Mice. We used homologous recombination in ES cells to create a conditional KO allele of WNK1 (WNK1cond allele) in which two loxp sites were inserted upstream of exon 4 and downstream of exon 4a and two FRT sites were inserted upstream and downstream of exon 4a (Fig. S1A4). This allele allows the inactivation of all WNK1 isoforms by recombination between the two loxp sites and of KS-WNK1 alone by recombination between the two FRT sites. Details on the generation of the targeting vector, ES cell clones, and corresponding mice are given in SI Methods. Chimeric males, obtained by injection of one ES cell clone, were crossed with C57BL/6N females to produce WNK1+−neo animals, which were then bred with hACTBFLPe mice (19) to remove both the neo resistance cassette and exon 4a, specific for KS-WNK1. Real-time quantitative

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Familial hyperkalemic hypertension (FHHt) is a rare Mendelian form of human hypertension (Online Mendelian Inheritance in Man 145260) characterized by hypertension, hyperkalemia, and hyperchloremic metabolic acidosis (1). FHHt-causing mutations were identified in the WNK1 and WNK4 genes (2), encoding proteins from the WNK [With No lysine (K)] family of serine-threonine kinases (3). Because FHHt associates net positive Na+ balance with K+ and H+ renal retention, identification of WNK1 and WNK4 led to a new paradigm explaining how aldosterone promotes Na+ reabsorption or K+ secretion in hypovolemic or hyperkalemic states, respectively (4).

Two WNK1 isoforms have been described (5): long-WNK1 (L-WNK1), a ubiquitous isoform containing the whole kinase domain, and kidney-specific WNK1 (KS-WNK1), a kinase-defective isoform. Whereas L-WNK1 is expressed in the whole nephron at a low level, KS-WNK1 is highly expressed in the distal convoluted tubule (DCT) and to a lesser extent in the connecting tubule (CNT). The implication of WNK1 isoforms and WNK4 in the regulation of Na+ and K+ transport has been mostly studied in vitro. Several of these studies focused on the regulation of the NaCl cotransporter (NCC), as its inhibitors, the thiazide diuretic agents (6), correct the blood pressure and metabolic abnormalities of FHHt. WNK4 inhibits NCC activity by repressing its cell surface targeting in vitro (7–9), whereas L-WNK1 activates NCC by relieving the WNK4-mediated inhibition (10). KS-WNK1 regulates NCC by inhibiting either L-WNK1 (11) or WNK3, known to stimulate NCC activity (12). Inhibition of the K+ channel ROMK by WNK4 and L-WNK1 was also described in vitro and, again, the activity of L-WNK1 was inhibited by KS-WNK1 (13–16).

To better define the physiological role of KS-WNK1, we generated a mouse model of KS-WNK1 inactivation. As expected, NCC expression and phosphorylation were increased in KS-WNK1−/− mice. Na+ balance was affected as evidenced by increased diastolic blood pressure and decreased urinary aldosterone level. In addition, the expression of ROMK and BKCa potassium channels was modified. Our data therefore show that KS-WNK1 is an important regulator of Na+ and K+ transport in the DCT. Moreover, this model shows that the activation of NCC is not sufficient to induce the development of FHHt by opposition to what was suggested following the characterization of transgenic mice expressing the mutated form of WNK4 (17, 18). The deregulation of other transporters/channels, such as the Epithelial Na+ channel (ENaC), could therefore be required for FHHt development.

transgenic animals | water-electrolyte balance
RT-PCR showed that KS-WNK1 expression was abolished and L-WNK1 expression was unaffected in the kidney (Fig. S1D).

KS-WNK1 Inactivation Did Not Lead to the Development of Hyperkalemic Hypertension. Systolic and diastolic blood pressure was recorded over a 24-h period in KS-WNK1+/+ and KS-WNK1−/− mice by telemetry (Fig. 1). As expected, locomotor activity was minimal during the day in both groups (KS-WNK1+/+ mice, 25.0 ± 8.1 during the day vs. 105.5 ± 41.9 during the night; KS-WNK1−/− mice, 50.4 ± 9.7 during the day vs. 143.8 ± 33.1 during the night). No difference was observed between the groups. Whereas the 24-h average for systolic blood pressure (109.4 ± 2.7 mm Hg in KS-WNK1+/+ vs. 109.2 ± 3.6 mm Hg in KS-WNK1−/−) and heart rate (472.9 ± 26 beats/min vs. 498.9 ± 11.3 beats/min for WT and mutant mice, respectively) were similar, diastolic blood pressure was slightly increased in KS-WNK1−/− mice (76.7 ± 2.3 mm Hg in WT vs. 82.4 ± 2.9 mm Hg in mutant mice; Fig. 1). This difference was more pronounced between 16:00 and 02:00 hours, when the animals are the most active (81.6 ± 1.2 in KS-WNK1+/+ vs. 89.3 ± 0.9 in KS-WNK1−/−).

KS-WNK1−/− and KS-WNK1+/+ males were maintained in metabolic cages to measure blood and urine electrolytes. No hyperkalemia, hyperchloremia, or metabolic acidosis was detected in TS-WNK1−/− animals, even when submitted to a Na− or K+ load (Table 1). However, there was a 40% decrease in the 24-h urinary aldosterone level in KS-WNK1−/− mice (11.67 ± 1.49 pmol/mg in KS-WNK1+/+ vs. 6.84 ± 0.99 pmol in KS-WNK1−/−; P = 0.01), indicating that Na− reabsorption was increased. This decrease was maintained when the animals were fed a low-Na+ or high-Na+ diet for 15 d or a high-K+ diet for 10 d (Table 1).

KS-WNK1 Inactivation Led to Increased NCC Expression and Phosphorylation. As NCC is supposed to be the main target of the WNKs, we quantified its expression in renal cortex protein extracts. Although mRNA expression was not affected (Fig. S2), NCC protein expression and phosphorylation on tyrosine residues T44, T53, and T58 were increased twofold in KS-WNK1−/− mice (Fig. 2A). These results are in agreement with in vitro studies showing that KS-WNK1 prevents L-WNK1 activation of NCC (11). In addition, immunoblotting experiments performed on cellular fractions showed that NCC trafficking to the apical membranes was increased in KS-WNK1−/− mice, thus confirming that NCC is relocated to the plasma membrane following WNK1 activation, as showed in vitro (7–9, 11), but also that global NCC abundance was increased by KS-WNK1 inactivation (Fig. S3).

We also quantified the level of expression and phosphorylation of NCC in KS-WNK1−/− mice submitted to Na− depletion or load (Fig. S4 A and B). Whereas the ratio of phosphorylated NCC (pNCC) to total NCC increased with Na− depletion in WT animals, no change was observed in KS-WNK1−/− mice. During Na− load, only the level of phosphorylation was significantly decreased in WT mice. Again, this decrease was not seen in KS-WNK1−/− mice. Regulation of NCC phosphorylation by Na+ intake is therefore lost in KS-WNK1−/− animals. This misregulation in mutant animals was not associated with changes in Na+ excretion in mice submitted to a short (Fig. S4 C and D) or prolonged (Table 1) Na− depletion or moderate load.

The absence of clear clinical and biological phenotype in KS-WNK1−/− mice prompted us to compare activation of NCC expression and phosphorylation with the previously described TgWNK3PhaH1i model (17). NCC expression and phosphorylation were increased 3.3- and 3.9-fold, respectively, in TgWNK4PhaH1i mice (Fig. 2B). NCC activation was therefore stronger following WNK4 mutation than KS-WNK1 inactivation. Accordingly, the DCT fractional volume was similar between KS-WNK1−/− and KS-WNK1−/− mice, suggesting that there was no DCT hyperplasia nor hypertrophy in KS-WNK1−/− animals (Fig. 2C and Fig. S5).

SPAK and OSR1 Phosphorylation Is Decreased in KS-WNK1−/− Cortex. NCC activity is controlled by a network composed of the two WNK1 isoforms, WNK4, and WNK3 (20). Immunoblotting experiments for WNK3 had inconclusive findings. The level of expression of L-WNK1 and WNK4 was not modified in KS-WNK1−/− protein extract of kidney cortex (Fig. S6). In addition, mouse NCC is activated by phosphorylation by SPAK and OSR1 (21, 22). Surprisingly, we found that SPAK phosphorylation was decreased by 25% in KS-WNK1−/− cortex extract (Fig. S6) whereas the expression of both kinases was not modified. This unexpected result could be explained by compensatory mechanisms developed by segments located downstream of the DCT (as described later), as SPAK and OSR1 are not only expressed in the DCT but also in the TAL and weakly in theCNT and CCD (21).

Decreased ENaC Expression and Activity in KS-WNK1−/− Mice. Compensatory mechanisms could occur in segments downstream to the DCT in KS-WNK1−/− mice to prevent a marked hydropenia and thus hypertension. Real-time RT-PCR quantification of Scnn1a and Scnn1b transcripts, encoding the α-ENaC and β-ENaC subunits, showed that inactivation of KS-WNK1 led to a 20% to 30% decrease in expression of these subunits in the renal cortex (Fig. 3A). Similarly, membrane expression of the 85-kDa and 30-kDa forms of α-ENaC and of the 70-kDa forms of γ-ENaC was decreased by 47%, 45%, and 66%, respectively (Fig. 3B). The cleaved form of the α- and γ-subunits are believed to reflect the activation of the channel (23, 24). This decreased ENaC expression could be consecutive to the decreased aldosterone level.

To determine if the decreased expression was associated with decreased activity of the channel, KS-WNK1−/− and KS-WNK1+/+ animals were submitted to i.p. injection of amiloride (4.8 mmol/g). The first difference observed between the two groups was the very weak stimulation of diuresis by amiloride in KS-WNK1−/− mice compared with WT littermates. Over the 6-h period following amiloride injection, we managed to collect urine from only three and one of the seven control animals for the first and second injections of amiloride, respectively, whereas urine was collected from five of the seven control animals for both injections. This resulted in a much lower average urine volume following amiloride injections in KS-WNK1−/− animals compared with WT littermates (Fig. 3C). Similarly, although natriuresis was increased 6 h after amiloride injection as expected in KS-WNK1+/+ mice, the response to this diuretic appeared to be blunted in KS-WNK1−/− animals (Fig. 3D). These
observations showed that ENaC activity is decreased following KS-WNK1 inactivation.

Modified Expression of ROMK and BKCa in KS-WNK1−/− Mice. The absence of hyperkalemia in KS-WNK1−/− animals was intriguing, as they displayed decreased urinary aldosterone level and ENaC activity, expected to promote renal retention of K+ by blocking ROMK-dependent K+ secretion by the collecting duct. We analyzed the level of expression of ROMK and of the large-conductance calcium-activated K+ channel BKCa (Fig. 4). The expression of the α- and β1-subunits of the BKCa channel was increased 1.7-fold whereas the expression of the β4-subunit was decreased by 50% (Fig. 4A). Immunohistofluorescence showed that apical expression of ROMK also tended to be increased in the DCT and CNT of KS-WNK1−/− animals compared with WT littermates (Fig. 4B).

Discussion
Recent studies have shown that the WNK family regulates Na+ and K+ transport in the distal nephron. Whereas the role of WNK4 has been well described, the roles of L-WNK1 and its dominant-negative inhibitor, KS-WNK1, remain to be defined in vivo. The present study provides evidence that this kinase-defective WNK1 isoform is an important regulator of Na+ and K+ transport in the DCT as its inactivation in mice modified the expression of the Na–Cl cotransporter NCC and the ROMK potassium channel. Our study also suggests that KS-WNK1 could regulate the activity of the BKCa potassium channel.

Although a clear effect on Na+ transport in the DCT was observed in KS-WNK1−/− mice, the mechanisms by which KS-WNK1 controls K+ balance were more difficult to decipher. The increased expression of ROMK in KS-WNK1−/− mice was surprising as in vitro and in vivo studies rather suggested that KS-WNK1 stimulates ROMK apical expression by inhibiting L-WNK1 (14–16, 25). The expression of the α- and β-subunits of BKCa is also increased in KS-WNK1−/− mice, whereas that of the iberiotoxin-insensitive β4-subunit, coexpressed with KS-WNK1 in the DCT, was decreased. The regulation of BKCa by WNK1 isoforms has not been described previously. Whether these

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**Fig. 2.** Expression and phosphorylation of NCC is increased in KS-WNK1−/− mice but to a lesser extent than in TgWNK4PNAH mice. (A) Immunoblots of samples dissected from the renal cortex of control and KS-WNK1−/− males (n = 6 per group; Left) or control and TgWNK4PNAH males (n = 4 and n = 5, respectively; Right) incubated with anti-NCC and anti-phospho-NCC antibodies. (B) Densitometric analysis showed that abundance and phosphorylation of NCC were significantly increased in KS-WNK1−/− mice (black bars; *P < 0.05 compared with controls [open bars], but this activation was lower than that seen in TgWNK4PNAH mice [gray bars; **P < 0.005] compared with their controls [open bars]. Data are mean ± SEM. (C) Crystalline sections of KS-WNK1−/− and KS-WNK1−/− male kidneys immunostained with anti-NCC antibody. The signal appears stronger in the DCT of KS-WNK1−/− mice compared with control littermates. (Scale bar: 50 μm.)

### Table 1. Blood and Urine Electrolytes and 24-h Urinary Aldosterone Level in KS-WNK1+/+ and KS-WNK1−/− Mice Submitted to Different Na+ and K+ Intake

| Measurement          | Standard diet | Low-Na+ diet | High-Na+ diet | High-K+ diet |
|----------------------|---------------|--------------|---------------|--------------|
|                      | α+/− (n = 11) | −/− (n = 8)  | α+/− (n = 6)  | −/− (n = 6)  |
| Body weight, g       | 30.0 ± 1.1    | 29.7 ± 1.3   | 27.3 ± 0.6    | 27.4 ± 0.4   |
| Food intake, g        | 4.1 ± 0.4     | 4.1 ± 0.2    | 2.3 ± 0.5     | 3.0 ± 0.2    |
| Water intake, g       | 3.6 ± 0.2     | 3.3 ± 0.5    | 3.1 ± 0.1     | 2.6 ± 0.2    |
| Urine Volume, mL      | 1.15 ± 0.19   | 1.08 ± 0.21  | 1.4 ± 0.1     | 1.1 ± 0.1    |
| Na+, mmol/L           | 144.8 ± 2.6   | 143.1 ± 2.5  | 146.3 ± 0.8   | 147 ± 0.9    |
| K+, mmol/L            | 4.3 ± 0.2     | 4.5 ± 0.3    | 4.1 ± 0.2     | 4.2 ± 0.1    |
| Cl−, mmol/L           | 107.4 ± 2.1   | 107.1 ± 1.9  | 112.7 ± 0.8   | 113.6 ± 0.5  |
| pH                    | 7.34 ± 0.02   | 7.34 ± 0.03  | ND ND         | ND ND        |
| pCO2, mm Hg           | 50.4 ± 1.5    | 49.2 ± 1.7   | ND ND         | ND ND        |
| HCO3−, mmol/L         | 26.8 ± 1.4    | 25.5 ± 1.3   | ND ND         | ND ND        |
| Na+, mmol/min         | 177.5 ± 29.2  | 142.7 ± 13.3 | 38.1 ± 8.7    | 25.3 ± 4.3   |
| K+, mmol/min          | 255.4 ± 37.7  | 226.4 ± 27   | 138.2 ± 9.7   | 122.3 ± 12.6 |
| Cl−, mmol/min         | 182.2 ± 28.3  | 161.2 ± 19.5 | 176.4 ± 29.7  | 213.1 ± 18.5 |
| pH                    | 5.58 ± 0.02   | 5.57 ± 0.02  | ND ND         | ND ND        |
| pCO2, mmol/L          | 26.5 ± 1.0    | 24.9 ± 1.1   | ND ND         | ND ND        |
| HCO3−, mmol/L         | 0.24 ± 0.01   | 0.22 ± 0.01  | ND ND         | ND ND        |
| 24 h aldosterone, pmol| 19.8 ± 1.9    | 9.4 ± 2.2    | 40.5 ± 2.4    | 27.9 ± 3.1   |

Animals were housed in metabolic cages and fed the low- and high-Na+ diets for 2 wks and the high-K+ diet for 10 d (Methods). Plasma was taken at the end of the experimental period. Urine samples were collected daily. ND, not defined.

*P < 0.05 vs. control group fed the same diet.

†P ≤ 0.005 vs. control group fed the same diet.
modifications are directly caused by KS-WNK1 inactivation or represent a compensatory mechanism remains to be defined.

L-WNK1-mediated activation of NCC is inhibited by KS-WNK1 in vitro. Accordingly, NCC expression and phosphorylation were increased in KS-WNK1<sup>−/−</sup> mice. Previous in vivo studies suggested that FHHt is mainly the consequence of increased NCC activity (17, 18). However, KS-WNK1<sup>−/−</sup> mice did not exhibit hyperkalemic hypertension. Several hypotheses could explain this result. The first one is that NCC is not activated strongly enough to lead to hyperkalemic hypertension. Comparison between KS-WNK1<sup>−/−</sup> and Scn1a<sup>−/−</sup> mice showed that NCC activation was stronger following WNK4 mutation than KS-WNK1 inactivation. The mechanisms underlying this difference remain to be defined. One could be a differential effect on NCC, which activates NCC (12) and is inhibited by and inhibits WNK4 in a dose-dependent manner (26). WNK4 mutants lose the ability to inhibit WNK3, thus providing an explanation why WNK4 mutations have such a powerful effect on NCC activity. However, KS-WNK1 was shown to inhibit not only L-WNK1 but also WNK3 (26). KS-WNK1 inactivation should therefore release L-WNK1 and WNK3 activity from this dominant-negative effect.

However, the fact that KS-WNK1<sup>−/−</sup> mice are slightly hypervolemic, as evidenced by a decreased urinary aldosterone level and slightly elevated diastolic blood pressure, is not in favor of this first hypothesis. Our second hypothesis is that NCC activation is not sufficient to induce hyperkalemic hypertension and that the deregulation of other Na<sup>+</sup> or K<sup>+</sup> channels/transporters is required. Three studies have indeed shown that ENaC activity is directly activated by WNK4 mutations. WNK4 inhibits ENaC and FHHt-causing WNK4 mutations eliminate ENaC inhibition in vitro (27). Patients carrying the WNK4 Q565E mutation displayed elevated nasal potential difference and a higher response to amiloride compared with controls (28). Finally, mutated WNK4 transgenic mice have an increased ENaC expression and activity in the collecting duct and colon (18, 27). Conversely, ENaC activity is decreased in KS-WNK1<sup>−/−</sup> mice, as evidenced by reduced proteolytic activation of ENaC and diuretic response to amiloride. This reduced ENaC activity probably reflects a compensatory mechanism to prevent the development of a marked hypertension in KS-WNK1<sup>−/−</sup> animals as a result of a massive increase in Na<sup>+</sup> reabsorption following NCC activation. Such a compensation was previously described in NCC<sup>−/−</sup> mice, in which an up-regulation of ENaC activity compensated for the absence of Na<sup>+</sup> reabsorption by NCC (29, 30). Taken together, these studies suggest that ENaC is directly up-regulated by WNK4 mutations, whereas loss of KS-WNK1 indirectly causes a compensatory down-regulation of ENaC. The implication of this phenomenon in the development of FHHt remains to be defined.
Locus in FHHt patients are load. Our study does not support this. Experiments were performed on mice. The stained tubuli are DCTs and K\(^{+}\) deficit. Lower KS-WNK1 and K\(^{+}\) diet for 10 d and urine collected as described. missense KS-WNK1 diet (7% KCl; 4% K\(^{+}\)). (KS-WNK1) urine samples were submitted to these diets for 15 d. Urine samples were collected every 12 h during the first 48 h and then over a 24-h period on days 3, 8, and 15.

**Methods**

**Physiological Studies.** All studies were conducted on 3- to 5-mo-old male mice and were performed in accordance with the relevant guidelines of the French Ministry of Agriculture (Authorization Executive Order 75–215) for scientific experimentation on animals, European Communities Council Directive, and international ethical standards.

**Basal conditions.** Animals were housed in metabolic cages and fed a standard diet (0.7% NaCl) with free access to tap water. After a 3-d adaptation period, urines were collected daily for electrolyte measurements for 2 d.

**Low- and high-salt studies.** All diets were made to order by Scientific Animal Food and Engineering. Animals were housed in metabolic cages and fed a control diet containing 0.3% NaCl. After a 3-d adaptation period, urine samples were collected every 12 h over a 24-h period. The diet was then changed to a low-salt (0.03% NaCl) or high-salt (3% NaCl) diet. The animals were submitted to these diets for 15 d. Urine samples were collected every 12 h during the first 48 h and then over a 24-h period on days 3, 8, and 15.

**High-K\(^{+}\) study.** Urine of animals fed a control diet was collected as described earlier. The diet was then changed to a high-K\(^{+}\) (7% KCl) diet. Urine samples were collected every 12 h during the first 48 h and then daily. The animals were submitted to the high-K\(^{+}\) diet for 10 d and urine collected as described earlier.

At the end of the experimental period, animals were killed with ketamine and xylazine (0.1 and 0.01 mg/kg body weight, respectively). Creatinine, plasma, and urine electrolyte levels were determined using an AU400 analyzer (Olympus). Urinary aldosterone was measured by RIA (Dade Behring).

**Telemetry.** Experiments were performed on five KS-WNK1\(^{+/+}\) and five KS-WNK1\(^{−/−}\) 5-mo-old male mice. The cather of the BP telemeter (model TA11PA-C10; Data Sciences International) was inserted into the aorta via left common carotid artery (32). The telemetric transmitter probe was positioned s.c. on the flank. After a 2-wk recovery period, cardiovascular parameters and locomotor activity were recorded continuously for 24 h in freely moving mice housed in individual cages placed on top of the telemetric receivers in a light/dark-cycled recording room (0800 to 2000 hours). Each recording was visualized to select one segment without erratic fluctuations of enough duration (S1.2 s) every 15 min (four segments per hour) for 24 h, i.e., 96 segments for each animal.

**Immunoblot Analyses.** Preparation of kidney or cortex protein extract and immunoblotting was performed as described (33). The following polyclonal antibodies were used: NCC (gift from D. Ellison, Oregon Health and Science...
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Real-Time Quantitative RT-PCR. Total RNA of whole kidney or kidney cortex was extracted using the Nucleospin RNAII kit (Macherey-Nagel). RNA (2 µg) was reverse-transcribed using SuperscriptII reverse transcriptase and 1 µg of random primers (Invitrogen) in a total volume of 20 µl. Real-time quantitative PCR assays were performed in triplicate for each sample on a real-time detector (MJ Research) using intercalation of SYBR Green (qPCR MasterMix Plus with fluorescein; Eurogentec) as a fluorescence reporter or TaqMan assays (Applied Biosystems). Primers sequences are given in Table S1.

**Statistical Analysis.** For body weight, food/water intake, urinary output, urine electrolytes, and telemetry data, comparison between groups was performed using repeated-measures ANOVA. For plasma electrolytes, urine aldosterone level, immunoblots and quantitative RT-PCR, comparison between groups was performed using an unpaired Student's t test. The difference was considered as significant when P ≤ 0.05.

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