

SHORT COMMUNICATION



Mineralocorticoids modulate the expression of the β -3 subunit of the Na^+ , K^+ -ATPase in the renal collecting duct

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ABSTRACT

Renal sodium reabsorption depends on the activity of the Na^+ , K^+ -ATPase α/β heterodimer. Four α (α_{1-4}) and 3 β (β_{1-3}) subunit isoforms have been described. It is accepted that renal tubule cells express α_1/β_1 dimers. Aldosterone stimulates Na^+ , K^+ -ATPase activity and may modulate α_1/β_1 expression. However, some studies suggest the presence of β_3 in the kidney. We hypothesized that the β_3 isoform of the Na^+ , K^+ -ATPase is expressed in tubular cells of the distal nephron, and modulated by mineralocorticoids. We found that β_3 is highly expressed in collecting duct of rodents, and that mineralocorticoids decreased the expression of β_3 . Thus, we describe a novel molecular mechanism of sodium pump modulation that may contribute to the effects of mineralocorticoids on sodium reabsorption.

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Introduction

The Na^+ , K^+ -ATPase is a basolateral transport protein that creates the electrochemical gradient across the plasma membrane, hydrolyzing ATP to pump Na^+ out of the cell in exchange for K^+ .¹ In the sodium-reabsorbing segments of the renal tubule, the transepithelial reabsorption of sodium from the apical to the basolateral fluid depends on Na^+ , K^+ -ATPase activity, which parallels apical sodium influx.

The functional Na^+ , K^+ -ATPase is a protein heterodimer, formed by α and β subunits.²⁻⁴ The heterodimer can further interact with the small γ (FXYP) regulatory subunit.⁵ Although the α subunit is responsible for the catalytic activity and contains the binding sites for ions and ATP, the association of the β subunit with the α subunit is obligatory for ion pumping and ATP hydrolysis.^{2-4,6} The α subunit has 4 isoforms with tissue specific expression, with the α_1 isoform being present in the renal tubule. On the other hand, 3 isoforms of the β subunit have been identified.^{3,6} It has been described that the renal tubule expresses the

β_1 subunit, and most of the published studies on sodium pump of renal tissue have focused only on the variability of α_1 and β_1 .^{2-4,7} However, Northern blot and Western blot results obtained at the time of cloning of the β_3 subunit suggest that the kidney may express this isoform in the nephron and/or in interstitial cells.⁸⁻¹¹

Changes in the expression of β subunits affect the sodium pump function. The β_1 and β_2 subunits are necessary for correct maturation of Na^+ , K^+ -ATPase, regulate the plasma membrane abundance of the enzyme, participate in the stabilization of intercellular junctions and act as cell-adhesion molecules.¹²⁻¹⁴ Although Northern blot and Western blot analyses of whole tissue samples showed β_3 isoform expression in the kidney,^{8,9} there are no studies addressing its possible function in this organ. The use of heterologous expression systems showed that β_3 modulates pump activation, the apparent affinity for K^+ and Na^+ , the sensitivity to ouabain and stabilized sodium binding to the α subunit.^{11,15-17}

The homeostatic adjustment of sodium reabsorption in the distal nephron by aldosterone contributes to extracellular fluid balance and blood pressure control.⁵ Aldosterone increases tubular sodium reabsorption acting on epithelial cells of the distal nephron that express the mineralocorticoid receptor (MR; *NR3C2*). This induces a coordinated increase of the apical sodium influx that is paralleled by the increase in the activity of the Na^+, K^+ -ATPase at the basolateral membrane.⁵ The mechanisms implicated in the modulation of the Na^+, K^+ -ATPase by mineralocorticoids are incompletely understood. Several studies have shown that aldosterone can increase α_1 and β_1 mRNA levels in distal nephron cells *via* the MR and increase the targeting to the basolateral membrane.¹⁸⁻²¹ Studies in isolated cortical collecting ducts have confirmed that aldosterone induces a rapid increase in the abundance of basolateral α_1 and β_1 subunits.^{19,22,23} Studies *in vivo* have shown either no changes or increase in the abundance of renal α and β subunits at the mRNA and protein levels in the kidney of animals with increased levels of plasma aldosterone.^{5,24-26}

Despite the data suggesting that the β_3 subunit may be present in the renal tubules, as far as we know there are no studies identifying the kidney regions and/or specific renal cells that express β_3 . Moreover, no data are reported on the potential modulation by aldosterone or any other hormone on the expression of the β_3 subunit in the kidney. Thus, the goal of the present study was to characterize the expression of the β_3 subunit in renal tissue and to study the effect of MR activity on renal β_3 expression.

Results

We first analyzed the expression of α and β sodium pump subunits in rat tissues and found that both kidney cortex and kidney medulla express β_1 and β_3 subunits (Fig. 1A). Western blot studies of kidney cortex and medulla showed the presence of α_1 , β_1 and β_3 proteins (Fig. 1B). Primary structure comparisons show that the Na^+, K^+ -ATPase β_3 subunit is more divergent among species than either the β_1 or β_2 subunits.⁸ Thus, we also analyzed the expression of the β_3 subunit in renal tissue from mice (Fig. 1C-1E). Previous studies detected a higher abundance of β_3 in testis, lower in lung and kidney, and almost complete absence of β_3 in liver.^{8,11} The qRT-PCR analyses of

RNA samples from mouse tissues showed that testis indeed expressed a higher β_3 transcript abundance, but it was followed by a very strong signal from kidney medulla. The renal cortex also expressed the β_3 transcript with similar abundance to that of lung. A more detailed analysis of renal tissue zones showed that the β_3 transcript was ≈ 12 times more abundant in the inner medulla as compared with cortex. The Western blot study confirmed a higher abundance of the β_3 isoform protein in the kidney medulla of mice.

To characterize the cellular expression of β_3 in the renal tissue we first performed *in situ* hybridization. As shown in Fig. 2, β_3 mRNA labeling was abundant in renal medulla collecting duct cells, whereas in the cortex the labeling was present in collecting ducts, mainly in principal cells. The preferential expression of the β_3 isoform in collecting ducts was further confirmed by qRT-PCR of isolated renal tubules (Fig. 2I-2K). The abundance of the β_3 transcript (Fig. 2K) was more than 4-fold higher in the medullary collecting ducts as compared with convoluted proximal tubules and 2.5-fold higher than cortical collecting ducts.

Considering the pattern of expression, we evaluated whether the absence of adrenal hormones modified the abundance of β_3 in the rat kidney. As shown in Fig. S1, the abundance of the β_3 subunit in the renal cortex was not modified by adrenalectomy (ADX). Similarly, α_1 and β_1 subunit abundance did not change after ADX in the renal cortex. However, in renal medulla we observed that ADX decreased the abundance of α_1 isoform. Surprisingly, as shown in Fig. S1B, the abundance of the β_3 subunit in renal medulla increased by 2-fold after ADX, as compared with control rats (sham surgery). The β_1 subunit abundance was not modified by ADX. Thus, the absence of adrenal hormones caused a specific increase in β_3 and a decrease in α_1 expression in renal medulla.

Next, we tested the role of mineralocorticoids in the modulation of β_3 in mice (Fig. 3). To evaluate the possibility of a genomic effect of mineralocorticoids, we measured the abundance of sodium pump isoform transcripts and proteins, and we also added an ADX group with mineralocorticoid (DOCA) replacement therapy. Similar to the results observed in the kidney obtained from ADX rats, ADX did not modify the abundance of α_1 , β_1 or β_3 mRNAs or proteins in the renal cortex of mice (Fig. 3A). In contrast, ADX increased the abundance of β_3 mRNA and protein in

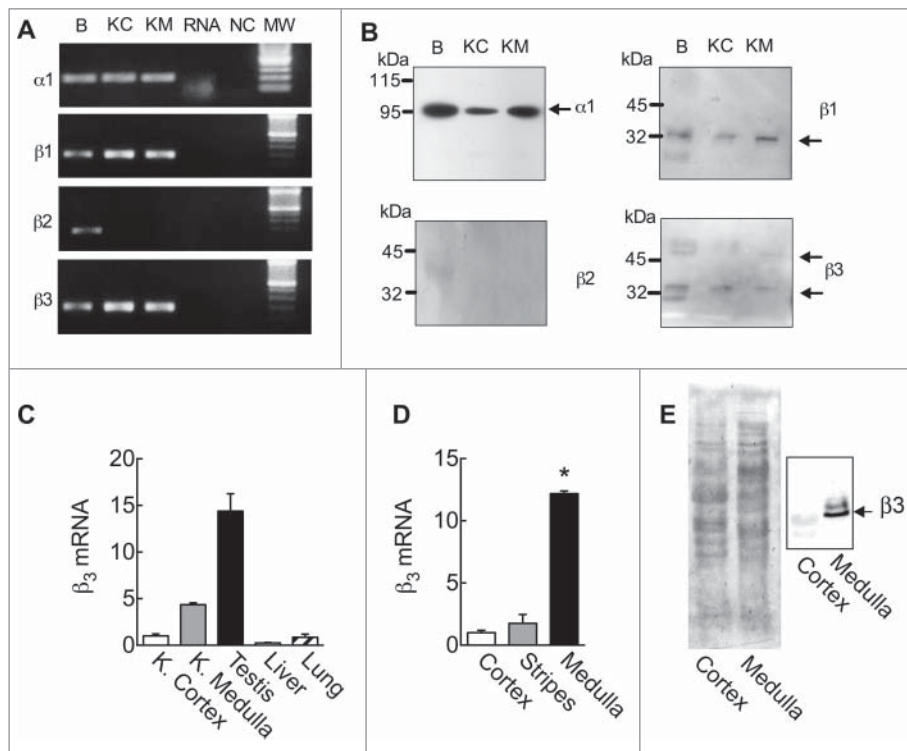


Figure 1. Pattern of expression of the β_3 subunit of the Na^+ , K^+ -ATPase in renal tissue. (A) Presence of β_3 mRNA in rat kidney, as compared with rat brain tissue (positive control). RT-PCR experiments show the presence of α_1 , β_1 and β_3 isoform transcripts in kidney cortex and kidney medulla. (B) The presence of the β_3 protein (bands of 35 kDa and 65 kDa) was confirmed by the Western blot of total protein homogenates from kidney cortex and medulla. (C) Total RNA was isolated from renal cortex, renal medulla, testis, liver and lung tissues. The abundance of β_3 mRNA was analyzed by real time qRT-PCR with specific primers, and expressed relative to the abundance of the ribosomal 18S (constitutive control). (D) Renal cortex, stripes and medulla were dissected under stereo microscope vision, and total RNA was isolated for measurement of β_3 mRNA by qRT-PCR. (E) Total protein homogenates prepared from renal cortex and renal medulla were analyzed by Western blot, to measure the presence of β_3 protein. Ponceau red staining was used to normalize the protein loading (right panel), left panel shows immunolabeling with anti- β_3 antibody.

renal medulla (Fig. 3B). Hormone replacement treatment with DOCA prevented the increase in β_3 mRNA and protein abundance in the renal medulla. In contrast, the abundance of α_1 or β_1 transcripts or proteins in renal medulla obtained from ADX or the ADX + DOCA mice did not show significant changes compared with control mice. Thus, the increase in β_3 expression after ADX was a consequence of mineralocorticoid deficiency.

The studies in ADX rodents suggested that the activity of the MR modulated the expression of the β_3 subunit in the renal medulla. We tested if the pharmacological blockade of the MR with spironolactone (Spiro) modulated the expression of β_3 in the renal medulla of mice (Fig. 4). Spironolactone treatment increased the abundance of β_3 mRNA and protein in renal medulla but not in renal cortex. In contrast, we did not observe statistically significant changes in the abundance of α_1

and β_1 isoforms transcripts or proteins in either renal cortex or medulla.

Discussion

In the present study, we found the differential expression of the β_3 subunit in the cortical and medullary collecting duct cells. The presence of some protruding cells in the cortical collecting duct region that were negative for β_3 , plus the enrichment toward the inner medulla region suggested a preferential expression in principal cells. The qRT-PCR experiments measuring β_3 mRNA also indicated a higher abundance of the β_3 transcript in the inner medullary collecting duct (IMCD). The results of experiments in adrenalectomized rodents with or without mineralocorticoid replacement therapy suggested that mineralocorticoids could decrease the expression of β_3 . The pharmacological blockade of the MR increased the abundance

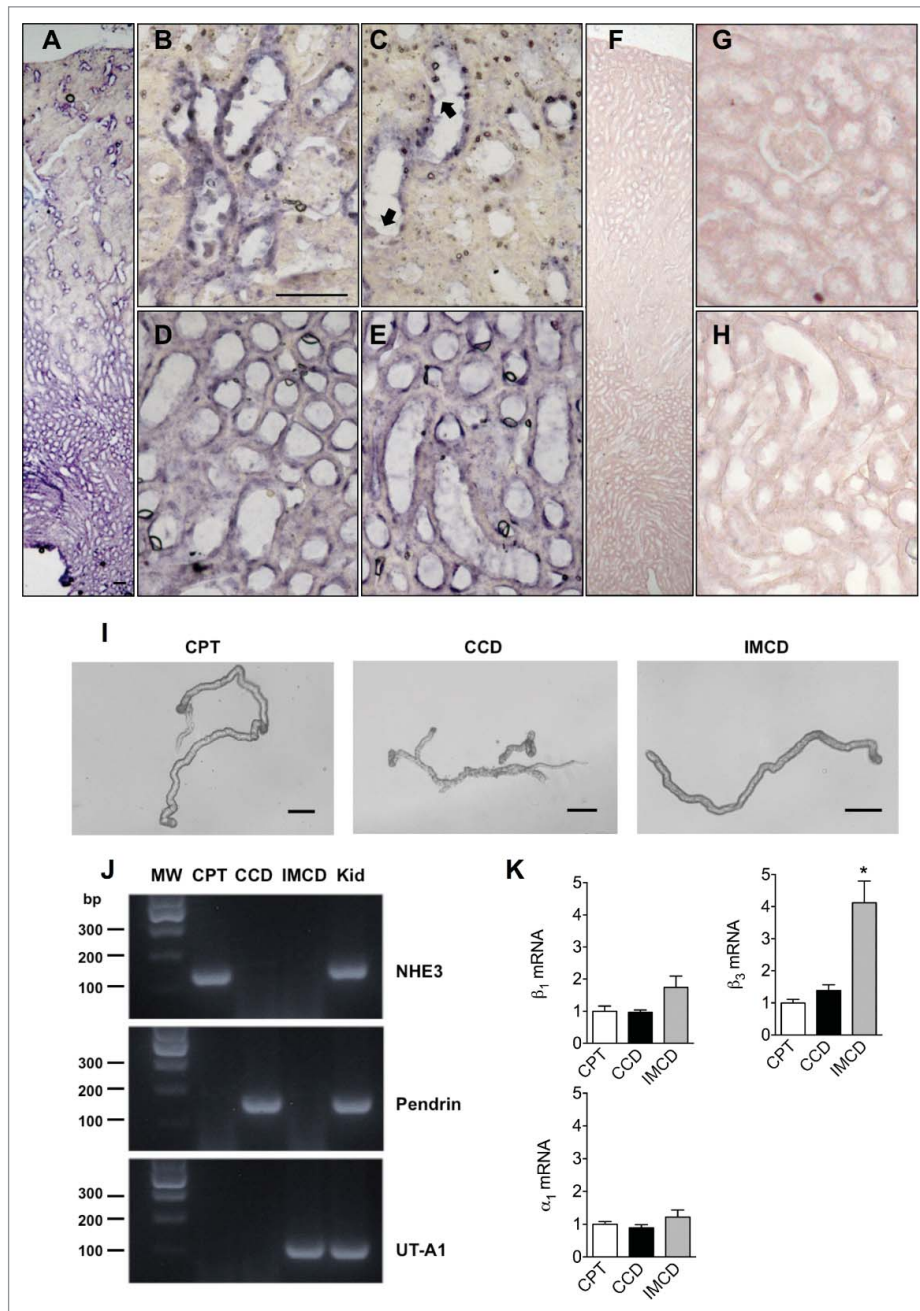


Figure 2. The β_3 sodium pump isoform is expressed by collecting duct cells. (A) Renal tissue was included in paraffin and tissue sections were used for *in situ* hybridization studies with a β_3 -specific probe attached to digoxigenin. The hybridization of the probe was detected by the NBT-BCIP method (blue color). The blue-violet labeling appeared mainly in the renal medulla (panel A), and in cortical collecting duct cells (panels B, C). Black arrows in panel C indicate negative cells with morphology characteristic of intercalated cells. In the renal medulla, the labeling was present in tubular cells (panels D and E). F, G and H panels, area negative control (scrambled probe). The black lines in panels A and B indicate 50 μm . (I) Representative pictures of isolated tubular segments; convoluted proximal tubule (CPT), cortical collecting duct (CCD) and inner medullary collecting duct (IMCD), scale bar = 200 μm . (J) Representative RT-PCR analysis for CPT, CCD and IMCD markers. Total RNA extracted from tubular segments was reverse transcribed and cDNA used for expression analysis. Transcripts for NHE3, Pendrin and UT-A1 were only present in CPT, CCD and IMCD respectively. All transcripts were present in cDNA from whole kidney (Kid). (K) Bars represent mean \pm SD of 4 independent experiments, * $p < 0.05$ vs. CPT and CCD.

of β_3 in the renal medulla of mice. Collectively, these data suggest that the increase in β_3 abundance that follows a decrease in aldosterone and MR activity after adrenalectomy may be the result of the modulatory

action of the MR present in principal cells from collecting ducts.

The $\alpha_1\beta_1$ heterodimer is considered the principal isozyme of the kidney.²⁷ In the initial studies after

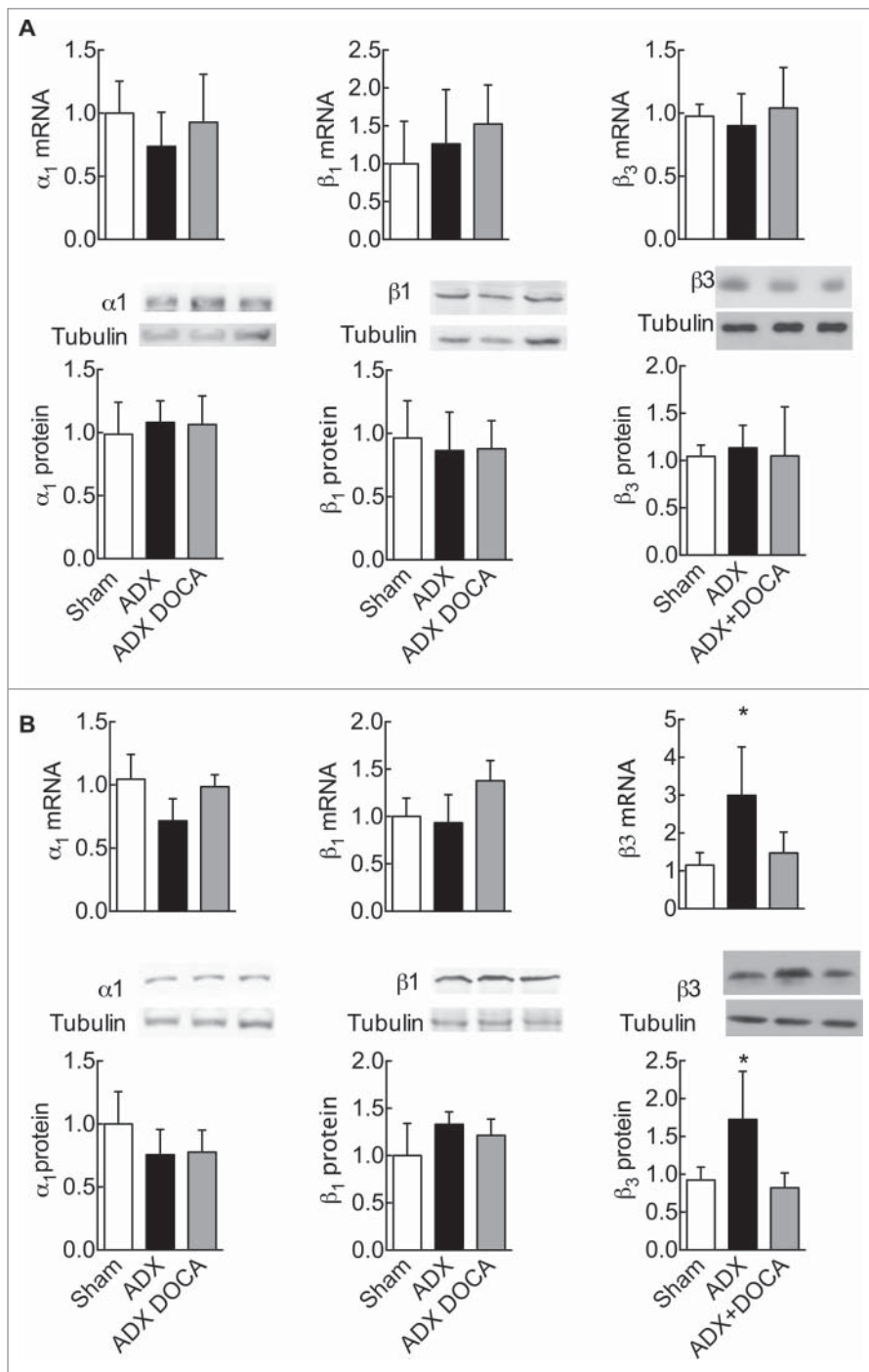


Figure 3. The upregulation of β_3 in ADX mice is prevented by mineralocorticoid replacement therapy. (A) Upper panels show the summary of studies in renal cortex and lower panels (B) show the results of studies in renal medulla. ADX in mice caused a significant upregulation of the β_3 subunit, which was prevented by DOCA treatment. Bars represent mean \pm SD of 4 independent experiments, * $p < 0.05$ vs. Sham and ADX + DOCA.

cloning, β_3 was described as an abundant isoform in testis, retina, liver and lung, with a much lower abundance in kidney.^{8,27} However, our results showed a strong basal expression in the renal medulla. We speculate that the preferential expression of β_3 in the renal medulla may explain the apparent discrepancy of our

data with the results of earlier studies, assuming that the tissue samples used in the previous studies were obtained from the renal cortex or were enriched in renal cortex material.

Our results show the cell-specific expression of β_3 in collecting duct principal cells, but not in

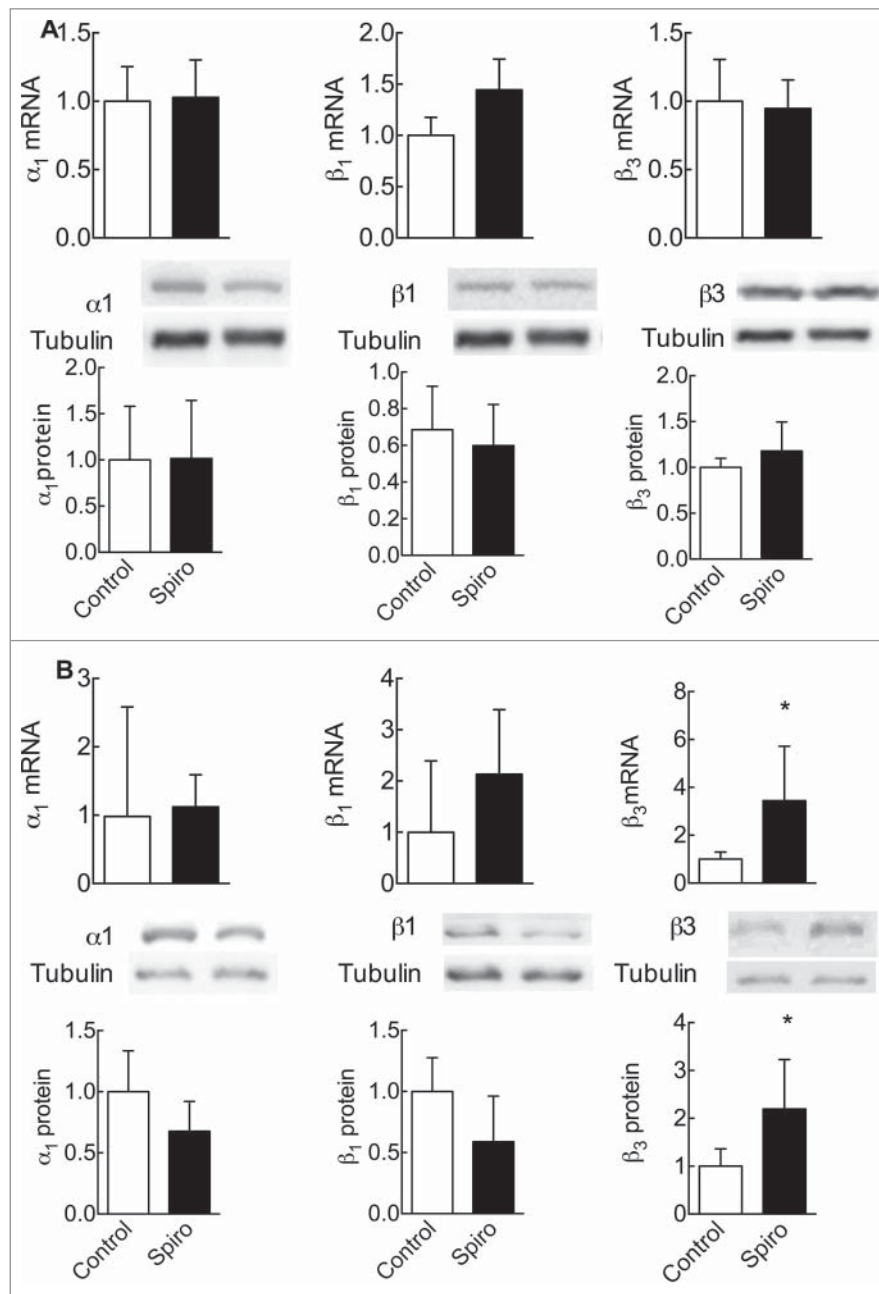


Figure 4. The pharmacological blockade of the MR cause the upregulation of β_3 in renal medulla of mice. (A) The treatment with the MR antagonist (Spironolactone, Spiro) did not modify β_3 expression in renal cortex. (B) The expression of β_3 is upregulated in the medulla of Spiro-treated mice. Bars represent mean \pm SD of 4 independent experiments, * $p < 0.05$ Spiro vs. Control.

intercalated cells. Contrasting with most animal cells that generate the sodium gradient that energizes the trans-epithelial sodium transport by the activity of the Na^+, K^+ -ATPase, collecting duct intercalated cells do not contain any detectable Na^+, K^+ -ATPase activity, and regulate intracellular volume by a mechanism depending on the vacuolar H^+ -ATPase (H^+ -ATPase; also referred to as V-ATPase in the literature).²⁸ Thus, the absence of in intercalated cells is consistent with

the role of the sodium pump in the mechanism mediating trans-epithelial sodium reabsorption in principal cells.

Previous studies indicate that specific tissue β subunit isoform expression may be due to targeting requirements and/or to β isoform-specific functional characteristics of the pump. The initial studies showed that the *Xenopus* β_3 can assemble with α_1 subunits to form a functional Na^+, K^+ -ATPase,²⁹ and that the

association of either β_1 or β_3 isoforms with the α_1 subunit was effective in promoting intracellular trafficking of α/β heterodimers, localization at the plasma membrane and expression of Na^+, K^+ -ATPase activity.³⁰ The study of K^+ kinetics of activation by the coexpression of *Bufo* (toad) α_1 subunit with β_1 , β_2 or β_3 in *Xenopus* oocytes¹⁰ showed that the K^+ half-activation constant ($K_{1/2}$) in the presence of sodium in the external medium was higher for α_1/β_3 than for α_1/β_1 , indicating that the β_3 subunit may reduce the kinetics of ion transport activity of the Na^+, K^+ -ATPase. Along these lines, the association of α_1 with β_1 or β_2 , isoforms found in muscular and brain cells can affect apparent K^+ affinities and turn-over-rate of the Na^+, K^+ -ATPase.¹⁷ Thus, it is plausible that one of the consequences of β_3 expression is reduced sodium pump activity in the inner medulla collecting duct principal cells, a region where PO_2 is low, as compared with proximal tubule cells in the cortex that are immersed in a high PO_2 milieu.

Surprisingly, our studies show a marked and specific modulation of the β_3 subunit abundance in renal medulla by mineralocorticoids and MR activity. The *in silico* analysis of the β_3 promoter region (http://epd.vitalt.ch/mouse/mouse_database.php) from $-5,000$ to $+100$ bp by using *INSILICASE*, showed the presence of 4 repressor glucocorticoid response elements (GRE; aAGgAcacaCtGt; -2526 , -527 , -488 , $+88$)³¹ but the absence of GRE activator elements (aGaACAcgtTGTtCt). Thus, the data suggest that active MR may modulate directly the expression of β_3 . It is well established that changes in dietary NaCl intake or the treatment with loop diuretics increase aldosterone plasma levels, reduce urinary sodium excretion and increase Na^+, K^+ -ATPase activity.^{32,33} Previous studies in cultured cells and isolated renal ducts have demonstrated a rapid increase of plasma membrane α_1/β_1 dimer after incubation with aldosterone.^{22,18,34,5} However, the potential genomic modulation of the sodium pump isoform abundance *in vivo* had been more controversial. The modification of dietary salt intake in rats, for periods ranging from overnight to 3 weeks, caused no significant changes in α_1 or β_1 total abundance, although urinary sodium excretion was modified according to dietary NaCl intake.^{35,36} Similarly, the treatment with furosemide did not affect the expression of α_1 .³⁶ Thus, the results of the present study suggest a new genomic mechanism of sodium pump modulation by

mineralocorticoids. Assuming that both β_1 and β_3 isoforms can associate with the α_1 subunit, the repression of β_3 expression under high plasma aldosterone may favor the formation of α_1/β_1 heterodimer. According to the results of characterization of the kinetic properties of different α/β dimers, the α_1/β_1 heterodimer may be more active than the α_1/β_3 heterodimer. Thus, the preferential expression of α_1/β_1 may favor sodium reabsorption. Further studies are needed to characterize the cell trafficking and destination of α_1/β_1 heterodimers versus α_1/β_3 heterodimer, and also the half-life of the proteins needs to be analyzed. In our experience, the available antibodies for β_3 lack the specificity necessary for such studies.

In summary, we described the expression of the β_3 sodium isoform in principal cells of the collecting duct, and obtained *in vivo* results that indicate that MR activity inhibits β_3 heterodimer in these cells. Future studies are needed to clarify whether the modulation of β_3 expression has a role in the aldosterone mediated increase of trans-epithelial sodium transport in β_3 collecting duct principal cells.

Materials and methods

Reagents

All reagents were from Sigma-Aldrich unless stated. 4-pregnen-21-ol-3,20-dione 21-acetate desoxycorticosterone acetate (DOCA, Q3461-000) was obtained from Steraloids Inc. Antibodies against Na^+, K^+ -ATPase α_1 -subunit (SC21712, 1:1,000), Na^+, K^+ -ATPase β_1 -subunit (SC25709, 1:1,000), anti-rat Na^+, K^+ -ATPase β_3 -subunit (SC66337, 1:1,000), α -tubulin (SC12462-R, 1:5,000), β -actin (SC130656, 1:5,000), donkey anti-mouse IgG-HRP (SC2318, 1:5,000) and goat anti-rabbit IgG-HRP (SC2030, 1:5,000) were from Santa Cruz Biotechnology. Anti Na^+, K^+ -ATPase β_3 -subunit (ab137055, 1:1,500) was obtained from Abcam.

Animals

Male Sprague-Dawley rats weighing 180–220 g with free access to water and standard rat chow were randomly assigned to the adrenalectomy (ADX) group or to the sham surgery (control) group. Rats were anesthetized (Ketamine/Xylazine 3/0.5 mg/100 g b.w., i.p.) and underwent sham surgery or bilateral adrenalectomy.³⁷ All rats had free access

to standard rat chow, the ADX group received NaCl 0.9 % solution and the control group had free access to water. Rats were killed at day 5 by an overdose of Ketamine/Xylazine (6.6/1.3 mg/100 g b.w., i.p.). Male C57BL/6 mice (20–25 g) were assigned to sham surgery, ADX, or ADX+DOCA (2 injections of DOCA, 7.6 mg/100 g b.w., i.m. in olive oil; immediately after surgery and at day 2). Sham and ADX+DOCA groups were maintained on regular diet with free access to water, whereas the ADX group was maintained on 0.9% NaCl solution. The protocols were approved by The Ethics Committee of the Faculty of Medicine of the University de Chile in accordance to The National Institutes of Health Guide for the Care and Use of Laboratory Animals (Protocol No. CBA-0540 FMUCH).

RNA isolation and qRT-PCR

Tissues were processed for total RNA extraction with TRIzol™ Reagent (15596026, Invitrogen™, Thermo Fischer Scientific) as per manufacturer instructions, followed by DNase digestion (TURBO DNA-free™ Kit AM1907, Thermo Fisher Scientific). RNA integrity was evaluated by measuring the S18/28 ratio, considering a value >1.8 as optimal. Reverse transcription was performed with ImProm-II™ Reverse Transcription System (A3800, Promega) using 100 ng of total RNA as template. cDNAs were amplified using SYBR Green and GoTaq® 2-Step RT-qPCR System (A6010, Promega) in a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA). Transcript levels were normalized to S18 rRNA subunit. Primers were synthesized by Integrated DNA Technologies and were as follows: Na⁺,K⁺-ATPase α_1 -subunit (*ATP1A1*) forward 5'-TGCGGAAGCTCATTATCAGG-3', reverse 5'-CACATGCTTTGGTGCTTTCC-3'; Na⁺,K⁺-ATPase β_1 -subunit (*ATP1B1*) forward 5'-AGGTTTGCTGTCCAAGGTGT-3', reverse 5'-ATC-GATGGGCCGTTACCTTT-3'; Na⁺,K⁺-ATPase β_3 -subunit (*ATP1B3*) forward 5'-TCAGCTGGACCTGCCAT TCT-3', reverse 5'-GCGACCGTACATGGGTCAA-3'; NHE3 (*SLC9A3*) forward 5'-GTTGCTGACACTGGTC TTCA-3', reverse 5'-TCCTTGACTTTCTTCTCATC CA-3'; Pendrin (*SLC26A4*) forward 5'-TCTGGACTGG CTCCCAAAT-3', reverse 5'-GGGCACAAGAAAG TGGTCATC-3'; UT-A1 (*SLC14A2*) forward 5'-GACAGTGAGACGCAGTGAAG-3', reverse 5'-ACG

GTCTCAGAGCTCTCTTC-3'; 18S rRNA forward 5'-CGGCTACCACATCCAAGGAA-3', reverse 5'-GCTG GAATTACCGCGGCT-3'.

Western blot

Total protein homogenates from renal and brain tissues were prepared in sucrose lysis buffer (50 mM Tris, 5 mM MgCl₂, 250 mM sucrose, pH 7.6) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail 11873580001, Roche Applied Science).³⁸ Then, samples were centrifuged at 4,400 g for 15 min at 4°C and the supernatant was recovered. Proteins were quantified by Bradford method (Bio-Rad Protein Assay Kit I 5000001, Bio-Rad Laboratories) according to the manufacturers protocol. Proteins (50 μ g) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-P, 10600023; GE Healthcare). Membranes were blocked with 5 % non-fat dry milk in PBS at room temperature during 1 hour. Primary antibodies were incubated overnight at 4°C, followed by incubation with a HRP-conjugated secondary antibody for 1 hour at room temperature. Protein bands were detected by chemiluminescence using EZ-ECL Kit (20–500–500, Biological Industries). Immunodetection was performed with a ChemiScope Series 3200-Mini photo-documentation system (Clinx Science Instruments, Shanghai, China). Protein content was normalized using α -tubulin and β -actin as loading controls.

Tubular segment isolation

Defined tubular segments were isolated by free-hand microdissection in Krebs-Ringer phosphate buffer pH 7.4 supplemented with 1% fetal bovine serum (Gibco™ 12483–020, Thermo Fisher Scientific) at 4°C under a high-magnification stereomicroscope (DZ.1100, Euromex, Arnhem, Netherlands). Approximately 20 mm of convoluted proximal tubule (CPT), cortical collecting duct (CCD) and inner medullary collecting duct (IMCD) were isolated and used for total RNA preparation as described previously. Expression of NHE3, Pendrin and UT-A1 were used as markers of tubule identity and purity for CPT, CCD and IMCD respectively. Representative pictures of each tubular segments were recorded using a 16-MP MU1603 digital camera (AmScope, Irvine, CA).

In-situ hybridization

Kidney tissues were hemisected coronally along the renal papilla fixed in 10% formalin and paraffin-embedded for *in situ* hybridization as described.³⁹ Briefly, 10- μ m tissue sections were prepared using a Leica CM1510-S Cryostat (Leica Biosystems, Wetzlar, Germany), cleared in xylenes and rehydrated using an ethanol gradient. Sections were treated with diethyl pyrocarbonate (DEPC) for 1 min, rinsed in DEPC-treated PBS, and then exposed to proteinase K (20 mg/mL) for 5 minutes at 37°C. Sections were mounted in DEPC-treated slides and fixed in freshly made 4% paraformaldehyde for 10 min, followed by several washes with DEPC-treated 0.02% Tween-20 PBS (PBS-T). The tissues were covered with 50 μ L of hybridization buffer (50% formamide, 5X SSC, 0.1% Tween, 9.2 mM citric acid, pH 6.0, 50 μ g/mL heparin, 500 μ g/mL yeast RNA) followed by RNase-free hybrislips (70329, Electron Microscopy Sciences) and placed in a hybridization oven for 2 hours at 54°C. Na⁺,K⁺-ATPase β_3 -subunit or control probes (ATP1B3: digoxigenin-5'-ATATGCAGTTACCAGT GTTAA-3'-digoxigenin, Control: digoxigenin-5'-GTGTAACACGTCTATACGCCCA-3'-digoxigenin; LNATM Detection Probes, Exiqon) diluted in hybridization buffer (40 μ M, 75 μ L/tissue section) were preheated at 65°C for 5 min to linearize and then added to the slides and incubated overnight at 54°C. Slides were rinsed in 50% deionized formamide/50% 2X SSC, followed PBS-T washes and then blocked for 1 h (2% goat serum, 2 mg/ml BSA in PBS-T at room temperature) followed by overnight incubation at 4°C with 1:500 anti-digoxigenin alkaline phosphatase (AP) antibody (11093274910, Roche Applied Science) in blocking solution. After extensive washing with PBS-T and then with AP buffer (100 mM Tris-HCl pH 9.5, 50 mM MgCl₂, 100 mM NaCl, 0.1% Tween-20), the slides were incubated in nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP Reagent Kit N6547, Thermo Fisher Scientific) diluted in AP buffer and then rinsed with AP buffer, to stop color development, before dehydration, clearing, and mounting.

Statistical analyses

Values are expressed as mean \pm SD for each experimental group with number of experiments (*n*) referring to number of animals. Comparisons between 2

groups were made by Student's *t*-test while comparisons between 3 or more experimental groups were made using one-way ANOVA and Tukey's post-test (Prism 6.0 software; GraphPad Software Inc. La Jolla, CA). A *p* value of <0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

No conflicts of interest were disclosed.

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