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Aldosterone regulates Na^+, K^+ ATPase activity in human renal proximal tubule cells through mineralocorticoid receptor

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ABSTRACT

The mechanisms by which aldosterone increases Na^+, K^+ ATPase and sodium channel activity in cortical collecting duct and distal nephron have been extensively studied. Recent investigations demonstrate that aldosterone increases Na–H exchanger-3 (NHE-3) activity, bicarbonate transport, and H^+ ATPase in proximal tubules. However, the role of aldosterone in regulation of Na^+, K^+ ATPase in proximal tubules is unknown. We hypothesize that aldosterone increases Na^+, K^+ ATPase activity in proximal tubules through activation of the mineralocorticoid receptor (MR). Immunohistochemistry of kidney sections from human, rat, and mouse kidneys revealed that the MR is expressed in the cytosol of tubules staining positively for *Lotus tetragonolobus* agglutinin and type IIa sodium-phosphate cotransporter (NpT2a), confirming proximal tubule localization. Adrenalectomy in Sprague–Dawley rats decreased expression of MR, ENaC α, Na^+, K^+ ATPase α1, and NHE-1 in all tubules, while supplementation with aldosterone restored expression of above proteins. In human kidney proximal tubule (HKC11) cells, treatment with aldosterone resulted in translocation of MR to the nucleus and phosphorylation of SGK-1. Treatment with aldosterone also increased Na^+, K^+ ATPase-mediated ^{86}Rb uptake and expression of Na^+, K^+ ATPase α1 subunits in HKC11 cells. The effects of aldosterone on Na^+, K^+ ATPase-mediated ^{86}Rb uptake were prevented by spironolactone, a competitive inhibitor of aldosterone for the MR, and partially by Mifepristone, a glucocorticoid receptor (GR) inhibitor. These results suggest that aldosterone regulates Na^+, K^+ ATPase in renal proximal tubule cells through an MR-dependent mechanism.

1. Introduction

Aldosterone regulates extracellular fluid volume and potassium metabolism by activation of the mineralocorticoid receptor in target tissues, such as the distal tubule and the cortical collecting duct of the kidney, where aldosterone increases sodium reabsorption and potassium excretion [1–5]. Reabsorption of sodium ions through NCC or ENaC on the apical membrane of the distal tubule or cortical collecting duct results in a fall in transmembrane potential, thus increasing the flow of positive ions, such as potassium, out of the cell into the lumen. The reabsorbed sodium ions are transported out of the tubular epithelium into the renal interstitial fluid through the action of basolateral Na^+, K^+ ATPase. Several studies suggest that the effects of aldosterone are not limited to distal and collecting tubules. Hierholzer and colleagues [6,7] demonstrated that aldosterone stimulates proximal tubular volume reabsorption in adrenalectomized rats using micropuncture techniques. More recent studies demonstrate that aldosterone increases NHE3 expression and function in the apical membranes of proximal tubules under pathophysiological conditions such as cirrhosis [8]. In adrenalectomized animals and in human proximal tubule cells, aldosterone has been shown to increase proximal tubular fluid reabsorption and NHE3 expression [9,10]. Aldosterone also increases NHE1 function [11], H^+-ATPase activity [12] and bicarbonate transport in renal proximal tubules [13]. The mechanisms for aldosterone-mediated actions in the proximal tubule, including the pathways stimulated by aldosterone in proximal tubule have not been identified. Based on treatment time (15 min) and blockade of the effects of aldosterone by RU486, the effects of aldosterone on bicarbonate transport and Na^+/H^+ exchange in renal proximal tubules were shown to be dependent upon non-genomic actions through GR [12,13]. Whether other aldosterone effects on proximal tubule are mediated through the classical mineralocorticoid/genomic pathway or through

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non-genomic mechanisms has not been determined. Several investigators have demonstrated the presence of MR and sodium and glucocorticoid-dependent kinase (SGK-1), a major MR target signaling molecule, in proximal tubules by quantitative PCR [14–16]. On the other hand, type 2 β-hydroxysteroid dehydrogenase (11-βHSD), the enzyme that confers specific tissue responsiveness to mineralocorticoid stimulation by metabolism of glucocorticoids, has not been identified in the proximal kidney tubule [17,18], while the type 1 form, which facilitates activation of glucocorticoid receptors by glucocorticoids, has been found [19]. However, Bonvalet et al. [20] showed that carbenoxolone completely prevented conversion of corticosterone to dihydrocorticosterone in both proximal convoluted and cortical collecting tubules suggesting expression of type-2 11-βHSD. Based on these findings, we tested the hypothesis that aldosterone regulates Na^+, K^+ ATPase activity and expression in human kidney proximal tubule cells through MR-dependent mechanisms. Our studies demonstrate the presence of MR in rat, mouse, and human kidney proximal tubules and confirm an essential role of MR in regulation of proximal tubule Na^+, K^+ ATPase by aldosterone.

2. Experimental procedures

2.1. Materials

Aldosterone, Mifepristone, spironolactone, Phosphatase inhibitor cocktail-1, and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). Antibodies against Na^+, K^+ ATPase α1 subunit (α6F) developed by Dr. D.M. Fambrough and against mineralocorticoid receptor (rMR1-18 1D5) developed by Dr. C. Gomez-Sanchez were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of NIHCD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Antibodies against phospho- and total SGK1 were purchased from Cell Technologies, and monoclonal antibodies against NHE-1 were purchased from BD Biosciences. Polyclonal antibodies against ENaCα, β and γ subunits were kindly provided by Dr. Carolyn Ecelbarger (Georgetown University, Washington DC). Polyclonal antibodies against mouse NpT2a were kindly provided by Dr. Moshe Levi (University of Colorado, Denver) and against NKCC2 were kindly provided by Dr. Mark Knepper (NIH). Polyclonal antibodies against human NpT2a (NaPi3) were kindly provided by Dr. Robert Gunn (Emory University). HRP-linked secondary antibodies were purchased from Vector Laboratories. AlexaFluor antibodies were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma, unless otherwise specified.

2.2. Animal preparation

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (100–150 g, Charles River Laboratories, Wilmington, MA), received free access to water and standard rat chow (Purina) containing 23% protein. Rats were adrenalectomized as previously described [21] and received 1/4 normal saline to drink thereafter. After 14 days, some rats received aldosterone replacement (12 μg/rat/day) via a 14-day minipump (Alzet, Durect). Paraffin blocks of mouse kidneys were kindly provided by Dr. Barbara Clark (Department of Biochemistry, University of Louisville). Paraffin blocks of de-identified human kidneys were provided by Dr. Susan Coventry (Department of Pathology, University of Louisville) after IRB approval from the University of Louisville.

2.3. Immunohistochemistry

Immunohistochemistry of kidney slices was performed as described previously [22] using monoclonal antibodies against rat mineralocorticoid receptor, Na^+, K^+ ATPase α1 subunit, NHE-1, polyclonal antibodies against ENaC α, β, and γ subunits, NpT2a, NKCC2, or appropriate isotype control IgG (negative control).

2.4. Basolateral membrane isolation

Kidney cortical BLMs were prepared from rats treated with or without aldosterone for 14 days by the method of Sacktor et al. [23] with slight modifications as described previously [22].

2.5. Western blot analysis

Western blot was performed exactly as described previously [24,25].

2.6. ATP hydrolysis assay

BLM vesicles were quickly frozen in liquid nitrogen and were slowly thawed on ice to make them permeable to ATP before measurement of Na^+, K^+ ATPase activity. Na^+, K^+ ATPase activity in basolateral membranes was assayed as ouabain (4 mM)-sensitive ATP hydrolysis as previously described [26]. The inorganic phosphate released was measured as described previously [26].

2.7. Cell culture

Human kidney cells (HKC-5 and HKC-11, a gift from Dr. Lorraine Racusen, John Hopkins University Baltimore, MD), human kidney proximal tubule cells (HK2, ATCC, Manassas, VA) and Madin–Darby Canine Kidney cells (MDCK, ATCC, Manassas, VA) were cultured as previously described [22]. Cells were maintained in DMEM-F12 (1:1) supplemented with 10% FBS, and 1% penicillin/streptomycin, and cultured to 90–95% confluence. Cells were washed with serum-free medium 24 h before use.

2.8. Confocal microscopy

Cells treated with vehicle or 10 nM aldosterone were fixed in 4% paraformaldehyde and confocal microscopy was performed exactly as described previously using antibodies against MR and phalloidin-Alexa488 (actin).

2.9. Ouabain-sensitive ^{86}Rb uptake

Ouabain-sensitive ^{86}Rb uptake in human kidney cells was measured at 37 °C exactly as described previously [24,26]. Cells were treated for 24 h at 37 °C with aldosterone at indicated concentrations. Cells were then treated at 37 °C with 5 μM monensin and half the cells were also treated with 4 mM ouabain 30 min prior to ^{86}Rb uptake. A trace amount of ^{86}Rb (~1 μCi/ml ^{86}Rb) in serum-free DMEM-F12 (1:1) was added and uptake was carried out for 10 min. The cells were washed 5–6 times with ice cold PBS and lysed overnight in 0.5 N NaOH containing 0.1% Triton X-100 at 37 °C. An aliquot (100 μl) of the lysate was used to measure radioactivity. The difference between ^{86}Rb uptake measured in the presence or absence of 4 mM ouabain was used as a measure of Na^+, K^+ ATPase-mediated transport activity. Uptake data are expressed as nanomoles rubidium accumulated per milligram of protein per 10 min.

2.9.1. Protein determination

Protein concentration was determined using a bicinchoninic acid protein assay kit (Sigma) using BSA standard.

2.9.2. Statistics

Data are shown as mean ± SE. The n values represent the number of independent experiments. Each experiment was performed in triplicate. p values were calculated by Student's *t*-test or by one-way

ANOVA, followed by Bonferroni analysis using GraphPad Prism software. A p value <0.05 was a priori considered statistically significant.

3. Results

3.1. Expression of the mineralocorticoid receptor in kidney proximal tubules

To determine if MR is expressed in kidney proximal tubules, we performed immunohistochemistry on human, rat, and mouse kidney sections and identified MR in both proximal and distal tubules. As shown in Fig. 1, cytosolic expression of MR was observed in proximal tubules of all the three species. Of note, staining was observed in the proximal tubules only in the juxtamedullary cortex and more specifically in the renal pyramids but not in the proximal tubules of the superficial cortex. To confirm proximal tubule localization of MR staining, human kidney sections were counterstained with *Lotus tetragonolobus* agglutinin (a marker for proximal tubules, purple stain). The arrows indicate proximal tubules showing the presence of the MR. Additionally, to confirm the presence of MR in proximal tubules, rat kidney sections were analyzed by immunofluorescence using NpT2a (red staining) and MR (green staining) antibodies. As shown in Fig. 1B, in juxtamedullary proximal tubules MR staining was present in cytosol of tubules positively stained with NpT2a (arrows) and nuclear staining was observed in cortical collecting ducts negative for NpT2a staining (arrowheads).

3.2. Effect of adrenalectomy on expression of MR and sodium transporters

To determine the effect of loss of aldosterone on expression of MR, rats were subjected to adrenalectomy (ADX) followed by treatment with vehicle or aldosterone via minipump for an additional 14 days.

Kidney sections from sham operated, ADX, or ADX rats treated for 14 days with aldosterone were analyzed for MR expression by immunohistochemistry. As shown in Fig. 2A, adrenalectomy decreased expression of MR in all nephron segments. Aldosterone replacement to ADX rats restored MR expression in all nephron segments. The immunohistochemistry findings were confirmed by Western blot analysis of homogenates from kidney cortex. As shown in Fig. 2B, adrenalectomy decreased MR expression in ADX rats. Aldosterone replacement to ADX rats restored MR expression.

As a positive control, the effects of adrenalectomy and of aldosterone replacement on ENaC expression were examined. Adrenalectomy decreased the expression of ENaCα without changing the expression of ENaC β or γ subunits in the cortical collecting duct. Aldosterone replacement to ADX animals restored ENaCα expression (Supplementary Fig. 1).

3.3. Effect of aldosterone on Na^+, K^+ ATPase α1 subunit and NHE1 expression

Aldosterone increases expression of Na^+, K^+ ATPase α1 subunit in kidney collecting tubules [27,28] and activity of NHE1 in S3 segment of proximal tubules [29]. We have previously demonstrated that NHE-1 is required for regulation of Na–K ATPase activity and membrane expression in proximal tubules [22]. We examined the effects of adrenalectomy on Na^+, K^+ ATPase α1 subunit and NHE1 expression in kidney sections from sham operated, ADX, or ADX rats treated with aldosterone by immunohistochemistry using antibodies against Na^+, K^+ ATPase α1 subunit or NHE1. As shown in Fig. 3A, adrenalectomy decreased Na^+, K^+ ATPase α1 subunit and NHE1 expression in all tubules. Aldosterone replacement to ADX animals restored expression of Na^+, K^+ ATPase α1 subunit and NHE1 in all kidney tubules. To confirm and quantify the immunohistochemistry data, expression of Na^+, K^+ ATPase α1 subunits in kidney cortical basolateral membranes was determined by Western blot. As shown in Fig. 3B, basolateral membranes from ADX animals showed decreased expression of Na^+, K^+ ATPase α1 subunit and NHE1 which was largely restored by treatment with aldosterone. To determine the effects of adrenalectomy on Na^+, K^+ ATPase activity, we measured activity in basolateral membranes from kidney cortex of the above rats. As shown in Fig. 3C, activity decreased in basolateral membranes from ADX animals. Aldosterone replacement to ADX animals restored Na^+, K^+ ATPase activity in basolateral membranes.

To determine if adrenalectomy had a global effect on all Na^+ transporters, we examined the expression of NKCC2 and NpT2a in the described animal groups. In contrast to Na^+, K^+ ATPase and NHE1, expression of NKCC2 or NpT2a did not change with adrenalectomy or aldosterone replacement to ADX rats (Fig. 3D).

3.4. Effect of aldosterone on MR expression in human kidney proximal tubule cells

The preceding animal studies demonstrate that MR is expressed in human, rat, and mouse kidney proximal tubules. To examine the mechanisms for aldosterone-mediated changes in proximal tubule sodium transporter function and expression, we turned to a cell culture model of renal proximal tubule, the human kidney cell line HKC11 cells [30]. Proximal tubular origin of HKC11 cells was confirmed by identification of NpT2a (NaPi-3) and NHERF-1 expression by confocal microscopy (Supplementary Fig. 2). To determine if MR is expressed and regulated in proximal tubule cells, HKC11 cells were treated for 30 min with 10 nM aldosterone and expression of MR was examined by confocal microscopy. As shown in Fig. 4A, cytosolic expression of MR was observed under basal conditions. Treatment with 10 nM aldosterone for 30 min resulted in a marked increase in nuclear translocation of the MR. The aldosterone-stimulated increase in nuclear translocation of MR was confirmed by Western blot of nuclear fraction in HKC11 cells (Fig. 4B).

To determine if aldosterone regulates SGK-1 in proximal tubule cells similar to cortical collecting ducts [2,5], HKC11 cells were treated with 10 nM aldosterone for different time intervals (0–6 h). As shown in Fig. 4C, treatment with aldosterone initially increased SGK-1 phosphorylation but the phosphorylation status returned to the baseline level after 1 h of aldosterone treatment. Total SGK-1 expression levels did not change with aldosterone treatment.

Nuclear-free cytosolic fractions from the above cells were also used to examine the effects of aldosterone on MR and Na^+, K^+ ATPase α1 expression. As shown in Fig. 4D, treatment with aldosterone for 4 h decreased cytosolic expression of MR consistent with the translocation of the receptor to the nucleus seen in the confocal studies. Treatment with aldosterone increased Na^+, K^+ ATPase α1 and β1 subunit expression by 4 h as expected (Fig. 4D and Supplementary Fig. 3). Studies by Bonvalet et al. [20] suggested 11-βHSD2 activity in proximal convoluted tubules. To determine if 11-βHSD2 is expressed in the proximal tubule cells, we performed Western blot in whole cell homogenates from HKC-11 cells before and after treatment with 10 nM aldosterone. As shown in the Supplementary data, 11-βHSD2 is expressed in HKC-11 cells similar to MDCK cells (Supplementary Fig. 3).

3.5. Effect of aldosterone on Na^+, K^+ ATPase-mediated ^{86}Rb uptake in human kidney cells

To determine if aldosterone stimulates Na^+, K^+ ATPase-mediated ion transport as well as expression of the α subunit, HKC11 cells were treated with 1 pM–10 nM aldosterone for 24 h and Na^+, K^+ ATPase-mediated ^{86}Rb uptake was measured. As shown in Fig. 5A, aldosterone increased Na^+, K^+ ATPase-mediated ^{86}Rb uptake in HKC11 cells in a dose dependent manner. Of note, the increase reached significance in cells treated with 10 pM aldosterone. The maximum increase in Na–K ATPase-mediated ^{86}Rb uptake was observed in cells treated with 1 nM aldosterone. ^{86}Rb uptake decreased in cells treated with 10 nM aldosterone compared to cells treated with 1 nM aldosterone but was

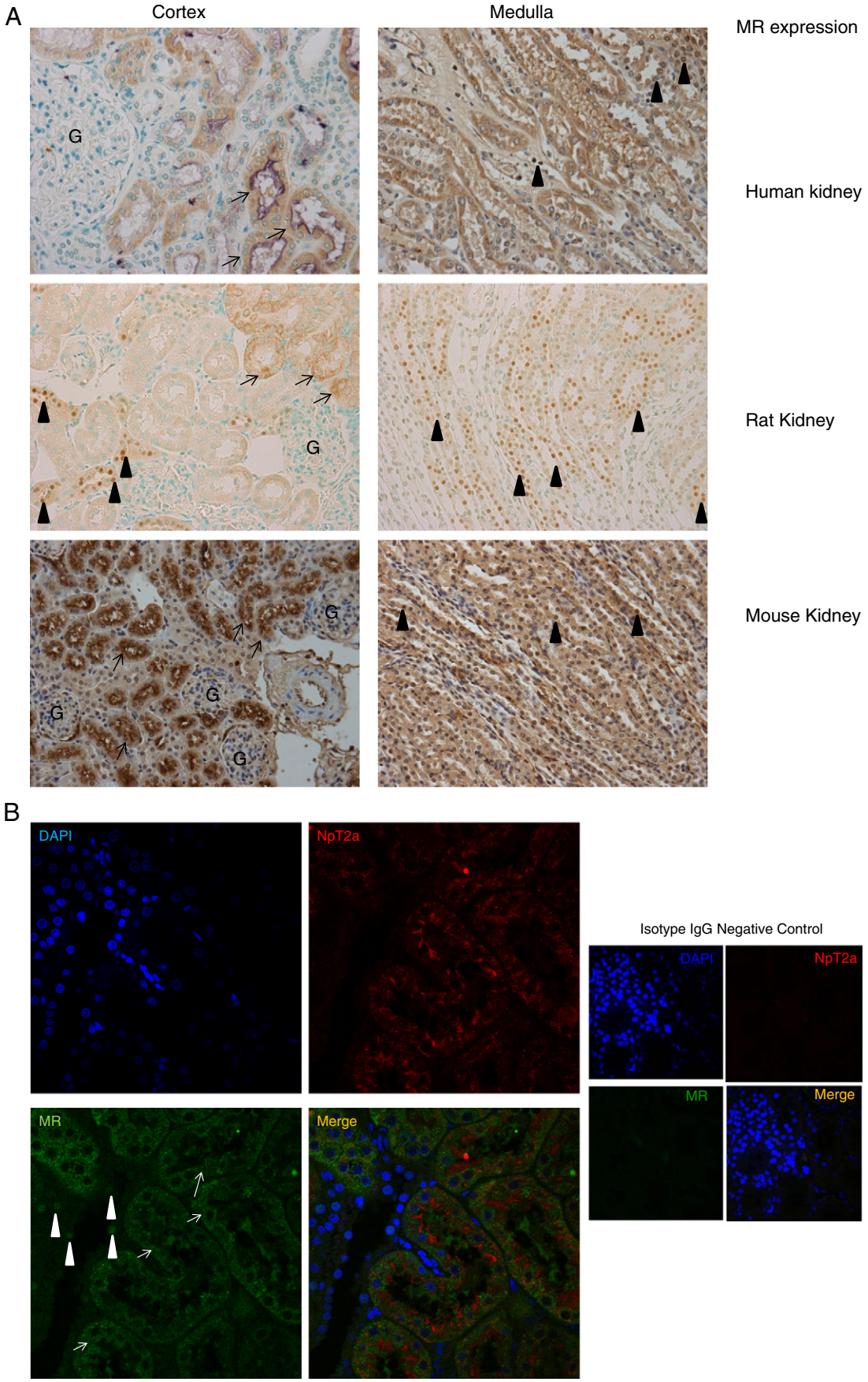

Fig. 1. Expression of mineralocorticoid receptor (MR) in kidney. Human, rat, or mouse kidney sections (cortex, left panel or medulla, right panel) were analyzed by immunohistochemistry using antibodies against MR. Human kidneys were counterstained with *Lotus tetragonolobus* (purple stain) to mark the proximal tubules (left top panel). Mouse and rat consecutive sections were stained with antibodies for NpT2a to mark proximal tubules (data not shown, n = 4). B, rat kidney sections were analyzed by immunofluorescence using antibodies against NpT2a (red staining) and MR (green staining). Arrows show intracellular expression in proximal tubules and arrowheads show nuclear expression in distal and collecting tubules.

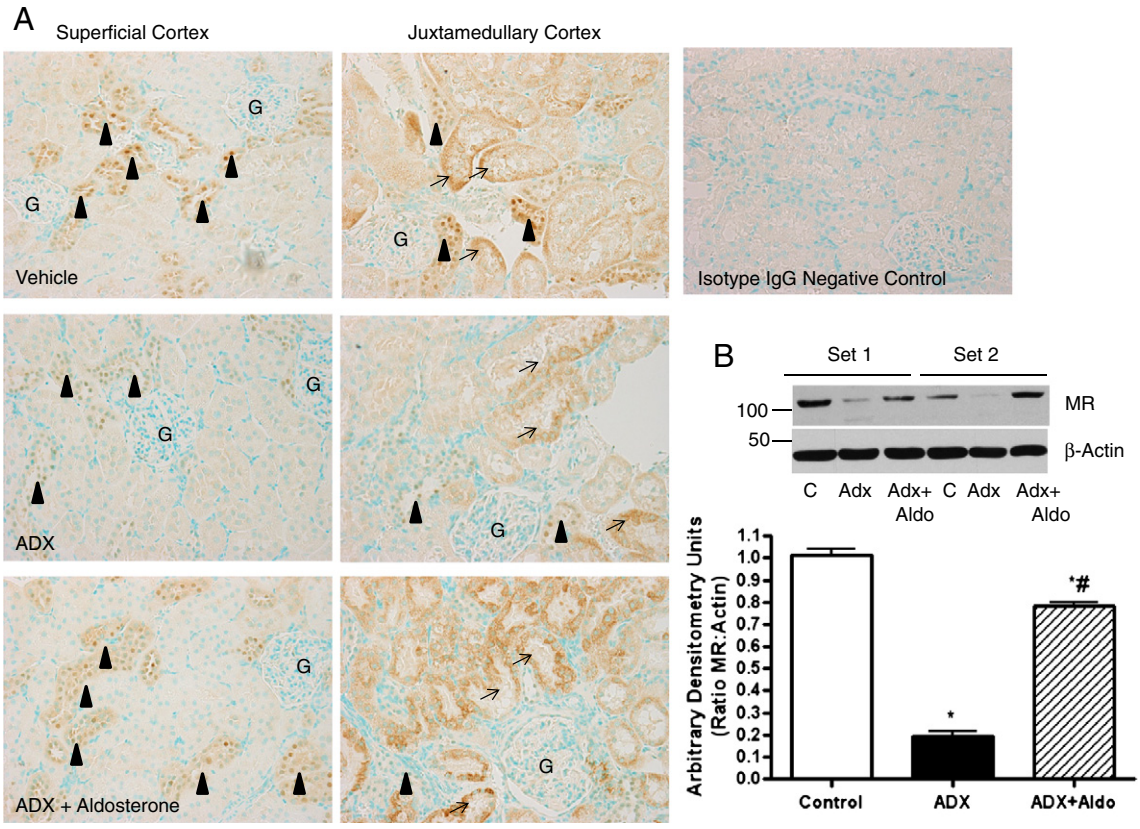

Fig. 2. Effect of ADX on MR expression in kidney. A, kidney sections from sham operated and vehicle treated (top panel), ADX and vehicle treated (middle panel) and ADX rats treated with aldosterone (bottom panel) were analyzed by immunohistochemistry using antibodies against MR. Left panels show histochemistry from the superficial cortex and the right panels show histochemistry from juxtamedullary cortex. Arrows indicate expression in proximal tubules. Arrowheads indicate expression in cortical collecting duct. All pictures were taken using a 40× objective lens. B, kidney cortical homogenates from the above animals were analyzed by Western blot for MR expression. Representative histochemistry or Western blots of kidneys from four individual animals in each group are shown. Bar graph shows arbitrary densitometry units as ratio of MR to actin in homogenates from four individual animals ($n = 4$ in each group). *Indicates $p < 0.05$ by ANOVA followed by Bonferroni analysis from control animals. #Indicates $p < 0.05$ by ANOVA followed by Bonferroni analysis from ADX animals.

significantly higher than the vehicle treated cells. To confirm that aldosterone increases Na^+, K^+ ATPase-mediated ^{86}Rb uptake in proximal tubules we treated two other human kidney proximal tubule cell lines, HK2, HKC-5, and a distal tubular cell line, MDCK cells. In all of the above cell lines, 24 h treatment with aldosterone increased Na^+, K^+ ATPase-mediated ^{86}Rb uptake (Supplementary Fig. 4).

To determine if the effects of aldosterone are mediated through MR or GR, cells were treated with aldosterone in the presence or absence of 1 μM spironolactone (MR antagonist) or Mifepristone (RU486, a GR antagonist). As shown in Fig. 5B, aldosterone increased Na^+, K^+ ATPase-mediated ^{86}Rb uptake in human kidney proximal tubule cells. Treatment with spironolactone completely prevented the aldosterone-induced increase in Na^+, K^+ ATPase-mediated ^{86}Rb uptake. Mifepristone blocked the aldosterone-induced increase in Na^+, K^+ ATPase-mediated ^{86}Rb uptake by less than 25%. To determine if treatment with dexamethasone similarly increases Na^+, K^+ ATPase-mediated ^{86}Rb uptake cells were treated for 24 h with 1 μM dexamethasone in the presence or absence of Mifepristone (1 μM) or spironolactone (1 μM). As shown in Fig. 5C, treatment with dexamethasone increased Na^+, K^+ ATPase-mediated ^{86}Rb uptake which was completely prevented by Mifepristone but not by spironolactone.

4. Discussion

In the present study, we report immunohistochemical demonstration of mineralocorticoid receptor expression in human, rat, and mouse kidney proximal tubules. Specifically, the expression was observed in the proximal tubules of the juxtamedullary cortex particularly in the renal pyramids. The proximal tubule localization of MR is confirmed by positive co-staining with the specific markers of proximal tubule, *L. tetragonolobus* agglutinin (human kidney sections) and NpT2a (rat kidney sections).

We have previously demonstrated that low dose ouabain, another steroid hormone, stimulates sodium pump activity in an NHE-1 dependent manner [22]. Our current data showing that expression of Na^+, K^+ ATPase and NHE-1 increases in ADX rats treated with aldosterone in proximal tubule along with previous studies showing aldosterone-stimulated upregulation of NHE3 [9,10] and increased sodium bicarbonate transport [12,13] suggest that aldosterone increases sodium uptake and transepithelial transport in proximal tubules. This suggestion is strengthened by the demonstration that the increase in alpha subunit expression corresponds to enhanced Na^+, K^+ ATPase activity. Thus this represents an increase in functional Na transport activity. The absence of an effect of aldosterone on expression of NKCC2 and NpT2a shows that the effects of aldosterone are specific to the proximal tubule proteins involved in transepithelial sodium transport and not simply a nonspecific effect on all transporters. Our results stand in contrast to previous reports suggesting no effect of exogenous aldosterone on proximal tubule ion transport. Notably, these prior studies were performed in adrenal-intact animals and with a shorter duration of aldosterone treatment suggesting that perhaps a major physiologic role for aldosterone in proximal tubule is in tonic basal expression of these transporters [33,34].

In a recent study Ackermann et al. demonstrated the expression of MR in the thick ascending limb, distal tubules, and cortical collecting duct but not in the proximal tubules of Wistar rats and mice both by PCR and immunohistochemistry [32]. In contrast, Leite-Dellova et al. [31] and Pinto et al. [11] demonstrated the expression of MR mRNA in

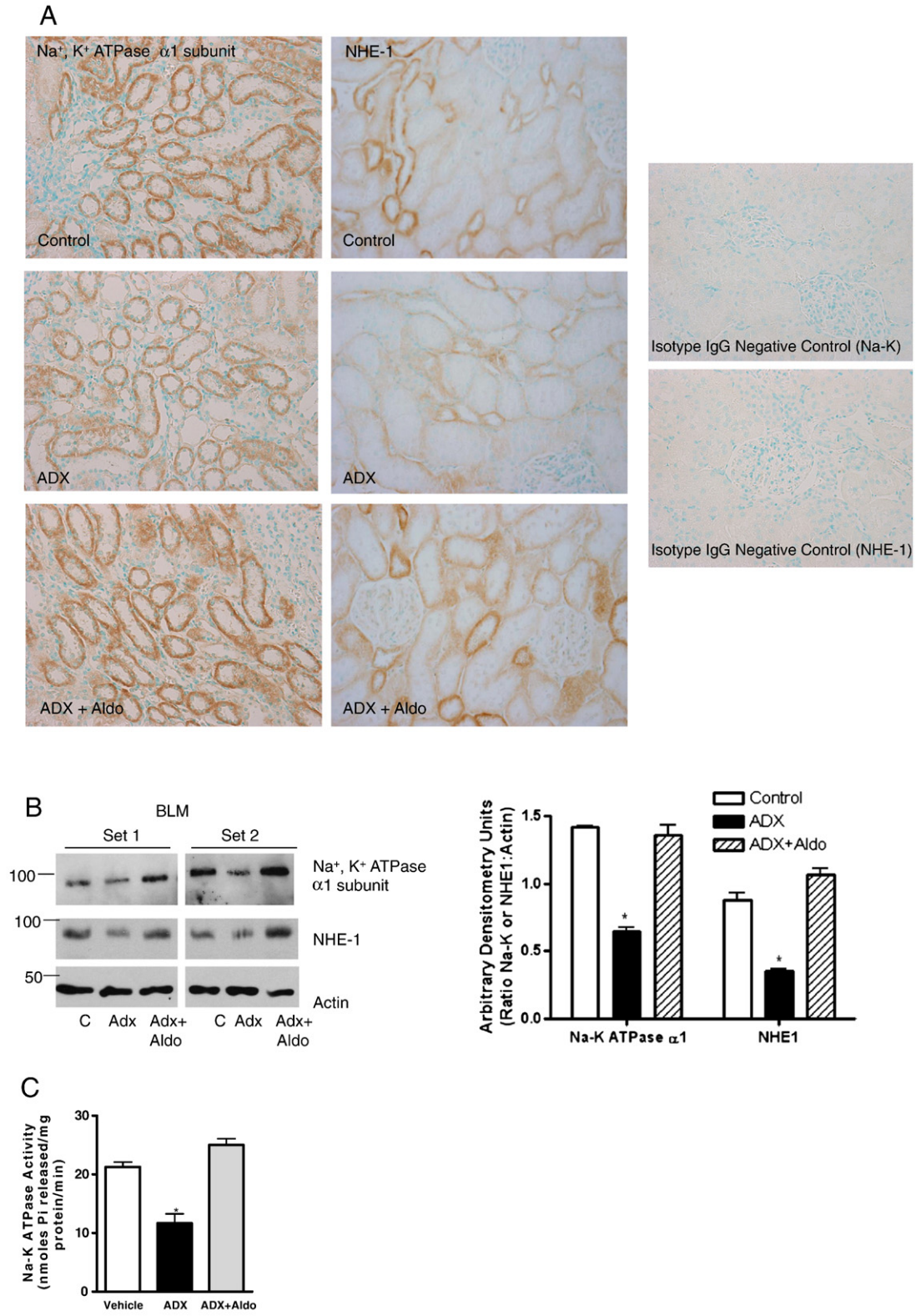

Fig. 3. Effect of ADX on Na^+, K^+ ATPase and NHE1 expression in kidney. A, kidney sections from sham operated and vehicle treated (top panels), ADX and vehicle treated (middle panels) and ADX rats treated with aldosterone (bottom panels) were analyzed by immunohistochemistry using antibodies against Na^+, K^+ ATPase α1 subunit (left panels) or NHE1 (right panels). B, kidney cortical basolateral membranes from the above animals were analyzed by Western blot for Na^+, K^+ ATPase α1or β1subunit or NHE1 expression. Representative histochemistry or Western blots of kidneys from four individual animals in each group are shown. Bar graph shows arbitrary densitometry units as ratio of Na^+, K^+ ATPase α1 subunit or NHE1 to actin in crude membranes from four individual animals (n = 4 in each group). *Indicates $p < 0.05$ by ANOVA followed by Bonferroni analysis from control animals. C, Na^+, K^+ ATPase activity determined as ouabain-sensitive ATP hydrolysis in kidney basolateral membranes from sham operated, ADX or ADX rats treated with aldosterone. Each bar represents activity as mean ± se from four individual animals performed in triplicate (n = 4 in each group). D, kidney sections from sham operated and vehicle treated (top panel), ADX and vehicle treated (middle panel) and ADX rats treated with aldosterone (bottom panel) were analyzed by immunohistochemistry using antibodies against NKCC2 (left panels) or NpT2a (right panels, n = 4 in each group).

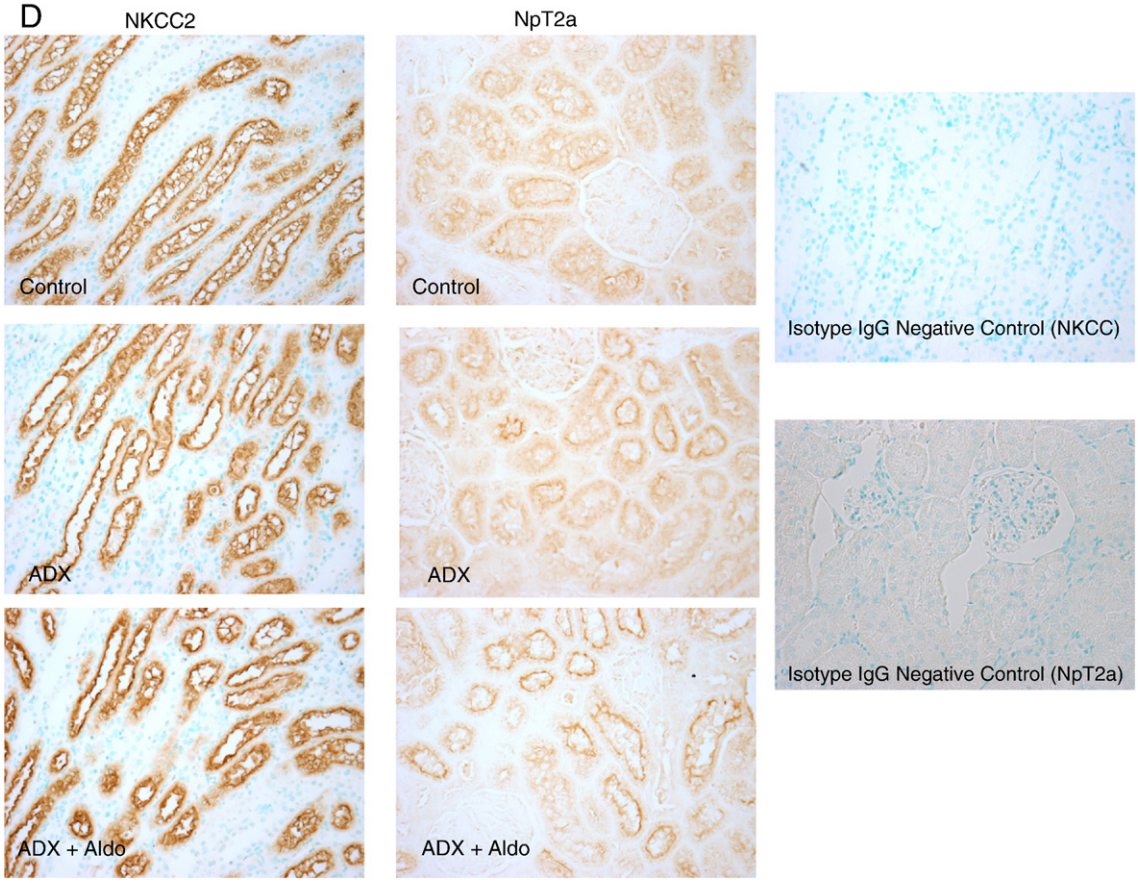

Fig. 3 (*continued*).

S3 segments of the proximal tubules from Wistar rats and WKY and SHR animals. Our results demonstrating protein expression confirm the results of Leite-Dellova [31] and Pinto et al. [11]. Differences in model systems may explain these apparently disparate results. Additionally, our studies together with studies by Leite-Dellova et al. [31] and Pinto et al. [11] demonstrating MR expression in JG nephrons but not superficial nephrons suggest heterogeneity of expression even within the population of proximal nephrons which may also explain conflicting findings.

Our data suggest that aldosterone regulates sodium transport in the proximal tubule through a classic mineralocorticoid receptor stimulated pathway. Aldosterone increased translocation of MR to the nucleus, stimulated SGK-1 phosphorylation, and increased expression of Na^+, K^+ ATPase in a time dependent manner in human kidney proximal cells similar to what is seen in cells from cortical collecting ducts [35,36]. Aldosterone-mediated increase in Na^+, K^+ ATPase-dependent ^{86}Rb uptake was completely prevented by pretreatment with spironolactone. Interestingly, treatment with RU486, an antagonist of GR partially prevented the increase in aldosterone-mediated increase in Na^+, K^+ ATPase activity. Unlike aldosterone, treatment with dexamethasone increases Na^+, K^+ ATPase through the classical GR mediated pathway. Although not all investigators have successfully demonstrated SGK1 in proximal tubule [14–16,37] our study clearly shows not only the presence of SGK1 but aldosterone-stimulated phosphorylation of SGK1 corresponding to the increase in sodium transporter expression. In total, these studies suggest that the well-known effects of aldosterone on Na, K, and acid base homeostasis may result from effects on proximal as well as distal tubules. Thus, inhibitors of aldosterone may have a more global effect on renal handling of these electrolytes than previously appreciated. A major question raised by these studies is under what circumstances does aldosterone exert these effects? The significant decrease in sodium transporter expression in proximal tubule cells after adrenalectomy and the subsequent resurgence of expression after aldosterone treatment suggest a role for aldosterone in regulation of chronic transporter expression. The ability of spironolactone to block that resurgence confirms an MR-dependent mechanism for this aldosterone action. However, the ability of the GR antagonist to partially block aldosterone-stimulated sodium transporter expression also suggests a role for chronic glucocorticoid regulation of proximal tubule sodium transporters. As critically reviewed by Funder in 2009 [38], under normal circumstances it is likely that up to 99% of proximal tubule MR is occupied by glucocorticoids which may actually serve as MR antagonists. There is evidence, however, that under pathologic conditions, the NAD/NADH ratio may be altered and thus enhance MR effects of glucocorticoids. In addition, several kidney diseases including diabetic nephropathy [39,40] or ischemia re-perfusion injury [41,42] show increased intra-renal production of aldosterone which may then play a more prominent role in regulation of proximal tubule function. Wang et al. [43] and Turban et al. [44] have shown that chronic aldosterone treatment in the presence of high sodium intake results in upregulation of proximal tubule NHE3 and sodium bicarbonate transport during the process of mineralocorticoid escape. These findings suggest that the aldosterone-stimulated increase in NHE3 and sodium pump expression could blunt the pressure-natriuresis response in proximal tubule transport thus contributing to the sustained increase in sodium retention and hypertension in individuals with mineralocorticoid excess.

Aldosterone is now recognized as a hormone with pleiotropic effects, not simply a regulator of Na and K homeostasis, and with effects on multiple organs, not just those involved in electrolyte transport. Specifically, aldosterone is increasingly seen as a mediator of progressive fibrosis in chronic kidney disease. The current work suggests that the deleterious effects of chronic elevations in aldosterone, as seen in many chronic kidney diseases, may result in part from effects of aldosterone on proximal tubule [45,46]. Further studies will be required to determine if the profibrotic effects of aldosterone in proximal

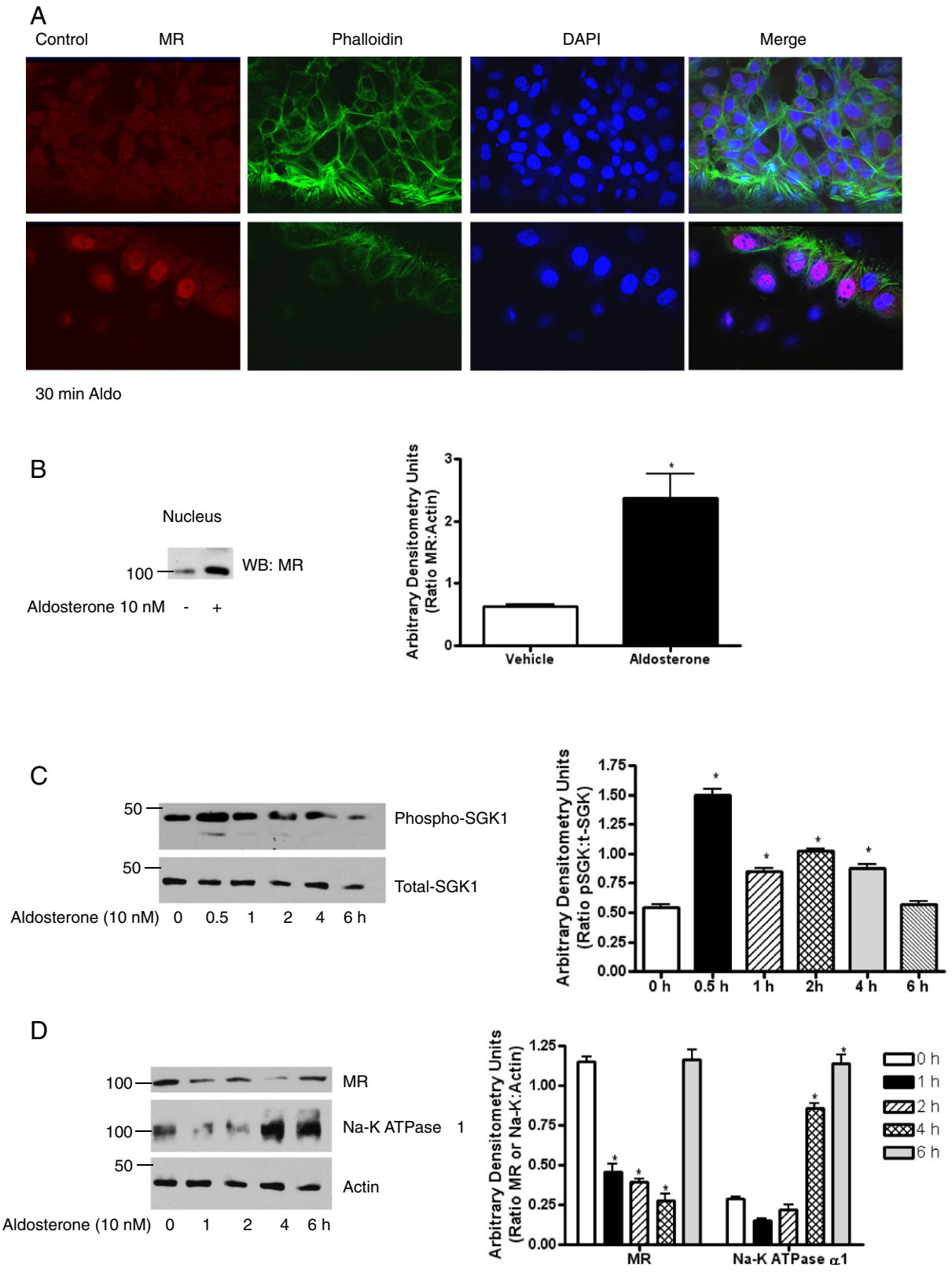

Fig. 4. Effect of aldosterone on MR expression in human kidney proximal tubule cells. HKC11 cells were treated for 30 min with vehicle or 10 nM aldosterone at 37 °C. A, cells were fixed and analyzed by confocal microscopy using antibodies against MR (red staining). Cells were also stained with DAPI (nucleus, blue staining) and phalloidin (F-actin, green staining). B, cells were treated for 30 min with 10 nM aldosterone at 37 °C and MR expression was analyzed by Western blot in nuclear fraction. Bar graph shows arbitrary densitometry units as ratio of MR to actin in nuclear fraction from five individual experiments (n = 5). *Indicates $p < 0.05$ by ANOVA followed by Bonferroni analysis from vehicle treated group. C, cells were treated for the indicated time with 10 nM aldosterone at 37 °C. Nuclear free cell lysates were analyzed using antibodies against phospho-SGK1 (top panel). The nitrocellulose membranes were stripped and reprobed using total-SGK1 antibodies (bottom panel). Bar graph shows arbitrary densitometry units as ratio of phospho- to total SGK-1 in nuclear free cell lysates from five individual experiments (n = 5). *Indicates $p < 0.05$ by ANOVA followed by Bonferroni analysis from 0 time point group. D, nuclear free cell lysates were also analyzed by Western blot using antibodies against MR (top panel), Na^+, K^+ ATPase α1 subunit (middle panel) or actin (bottom panel). Bar graph shows arbitrary densitometry units as ratio of MR or Na^+, K^+ ATPase α1 subunit in nuclear free cell lysates from five individual experiments (n = 5 in each group). *Indicates $p < 0.05$ by ANOVA followed by Bonferroni analysis from 0 time point group. Representative confocal images or Western blots from five individual experiments are shown.

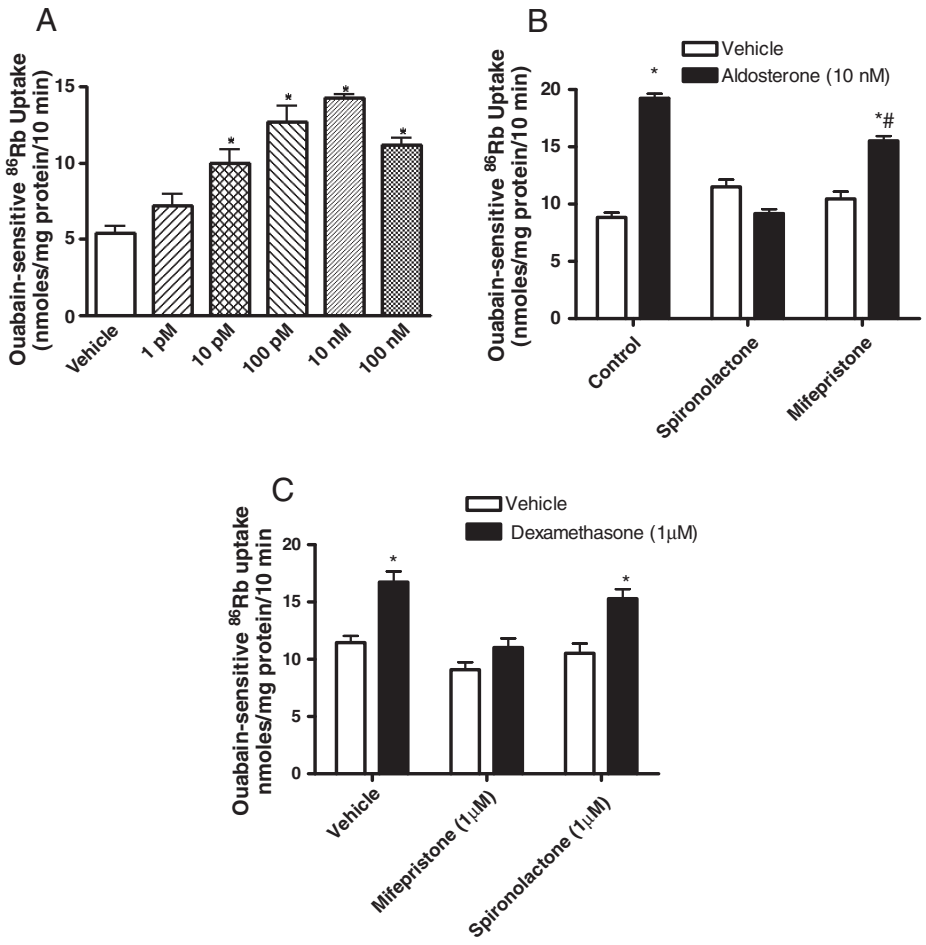

Fig. 5. Effect of aldosterone on Na^+, K^+ ATPase-mediated ion transport. A, HKC11 cells were grown on 24 well cell culture plates. Cells were treated for 24 h with different concentrations of aldosterone. Na^+, K^+ ATPase-mediated ion transport as ouabain-sensitive ^{86}Rb uptake was measured as described in Experimental procedures. Each bar represents data as nmoles ^{86}Rb uptake/mg protein/10 min (mean ± se) from six individual experiments performed in triplicate (n = 6). *Indicates $p < 0.05$ by ANOVA followed by Bonferroni analysis. B, HKC11 cells were pretreated with 1 μM spironolactone or 1 μM Mifepristone for 30 min followed by treatment with 10 nM aldosterone for 24 h in the presence or absence of the above inhibitors. Na^+, K^+ ATPase-mediated ^{86}Rb uptake was measured as above. Each bar represents data as nmoles ^{86}Rb uptake/mg protein/10 min (mean ± se) from six individual experiments performed in triplicate (n = 6). *Indicates significantly different from vehicle and # indicates significantly different from aldosterone alone treated group ($p < 0.05$) by ANOVA followed by Bonferroni analysis. C, HKC11 cells were pretreated with 1 μM Mifepristone or 1 μM Spironolactone for 30 min followed by treatment with dexamethasone (1 μM) for 24 h in the presence or absence of the above inhibitors. Na^+, K^+ ATPase-mediated ^{86}Rb uptake was measured as above. Each bar represents data as nmoles ^{86}Rb uptake/mg protein/10 min (mean ± se) from six individual experiments performed in triplicate (n = 6). *Indicates significantly different from vehicle treated group ($p < 0.05$) by ANOVA followed by Bonferroni analysis.

tubule are related to or dependent on the effects on electrolyte transport or through completely separate mechanisms.

In summary, our results for the first time demonstrate the presence of MR in proximal tubules in human, rat, and mouse kidneys and the evidence for regulation of the expression and function of sodium transport proteins by aldosterone through a MR dependent mechanism. These studies suggest an important role for the renal proximal tubule in mineralocorticoid regulation of sodium homeostasis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.05.009.

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