Aldosterone Stimulates Activity and Surface Expression of NHE3 in Human Primary Renal Proximal Tubule Epithelial Cells (RPTEC)

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Key Words

Aldosterone • EGF-receptor • NHE3 • Proximal tubule epithelial cells

Abstract

The steroid hormone aldosterone is a major regulator of extracellular volume and blood pressure. Aldosterone effectors are for example the epithelial Na⁺ channel (ENaC), the Na⁺-K⁺-ATPase and the proximal tubule Na⁺/H⁺ exchanger isoform 3 (NHE3). The aim of this study was to investigate whether aldosterone acts directly on proximal tubule cells to stimulate NHE3 and if so whether the EGF-receptor (EGFR) is involved. For this purpose, primary human renal proximal tubule cells were exposed to aldosterone. NHE3 activity was determined from Na⁺-dependent pH-recovery, NHE3 surface expression was determined by biotinylation and immunoblotting. EGFR-expression was assessed by ELISA. pH_i-measurements revealed an aldosterone-induced increase in NHE3 activity, which was inhibited by the mineralocorticoid receptor blocker spironolactone and by the EGFR-kinase inhibitor AG1478. Immunoprecipitation and immunoblot analysis showed an aldosterone-induced increase in NHE3 surface

expression, which was also inhibited by spironolactone and AG1478. Furthermore, aldosterone enhanced EGFR-expression.

In conclusion, aldosterone stimulates NHE3 in human proximal tubule cells. The underlying mechanisms include AG1478 inhibitable kinase and are paralleled by enhanced EGFR expression, which could be compatible with EGF-receptor-pathway-dependent surface expression and activity of NHE3 in human primary renal proximal tubule epithelial cells.

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Introduction

Aldosterone plays a crucial role in hormonal control of Na⁺-homeostasis which is essential for extracellular volume and blood pressure regulation [1-3]. In addition, aldosterone seems to affect tissue homeostasis and may contribute to cardiovascular dysfunction [4, 5]. Aldosterone controls Na⁺-homeostasis by stimulation of Na⁺-absorption across tight epithelia, where the epithelial sodium channel (ENaC) and the Na⁺-K⁺-ATPase act as effectors [6, 7]. In the aldosterone-sensitive distal nephron (ASDN) aldosterone enhances the activity and expression

of ENaC and the Na⁺-K⁺-ATPase. In addition to the ASDN, other segments of the nephron play an important role in fluid and electrolyte reabsorption. 60-70 % of the filtered NaCl and volume are reabsorbed in the proximal tubule. The main transporters for this reabsorption are the apical NHE3 and the basolateral Na⁺-K⁺-ATPase. Both transporters show a volume-contraction-dependent abundance in the proximal tubule [8, 9].

Aldosterone affects Na⁺ reabsorption in the collecting duct after binding to the mineralocorticoid receptor (MR), which modulates the expression of the serum- and glucocorticoid-regulated kinase, ENaC and the Na⁺-K⁺-ATPase [6, 7, 10-12]. In addition, aldosterone stimulates epidermal growth factor receptor (EGFR) expression in the kidney [21]. Interestingly, activation of EGFR can stimulate NHE3 and thereby reabsorption of NaCl and fluid [13-16].

40 years ago, it has been shown that aldosterone enhances proximal tubule NaCl and fluid reabsorption in rats by a spironolactone-sensitive mechanism [17]. Recently, we demonstrated in adrenalectomized rats that aldosterone stimulates NHE3-dependent proximal tubule volume reabsorption and the abundance of NHE3 in the brush border membrane without changing the total amount of NHE3 in cortical homogenates, but the underlying mechanisms have not been elucidated [8]. Therefore the aim of this study was to determine the mechanism of aldosterone action on NHE3 in proximal tubule epithelial cells, especially whether the action of aldosterone on the proximal tubule function is direct and whether it is mediated by EGFR kinase.

Materials and Methods

Materials

Dulbecco's minimal essential medium (DMEM), HAM-F12, and fetal calf serum were from Biochrom (Berlin, Germany). Trypsin and N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) were obtained from Serva (Heidelberg, Germany). BCECF-AM and nigericin were obtained from Alexis (Grünberg, Germany). Aldosterone was bought from Fluka (Buchs, Swiss). Spironolactone was purchased from Sigma (Deisenhofen, Germany), AG1478 was obtained from Calbiochem (Bad Soden, Germany), S3226 was a generous gift from Dr. Lang (Aventis Pharma Deutschland GmbH, Frankfurt a.M., Germany). All the other chemicals were purchased from Merck (Darmstadt, Germany).

The Ringer solutions were composed as follows:

HEPES-Ringer (pH 7.4 at 37°C): 122.5 mmol/l NaCl, 5.4 mmol/l KCl, 1.2 mmol/l CaCl₂, 0.8 mmol/l MgCl₂, 1 mmol/l NaH₂PO₄, 5.5 mmol/l glucose, and 10 mmol/l HEPES.

Na⁺-free HEPES-Ringer (pH 7.4 at 37°C): 122.5 mmol/l NMDG, 1 mmol/l KH₂PO₄, 4.4 mmol/l KCl, 1.2 mmol/l CaCl₂, 0.8 mmol/l MgCl₂, 1 mmol/l NaH₂PO₄, 5.5 mmol/l glucose, 10 mmol/l HEPES, 122.5 ml/l 1M HCl.

Na-free NH₄Cl-HEPES-Ringer (pH 7.4 at 37°C): 80.5 mmol/l NMDG, 1 mmol/l KH₂PO₄, 4.4 mmol/l KCl, 1.2 mmol/l CaCl₂, 0.8 mmol/l MgCl₂, 1 mmol/l NaH₂PO₄, 5.5 mmol/l glucose, 10 mmol/l HEPES, 40 mmol/l NH₄Cl, 80.5 ml/l 1M HCl.

Calibration solution I or II (pH 6.8 or pH 7.6 at 37 °C): 132 mmol/l KCl, 1 mmol/l CaCl₂, 1 mmol/l MgCl₂, 10 mmol/l HEPES.

Protein extraction buffer: 50 mmol/l TRIS-HCl, 150 mmol/l NaCl, 1 % Nonidet P40, 0.5% Na-deoxycholate, pH 7.5, protease inhibitor mix I (Calbiochem, Bad Soden, Germany).

Cell culture

Primary human renal proximal tubule cells (RPTEC, obtained from Clonetics, Walkersville, MD, USA) were cultivated as described before [18]. The RPTEC model is a valid model for differentiated renal proximal tubule epithelial cells, as these cells stain positive for gamma-glutamyl transpeptidase and for alkaline phosphatase. RPTEC form tubules on Matrigel and under specific culture conditions these cells may polarize and form brushborders with microvilli (characterization of RPTEC by the manufacturer Clonetics, Walkersville, MD, USA). Subconfluent (80 % confluence) RPTEC monolayers were split once a week by 5 min trypsinization (0.25 g/l) in Ca²⁺- and Mg²⁺-free, EDTA-containing Ringer's solution. Cells were incubated in serum- and hormone-free medium 24 hrs prior to experiments.

A) pH_i-measurements (ammonium chloride prepulse technique). RPTEC cells on coverslip mounting chambers were loaded with the pH-sensitive dye BCECF-AM (2 μM) at 37 °C for 3 min. Intracellular pH (pH_i) was calculated from the fluorescence emission ratio collected with an ICCD camera (Hamamatsu, Herrsching, Germany) from 5 - 10 cells/coverslip during excitation at 450 nm and 490 nm as described earlier [19]. The emitted light was filtered through a bandpass filter (515-565 nm). Finally, pH_i was calibrated at pH 6.8 and pH 7.6 with 10 μM nigericin (K⁺/H⁺ exchanger) on each coverslip. The data acquisition rate was one fluorescence intensity ratio every 5 s. Images were digitized on-line using video-imaging software (Hamamatsu, Herrsching, Germany). After background subtraction, fluorescence intensity ratios were converted to pH_i. Recovery rates of pH_i in HEPES-Ringer after experimentally induced alkalization by 40 mM NH₄Cl pre-pulse following acidification in Na⁺-free HEPES-Ringer were calculated from the initial slopes of pH_i changes after addition of Na⁺ and expressed as dpH/t [min]. Na⁺-dependent pH-recovery was almost completely prevented by the NHE-specific inhibitor S3226, indicating that under our experimental conditions pH-recovery is a valid measure for NHE3 activity (figure 1).

B) Biotinylation. Adherent RPTEC cells were mechanically detached from cell culture plates and washed 3 times with PBS. The cell pellets (5 x 10⁷ cells) were treated with NHS-SS-biotin (cell surface biotinylation; Molecular Biosciences Inc., Boulder, Colorado, USA) or with NHS-biotin (intracellular biotinylation; Molecular Biosciences Inc., Boulder, Colorado, USA) at 4 °C for 1 hr in biotinylation buffer (0.5 mg/

Fig. 1. pH_i-sensitive video imaging. RPTEC exposed to NHE3 inhibitor S3226 (3 μM) were not able to recover in Hepes Ringer indicating that NHE3 mediates the pH_i recovery of the cells (n = 5; * = p = 0.5). Therefore it is postulated that the H⁺-ATPase or Na⁺/H⁺ exchanger isoform 1 play a minor role in the observed pH_i recovery of RPTEC.

1 NHS-SS- or NHS-biotin in PBS). Thereafter cells were washed 3 times with PBS, resuspended in protein extraction buffer and extracted by using an ultrathurax (20 strokes) at 4°C. Protein content was determined by the bicinchoninic acid protein assay according to Lane *et al.* [20].

C) NHE3 specific immunoprecipitation and immunoblotting. Equal amounts of protein extracts were preincubated with protein G agarose to minimize non specific binding. 1.5 μg/ml NHE3 antibodies (NHE31-A; Biotrend Chemikalien GmbH, Cologne, Germany) were bound to protein G agarose (Roche, Mannheim, Germany) and thereafter the protein extracts were exposed to the protein G agarose bound NHE3 antibodies, the protein G agarose was washed and NHE3-antibody complexes were eluted. The composition of the buffers and the procedure used for incubation, washing and elution accorded to the user manual of the protein G agarose (Roche, Mannheim, Germany).

Proteins were separated by 12 % SDS-PAGE and blotted on a nitrocellulose membrane. The blots were blocked (1 % Tween 20, 5 % BSA), washed and exposed to streptavidin-HRP (200 ng/ml; Pierce Biotechnology Inc., Rockford, USA) for 1 hr. After washing the blots extensively, the bound biotinylated NHE3 was detected by enhanced chemiluminescence (ECL; Amersham Life Science, Arlington Heights, USA). Blots were scanned and densitometry was performed by ImageJ version 1.33 software.

D) EGFR specific ELISA. RPTEC were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM Na₄P₂O₇, 2 mM Na₃VO₄, 10% glycerol, 0.5% deoxycholate, 0.1% SDS, protease inhibitor mix I (Calbiochem, Bad Soden, Germany), 1% Triton X-100) for 25 min at 4 °C. Insoluble material was removed by centrifugation at 12.000 × g for 15 min at 4 °C. Cell lysates were analysed by EGFR specific ELISA using the human EGFR DuoSet ELISA Development System (R&D Systems (Abingdon, United Kindom)) according to the manufacturer's manual.

Statistics

All data are given as mean values ± SEM. Columns represent the means and error bars the SEM. The significance of difference was determined by the unpaired Student *t* test. *P* < 0.05 was considered to be statistically significant. To compare the dependent data, an analysis of variance with appropriate post-hoc analysis was made (ANOVA test, Prophet 5.0 software).

Results

Aldosterone induces NHE3 activity and expression

The precise mechanism of aldosterone action on proximal tubule NHE3 has not yet been elucidated. Therefore we performed the actual studies in isolated primary human renal proximal tubule epithelial cells (RPTEC) to address the question of a direct versus an indirect effect of aldosterone on proximal tubule NHE3.

Analysis of the pH_i recovery rates of vehicle treated and aldosterone treated RPTEC demonstrated that aldosterone stimulates NHE3 activity significantly (figure 2B). Simultaneous treatment of RPTEC with 100 nM aldosterone and 10 μM aldosterone receptor antagonist spironolactone for 72 hrs resulted in a significant inhibition of the aldosterone induced NHE3 activation (figure 2A). These data suggest that aldosterone mediates the observed NHE3 activation in proximal tubular epithelial cells via the mineralocorticoid receptor.

Figures 3A and B show that exposure to aldosterone increases NHE3 cell surface expression. In contrast to the aldosterone-dependent NHE3 activity achieving its maximum effect between 1 and 10 nM, aldosterone induced NHE3 expression shows its maximum effect at 10 nM. This discrepancy between aldosterone dose dependent NHE3 activity and expression might be due to the aldosterone mediated activation of transporters already located in the cell membrane.

Aldosterone mediates an increase in EGFR expression

In addition to the stimulation of NHE3 cell surface expression, aldosterone significantly stimulates epidermal growth factor receptor (EGFR) expression in RPTEC (figure 4). The aldosterone-induced EGFR expression is inhibited by spironolactone, suggesting that aldosterone induces EGFR expression also via the mineralocorticoid receptor in primary human proximal tubule cells.

These results are in agreement with data of Krug *et al.* reporting an aldosterone stimulated EGFR expression in the renal tissue of adrenalectomized rats, in primary human proximal tubule cells, and in cells transfected with the human mineralocorticoid receptor [21].

Aldosterone stimulated NHE3 activity and NHE3 expression depends on EGFR

The aldosterone stimulated NHE3 activity and NHE3 cell surface expression in RPTEC depends on the EGFR pathway, as it is prevented by the EGFR tyrosine kinase inhibitor AG1478 (tyrphostin) (figures 2A and 3D).

These data suggest an EGFR-pathway-dependent stimulation of NHE3 activity in proximal tubule cells, as it has been reported by Khurana *et al.* for ileal brush border membranes and an ileal cell line (Caco-2) [14]. Khurana *et al.* demonstrated that EGF treatment increases the tyrosine phosphorylation of the phosphatidylinositol 3-kinase (PI 3-kinase) stimulating the intestinal Na⁺ absorption via an increased NHE3 activity [14].

Discussion

In this study we demonstrate, that aldosterone directly induces activity and cell surface expression of NHE3 in human primary proximal tubule cells in culture. The underlying mechanism involves EGFR. Therefore, a direct mechanism of aldosterone action on NHE3 in the proximal tubule can be postulated.

These data confirm the *in vivo* data of Krug *et al.*, namely that aldosterone induces NHE3 expression in brush border membrane [8]. In addition, Fernandez-Llama *et al.* reported a significant increase in proximal tubule brush border NHE3 expression despite a decrease of total cortical NHE3 abundance in kidneys of rats with CCl₄-induced cirrhosis with a consecutive decrease in renal Na⁺ excretion and elevated plasma aldosterone levels [22]. These data suggest that aldosterone modulates subcellular trafficking of NHE3, whereby more cellular NHE3 is shifted into the brush border membrane. Such a

Fig. 2. A) pH_i-sensitive video imaging. RPTEC were exposed to control (vehicle), 10 μM spironolactone, 100 nM aldosterone ± 10 μM spironolactone, 100 μM AG1478 or 100 nM aldosterone ± 100 μM AG1478 for 72 hrs. Spironolactone and AG1478 significantly inhibit the aldosterone dependent NHE3 activation in RPTEC (* = p = 0.5). B) pH_i-sensitive video imaging. RPTEC were exposed to control (vehicle), 1, 10 and 100 nM aldosterone for 72 hrs. RPTEC show an aldosterone dependent NHE3 activity (n = 22 - 62).

mode of action of aldosterone is consistent with its effect on the collecting duct where part of its antinatriuretic effect results from the induction of proteins regulating the membrane trafficking of ENaC [11].

Fig. 3. A) NHE3 specific immunoprecipitation and immunoblot. RPTEC were exposed to control (0), 1, 10 and 100 nM aldosterone for 72 hrs. Aldosterone induces a dose dependent increase in NHE3 cell surface expression (cell surface biotinylation), but has no influence in total cellular NHE expression (cytoplasmatic biotinylation). B) NHE3 specific immunoblot analysis. RPTEC were exposed to control (vehicle), 1, 10 and 100 nM aldosterone for 72 hrs. Aldosterone induces a significant increase in NHE3 cell surface expression of RPTEC (n = 5 - 14). C) NHE3 specific immunoprecipitation and immunoblot. RPTEC were exposed to control (vehicle), 10 μ M spironolactone or 100 nM aldosterone $\pm$ 10 μ M spironolactone for 72 hrs. Spironolactone inhibits the aldosterone-induced NHE3 cell surface expression of RPTEC. D) NHE3 specific immunoblot analysis. RPTEC were exposed to control (vehicle), 10 μ M spironolactone, 100 nM aldosterone $\pm$ 10 μ M spironolactone, 100 μ M AG1478 or 100 nM aldosterone $\pm$ 100 μ M AG1478 for 72 hrs. Spironolactone (aldosterone antagonist) and AG1478 (EGFR tyrosine kinase inhibitor) inhibit significantly the aldosterone-induced NHE3 cell surface expression of RPTEC (* = p = 0.5).

Aldosterone induced NHE3 cell surface expression seems to be a mineralocorticoid specific effect, because the aldosterone receptor antagonist spironolactone significantly inhibited the aldosterone-induced NHE3 cell surface expression of RPTEC (figures 3C and D) and NHE3 activity (figure 2A).

The presented data are consistent with those reported by Stolte *et al.*, who demonstrated a mineralocorticoid (aldosterone) but not glucocorticoid (dexamethasone) dependent increase in proximal tubule NaCl and fluid reabsorption in adrenalectomized rats [17]. Although there is a controverse discussion about the mineralocorticoid receptor expression in the proximal tubule, Krug *et al.* reported mRNA and protein expression of the mineralocorticoid receptor in primary human renal proximal tubule epithelial cells (RPTEC) [21]. In addition, mRNA for mineralocorticoid receptors has been described in proximal tubule cells, although at a lower amount than in collecting duct cells [23]. These data indicate that mineralocorticoid receptors may not have the same importance in proximal tubule as compared to collecting duct. But how are the mineralocorticoid receptors protected from glucocorticoids (which have much higher plasma concentrations) in order to confer a certain degree of specificity? In collecting duct cells the glucocorticoids are hydrolysed by the 11 β -hydroxy steroid dehydrogenase-2. However, the 11 β -hydroxy steroid dehydrogenase 1, but not isoform 2, is expressed in the proximal tubule [24, 25], which has a 10- to 100-fold lower affinity for cortisol

Fig. 4. EGFR specific ELISA. Aldosterone stimulates significantly EGFR expression of RP-TEC, which is inhibitable by spironolactone (n = 6 - 9).

and therefore might not be a sufficiently protective enzyme [24]. Additional “specificity-conferring” mechanisms must play a role in the proximal tubule. Such mechanisms include e.g. cytochrome p-450 oxidases, a higher degree of protein binding of glucocorticoids or different transport rates across the cell membrane [25]. In addition, a functional preference of the mineralocorticoid receptor for aldosterone as compared to glucocorticoids, despite comparable equilibrium binding constants, has been described. This could be the result of different K_{on} and K_{off} constants, resulting finally in an enhanced aldosterone-receptor half-life [24-26]. The response to mineralocorticoid receptor activation also depends on the coactivation of other signaling cascades (e.g. protein kinases) which may be different for mineralocorticoids and glucocorticoids [27, 28]. Future studies have to address this issue in more detail, as for example, to use a cellular mineralocorticoid receptor overexpression or knock down *in vitro* model for future studies to elucidate the role of the mineralocorticoid receptor on NHE3 activity.

Another hormonal stimulus of proximal tubule NHE3 is angiotensin II, which is also the main inductor for aldosterone secretion during volume concentration [29]. Angiotensin II exerts acute, second messenger mediated effects, whereas aldosterone acts slower, mainly through altered gene expression. Therefore it is conceivable that aldosterone and angiotensin II might act synergistically in the proximal tubule, where angiotensin II might mediate short term effects and aldosterone might mediate long term effects for adaptation to Na^+ and volume depletion *in vivo*. Of course, this hypothesis has to be tested *in*

vivo. In addition, ongoing studies will address the molecular links of aldosterone induced, EGFR pathway dependent NHE3 cell surface expression and activity in proximal tubule cells. There are several studies suggesting a link between aldosterone application to animals and EGFR expression [30-32]. Various cellular signaling pathways are involved in the regulation of EGFR expression, but further studies are needed to determine the precise mechanism(s) involved. At present, it is not known whether the mineralocorticoid receptor binds directly to the EGFR promoter. Aldosterone could also be linked indirectly to EGFR expression, for example, via activation of AP1 [5]. Future studies have to elucidate the mechanism of aldosterone on NHE3 trafficking, especially the effect of aldosterone on cytoskeletal proteins.

Aldosterone is commonly seen as a major stimulator of Na^+ reabsorption in the distal tubule and the collecting duct. Interestingly, the collecting duct reabsorbs no more than 5 % of the filtered Na^+ , but 60 - 70 % of the filtered Na^+ are reabsorbed in the proximal tubule and 20 - 30 % are reabsorbed in the loop of Henle. Therefore the question arises, whether the regulation of glomerular filtration and tubular Na^+ reabsorption in the early nephron segments serve to adjust Na^+ delivery to the limited regulatory ranges of the distal tubule and collecting duct [33]. The present study demonstrates an ideal mechanism by which the early and the late tubular Na^+ reabsorption capacities could be at least partly coupled by the same aldosterone mediated mechanism. Aldosterone regulates the activity and expression of ENaC in the distal tubule

and, as we have demonstrated in this study, it mediates the abundance and activity of NHE3 in the apical membrane of proximal tubule epithelial cells. Considering the *in vivo* data of Krug *et al.* and the results reported in the present study we suggest that under physiological conditions (normal Na⁺ intake) the plasma aldosterone levels are in a concentration range, where the Na⁺ reabsorption in distal tubule and collecting duct is not saturated and the NHE3 dependent mechanism is probably not activated [8]. Volume depletion caused for example by chronically low Na⁺ intake or loss of blood, increases the plasma aldosterone level to a range stimulating the proximal tubule Na⁺ reabsorption.

In conclusion, aldosterone induces EGFR-dependent NHE3 cell surface expression and activity in human proximal tubule cells. The underlying mechanisms include AG1478 inhibitable kinase and are paralleled by enhanced EGFR expression, which could be compatible with EGF-receptor-pathway-dependent surface expression and activity of NHE3 in human primary renal proximal tubule epithelial cells. There are several studies suggesting a link between aldosterone application to animals and EGFR expression [30-32]. Various cellular signaling pathways

are involved in the regulation of EGFR expression, but further studies are needed to determine the precise mechanism(s) involved. At present, it is not known whether the mineralocorticoid receptor binds directly to the EGFR promoter. Aldosterone could also be linked indirectly to EGFR expression, for example, via activation of AP1 [5]. However, this might represent an additional mechanism for aldosterone-mediated control of Na⁺-homeostasis besides the stimulation of Na⁺-uptake via ENaC and Na⁺-K⁺-ATPase in the distal tubule and the collecting duct [6-8]. Therefore the direct effect of aldosterone on NHE3 in the proximal tubule can have potential physiological and pathophysiological relevance considering the renal function in Na⁺, pH and volume homeostasis.

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