Rapid aldosterone-mediated signaling in the DCT increases activity of the thiazide-sensitive NaCl cotransporter

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**Significance statement**

The NaCl cotransporter NCC in the kidney distal convoluted tubule (DCT) regulates urinary NaCl excretion and blood pressure. The importance of NCC is highlighted by the clinical use of NCC-targeting diuretics and in the salt-wasting Gitelman syndrome or hypertensive Gordon’s syndrome. The long-term effects of the mineralocorticoid aldosterone on modulating NaCl reabsorption via NCC are well established. Now, in this study the authors demonstrate that aldosterone has rapid effects in the DCT, partly via the membrane receptors EGFR and GPR30. Signaling from these receptors affect NCC activity such that when the DCT encounters aldosterone released in response to hypovolemia, aldosterone rapidly increases NaCl reabsorption to help restore blood volume. These rapid mechanisms likely help regulate blood pressure.
Rapid aldosterone-mediated signaling in the DCT increases activity of the thiazide-sensitive NaCl cotransporter

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Running title: Rapid aldosterone actions in DCT

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Abstract

Background: The NaCl cotransporter NCC in the kidney distal convoluted tubule (DCT) regulates urinary NaCl excretion and blood pressure. Aldosterone increases NaCl reabsorption via NCC over the long-term by altering gene expression. But the acute effects of aldosterone in the DCT are less well understood. Methods: Proteomics, bioinformatics, and cell biology approaches were combined with animal models and gene targeted mice. Results: Aldosterone significantly increases NCC activity within minutes in vivo or ex vivo. These effects were independent of transcription and translation, but were absent in the presence of high potassium. In vitro, aldosterone rapidly increased intracellular cAMP and inositol phosphate accumulation, and altered phosphorylation of various kinases/kinase substrates within the MAPK/ERK, PI3K/AKT and cAMP/PKA pathways. Inhibiting GPR30, a membrane-associated receptor, limited aldosterone effects NCC activity ex vivo and NCC phosphorylation was reduced in GPR30 knockout mice. Phosphoproteomics, network analysis and in vitro studies determined that aldosterone activates EGFR-dependent signaling. The EGFR immunolocalized to the DCT and EGFR tyrosine kinase inhibition decreased NCC activity ex vivo and in vivo. Conclusion: Aldosterone acutely activates NCC to modulate renal NaCl excretion.
**Introduction**

Aldosterone modulates body sodium (Na$^+$) and potassium (K$^+$) balance by regulating renal Na$^+$ reabsorption and K$^+$ secretion. Aldosterone primarily mediates its effects on the aldosterone-sensitive distal nephron (ASDN) $^1$, comprising the late distal convoluted tubule (DCT), connecting tubule (CNT) and collecting duct (CD). The classic mechanism for aldosterone actions involves the binding of aldosterone to the mineralocorticoid receptor (MR), translocation of the MR to the nucleus and subsequent MR-mediated alterations in gene transcription of various ion-transporting proteins such as the epithelial sodium channel ENaC $^2$. These so called “genomic” effects influence Na$^+$ and K$^+$ transport within 2-3 hours and play an important role for electrolyte balance and the maintenance of blood pressure. Additionally, aldosterone can rapidly cause subcellular redistribution of ENaC to the plasma membrane and increase amiloride-sensitive sodium currents within minutes $^3$-$^5$.

Aldosterone also has the capacity for rapid (seconds/minutes) “non-genomic” effects on target cells, which occur in concurrence with alterations in cytosolic Ca$^{2+}$ levels (via PLC, DAG, IP3 pathway), or cAMP-mediated signaling $^2$. Whether these rapid effects are mediated via the MR alone $^6$, by interaction of the MR with the epidermal growth factor receptor (EGFR), IGF1R, PDGFR, AT-1 or GPR30 (also known as GPER1$^7$) or via direct activation of alternative membrane bound receptors are controversial $^2$. For example, although aldosterone can increase intracellular cAMP and Ca$^{2+}$ levels in MR-deficient mice $^8$ and non-cell permeable aldosterone analogues can trigger PKC signaling or ERK activation $^9$-$^{10}$, the existence of a plasma membrane-bound receptor that responds consistently to aldosterone is hotly debated $^7$-$^{11}$-$^{12}$. However, one such candidate, GPR30 is functionally expressed in kidney epithelial cells and responds to aldosterone stimulation $^{13}$-$^{14}$.
Although chronic aldosterone administration increases abundance and phosphorylation (activation) of the thiazide-sensitive NaCl cotransporter NCC\textsuperscript{15-18} in cells of the late DCT, it is unknown whether these effects are direct. Furthermore, although in a timeframe of 12-24 h aldosterone can activate NCC in a SPAK-dependent manner \textsuperscript{19}, whether aldosterone exerts acute (seconds/minutes) effects in DCT cells has never been examined. The aim of this study was to investigate whether aldosterone can have rapid effects on DCT cells to regulate NCC and to identify the potential signaling mediators behind this effect. The major finding is that within minutes, aldosterone is able to activate a variety of different signaling cascades in DCT cells that ultimately results in increased NCC activity. The results demonstrate for the first time an important new role of non-genomic aldosterone actions in the DCT that is likely to have major implications for the rapid regulation of NaCl transport and consequently blood pressure regulation.
Methods

Extensive methods are provided in the Supplemental Material.

**Antibodies.** The majority of utilized antibodies were commercial rabbit monoclonal antibodies, the details of which are listed in the Supplemental Material. Specificity of the commercial antibodies was based on that they either gave a single unique band on an immunoblot corresponding to the target proteins predicted molecular weight, or the most prominent band on the immunoblot was at the target proteins predicted molecular weight (with no other bands of similar size). Other antibodies were SPAK and pSPAK \(^{20}\), and pNCC-T58 \(^{20}\).

**Ca\(^{2+}\) and cAMP measurements.** The mouse kidney DCT cell line (mpkDCT) has been characterized previously \(^{21,22}\). Cells were routinely cultured at 37°C in 5% CO\(_2\) in mpkDCT medium (DMEM/F12- media (Invitrogen) containing 60 nM sodium selenite, 5 μg/ml transferrin, 2 mM glutamine, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose and 20 mM HEPES (pH 7.4)). For Ca\(^{2+}\) assays, cells were cultured in black Visiplates (Wallac) until confluent, with the last 24 h in pure phenol red free DMEM-F12 media (Invitrogen). Cells were washed with HBSS before incubating in dye loading solution (5 μM Fluo-4, 2.5mM probenecid, 1x PowerLoad in HBSS) for 30 min at 37°C. Cells were washed in assay buffer (HBSS containing 2.5 mM probenecid) and overlaid with 200 μl assay buffer. Fluorescence was measured using an EnSpire plate reader (PerkinElmer), with an excitation wavelength of 495 nm and an emission wavelength of 510 nm continuously for 2 min (background fluorescence) and for 10 min after addition of 50 μl of agonist (to reach final concentrations as indicated) or control solution. For cAMP assay
experiments, cells were grown on a semi-permeable filter support (Transwell, Corning) until a fully confluent polarized monolayer was formed (transepithelial resistance (TER) >5 kΩ.cm²). Cells were stimulated as indicated for 30 mins, lysed and intracellular cAMP levels were measured using a cAMP enzyme immunoassay kit (Enzo) according to manufacturer's instructions. All measurements were carried out in triplicates on at least 5 separate days.

**Inositol Phosphate (IP) measurements.** IP accumulation was measured using a scintillation proximity based inositol-phosphate accumulation assay (SPA-IP ²³). In brief, mpkDCT cells were seeded at 35,000 cells/well in 100 µL mpkDCT medium in 24-well filter plates and incubated for 24 h at 37°C with 0.5 µCi of myo-[³H]inositol (PerkinElmer). Medium without myo-[³H]inositol was added to the basolateral compartment. The following day, cells were washed twice in pure DMEM-F12 (Invitrogen) and incubated in pure DMEM-F12 supplemented with 10 mM LiCl at 37°C for 30 min in the presence of diluent (DMSO), aldosterone (1nM) or the activator of phospholipase C m-3M3FBS (Tocris) as a positive control. Cells were incubated in 10 mM formic acid for 60 min. 35 µl from each well was transferred to a 96-well plate, 1 mg of yttrium silicate SPA beads (SPA-Ysi; RPNQ, PerkinElmer) added and the plate mixed for 30 min by high-speed agitation. Beads were centrifuged (5 min, 400 g) and [³H]inositol phosphate binding was measured on a TopCount NXT (PerkinElmer, Waltham, MA).

**Immunoblotting experiments.** mpkDCT cells were grown on filter plates in mpkDCT media until TER >5 kΩ.cm². The evening prior to experiments, cells were cultured in pure phenol red free DMEM-F12 media (Invitrogen). Cells were treated with agonists/antagonists or
relevant control solutions as indicated for 30 min at 37°C. For G-36 or erlotinib antagonist experiments, cells were pre-incubated with G-36 or pure media for 20 min prior to subsequent stimulation. Standard procedures were utilized for SDS-PAGE and immunoblotting. For detection of GPR30 and EGFR in native DCT cells, protein lysates from DCT cells isolated from parvalbumin-GFP mice were used as previously described 24. Extensive details are provided in Supplemental Material. EGFP purity (DCT cell purity) in the sample was 94%.

**Immunohistochemistry and confocal microscopy analysis.** Archived paraffin-embedded male mouse kidney tissue was processed and immunolabeled for light- or confocal laser scanning microscopy as previously described 25.

**Reverse-transcriptase PCR (RT-PCR).** RNA purification and RT-PCR was performed on 1 µg RNA as previously described 26. Archived cDNA from male mouse kidney cortex was used as a positive control. Primer sequences are in supplemental material.

**Mouse cortical tubule suspensions.** Mouse cortical tubule suspensions from male C57BL/6J mice were prepared as previously described 22. Equal volumes of the suspensions were plated into 24-well tissue culture plates and pre-incubated in pure DMEM-F12 media (Invitrogen) for 2½ h at 37°C / 5% CO₂. For erlotinib and G36 studies, the antagonists were added to the suspensions for the last 30 min of pre-incubation. Suspensions were treated using antagonists, aldosterone (1 nM) or relevant controls as indicated in text for 30 min at
37°C / 5% CO₂. In some studies, 5 µM actinomycin D and 100 µM cycloheximide (Sigma-Aldrich) were included throughout the 2½ h pre-incubation and stimulation steps. At the end of the experiment media was removed and cells lysed in Laemmli sample buffer containing DTT (15 mg/mL), sonicated and heated at 65°C for 15 min before immunoblotting. For high potassium experiments, KCl was added to the media where indicated to make the final K⁺ concentration 8 mM.

**Acute aldosterone effects in mice.** All animal protocols comply with the European community guidelines for the use of experimental animals and were approved and performed under a license issued by the Danish Ministry of Justice (Dyreforsøgstilsynet). Male C57BL/6J mice (approx. 30 g bodyweight) were administered 50 µl of an aldosterone solution (43 µM in DMSO) by intramuscular injection. This resulted in a 3-5 fold increase in plasma aldosterone levels (Fig. 1 and Supplemental Fig. 1). After 30 or 60 min, animals were anesthetized using isofluorane and blood sampled from the retro-orbital plexus. Kidneys were removed and processed for immunoblotting as previously described 20.

**In vivo role of EGFR and GPR30.** In study 1, 10 week old male FVB mice (Janvier Labs, France) were used and their bladder emptied at the beginning of the experiment. Mice received either Gefitinib (0.8 mg/g bodyweight, Selleckchem) or vehicle (20% PEG300) by gavage (250 µl). The experimental period was 3 h, during which the mice were housed in their usual cages. Spot urine was collected during the last 1 h period. Blood was removed from the vena cava under isoflurane anesthesia into lithium heparin tubes, the animals euthanized and the kidneys collected and snap frozen in liquid N₂. Immunoblotting samples
were prepared as previously described \(^{27}\). For study 2, 10 week old female FVB mice (Janvier Labs, France) were used and the experimental period was 1 h or 2 h. For study 3, 10 week old male FVB mice (Janvier Labs, France) were used. After 1 h treatment with Gefitinib or vehicle, mice were administered 50 µl of an aldosterone solution (43 µM Aldosterone) or vehicle by intramuscular injection. The experiment was terminated 1 h later by decapitation and trunk blood collected. Generation of the GPR30\(^{-/-}\) mice has been previously described \(^{28}\). Archived kidneys isolated from three-month-old female GPR30\(^{-/-}\) and wildtype control mice were used \(^{29}\). Both groups were subject to ovariectomy four weeks before tissue was collected. All animal experiments were approved by the Ethics Committees for Animal Research at Gothenberg University and Karolinska Institutet.

**SILAC labeling, LC-MS/MS sample preparation and running conditions and LC-MS/MS data analysis.** These were performed in a similar manner to our previous study \(^{24}\). Extensive details are provided in Supplemental Material. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE \(^{30}\) partner repository with the dataset identifier PXD010032 (Username: reviewer12098@ebi.ac.uk Password: HS8uC5pG).

**Bioinformatics.** Regulated phosphorylation motifs were predicted using standard settings in MotifX (http://motif-x.med.harvard.edu/motif-x.html). Go-term analysis was carried out using app ClueGo v2.5.1 in Cytoscape. Proteins (uniprot accessions used) with significant changes in phosphorylation levels were subjected for phosphorylation core analysis using Ingenuity Pathway Analysis (IPA, Qiagen) to generate functional pathways.
**Statistics.** Immunoblotting and second messenger data are expressed as mean ± S.E.M. For two groups, data meeting the statistical assumptions of normality were assessed using an unpaired Student’s t-test. Comparisons of more than two groups were performed using a one-way ANOVA followed by Tukey’s or Dunnett’s multiple comparison tests. Significance was considered at $P < 0.05$. 
Results

*Aldosterone rapidly modulates NCC activity in native DCT cells*

Kidneys were isolated from mice 60 min subsequent to a single intramuscular injection of aldosterone, and the phosphorylation levels of NCC at an activating site (T58) were examined (Fig 1A). Relative to total NCC, the levels of pT58-NCC were significantly increased with aldosterone (Fig 1B), with a positive and significant correlation between pT58-NCC and plasma aldosterone concentrations (Fig 1C and 1D). These changes in pT58-NCC levels occurred independently of alterations in plasma Na\(^+\) or K\(^+\) levels (Fig 1E to G). Similar observations were apparent in another cohort of mice 30 min after aldosterone injection (Supplemental Fig. 1). To study the isolated effects of aldosterone, *ex vivo* cortical tubule suspensions were isolated from mouse kidney and incubated with aldosterone (1 nM) for 30 minutes. pT58-NCC levels normalized for total NCC levels were significantly elevated in the treated group relative to controls (Fig 1H to I). Similar effects of aldosterone (1 nM) were observed in cortical tubule suspension experiments performed in the presence of the transcriptional inhibitor actinomycin D and the protein translation inhibitor cycloheximide (Fig. 1J and 1K), excluding a genomic role of aldosterone in the activation of NCC. Additional cortical tubule experiments determined that the effect of aldosterone on pT58-NCC levels occurred within 15 min (Fig. 2A and 2B) and that lower aldosterone concentrations (1 pM – 10 nM) had a greater effect on pT58-NCC than 1 μM (Fig. 2C and 2D). However, the effects of aldosterone were not observed when similar cortical tubule experiments were performed in high K\(^+\) (8 mM) containing media (Fig. 2E and 2F).
Aldosterone rapidly increases cAMP and IP levels and phosphorylation of kinases/kinase substrates within the MAPK/ERK, PI3K/AKT and cAMP/PKA signaling pathways.

Although in our hands mpkDCT cells do not reliably express NCC, they are a good model to study signaling mechanisms in the DCT. The levels of 3 major intracellular second messengers in mpkDCT cells after 30 min of aldosterone treatment are shown in Fig 3. Intracellular cAMP levels were consistently increased approximately 50% after aldosterone treatment (Fig 3A). Levels of total cellular inositol phosphate (IP) were significantly increased after 30 min of aldosterone treatment (Fig 3B), suggesting aldosterone can activate an IP$_3$-Ca$^{2+}$ signaling pathway. However, within a similar timeframe no measurable differences in total cellular Ca$^{2+}$ levels were observed (Fig 3C).

Alterations in intracellular cAMP and IP levels suggest that aldosterone signaling involves a variety of different pathways e.g. phosphoinositide 3-kinase (PI3K), PLC, PKC and PKA. To examine this further, mpkDCT cells were treated with aldosterone (1µM and 1nM) for 30 min and phosphorylation levels (as an indicator of activity) relative to total protein abundances of various components of these major pathways were examined. Phosphorylation levels of ERK1/2, were increased (Fig 3D and 3E), and in line with increased IP levels, phosphorylation of the inositol 1,4,5-triphosphate receptor (InsP3R) was increased at S1756 (Fig 3D and 3E), a site known to modulate sensitivity of the receptor to IP3. Phosphorylation at Y458 on the p85 regulatory subunit, a site that has previously been reported to track with the activation of the PI3K complex, was also greatly increased by aldosterone (Fig 3D and 3E). Combined, these data suggest that the rapid effects of aldosterone in mpkDCT cells involve activation of the MAPK/ERK pathway and the PI3K pathway. In line with this, aldosterone increased phosphorylation levels of two downstream targets of PI3K, namely PDK S241 and AKT S473 (Fig 3F and 3G). Furthermore,
aldosterone increased phosphorylation of SGK1 at S422 (Fig 3F and 3G), which may be a converging node between the PKA pathway, likely activated due to the increased cAMP levels and the PDK pathway. PKA activation and convergence of PDK/PKA pathway are further suggested by increased phosphorylation of SPAK on S373 within its regulatory domain (Fig 3F and 3G) and S133 of CREB (Fig 3H and 3I). Supporting activation of AKT are increased levels of GSK3β phosphorylation at S9 (Fig 3H and 3I). In addition, a small increase in phosphorylation of P70S6K at T389 (Fig 3H and 3I) suggests that AKT-mediated activation of mTORC1 is also involved in the acute response of mpkDCT cells to aldosterone. Interestingly, in line with the ex vivo effects of aldosterone on pT58-NCC, for the majority of signaling molecules examined, a greater response to the lower aldosterone concentration (1 nM) was observed. This concentration was used in subsequent studies.

**GPR30 partially mediates the rapid effects of aldosterone in mpkDCT cells.**

Rapid aldosterone effects are attributed to a variety of different receptors, including GPR30. RT-PCR confirmed GPR30 expression in mouse kidney and mpkDCT cells (Fig 4A). GPR30 was also detected in ex vivo mouse DCT cells purified by FACS (Fig 4B). To functionally address the role of GPR30, mpkDCT cells were treated for 30 minutes with aldosterone, the GPR30 agonist G-1, or aldosterone in the presence of the GPR30 antagonist G-36 (Fig 4C). Treatment with G-1 significantly increased the phosphorylation status of ERK1/2, InsP3R, AKT, CREB and SGK (Fig 4D). In the presence of G-36, aldosterone was unable to significantly increase phosphorylation of InsP3R, PDK1, AKT and CREB (Fig 4D). In the presence of G-36, phosphorylation of ERK1/2, SGK1, SPAK and CREB was significantly increased by aldosterone, but the relative phosphorylation levels
were attenuated versus aldosterone alone treatment (Fig 4D). Some of the effects of G-1 on mpkDCT cells may be due to altered cAMP levels, but the responses were highly variable (Supplemental Fig 2). Furthermore, in cortical tubule suspensions, the effects of aldosterone (1 nM) on pT58-NCC levels were partially blunted in the presence of G-36 (Fig 4E and F). Together this *ex vivo* and *in vitro* data indicate that although some of the acute effects of aldosterone to activate NCC appear to be mediated via GPR30, alone it cannot fully account for the signaling networks activated by aldosterone. Supporting a role of GPR30 in modulating NCC activity *in vivo*, the levels of phosphorylated NCC were significantly reduced in ovariectomized (both GPR30 and NCC respond to estradiol) GPR30 knockout mice (Supplemental Fig 3).

**Systems level analysis of rapid aldosterone-mediated signaling**

To uncover alternative pathways relaying the rapid aldosterone effects, a quantitative phosphoproteomics strategy was used to investigate the global cell signaling changes in mpkDCT cells (Workflow in Supplemental Fig 4). Combining the LC-MS/MS data from 4 biological replicates, 4723 unique protein groups were identified in mpkDCT cells (Supplemental Table 1). A total of 11979 unique phosphorylation sites were identified on 1513 different proteins (Supplemental Table 2), of which 4432 sites were confidently assigned with a phosphoRS score above 80. Of these, 3275 sites have a CPhos site conservation score greater than 0.9, suggestive of biological and functional importance. The distribution of phosphopeptides are represented as a volcano plot in Fig 5A. Phosphopeptides with large changes (LOG2 ratios all higher than 0.75 or all lower than -0.75) were also retained for further analysis. In total, 602 phosphorylation sites significantly
increased in abundance and 1178 sites were decreased significantly upon aldosterone treatment (Supplemental Table 2). Several of these sites are targets of the MAPK/ERK, cAMP/PKA or PI3K/AKT signaling pathways. A similar proline-directed motif was suggested using Motif-X 42,43 (Supplemental Fig 5A) or Icelogo (Supplemental Fig 5B) 44 for the aldosterone regulated phosphorylation sites. GO-term analysis of the proteins with regulated phosphorylation sites identified a predominance of proteins involved in biological processes such as nucleic acid metabolic process, cytoskeleton organization as well as transcription or translation (Supplemental Fig 6A). GO-term analysis of proteins with greatly changed phosphorylation sites (LOG2 fold change higher than 0.5 or lower than -0.5) suggest aldosterone actions are linked to establishment of epithelial polarity, signal transduction and the regulation of sodium ion transmembrane transporter activity (Supplemental Fig 6B). This analysis was further presented as a CluePedia 45 view showing enriched genes that belong to the same biological process groups or shared between processes (Fig 5B). Ingenuity® Pathway Analysis using the regulated phosphorylation sites identified nine complex interlinked networks (Supplemental Fig 7A). The EGFR was predicted from the analysis of all complex networks to be an activated upstream signaling molecule (Supplemental Fig 7B). When EGFR was added to the top 4 predicted networks, it became a central network node connecting key kinases and subsequent substrates (Supplemental Fig 8 and 9).

The EGFR is localized to mouse DCT and is involved in the rapid aldosterone response

The EGFR was identified in mpkDCT cells, mouse kidney and ex vivo FACS purified mouse DCT cells Fig 6A and B). Immunohistochemistry on mouse kidney localized the EGFR to
the majority of renal tubules, including the distal tubule (Fig 6C). Double-labeling with antibodies against NCC confirmed localization of the EGFR in the DCT (Fig 6D and E), where it resides predominantly in the basolateral membrane domain. In mpkDCT cells treated with aldosterone for 30 min, phosphorylation of the EGFR at Y1068 (active site) was significantly increased (Fig 6F and H). However, in the presence of G-36, aldosterone-induced phosphorylation of the EGFR was attenuated (Fig 6G and I), suggesting some involvement of GPR30 in the aldosterone-induced activation of EGFR. Furthermore, when similar experiments were performed with dexamethasone in the cell culture media (limiting aldosterone selectivity for the MR), several of the effects of aldosterone, including increased phosphorylation of the EGFR, were diminished (Supplemental Fig 10). This suggests that the MR is involved in transactivation of the EGFR 46, or there is some crosstalk with the glucocorticoid receptor 47. To further assess the role of the EGFR, mpkDCT cells were treated either with aldosterone (1 nM) or aldosterone plus the EGFR inhibitor erlotinib (100 nM) for 30 min and phosphorylation levels of various signaling components examined. As seen previously (Fig 3), aldosterone alone significantly increased phosphorylation of the EGFR, ERK1/2, InsP3R, PDK1, AKT, SGK, SPAK and CREB (Fig 7). Erlotinib treatment alone suppressed phosphorylation of the EGFR, ERK1/2 and AKT, confirming inhibition of EGFR-dependent signaling. In the presence of erlotinib, aldosterone was unable to significantly increase phosphorylation of the EGFR, ERK1/2, InsP3R, SGK1 and SPAK. Furthermore, in cortical tubule suspensions, the effects of aldosterone (1 nM) to increase pT58-NCC levels were absent in the presence of erlotinib (Fig 7C and D). Together these data indicate that the EGFR is involved in rapid aldosterone effects in DCT cells.

The EGFR regulates NCC activity in vivo
To examine if the EGFR plays a role for modulation of NCC activity *in vivo*, mice were treated for 3 h with either the EGFR antagonist Gefitinib or vehicle. Phosphorylation levels of the EGFR were significantly decreased following Gefitinib (**Fig 8A and B**), confirming inhibition of the receptor. Concurrently ERK1/2, AKT, and NCC phosphorylation levels were decreased (**Fig 8A and B**). These changes in NCC phosphorylation subsequent to EGFR inhibition correlated with a significantly raised urinary sodium/creatinine excretion and a marked trend for higher fractional excretion of sodium (**Fig 8C-F**). Similar observations on pT58-NCC levels were apparent in mice treated for 1 h or 2 h with Gefitinib (**Supplemental Fig. 11**). To further explore a role of the EGFR, mice were treated with either Gefitinib or vehicle for 1 h, plus either aldosterone or vehicle for 1 h. Relative to vehicle, aldosterone significantly increased NCC-pT58 levels, but this was blunted by Gefitinib (**Fig 8G and H**), even though aldosterone levels in this group of mice were highest (**Fig 8I**). A significant positive correlation was observed between plasma aldosterone concentration and the NCC-pT58/total NCC ratio only in the absence of gefitinib (**Fig 8J**).

**Discussion**

The genomic actions of aldosterone to alter renal Na\(^+\) absorption and K\(^+\) secretion play an essential role in maintaining overall body Na\(^+\) and K\(^+\) balance. Although these aldosterone-induced genomic effects can occur within 60 min of aldosterone administration, the alterations in second messengers, the redistribution of ENaC and increases in amiloride-sensitive sodium currents that occur within seconds to minutes of aldosterone
exposure cannot be explained by these mechanisms \(^3\)-\(^5\). Thus, other rapid aldosterone-mediated signaling mechanisms must exist. Given the important role of NCC in relaying aldosterone-dependent effects, we sought to determine whether aldosterone exerts acute modulation of this cotransporter and the signaling networks employed. A unique finding from this study is that within minutes, aldosterone increases the levels of intracellular cAMP and IP, and activates several proteins in mpkDCT cells including ERK1/2, SGK, AKT, PDK1 and SPAK that have well-established roles in modulating Na\(^+\) transport in the kidney. This ultimately leads to activation of the NaCl cotransporter NCC, a finding confirmed \textit{ex vivo} and \textit{in vivo}. Furthermore, altered phosphorylation of a variety of different transcription factors and translation initiation factors suggests that these early aldosterone effects act in concert with additional, but later onset genomic effects to modulate transcriptional and translational regulation in DCT cells. A simplified hypothetical signaling pathway for rapid aldosterone effects is illustrated in Fig 9.

The mechanisms by which aldosterone elicits rapid signaling responses in target cells are evolving. Since rapid effects occur using non-cell permeable aldosterone analogues \(^9\)-\(^{10}\), in the presence of transcriptional and translation inhibitors \(^{49}\) or in MR knockout mice \(^8\), they are, at least in part, mediated by a plasma membrane-bound receptor. The increased total cellular inositol phosphate and cAMP levels in mpkDCT cells within minutes of aldosterone application also suggests that a membrane-bound receptor is playing a role in the DCT. GPR30 \(^7\), which can signal via cAMP or IP3 and responds to aldosterone stimulation \(^{13,14}\), was detected in both mpkDCT cells and the DCT \textit{in vivo}, making this a possible candidate. The EGFR, which can be transactivated following stimulation of GPR30 or the MR \(^{46}\), was also expressed in the DCT. Supporting a role of the EGFR and GPR30, in mpkDCT cells aldosterone acutely altered phosphorylation of various kinases/kinase substrates within the
MAPK/ERK, PI3K/AKT or cAMP/PKA signaling pathways, including ERK, PI3Kp85, PDK1, SGK1, AKT, CREB and GSK3β, which are known to be modulated by GPR30 and/or EGFR-mediated signaling. A role of GPR30 was further strengthened by the inhibition of aldosterone-mediated InsP3R, PDK1 and AKT phosphorylation in the presence of the GPR30 antagonist G-36, whereas an EGFR antagonist erlotinib inhibited aldosterone-mediated increases in ERK1/2, InsP3R, SGK1 and SPAK phosphorylation. These data suggest that aldosterone-mediated signaling via the EGFR and GPR30 are not mutually exclusive, and some signaling pathways may be activated by one, or both receptors simultaneously. Alternatively, presuming the antagonists fully inhibit EGFR or GPR30-dependent signaling, these results indicate that GPR30 and/or EGFR alone are not fully accountable for the acute aldosterone effects in mpkDCT cells and that other mediators of rapid aldosterone effects in these cells must exist. The existence of alternative membrane receptors that may relay aldosterone effects, including IGF1R and PDGFR, were identified in our non-biased signaling networks generated from phosphoproteomics data. Nonetheless, as the aldosterone effects on phosphorylated NCC are reduced following inhibition of GPR30 or the EGFR in ex vivo cortical tubule suspensions, and pT58-NCC levels are reduced following genetic or pharmacological inhibition of GPR30 or the EGFR in vivo, these receptor-dependent pathways are a novel mechanism by which NaCl transport is regulated in the DCT. Further studies, potentially including the generation of mice with DCT-specific deletion of the EGFR, are required to further assess the contributions of these receptors in the acute aldosterone response.

Another observation is that the largest changes in signaling pathways occurred at lower concentrations of aldosterone, suggesting that the acute actions of aldosterone to activate NCC occur within the normal physiological range of aldosterone.
A higher concentration of aldosterone has a reduced response is unclear, but a similar biphasic effect on NCC activity occurs with Angiotensin II. Another point of note is that although aldosterone consistently increased SPAK phosphorylation, a key mediator of NCC activation, these effects are relatively modest. This suggests that other SPAK-independent mechanisms may be involved, such as alterations in the rate of NCC dephosphorylation.

The effects of aldosterone on NCC were initially assumed to be MR-dependent. However, recent studies indicate that the MR may be dispensable for the long-term effects of aldosterone on NCC and changes in NCC have instead been attributed to changes in plasma $K^+$ and $K^+$ concentrations. Our studies demonstrate that rapidly aldosterone can increase phosphorylated NCC levels, independently of total NCC levels, in ex vivo cortical tubule suspensions, or in vivo. This increase in phosphorylated NCC in mouse kidney was positively correlated with plasma $[K^+]$ under these conditions. However, in cortical tubule suspensions pT58-NCC levels were significantly reduced in the presence of high $[K^+]$ and significant effects of short-term aldosterone treatment on phosphorylated NCC were inhibited. Together, this adds a new dimension to both the mechanisms and the time-course within which aldosterone affects the DCT to modulate NCC activity. When the DCT encounters aldosterone released due to hypovolemia, its main function is to rapidly increase NaCl reabsorption to help restore blood volume. Thus, it is of physiological benefit that the positive effects of aldosterone on NCC activity occur within minutes. Conversely, when the DCT encounters aldosterone and high $K^+$ concentrations in hyperkalemic states, the effects of high $K^+$ appear to "override" the acute effects of aldosterone and reduce NCC activity, promoting $K^+$ secretion in downstream portions of the renal tubule.

In conclusion, we have identified novel signaling pathways by which aldosterone can exert rapid effects in the DCT. This study identifies an important new role of non-genomic
aldosterone actions in the DCT that are likely to have major implications for modulation of NaCl transport and ultimately blood pressure regulation.

**Author contributions.** R.A.F made the conception and initial design of the work. L.C, Q.W, E.T.B.O, S. B. P, L. P, B. O, F. L. L, T.P, H. D, T. R, C. E. F and R.A.F acquired data, analyzed and interpreted data or developed new reagents. L. C. and R.A.F drafted the manuscript. All authors critically revised manuscript for intellectual content, approved the final version of the manuscript and agree to be accountable for all aspects of the work.

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**Supplemental Material**

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Figures and legends

Fig. 1. Aldosterone rapidly modulates NCC activity in native DCT cells. A) Mice were injected with aldosterone and after 60 min the phosphorylation levels of NCC at T58 (NCC-pT58, surrogate marker of NCC activation) was assessed by immunoblotting. Group 1 and 2 refer to independent experimental cohorts. B) Summary of data (n=12) from two independent experiments. Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. C) Plasma aldosterone levels. D) A significant positive correlation exists between plasma aldosterone concentration and the NCC-pT58/total NCC ratio. E) Plasma Na\(^+\) levels are not significantly different. F) Plasma K\(^+\) levels are not significantly different. G) No significant correlation was observed between plasma K\(^+\) levels and the NCC-pT58/total NCC ratio after 60 min of aldosterone treatment. H) Cortical tubule suspensions were treated with aldosterone (1 nM) for 30 min and NCC-pT58 levels were assessed by immunoblotting. I) Summary of data (n=17/group). J) Cortical tubule suspensions were treated for 150 min with actinomycin D and cycloheximide (A+C) where indicated before treatment with aldosterone (1 nM) for 30 min. NCC-pT58 levels were assessed by immunoblotting. K) Summary of data. Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. *: 0.01<p<0.05; **: 0.001<p<0.01; ***: 0.0001<p<0.001 relative to control. Aldo = aldosterone, Con = control.

Fig. 2. Aldosterone effects on NCC activity in ex vivo cortical tubule suspensions are time and concentration dependent but absent in high K\(^+\) media. A) Cortical tubule suspensions from male mice were treated with aldosterone (1 nM) for various times and
NCC and NCC-pT58 levels were assessed by immunoblotting. B) Summary of data (n=8/group). *: 0.01<p<0.05; **: 0.001<p<0.01 relative to control. C) Cortical tubule suspensions from male mice were treated with various aldosterone concentrations (1 pM to 1 μM) for 30 min and NCC-pT58 levels were assessed by immunoblotting. D) Summary of data (n=14/group). *: 0.01<p<0.05; **: 0.001<p<0.01 relative to time-matched control. E) Cortical tubule suspensions from male mice were pre-incubated in media containing 8 mM K+ before incubation with aldosterone (1 nM) for 30 min and NCC-pT58 levels were assessed by immunoblotting. B) Summary of data (n=6/group). *: 0.01<p<0.05 relative to 4mM control conditions. All data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. Aldo = aldosterone, Con = control.

Fig.3. Aldosterone rapidly increases cAMP and IP levels and induces phosphorylation of various kinases/kinase substrates within the MAPK/ERK, PI3K/AKT and cAMP/PKA signaling pathways. A) Intracellular cAMP levels were significantly increased in mpkDCT cells stimulated with aldosterone for 30 min. B) Total cellular IP levels were significantly raised in mpkDCT cells stimulated with aldosterone for 30 min. C) No significant differences in total intracellular Ca2+ levels were detectable following 30 min aldosterone treatment. ATP was used as a positive control. D) mpkDCT cells were treated with aldosterone for 30 min and protein homogenates examined by immunoblotting with antibodies targeting phosphorylation sites of ERK1/2, PI3K and InsP3R. E) Summary of data from 3 independent experiments (n=10/group). F) Immunoblotting with antibodies targeting phosphorylation sites of PDK1, SGK1, AKT and SPAK. G) Summary of data from 3 independent experiments (n=10/group). H) Immunoblotting with antibodies targeting phosphorylation sites of CREB,
GSK3β and p70S6K. I) Summary of data from 3 independent experiments (n=10/group).

Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. *: 0.01<p<0.05; **: 0.001<p<0.01; ***: 0.0001<p<0.001 relative to individual control group. Aldo = aldosterone, Con = control.

**Fig.4. GPR30 is expressed in mouse kidney DCT cells and partly mediates the rapid effects of aldosterone.**

A) GPR30 is detected in mpkDCT or mouse kidney (Kid) samples by RT-PCR. B) GPR30 is detected in protein homogenates from mouse kidney, mpkDCT cells or DCT cells purified from mouse kidney (DCT) by immunoblotting. C) mpkDCT cells were treated for 30 minutes with aldosterone, the GPR30 specific agonist G-1 or aldosterone (1 nM) in the presence of the GPR30 antagonist G-36 and phosphorylation levels of various signaling proteins examined by immunoblotting. D) Summary of data (n=8/group). Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. *: 0.01<p<0.05; **: 0.001<p<0.01; ***: 0.0001<p<0.001. Aldo = aldosterone, Con = control. E) Cortical tubule suspensions were treated for 30 min with G-36 where indicated before treatment with aldosterone (1 nM) for 30 min. NCC-pT58 levels were assessed by immunoblotting. F) Summary of data. Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. *: 0.01<p<0.05; **: 0.001<p<0.01; ***: 0.0001<p<0.001 relative to individual control group. Aldo = aldosterone.

**Fig.5. Systems level analysis of rapid aldosterone-mediated signaling.** mpkDCT cells were treated with aldosterone (1nM) for 30 min and global phosphorylation levels...
determined using SILAC based LC-MS/MS. A) Volcano plot of phosphopeptide quantification from 4 independent experiments. Y-axis indicates -log10 (p-value) while the horizontal axis indicates the base 2 logarithmic value of the mean peptide abundance ratio (aldosterone vs. vehicle). Horizontal dashed line represents the Benjamini-hochberg FDR threshold of 10% used for statistical significance. Phosphopeptides significantly increased are shown in red, and those decreased are shown in green. B) CluePedia view of the Go-term analysis using regulated phosphopeptides with LOG 2 ratios higher than 0.5 or lower than -0.5. Genes belong to biological process groups or shared between processes are shown.

Fig.6. The EGFR is localized to the DCT and activated by aldosterone. A) EGFR is detected in mpkDCT or mouse kidney (Kid) samples by RT-PCR. B) EGFR is detected by immunoblotting in protein homogenates from mouse kidney, mpkDCT cells or DCT cells purified from mouse kidney (DCT). C) Immunohistochemistry of EGFR in mouse kidney sections. Staining is strongest in segments morphologically resembling distal tubules (DT) and is weaker in proximal tubules (PT). D-E) Confocal images of mouse kidney sections immunolabeled for EGFR and colocalized with the DCT expressed protein NCC. F) In mpkDCT cells treated with aldosterone for 30 min, phosphorylation of the EGFR at Y1068 (active site) was significantly increased. G) In the presence of G-36, aldosterone had a reduced effect on EGFR phosphorylation levels. H-I) Summary of data. Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. *: 0.01<p<0.05; ***: 0.0001<p<0.001 relative to individual control group. Aldo = aldosterone, Con = control.
Fig. 7. The EGFR partially mediates the acute effects of aldosterone. A) mpkDCT cells were treated with aldosterone (1nM) or aldosterone plus the EGFR inhibitor erlotinib (100nM) for 30 min and phosphorylation levels of various signaling proteins examined by immunoblotting. B) Summary of data (n=8/group). Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. C) Cortical tubule suspensions were treated for 30 min with erlotinib where indicated before treatment with aldosterone (1 nM) for 30 min. NCC-pT58 levels were assessed by immunoblotting. D) Summary of data. Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. *: 0.01<p<0.05; **: 0.001<p<0.01; ***: 0.0001<p<0.001 relative to individual control group. Aldo = aldosterone.

Fig. 8. In vivo inhibition of the EGFR reduces NCC activity and attenuates the effects of aldosterone on NCC activity. A) Mice were treated for 3 h with either the EGFR antagonist Gefitinib or vehicle and phosphorylation levels of various signaling proteins in the kidney examined by immunoblotting. B) Summary of data (n=11/group). Data are presented as phosphorylated levels relative to total protein levels and normalized relative to vehicle. Phosphorylation levels of NCC at T58 were significantly reduced alongside phosphorylation of the EGFR, ERK1/2, AKT. C) Spot urine collected during the last 1 h period (n=5-7/group) of EGFR inhibition demonstrated increased fractional excretion of sodium (Na+). D) Significantly higher urinary sodium/creatinine excretion. E) Urinary potassium/creatinine excretion. F) Urinary sodium excretion relative to potassium. *: 0.01<p<0.05; **: 0.001<p<0.01; ***: 0.0001<p<0.001 relative to control. G) Mice were treated with either the EGFR antagonist Gefitinib (Gef) or vehicle (Veh) for 2 h. 1 h before the experiment was
terminated animals were injected with aldosterone (aldo). Phosphorylation levels of NCC at T58 (NCC-pT58, surrogate marker of NCC activation) were assessed by immunoblotting. H) Summary of NCC data (n=6). Data are presented as phosphorylated levels relative to total protein levels and normalized relative to vehicle. Aldosterone significantly increased NCC-pT58 levels, but this was limited by Gefitinib. Similar samples were assessed for ERK phosphorylation as an indirect indication of successful EGFR inhibition. Summary data is shown. I) Plasma aldosterone levels. J) A significant positive correlation exists between plasma aldosterone concentration and the NCC-pT58/total NCC ratio in the absence of gefitinib (solid correlation line). In contrast, there was no correlation in the presence of gefitinib (dashed correlation line). *: 0.01<p<0.05; **: 0.001<p<0.01; ****: 0.0001<p<0.001; relative to vehicle group.

**Fig.9. Summary of potential rapid signaling mechanisms of aldosterone in the DCT.**

The non-biased proteomics data were combined with data from immunoblotting experiments and current literature to generate a simplified hypothetical signaling pathway for rapid aldosterone effects in DCT cells. GPR30 and EGFR are involved in mediating some of the rapid effects of aldosterone, but a role for the mineralocorticoid receptor (MR), or other seven transmembrane receptors (7TMR) or matrix metalloproteinases (MMPs) requires further investigation. Dotted lines or question marks indicate potential pathways of aldosterone signaling that need further investigation.
A. mpkDCT and Kid

B. Kid DCT mpkDCT

C. Immunohistochemistry

D. Confocal microscopy

E. Immunofluorescence

F. Western blot for EGFR-pY1068 and EGFR

G. Western blot for EGFR-pY1068 and EGFR

H. Graph showing phosphorylation levels

I. Graph showing phosphorylation levels
Altered phosphorylation of 242 proteins involved in regulation of transcription/translation
SUPPLEMENTAL MATERIAL

Rapid aldosterone-mediated signaling in the DCT increases activity of the thiazide-sensitive NaCl cotransporter

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Supplemental Fig. 1. Aldosterone rapidly modulates NCC activity in native DCT cells. A) Mice were injected with aldosterone and after 30 min the phosphorylation levels of NCC at T58 (NCC-pT58, surrogate marker of NCC activation) was assessed by immunoblotting. B) Summary of data (n=8). Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control (Con) conditions. C) Plasma aldosterone levels. D) A significant positive correlation exists between plasma aldosterone concentration and the NCC-pT58/total NCC ratio. E) Plasma Na⁺ levels are not significantly different. F) Plasma K⁺ levels are not significantly different. G) No significant correlation was observed between plasma K⁺ levels and the NCC-pT58 / total NCC ratio after 30 min of aldosterone treatment. *: 0.01<p<0.05; ****: 0.0001<p<0.001.
Supplemental Fig. 2. Effects of G-1 and G-36 on cAMP levels in mpkDCT cells. mpkDCT cells were stimulated for 30 min with the GPR30 specific agonist G-1 or the GPR30 antagonist G-36. ***: 0.0001<p<0.001.
Supplemental Fig. 3. Phosphorylated NCC levels are reduced in GPR30 knockout mice. A) NCC levels were assessed by immunoblotting of kidney samples from ovariectomized GPR30−/− and wildtype control mice. B) Summary of data (n=6/group). Data are normalized relative to control conditions. Phosphorylation levels of NCC at T58 (surrogate marker of NCC activation) were significantly reduced in GPR30−/− mice whereas total NCC levels were unchanged. **: 0.001<p<0.01.
Supplemental Fig. 4. Schematic overview of the experimental phosphoproteomics workflow. Cells were grown in either SILAC light (Lys+0, Arg+0) or heavy media (Lys+6, Arg+10) on filter plates until confluent and stimulated with aldosterone (1nM) for 30 min. Four passages of labelled cells were used to generate four biological replicates for statistical analysis. Cells were harvested, equally pooled and subjected to offline high-pH fractionation based two dimensional LC-MS/MS analysis (Q-Exactive). Peptide quantification was performed at the MS1 level.
Supplemental Fig. 5. Information based sequence logo of the up- and downregulated phosphorylation motifs following aldosterone stimulation of mpkDCT cells. A) Predicted phosphorylation motifs using Motif-X. B) Predicted phosphorylation motifs using the Iceolog software.
Supplemental Fig. 6. GO-term biological processes analysis by Clue-Go. The terms represent processes that are significantly over-represented in aldosterone regulated genes relative to the whole mouse genome. A) All significantly changed phosphopeptides were included in the analysis. B) Only phosphopeptides with LOG2 ratios higher than 0.5 or lower than -0.5 were included in the analysis. This analysis suggests that aldosterone actions are linked to establishment of epithelial polarity, signal transduction and regulation of sodium ion transmembrane transporter activity. **: 0.001<p<0.01
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Supplemental Fig. 7. IPA pathway analysis of aldosterone signaling events in mpkDCT cells. mpkDCT cells were treated with aldosterone (1nM) for 30 min and global phosphorylation levels determined using LC-MS/MS. A) IPA core analysis using significantly up or down regulated phosphorylation sites from the phosphoproteomics datasets gave nine complex interlinked networks. B) Upstream regulator analysis of all complex networks highlighted the EGFR to be an activated upstream signaling node.
Supplemental Fig. 8. Interaction Network Analysis of the top 4 networks demonstrates that aldosterone stimulation mediates a highly interlinked signaling network. When the EGFR (blue) was added to these networks, it became a central network node connecting key kinases and subsequent
Supplemental Fig. 8. Interaction Network Analysis of the top 4 networks demonstrates that aldosterone stimulation mediates a highly interlinked signaling network. When the EGFR (blue) was added to these networks, it became a central network node connecting key
Supplemental Fig. 9. The MR may be involved in the rapid aldosterone mediated transactivation of the EGFR. 

A) mpkDCT cells were grown in dexamethasone containing media (see methods) and dexamethasone (dex) was removed in some groups for 16h before the experiment. Cells were subsequently treated with aldosterone (1 nM and 10 μM) for 30 min and protein homogenates examined by immunoblotting with antibodies targeting phosphorylation sites of selected proteins. Effects of aldosterone on the EGFR, ERK1/2, InsP3R, CREB and p70S6K were diminished in the presence of dexamethasone.

B) Summary of data from 2 independent experiments (n=6/group). Data are presented as phosphorylated levels relative to total protein levels and normalized relative to control conditions. *: 0.01<p<0.05; ***: 0.0001<p<0.001 relative to control conditions.
Supplemental Fig.10. Inhibition of the EGFR in vivo acutely reduces NCC activity. A) Mice were treated for 1 h or 2 h with either the EGFR antagonist Gefitinib or vehicle and phosphorylation levels of various proteins in the kidney examined by immunoblotting. B) Summary of data (n=6 / group). Data are presented as phosphorylated levels relative to total protein levels and normalized relative to vehicle conditions. *: 0.01<p<0.05; **: 0.001<p<0.01 relative to vehicle group.
Extended methods and materials

Chemicals. G-1 and G-36 (Tocris), aldosterone and ATP (Sigma-Aldrich), and Erlotinib and Gefitinib (Selleckchem) were used at the concentrations as indicated in the text.

Antibodies. The following antibodies were used: SGK1 (Cat # 12103), InsP3R (Cat # 3763), pInsP3R-S1756 (Cat # 3760) CREB (Cat # 9197), pCREB-S133 (Cat # 9198), AKT (Cat # #4685), pAkt-S473 (Cat # 9271), ERK1/2 (Cat # 4695), pERK-T202/Y204 (Cat # 4370), p70S6K (Cat # 9202), p-p70S6K-T389 (Cat # 9234), pPI3K p85-T458 (Cat # 4228), PI3K p85 (Cat # 4257), PDK1 (Cat # 5662), pPDK1-S241 (Cat # 3438), GSK3β (Cat # 12456) and pGSK3β-S9 (Cat # 9323) were from Cell Signaling. EGFR Y1092 (ab40815) and EGFR (ab52894) were from Abcam. pSGK-S422 (sc16745) and GPR30 (sc48524R) were from Santa Cruz. Specificity of the commercial antibodies was based on that they either gave a single unique band on an immunoblot corresponding to the target proteins predicted molecular weight, or the most prominent band on the immunoblot was at the target proteins predicted molecular weight (with no other bands of similar size). Other antibodies were SPAK and pSPAK 1 and pNCC-T58 1.

Ca²⁺ and cAMP measurements. The mouse kidney DCT cell line (mpkDCT) has been demonstrated to be an excellent model of the DCT 2. Cells were routinely cultured at 37°C in 5% CO₂ in mpkDCT medium (DMEM/F12- media (Invitrogen) containing 60 nM sodium selenite, 5 μg/ml transferrin, 2 mM glutamine, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM D-glucose and 20 mM HEPES (pH 7.4)) as
previously described. For Ca^{2+} assays, cells were cultured in black Visiplates (Wallac) until confluent, with the last 24 h in pure phenol red free DMEM-F12 media (Invitrogen). Cells were washed with HBSS before incubating in dye loading solution (5 µM Fluo-4, 2.5mM probenecid, 1x PowerLoad in HBSS) for 30 min at 37°C. Cells were washed in assay buffer (HBSS containing 2.5 mM probenecid) and overlaid with 200 µl assay buffer. Fluorescence was measured using an EnSpire plate reader (PerkinElmer), with an excitation wavelength of 495 nm and an emission wavelength of 510 nm continuously for 2 min (background fluorescence) and for 10 min after addition of 50 µl of agonist (to reach final concentrations as indicated) or control solution. For cAMP assay experiments, cells were grown on a semi-permeable filter support (Transwell, Corning) until a fully confluent polarized monolayer was formed (transepithelial resistance (TER) >5 kΩ.cm^2). Cells were stimulated as indicated for 30mins, lysed and intracellular cAMP levels were measured using a cAMP enzyme immunoassay kit (Enzo) according to manufacturer’s instructions. All measurements were carried out in triplicates on at least 5 separate days.

**Inositol Phosphate (IP) measurements.** IP accumulation was measured using a scintillation proximity based inositol-phosphate accumulation assay (SPA-IP). In brief, mpkDCT cells were seeded at 35,000 cells/well in 100 µL mpkDCT medium in 24-well filter plates and incubated for 24 h at 37°C with 0.5 µCi of myo-[^3H]inositol (PerkinElmer). Medium without myo-[^3H]inositol was added to the basolateral compartment. The following day, cells were washed twice in pure DMEM-F12 (Invitrogen) and incubated in pure DMEM-F12 supplemented with 10 mM LiCl at 37°C for 30 min in the presence of diluent (DMSO), aldosterone (1nM) or the activator of phospholipase C m-3M3FS (Tocris) as a positive control. Cells were incubated in 10 mM formic acid for 60 min. 35 µl from each
well was transferred to a 96-well plate, 1 mg of yttrium silicate SPA beads (SPA-Ysi; RPNQ, PerkinElmer) added and the plate mixed for 30 min by high-speed agitation. Beads were centrifuged (5 min, 400 g) and [3H]inositol phosphate binding was measured on a TopCount NXT (PerkinElmer, Waltham, MA).

**Immunoblotting experiments.** mpkDCT cells were grown on filter plates in mpkDCT media until a fully confluent polarized monolayer was formed (TER > 5 kΩ.cm²). The evening prior to experiments, cells were cultured in pure phenol red free DMEM-F12 media (Invitrogen). Cells were treated with agonists/antagonists or relevant control solutions as indicated for 30 min at 37°C. For G-36 antagonist experiments, cells were pre-incubated with G-36 or pure media for 20 min prior to subsequent stimulation. Cells were lysed in Laemmli sample buffer containing DTT (15 mg/mL), sonicated and heated at 65°C for 15 min. Standard procedures were utilized for SDS-PAGE. Immunoblots were developed using ECL detection and signal intensity in specific bands were quantified using Image Studio Lite (Qiagen) densitometry analysis. For detection of GPR30 and EGFR in native DCT cells, purified DCT cells were isolated from parvalbumin-GFP mice as previously described ⁵.

**Immunohistochemistry and confocal microscopy analysis.** Archived paraffin-embedded male mouse kidney tissue was processed and immunolabeled for light- or confocal laser scanning microscopy as previously described ⁶. Microscopy was carried out with a Leica DMRE light microscope or a Leica TCS SL confocal microscope with an HCX
PL APO 63 x oil objective lens (numerical aperture: 1.40) (Leica Microsystems).

Brightness was digitally enhanced on presented images.

**Reverse-transcriptase PCR (RT-PCR).** RNA purification and RT-PCR was performed on 1 µg RNA as previously described \(^7\). Archived cDNA from male C57BL/6J mouse kidney cortex was used as a positive control. Primers are; GPR30 forward 5’-CACGTGACATTGACCTCTGACCT and GPR30 reverse 5’-TCACTCAGGAGTTAGGAGTGGCA, EGFR forward 5’-CTTCAAGGATGTGAAGTGTG and EGFR reverse 5’-TGTACGCTTTGGAACACTGT.

**Mouse cortical tubule suspensions.** Mouse cortical tubule suspensions were prepared as previously described \(^2\). Equal volumes of the suspensions were plated into 24-well tissue culture plates and pre-incubated in pure DMEM-F12 media (Invitrogen) for 150 min at 37°C / 5% CO\(_2\). For erlotinib and G36 studies, the antagonists were added to the suspensions for the last 30 min of pre-incubation. Suspensions were treated using antagonists, aldosterone (1 nM) or relevant controls as indicated in text for 30 min at 37°C / 5% CO\(_2\). In some studies, 5 µM actinomycin D and 100 µM cycloheximide (Sigma-Aldrich) were included throughout the pre-incubation and stimulation steps. At the end of the experiment media was removed and cells lysed in Laemmli sample buffer containing DTT (15 mg/mL), sonicated and heated at 65°C for 15 min before immunoblotting. For high potassium experiments, KCl was added to the media where indicated to make the final K\(^+\) concentration 8 mM.
**Isolation of Enhanced Green Fluorescent Protein (EGFP) expressing mouse DCT cells.** All animal protocols comply with the European community guidelines for the use of experimental animals and were approved and performed under a license issued by the Danish Ministry of Justice (Dyreforsøgstilsynet). Mice had free access to standard rodent chow and water. Transgenic mice expressing EGFP driven by the parvalbumin promoter were euthanized by cervical dislocation and kidneys quickly removed. The kidneys were sliced into approximately 1 mm pieces and incubated for 40 min in buffer B (125 mM NaCl, 0.4 mM KH2PO4, 1.6 mM K2HPO4, 1 mM MgSO4, 10 mM Na-acetate, 1 mM α-ketoglutarate, 1.3 mM Ca-gluconate, 5 mM glycine, 30 mM glucose and 5 μg/mL DNase I (Sigma), pH 7.4) containing 2 mg/ml collagenase B (Roche). Samples were mixed continuously at 850 rpm at 37°C. After 10 min, half of the enzyme solution was removed and replaced with buffer B without collagenase, and samples were incubated for a further 10 min. This procedure was repeated for another 10 min. After a total incubation of 40 min, the tubular suspensions were passed through a 100 μM cell strainer (BD Falcon), and centrifuged for 3 min at 200 g. Cells were washed with a trypsin/EDTA solution (Lonza) containing 10 mM HEPES, 30 mM glucose and 50 μg/ml DNase I. Cells were again resuspended in trypsin/EDTA solution and incubated for 15 min at 37°C. Cells were washed in DMEM/HamF12 cell culture medium (Gibco) containing 5% FBS, 30 mM glucose, 10 mM HEPES and 50 mg/ml DNase I and subsequently resuspended in 1.5 ml medium. Cells were passed through a 40 μM cell strainer and kept at 4°C. EGFP-positive and negative cells were isolated on a FACS Aria III (BD Biosciences) machine at the FACS Core Facility, Aarhus University, Denmark. Sorted cells were centrifuged for 10 min at 3,000 g at 4°C and the pellet was resuspended in 1x Laemmli sample buffer (62.5 mM Tris, 8.75% Glycerol, 3% SDS, 89.5 μM Bromphenolblue, 15 mg/ml DTT, pH 6.8).
Samples were heated for 10 min at 65°C. EGFP purity changed from 4% before sorting to 94% after sorting in the EGFP-positive sample (DCT cells).

**Acute aldosterone effects in mice.** All animal protocols comply with the European community guidelines for the use of experimental animals and were approved and performed under a license issued by the Danish Ministry of Justice (Dyreforsøgstilsynet). Male C57BL/6J mice (approx. 30 g bodyweight) were administered 50 µl of an aldosterone solution (43 µM in DMSO) by intramuscular injection. This resulted in a 3-fold increase in plasma aldosterone levels as measured using an enzyme immunoassay kit (EIA-5298; DRG International, Springfield, NJ; range 20–1,000 pg/ml; QC: standards) ([Fig. 1](#)). After 30 or 60 min, animals were anesthetized using isofluorane and blood sampled from the retro-orbital plexus. Kidneys were removed and processed for immunoblotting as previously described ¹. Sodium and potassium levels in the urine were determined using flame photometry (Sherwood Model 420).

**In vivo role of EGFR.** All animal protocols were approved and performed under a license issued by the Danish Ministry of Justice (Dyreforsøgstilsynet, permit #2014-15-0201-00043). 10 week old male FVB mice (Janvier Labs, France) were used and their bladder emptied at the beginning of the experiment. Mice received either Gefitinib (0.8 mg/g bodyweight, Selleckchem) or vehicle (20% PEG300) by gavage (250 µl). The experimental period was 3 h, during which the mice were housed in their usual cages. Spot urine was collected during the last 1 h period. Blood was removed from vena cava under isoflurane anesthesia into lithium heparin tubes, the animals euthanized and the kidneys collected
and snap frozen in liquid N\textsubscript{2}. Immunoblotting samples were prepared as previously described \textsuperscript{9}. Creatinine in urine and plasma was determined using a colorimetric assay (ABX Pentra Creatinine 120 CP kit, Horiba ABX SAS, Montpellier, France) according to the manufacturer’s instructions. Sodium and potassium levels in the urine were determined using flame photometry (Sherwood Model 420). For study 2, 10 week old female FVB mice (Janvier Labs, France) were used and the experimental period was 1 h or 2 h. For study 3, 10 week old male FVB mice (Janvier Labs, France) were used. After 1 h treatment with Gefitinib or vehicle, mice were administered 50 \textmu{l} of an aldosterone solution (43 \textmu{M} Aldosterone) in 3.2 ul DMSO in 1.5 ml saline or vehicle by intramuscular injection. The experiment was terminated 1 h later by decapitation and trunk blood collected.

\textbf{In vivo role of GPR30.} Generation of the GPR30\textsuperscript{-/-} mice has been previously described \textsuperscript{10}. Animal care was in accordance with institutional guidelines. Archived kidneys isolated from three-month-old female GPR30\textsuperscript{-/-} and wildtype control mice four weeks after ovariectomy were used \textsuperscript{11}. All animal experiments were approved by the Ethics Committees for Animal Research at Gothenburg University and Karolinska Institutet.

\textbf{SILAC labeling.} mpkDCT cells were grown in SILAC advanced DMEM/F12-Flex media (Invitrogen) in light (12C\textsubscript{6} lysine, 12C\textsubscript{6} 14N\textsubscript{4} arginine) or heavy (13C\textsubscript{6} lysine, 13C\textsubscript{6} 15N\textsubscript{4} arginine) conditions for at least 21 days (3-5 passages). Mass spectrometry (MS) confirmed labeling efficiency >97\%. Cells were grown on filter plates until a fully confluent polarized monolayer was formed. On the day of experiment, cells were incubated in phenol red and serum-free pure media for 4 hrs. Aldosterone (1 nM) was added to both
apical and basolateral compartments of experimental group, controls received an
equivalent volume of DMSO. After 30 min cells were washed twice in ice-cold PBS and
scraped in cell lysis buffer (8 M urea, 2 M thiourea, 50 mM Tris, pH 7.5) containing
protease and phosphatase inhibitors (Halt protease and phosphatase inhibitors). After 20
min incubation, lysates were sonicated on ice and centrifuged at 16,000g for 10 min at
4ºC. Protein concentrations were determined by BCA assay (Pierce) and equal quantities
of differentially labelled control or hormone-treated samples were pooled. In all four
generation of DCT cells (S1, S2, S3 and S4), heavy labelled cells were treated with
aldosterone while light labelled cells were as control.

**LC-MS/MS sample preparation and running conditions.** 1 mg lysates from each
biological replicate (light and heavy combined) were reduced, alkylated, pre-digested by
lys-c, followed by trypsin digestion overnight. Peptides were then desalted and fractionated
offline into 7 fractions using Oasis PRiME HLB columns (Waters) under high pH. Elution
solution used for the 7 fractions are 3% ACN, 5% ACN, 7.5% ACN, 10% ACN, 12.5%
ACN, 15% ACN and 50% ACN in 0.1% trimethylamine. The phosphopeptides from each
fraction were enriched using home-made TiO₂ columns (GL Science). Samples were
analyzed by Thermo EASY nLC 1000 coupled with QExactive, through an EASY-Spray
nano-electrospray ion source. Peptides were trapped by a pre-column (Acclaim PepMap
100, 75um*2cm, nanoviper fitting, C18, 3um, 100Å, Thermo Scientific) and analyzed by an
analytical column (EASY-Spray column PepMap, 75um*25cm, nanoviper fitting, C18, 3um,
100 Å, Thermo Scientific). Buffer A was 0.1% FA and Buffer B was 100% ACN/0.1% FA. A
60 min gradient of 0-35% B was used for peptide separation. The MS was set up as full
scan (m/z 300-1700) at the resolution of 70,000; 10 data dependent MS/MS scans at the
resolution of 17,500; HCD collision energy, 29%; dynamic exclusion time 30s; charge state
exclusion, less than +1 and above +8. Phospho-enriched samples were run in
duplicates/triplicates and flowthrough samples (total protein) were run only once per
sample.

**LC-MS/MS data analysis.** Proteome Discoverer (version 1.4, Thermo Scientific) database
searching used both SEQUEST and MASCOT against a mouse protein database
(mouse.protein.v20180126.fasta, Uniprot). The parameters for Proteome Discoverer were:
precursor mass tolerance, 10 ppm; fragment mass tolerance, 0.02 Da; maximum miss
cleavage, 2; static modification, cysteine carbamidomethylation; variable modification: N-
terminal acetylation, methionine oxidation, 10+ for heavy arginine, 6+ for heavy lysine,
phosphorylation of STY. Percolator was used to calculate false discovery rate (FDR);
phosphoRS 3.0 algorithm was used to evaluate phosphorylation site probability score.
Only rank 1 and high confidence (with a target false discovery rate (FDR) q-value below
0.01) peptides were included in the final results. The quantification of each unique peptide
was obtained from the sum of raw values from different peptide charge states, different
fractions and different technical replicates. The heavy-to-light ratio of each unique peptide
was calculated from the summed raw intensities of heavy or light peptide from different
charge states and different fractions. Quantification ratios were normalized based on
median log₂ ratios of each biological replicate. Normalization was done using all peptides,
including phosphopeptides and non-phosphopeptides. Phosphopeptides identified and
quantified in at least three replicates were subjected to Benjamini-Hochberg (BH) FDR
analysis, and those that passed the 10% BH-FDR threshold plus a few phosphopeptides
with LOG2 ratios all higher than 0.75 or all lower than -0.75 were retained for further
analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010032 (Username: reviewer12098@ebi.ac.uk Password: HS8uC5pG). Total proteome and identified phosphosites can be viewed from http://interpretdb.au.dk/database/mpkDCT_acute_Aldo/mpkDCT_proteome.html or http://interpretdb.au.dk/database/mpkDCT_acute_Aldo/Phosphosite.html.

**Bioinformatics.** Regulated phosphorylation motifs were predicted using standard settings in MotifX (http://motif-x.med.harvard.edu/motif-x.html). Go-term analysis was carried out using app ClueGo v2.5.1 in Cytoscape. Proteins (uniprot accessions used) with significant changes in phosphorylation levels were subjected for phosphorylation core analysis using Ingenuity Pathway Analysis (IPA, Qiagen) to generate functional pathways. Both direct and indirect relationships were considered in the analysis. Molecules per network was set to 140, and networks per analysis was set to 10. Confidence was set to experimentally observed.

**Statistics.** Immunoblotting and second messenger data are expressed as mean ± S.E.M. For two groups, data meeting the statistical assumptions of normality were assessed using an unpaired Student’s t-test. Comparisons of more than two groups were performed using a one-way ANOVA followed by Tukey’s multiple comparison tests. Significance was considered at $P < 0.05$. 
**Associated References**


Additional information Table S1 and Table S2

**Table S1** uploaded excel file

Table S1_mpkDCT-proteome.xlsx

(http://interpretdb.au.dk/database/mpkDCT_acute_Aldo/mpkDCT_proteome.html)

**Table S2** uploaded excel file

Table S2_mpkDCT-phosphoproteome.xlsx

(http://interpretdb.au.dk/database/mpkDCT_acute_Aldo/Phosphosite.html)
Rapid aldosterone-mediated signaling in the DCT increases activity of the thiazide-sensitive NaCl cotransporter

METHODS

Ex vivo

In vivo

Effect of short-term (15-60 min) aldosterone stimulation on phosphorylation of the NaCl cotransporter (NCC): phosphorylation was determined in cultured DCT cells, in ex vivo tubule suspensions and in mice.

In vitro phosphoproteomics

Novel signaling pathways activated by aldosterone were uncovered using silac-based phosphoproteomics of cultured DCT cells.

CONCLUSION

Aldosterone acutely activates NCC to modulate renal NaCl excretion

• Aldosterone rapidly increased intracellular cAMP and IP3 accumulation, and altered phosphorylation of various kinases/kinase substrates.
• Inhibition of GPR30, limited the actions of aldosterone on NCC activity.
• Phosphoproteomics, network analysis and in vitro studies determined that EGFR-dependent signaling was activated by aldosterone.
• NCC activity was decreased by EGFR tyrosine kinase inhibition ex vivo and in vivo.