Mineralocorticoid Effects in the Kidney: Correlation between αENaC, GILZ, and Sgk-1 mRNA Expression and Urinary Excretion of Na⁺ and K⁺

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Abstract. Aldosterone exerts its effects through interactions with two types of binding sites, the mineralocorticoid (MR) and the glucocorticoid (GR) receptors. Although both receptors are known to be involved in the anti-natriuretic response to aldosterone, the mechanisms of signal transduction leading to modulation of electrolyte transport are not yet fully understood. This study measured the Na⁺ and K⁺ urinary excretion and the mRNA levels of three known aldosterone-induced transcripts, the serum and glucocorticoid-induced kinase (Sgk-1), the α subunit of the epithelial Na⁺ channel (αENaC), and the glucocorticoid-induced-leucine-zipper protein (GILZ) in the whole kidney and in isolated cortical collecting tubules of adrenalectomized rats treated with low doses of aldosterone and/or dexamethasone. The resulting plasma concentrations of both steroids were close to 1 nmol/L. Aldosterone, given with or without dexamethasone, induced anti-natriuresis and kaliuresis, whereas dexamethasone alone did not. GILZ and αENaC transcripts were higher after treatment with either or both hormones, whereas the mRNA abundance of Sgk-1 was increased in the cortical collecting tubule by aldosterone but not by dexamethasone. We conclude the increased expression of Sgk-1 in the cortical collecting tubules is a primary event in the early antinatriuretic and kaliuretic responses to physiologic concentrations of aldosterone. Induction of αENaC and/or GILZ mRNAs may play a permissive role in the enhancement of the early and/or late responses; these effects may be necessary for a full response but do not by themselves promote early changes in urinary Na⁺ and K⁺ excretion.

Transepithelial Na⁺ and K⁺ transport is regulated by mineralocorticoid hormones in the distal nephron and other “tight” epithelia. The resulting changes in urinary Na⁺ and K⁺ excretion are responsible for the regulation of the sodium and potassium balance of the organism. Although the effects of mineralocorticoid hormones have been well described for about 50 yr, their underlying molecular mechanisms are only beginning to be understood in detail (1,2).

In its target tissues, aldosterone is known to bind to two types of receptor (3), a high-affinity type I receptor with a Kd = 0.5 to 3 nmol/L and a lower-affinity (Kd = 14 to 60 nmol/L) type II receptor, which have been identified with the proteins known as the cloned mineralocorticoid (MR) (4) and glucocorticoid (GR) (5) receptors, respectively (6–9).

Mineralocorticoid receptors, like GR and other steroid hormones receptors, are transcriptional regulators that, upon ligand binding, induce or repress the transcription of specific genes, which are transcribed into the so called aldosterone-induced (AIT) and aldosterone-repressed transcripts (ART), respectively. A number of AIT have been identified. These include subunits of the epithelial Na⁺ channel (ENaC) and of the Na,K-ATPase, which have obvious roles in Na⁺ transport as well as the small G-protein K-Ras2 (10), whose role in sodium transport is not yet fully understood (1,2). The serum and glucocorticoid-regulated kinase (Sgk-1) has been shown to be a major effector of the aldosterone action (11,12) by controlling the surface density of ENaC through phosphorylation of the ubiquitin-ligase Nedd4–2 (13). Recently, serial analysis of gene expression (SAGE) in an aldosterone-responsive CCD cell line has allowed the identification of many potential AIT and ART and particularly the mRNA of the protein GILZ (14). This mRNA was shown to be increased by more than tenfold within 4 h of aldosterone treatment (15), although the effect of this protein on Na⁺ or K⁺ transport is not yet understood.

In most of the studies in which these AIT have been identified, rather large concentrations of aldosterone have been used, concentrations that may have resulted in the occupancy of both type I and type II receptors. Thus it is not clear if the induction of the various known AIT are related to the occupancy of type I, type II, or both mineralocorticoid receptors. The high affinity of type I MR and the rather low concentrations of mineralocorticoid hormones measured under physio-
logic conditions suggest that day-to-day Na\(^+\) balance regulation might be affected by variation of the aldosterone level in the sub-nanomolar range.

Previously, we have shown that concentrations of a few nanomolar of aldosterone were able to induce large effects on the urinary Na\(^+\) and K\(^+\) excretion in adrenalectomized rats, even in the presence of a background level of glucocorticoid (16). The effect of such aldosterone levels on the expression of AIT in vivo have not yet been reported, and the relationship between the molecular (AIT expression) and the physiologic effects of aldosterone is still unclear. In this study, we have used the same experimental model to assess the effect of nanomolar aldosterone on the mRNA level of three known AIT, namely αENaC, Sgk-1, and GILZ, at the level of the whole kidney and in isolated cortical collecting ducts to correlate the modulation of these transcripts with physiologic effects.

Materials and Methods

Urinary Na\(^+\) and K\(^+\) Excretion Studies

Urinary electrolyte excretion was measured in a similar manner to the protocol described in Horisberger and Diezi (16). Briefly, male Wistar rats were adrenalectomized under pentobarbital (50 mg/kg) anesthesia. Upon recovery, they were allowed free access to a drinking solution of NaCl 35 mM + sucrose 50 g/L but were deprived of solid food. Twenty-four hours after adrenalectomy, the animals were anesthetized with pentobarbital. Four groups of animals were studied: one group received no steroid hormone (CONTROL), a second group received only aldosterone (ALDO), a third group was treated with dexamethasone (DEX), and a fourth group received both (ALDO+DEX). The animals in the DEX and ALDO+DEX groups received 5 μg/kg of dexamethasone intraperitoneally at the time of anesthesia. Surgical preparation included tracheotomy, cannulation of a jugular vein for intravenous infusion, cannulation of a femoral artery for BP monitoring through a mercury manometer and blood sampling, and suprapubic cannulation of the urinary bladder for urine collection. After cannulation of the jugular vein, an intravenous infusion was started at the rate of 0.72 ml · kg\(^{-1}\) · min\(^{-1}\) for 30 min and then maintained at the rate of 0.36 ml · kg\(^{-1}\) · min\(^{-1}\) for the rest of the experiment. For the DEX and ALDO+DEX groups, the infused solution contained 49 nmol/L (23 g/L), resulting in a steady-state delivery of 1.04 μg · kg\(^{-1}\) · h\(^{-1}\) (2.12 nmol · kg\(^{-1}\) · h\(^{-1}\)) for the first 30 min and 0.57 μg · kg\(^{-1}\) · h\(^{-1}\) (1.06 nmol · kg\(^{-1}\) · h\(^{-1}\)) throughout the rest of the experiment.

A scheme of the experimental protocol is presented in Figure 1. Two hours after the infusion was started (t = 120 min), urine was collected for six 30-min periods. At time t = 150 min (end of the control period), aldosterone was added to the perfusion solution at a concentration of 129 nmol/L (46 μg/L), resulting in a constant delivery of 1 μg · kg\(^{-1}\) · h\(^{-1}\) (or 2.78 nmol · kg\(^{-1}\) · h\(^{-1}\)). Blood samples were collected as shown in Figure 1A. Plasma and urinary Na\(^+\) and K\(^+\) concentrations were measured using a flame spectrometer (Instrument Laboratory IL943).

Aldosterone and Dexamethasone Plasma Concentrations

The plasma concentrations of aldosterone and dexamethasone resulting from the administration scheme described above were evaluated in another set of experiments. Six rats were prepared exactly according to the same protocol and administered the same amounts of aldosterone and dexamethasone as described for the ALDO group or DEX group, except that radiolabeled steroids were used. The total concentration and specific activity in the infusion solution was 129 nmol/L and 4.94 · 10\(^6\) DPM/nmol, respectively, for aldosterone. For dexamethasone, the total concentration and specific activity in the infusion solution was 49 nmol/L and 115.8 · 10\(^6\) DPM/nmol, respectively. After 150 min, 210 min, and 270 min, blood was collected for analysis. Two hundred microliters of serum or 200 μl of the infused solution were added to 5 ml of scintillation liquid and counted in a Packard β counter. Mean aldosterone counts were at 210 min 1604 ± 186 DPM and at 270 min 1569 ± 167 DPM. Mean dexamethasone count was 14187 ± 267 DPM at 150 min, 14944 ± 54 DPM at 210 min, and 15690 ± 194 DPM at 270 min after a mean background value of 28 (± 3) DPM had been subtracted. The plasma steroid concentrations were calculated from known concentrations in the infused solutions and the ratio of the CPM counted in the blood to the CPM counted in the infused solution.

Whole Organ Northern Blot

At the end of the experiment (t = 300 min), animals were killed and the kidneys were removed. Total RNA from frozen kidneys was purified according to Chomczynski and Sacchi (17). Twenty micrograms of RNA was separated on a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane (Ambion), UV cross-linked, and hybridized with the PCR-amplified randomly radiolabeled cDNA probe. cDNA probes for αENaC, GILZ, and Sgk-1 were amplified with the same primers as for the RT-PCR (Table 1), and GAPDH probe was amplified with the following primers 5' cggccatcacgccacagttt. GAPDH was used as a loading control. The blot was then exposed to MolecularImager screen (BioRad). Signals were quantified using an PhosphoImager (BioRad).

Quantitative PCR on Dissected Tubules

For isolation of cortical collecting ducts (CCD), the left kidney was perfused with Dulbecco modified Eagle medium:Ham’s F12 (1:1) (Life Technologies) and 0.07% collagenase (Roche). Microdissection and RNA extraction were performed exactly as described by Elalouf et al. (18). CCD samples were identified by the presence of branching connections, which define the end of the connecting tubule and the
Unless stated otherwise, the difference between mean values were... 

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Both steroids (ALDO/DEX). 

To establish a correlation between the physiologic effects of aldosterone and the modulation of the transcript level, we performed Northern blot analysis on the whole kidney and hybridized the blot with two well-known aldosterone-induced transcripts, namely the α subunit of ENaC and Sgk-1, and with the more recently described GILZ (15). The effects of aldosterone and dexamethasone on the mRNA abundance of these three proteins in the whole kidney are depicted in Figure 4. GILZ mRNA levels were threefold to fourfold higher in the ALDO (P < 0.05) and DEX (P < 0.001) groups than in CONTROL, and both hormones together induced a sevenfold increase (P < 0.001). The ENαC α subunit was induced approximately fourfold over the control by aldosterone (P < 0.05), approximately twofold with dexamethasone (P < 0.05), and approximately threefold with both hormones together (P < 0.01). Sgk-1 was induced eightfold over the control by aldosterone (P < 0.005), fivefold with dexamethasone (P < 0.005), and sevenfold with both hormones together (P < 0.001). The small apparent decrease in GAPDH mRNA content observed in the Aldo group was probably due to a lower loading of the gel rather than to a real change of the GAPDH mRNA abundance by aldosterone treatment as GAPDH is known to be expressed at a constant level (20). This experiment shows that αENaC and Sgk-1 mRNA abundance is increased in the whole kidney by both mineralocorticoids and glucocorticoids and that dexamethasone does not induce an additional effect over that reached with 1 nmol/L aldosterone.

Effect of Aldosterone and Dexamethasone on Transcript Abundance in the Whole Kidney

To establish a correlation between the physiologic effects of aldosterone and the modulation of the transcript level, we performed Northern blot analysis on the whole kidney and hybridized the blot with two well-known aldosterone-induced transcripts, namely the α subunit of ENaC and Sgk-1, and with the more recently described GILZ (15). The effects of aldosterone and dexamethasone on the mRNA abundance of these three proteins in the whole kidney are depicted in Figure 4. GILZ mRNA levels were threefold to fourfold higher in the ALDO (P < 0.05) and DEX (P < 0.001) groups than in CONTROL, and both hormones together induced a sevenfold increase (P < 0.001). The ENαC α subunit was induced approximately fourfold over the control by aldosterone (P < 0.05), approximately twofold with dexamethasone (P < 0.05), and approximately threefold with both hormones together (P < 0.01). Sgk-1 was induced eightfold over the control by aldosterone (P < 0.005), fivefold with dexamethasone (P < 0.005), and sevenfold with both hormones together (P < 0.001). The small apparent decrease in GAPDH mRNA content observed in the Aldo group was probably due to a lower loading of the gel rather than to a real change of the GAPDH mRNA abundance by aldosterone treatment as GAPDH is known to be expressed at a constant level (20). This experiment shows that αENaC and Sgk-1 mRNA abundance is increased in the whole kidney by both mineralocorticoids and glucocorticoids and that dexamethasone does not induce an additional effect over that reached with 1 nmol/L aldosterone.

Effect of Aldosterone and Dexamethasone on Transcript Abundance in Isolated CCD

It is well known that mineralocorticoid action is restricted to the distal part of the nephron. Therefore, we also performed microdissection experiments to examine the effect of steroids on the level of several AIT in the mineralocorticoid target cells using quantitative RT-PCR with Dodecenoyl-CoA δ Isomerase (DCI) as an endogenous internal control for mRNA extraction and PCR amplification. DCI was used as an internal standard because this gene was found to be expressed at about the same transcript copy level as the three genes of interest in CCD cells (15). We also verified by Northern blot that the GAPDH/DCI ratio was not altered by either steroid treatment (CONTROL: 3.8 ± 0.7, n = 10; Aldo: 2.7 ± 0.3, n = 11; Dex: 3.5 ± 0.5, n = 9; Aldo+Dex: 3.1 ± 0.4, n = 11). This allowed us to co-amplify each gene of interest with DCI as an internal standard while checking for saturation of the amplification.

The validity of the PCR amplified DNA signal requires similar RT-PCR amplification efficiency for the four genes. Therefore, in preliminary experiments, we checked the linearity and the similarity of the amplification by measuring the amount of DNA formed as a function of the number of PCR cycles. Figure 5 shows constant and similar amplification rates for the four targets up to 32 cycles. We therefore chose a...
32-cycle protocol for all the quantitative PCR experiments and compared the mRNA amounts of the three genes of interest with DCI. The GILZ/DCI ratio was the lowest among the three tested transcripts, probably because GILZ is a low-abundance transcript that is detectable by Northern hybridization only in thymus and spleen (14).

As shown in Figure 6, GILZ mRNA was induced threefold by aldosterone (P < 0.01), more than twofold by dexamethasone (P < 0.05), and fivefold by aldosterone and dexamethasone together (P < 0.001). The α subunit of ENaC mRNA was induced threefold by aldosterone (P < 0.01), more than twofold by dexamethasone (P < 0.05), and 3.5-fold by aldosterone and dexamethasone together (P < 0.001). Finally, Sgk-1 mRNA was increased by about threefold by aldosterone (P < 0.001), but dexamethasone alone had no effect. The effect of aldosterone was similar with or without dexamethasone.

**Discussion**

The existence of two types of receptors for mineralocorticoid hormones has been known and well demonstrated for a long time, and there is good evidence supporting a role for both of them in the natriuretic response to aldosterone (3,21,22). The question of the specific signal transduction pathway downstream of the binding of the steroid to each of these receptors has been much more difficult to solve. One problem has been the difficulty to obtain precise values of the binding affinities of mineralocorticoid and glucocorticoid steroids to both types of receptor. The values of the affinity of aldosterone reported in the literature vary considerably, from 0.3 to 4 nM for the MR and from 14 to 60 nM for the GR. Dexamethasone, a pharmacologic glucocorticoid, has been shown to have a high affinity for the GR, around 1 nM, but its affinity for the MR has been more difficult to define due to the much lower number of MR sites in natural tissues. Recent studies with artificial expression of MR and GR (8,23) have allowed more precise determination of binding parameters; however, some issues remain open. While the equilibrium binding measurements demonstrate a roughly similar K_d for MR of aldosterone and dexamethasone (0.5 and 0.7 nM, respectively), other parameters such as protection from proteolysis or transactivation activities demonstrated an apparent affinity two or three orders of magnitude lower for dexamethasone (8). It is probable that the effective binding of dexamethasone to MR leading to activation of the receptor is strongly limited by a fast dissociation rate (8).
Our present approach to answering these questions was to correlate plasma level of steroids with physiologic effects and modulation of mRNA abundance. A plasma concentration of 1 nmol/L of aldosterone was sufficient to produce large effects on the urinary electrolyte excretion.

Due to the limitations of the present study that uses a single concentration in vivo, it is not possible to reach firm conclusions about the occupancy of the two types of receptor. However, receptor occupancy under our experimental circumstances can be roughly estimated from published affinity values with the assumption that the intracellular and the extracellular concentrations of aldosterone were similar. These estimations are consistent with the known high affinity of aldosterone for MR (3,6–8). The 1 nmol/L concentration of aldosterone should occupy between 77% and 25% of the MR (calculated from a 0.3 to 3 nmol/L Kd range, respectively). Assuming a linear relationship between occupancy and effect, the contribution of GR in the aldosterone response is probably small because the used concentration of aldosterone should occupy only 1.5% to 6% of GR (that can be estimated from the 15 to 60 nmol/L range of Kd values [3,4]). Furthermore, this contribution seems even less likely considering the fact that the presence of dexamethasone, which clearly has a higher affinity than aldosterone for GR (8), makes essentially no difference to the aldosterone effect on the urinary Na/K excretion, while dexamethasone has by itself significant effects on mRNA

Figure 5. Comparative RT-PCR co-amplification efficiency of GILZ, αENaC, and Sgk-1 mRNA with DCI. PCR assays were performed in the presence of [α32P]dCTP, aliquots were taken every three cycles from 26 to 35 cycles. (A) Aliquots were loaded in a 3% agarose gel, fixed in 10% acetic acid, dried, and exposed on a MolecularImager screen. ENaC α subunit is 528-bp-long, Sgk-1 is 606-bp-long, GILZ is 261-bp-long, and DCI is 695-bp-long. (B, C, and D) Amounts of incorporated α32P expressed as a function of the number of PCR cycles. 32 cycles has been used for the rest of the experiment.
abundance of several proteins (see below). These short-term observations do not exclude the possibility that occupancy of the GR at higher aldosterone concentrations could enhance the mineralocorticoid response or may be necessary for maintenance of a long-term response.

Exposure to a 1 nM concentration of dexamethasone resulted in the CCD in a rise of the GILZ and αENaC mRNA levels, but no antinatriuretic effect was observed, suggesting that these responses may be permissive, i.e., may be needed for an enhanced early and/or prolonged response, but are not sufficient by themselves to generate the early mineralocorticoid response. Dexamethasone has been reported to be metabolized by the 11β-OH-steroid dehydrogenase type II, but rather inefficiently (24). The observed effects on transcripts abundance in the isolated CCD show that our administration scheme, and the resulting plasma concentrations, resulted in intracellular dexamethasone concentrations that were sufficient to occupy GR, at least partially. The small, but significant natriuretic effect of dexamethasone that was observed could be due to a glucocorticoid action in any segment of the nephron or be secondary to a cardiovascular effect of glucocorticoids, but we cannot determine from the present data the mechanism of this effect. It is probably not due to mineralocorticoid antagonism at the level of the CCD, because the same higher level of Na⁺ excretion was observed in the DEX versus CONTROL group and in the ALDO+DEX versus ALDO group. In addition, the antinatriuretic and kaliuretic effects of aldosterone were not decreased by the presence of dexamethasone.

It is interesting to compare the pattern of stimulation in the whole kidney and in isolated CCD. This pattern is different for Sgk-1 and for αENaC or GILZ. In the CCD, and within the physiologic range of doses explored in the present study, Sgk-1...
stimulation is clearly aldosterone-specific. In contrast, in the whole kidney, dexamethasone is also able to induce Sgk-1. Because MR is present only in the distal nephron, these observations indicate that the stimulation observed in the whole kidney is mostly due to GR occupancy. It thus seems most probable that the increased expression of Sgk-1 observed at the level of the whole kidney after either glucocorticoid treatment or high doses of aldosterone is not related to the mineralocorticoid effects.

The absence of effect of dexamethasone on the level of Sgk-1 mRNA in the isolated CCT is surprising considering that this steroid can clearly increase this mRNA species in other part of the kidney, as seen on the whole kidney analysis. As other mRNA species are modified in the CCT by dexamethasone, indicating, as discussed above, a significant level of receptor occupancy, it seems improbable that this lack of effect is due to a low intracellular level of this steroid resulting from the effect 11β-OH-steroid dehydrogenase type II. As the level of RNA was examined only 5 h after the initial administration of dexamethasone, it can be argued that a transient increase in Sgk-1 mRNA may have been missed. Indeed the short half-life of aldosterone and of Sgk-1 mRNA results in a transient expression of Sgk-1 upon single-dose administration of aldosterone (25–27). However, when aldosterone was administered continuously, a high level of Sgk-1 mRNA was observed for 6 and 21 d (28). Rabbit CCD cells continuously exposed to 10 nM aldosterone showed a sustained response of Sgk-1 mRNA abundance with a level at 24 h still about 50% of the maximal response recorded at 2 h (12), and a significant increase of Sgk-1 mRNA was seen in A6 cells 6 h after treatment with 100 μM dexamethasone (29). In addition, even a single dose of dexamethasone (0.5 mg/kg) resulted in a sustained increase in Sgk-1 mRNA in rat kidney (25). Thus, it seems very unlikely that the continuous intravenous administration of steroids used in our experiments would result in only a transient increase of Sgk-1 mRNA that then returns to baseline value within 5 h. One has to conclude that the Sgk-1 mRNA level is not under the control of GR alone in the CCT cells as it seems to be in other kidney cells. The requirement for homodimerization or heterodimerization of the activated steroid receptors and for cell-specific cofactors (30) may allow for a cell type–specific modulation of their transcriptional effects. It will be difficult to investigate these questions until a CCT cell line with biochemical and physiologic responses to corticosteroid similar to those observed in vivo (i.e., a strong electrolyte transport response to nM concentrations of aldosterone and no response to glucocorticoids) is available.

Our experiments only detected an increase of the mRNA level in the isolated CCT. They do not allow to determine the mechanism, transcriptional or post-transcriptional, of these effects, nor to conclude to a change of the protein expression level, which would need methods allowing to determine quantitatively the level of the Sgk-1 protein at the tubule segment level.

With ENaC, a similar pattern of stimulation was observed in the whole kidney and in the isolated CCD. However, as ENaC is expressed mostly in the distal segments of the nephron (31), the results on the whole kidney probably reflect essentially what happens in these segments.

The observation that the level of the mRNA coding for one ENaC subunit can be increased by glucocorticoid in the kidney is consistent with the recent demonstration that glucocorticoid treatment was able to increase the mRNA abundance for the three subunits of ENaC and to stimulate Na+ transport in isolated CCD and colon epithelium from MR knockout mice (32) and with absence of changes in the ENaC subunit transcripts in MR knockout mice (9). In the near future, the relative role of mineralocorticoid and glucocorticoid in triggering the Na/K response via MR and/or GR should be studied in nephron segment-specific (i.e., CCD) knockout animals using the Cre-lox technology (33), allowing a much more precise evaluation of their respective physiologic roles in sodium balance and homeostasis.

Variations of the aldosterone plasma level in the 0 to 1 nmol/L range have major effects on the urinary Na+ and K+ excretion, while similar concentrations of glucocorticoid do not. These aldosterone concentrations occur through the circadian cycle (34) and are due to “normal” day-to-day variations of the ECF volume. We show that, in this physiologic range of concentrations, aldosterone modulates mRNA level for several proteins, including Sgk-1, αENaC, and GILZ. While the increased level of Sgk-1 mRNA in the CCT is correlated with the early antinatriuretic and kaliuretic effects of aldosterone, the higher level of αENaC and GILZ mRNAs is not sufficient by itself to modify urinary electrolyte excretion. These proteins may rather be involved in the late phase response to aldosterone. Higher levels of aldosterone may further induce other proteins such as the αENaC subunit and GILZ (as demonstrated in this work) or the Na,K-ATPase α and β subunits (35) and energy metabolism enzymes (22,36) through activation of type II receptors (GR).

Acknowledgments

This work was supported by the Human Frontier Science Foundation (HFSP) grant RG 0261 to JDH and by the Swiss “Fonds National de la Recherche Scientifique,” Grant # 3100–065140.1 to DF. OM was supported in part by a fellowship from the Botnar Foundation. We are grateful to K. Geering, L. Schild, and M. A. Thomas for careful reading of the manuscript and providing helpful suggestions.

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