Renal Na/H Exchanger NHE-3 and Na-P04 Cotransporter NaP1-2 Protein Expression in Glucocorticoid Excess and Deficient States

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Abstract. Administration of pharmacologic doses of glucocorticoid in vivo increases renal proximal tubule apical membrane Na/H exchange and decreases Na/P04 cotransport activity (I). Current data suggest that the NHE-3 and NaP1-2 proteins mediate significant fractions of proximal tubule apical membrane Na/H exchange and Na/P04 cotransport, respectively. This study examines whether glucocorticoid excess or deficiency affects NHE-3 and NaP1-2 protein abundance and the intrarenal distribution of these transporters. Protein abundance of NHE-3 and NaP1-2 in control rats was compared to rats rendered glucocorticoid-deficient by bilateral adrenalectomy, and to rats receiving pharmacologic doses of dexamethasone using immunoblots and immunohistochemistry. Adrenalectomy had modest effects on NHE-3 protein abundance, but dexamethasone administration to either adrenalectomized or sham-operated rats significantly increased NHE-3 protein abundance in both the proximal tubule and thick ascending limb, but not the thin descending limb. Adrenalectomy increased NaP1-2 protein abundance in the proximal tubule, whereas dexamethasone administration dramatically suppressed NaP1-2 protein on the apical membrane in both adrenalectomized and sham-operated animals. No significant reciprocal increase in subapical NaP1-2 staining was seen in the dexamethasone-treated rats. The present study shows that glucocorticoids regulate proximal tubule apical membrane Na/H exchange and NaP1 cotransport by changes in protein abundance of NHE-3 and NaP1-2, respectively.

Glucocorticoid excess increases and glucocorticoid deficiency decreases net acid excretion by the kidney (2,3). The increased net acid excretion involves increased tubular H secretion and HCO3 absorption and enhanced urinary buffer excretion due to increased ammoniagenesis and excretion, and increased phosphate excretion (3–6). Glucocorticoids exert their effects on proximal tubule HCO3 and PO4 absorption by stimulating apical membrane Na/H exchange and inhibiting Na-P04 cotransport, respectively (1,7,8).

Immunohistochemical data suggest that proximal tubule apical membrane Na/H exchange and Na-P04 cotransport are mediated at least in part by NHE-3 and NaP1-2 proteins, respectively (9–12). Glucocorticoid excess increases renal cortical NHE-3 mRNA in the rabbit (13), but its effect on NHE-3 protein in various nephron segments in the intact animal has not been examined. It is also unclear whether glucocorticoid deficiency affects NHE-3 protein expression. Glucocorticoid excess decreases NaP1-2 mRNA and protein in whole renal cortex (14), but the intranephron and cellular distribution of NaP1-2 has not been examined in either glucocorticoid excess or deficient states. This is particularly relevant because plasma membrane insertion has been shown to be a mechanism of regulation of apical membrane NaP1-2 abundance (15,16). In the present study, we examined the abundance of these two transporter proteins with variations in the glucocorticoid status in rats by immunohistochemistry and immunoblot. We showed that glucocorticoids increase NHE-3 and decrease NaP1-2 protein expression in the proximal tubule, and increase NHE-3 protein expression in the thick ascending limb.

Materials and Methods

Animal Models

Male Sprague Dawley rats weighing 200 to 300 g were subjected to either bilateral adrenalectomy (ADX group) or sham operations (SHM group), and were allowed free access to water and rat chow. ADX animals were supplemented with 0.9% saline in their drinking water for at least 3 d before administration of either vehicle or glucocorticoid. Both ADX and SHM animals were given either 60 μg/100 g body wt subcutaneous injections of dexamethasone (DEX) twice daily or vehicle (phosphate-buffered saline [PBS]) for 2 d (four doses), and a fifth dose was given 2 h before sacrifice. Initially, four experimental groups (ADX, SHM, ADX + DEX, SHM + DEX) with four animals in each group were used for NHE-3 and NaP1-2 staining.
by immunohistochemistry and immunoblots of cortical and apical membranes. Subsequently, an additional set of experiments was performed using the same four conditions (ADX, n = 5; SHM, n = 4; ADX + DEX, n = 6; SHM + DEX, n = 4) to examine NHE-3 expression in both cortical and medullary membranes. The animals were sacrificed and kidneys were harvested for either renal membrane preparation or for immunohistochemistry after perfusion fixation. Renal NHE-3 and NaP2-2 protein expression was quantified by immunoblots in all four experimental groups and by immunohistochemistry in SHM, ADX, and ADX + DEX animals.

**Immunoblots**

Renal cortex or the inner stripe of the outer medulla was dissected and homogenized (buffer containing in mM: 300 mannitol, 20 Hepes, pH 7.50, and 5 ethyleneglycol-bis(β-aminomethyl ether)N,N’-tetra-acetic acid; in μg/ml: 100 phenylmethylsulfonyl fluoride, 2 leupeptin, 2 aprotinin, and 2 pepstatin A; Brinkman polytron), and the membrane fraction was obtained by centrifugation (Beckman J2–21 M, JA-20 rotor, 20,000 rpm, 40 min, 4°C). Apical membrane vesicles were prepared from the cortical homogenate by exposing cortical membranes to three consecutive precipitations in 15 mM MgCl2, and the final apical membrane-enriched vesicles were pelleted from the supernatant (Beckman J2–21 M, JA-20 rotor, 20,000 rpm, 40 min, 4°C). Twenty micrograms of either cortical membranes, medullary membranes, or cortical apical membranes was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. For immunoblotting, anti-NHE-3 antiserum (no. 1568 against epitope DSFLQADGPPEEQQL at 1:200 dilution) (9) and anti-NaP2-2 antiserum (against epitope LALPAHHTATRL at 1:1000 dilution) (11,12) were used as primary antisera. The specificity of both antisera have been characterized previously (9,12,15). Briefly, kidneys were perfused with 3% paraformaldehyde/0.05% picric acid in a 6:4 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol/kg water with sucrose) and 10% hydroxyethyl starch. After 5 min of fixation, the fixative was washed out by perfusion with 0.1 M cacodylate/sucrose buffer. Coronal slices of the fixed kidneys were mounted on cork disks, frozen in liquid propane cooled by liquid N2, and stored at -80°C until use. Serial sections (3 to 4 μm thick) were cut in a cryostat and placed on chrome alum gelatin-coated slides. Immunohistochemistry for NHE-3 and NaP2-2 was performed as described previously (9,12,15). For NHE-3, sections were pretreated with 10% normal goat serum in PBS, and for NaP2-2, with 3% milk powder and 0.3% Triton X-100 in PBS. Sections were then incubated with anti-NHE-3 antisemur (no. 1566 YSRHELTPNEDEKQ, 1/800 in PBS/BSA) or with anti-NaP2-2 antisemur (against epitope LALPAHHTATRL, 1:500 in PBS/milk powder) overnight in a humidified chamber at 4°C. After repeated rinsing in PBS, binding of the primary antibodies was detected with FITC-conjugated swine-anti-rabbit IgG (1:40 PBS/BSA; Dakopatts, Glostrup, Denmark). Rhodamine-conjugated phalloidin (1/100 dilution; Molecular Probes, Eugene, OR) was added to the secondary antibody for staining of actin filaments. Finally, the sections were rinsed with PBS, coverslips were applied with DAKO-Glycergel (Dakopatts) containing 2.5% 1,4-diazabicyclo-(2.2.2)-octane (DABCO; Sigma, St. Louis, MO) as a fading retardant, and the sections were studied by epifluorescence microscopy (Polyvar, Reichert-Jung, Vienna, Austria).

**Immunohistochemistry**

Localization and abundance of NHE-3 and NaP2-2 protein in the kidney were studied by immunohistochemistry in three experimental groups (SHM, ADX, and ADX + DEX). The kidneys were fixed by vascular perfusion through the abdominal aorta, as described previously (9,12,15). Briefly, kidneys were perfused with 3% paraformaldehyde/0.05% picric acid in a 6:4 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol/kg water with sucrose) and 10% hydroxyethyl starch. After 5 min of fixation, the fixative was washed out by perfusion with 0.1 M cacodylate/sucrose buffer. Coronal slices of the fixed kidneys were mounted on cork disks, frozen in liquid propane cooled by liquid N2, and stored at -80°C until use. Serial sections (3 to 4 μm thick) were cut in a cryostat and placed on chrome alum gelatin-coated slides. Immunohistochemistry for NHE-3 and NaP2-2 was performed as described previously (9,12,15). For NHE-3, sections were pretreated with 10% normal goat serum in PBS, and for NaP2-2, with 3% milk powder and 0.3% Triton X-100 in PBS. Sections were then incubated with anti-NHE-3 antisemur (no. 1566 YSRHELTPNEDEKQ, 1/8000 in PBS/BSA) or with anti-NaP2-2 antisemur (against epitope LALPAHHTATRL, 1:8000 in PBS/BSA) containing 2.5% 1,4-diazabicyclo-(2.2.2)-octane (DABCO; Sigma, St. Louis, MO) as a fading retardant, and the sections were studied by epifluorescence microscopy (Polyvar, Reichert-Jung, Vienna, Austria).

**Statistical Analysis**

Quantitative differences were assessed by ANOVA.

**Results**

**Effect of Glucocorticoids on NHE-3**

Figure 1, A and B, shows representative immunoblots examining NHE-3 protein abundance in cortical apical mem-

![Immunoblot of renal NHE-3 and β-actin protein abundance.](image-url)

Figure 1. Immunoblot of renal NHE-3 and β-actin protein abundance. Mobility in kilodaltons is indicated on the right. (A) Cortical apical membranes: ADX slightly decreased, whereas DEX significantly increased NHE-3 expression. Number of experiments: ADX, 9; SHM, 8; ADX + DEX, 10; SHM + DEX, 8. (B) Medullary membranes: ADX decreased, whereas DEX increased NHE-3 expression. Number of experiments: ADX, 5; SHM, 4; ADX + DEX, 6; SHM + DEX, 4. DEX, dexamethasone-treated; ADX, adrenalectomized; SHM, sham-operated.
branes and medullary membrane from animals from the four experimental groups. In renal cortex, NHE-3 abundance was not significantly different in the ADX and SHM animals, but was increased in both the ADX + DEX and the SHM + DEX animals \((P < 0.05\) for both by ANOVA). Labeling of \(\beta\)-actin, which served as a loading control, was not different in the four groups. Summary of four experiments showed the following relative NHE-3 signals: SHM, 100 ± 51%; ADX, 103 ± 20%; SHM + DEX, 245 ± 7%; ADX + DEX, 280 ± 16%. Immunoblots of cortical membranes showed similar results (not shown). Figure 1B shows a representative immunoblot of NHE-3 protein abundance in medullary membranes. The effect of ADX on NHE-3 abundance is more variable than in cortex, but overall ADX suppressed NHE-3 expression in the medulla \((P < 0.05\) by ANOVA). DEX administration to either SHM or ADX animals significantly increased NHE-3 protein abundance compared with SHM and ADX \((P < 0.05\) for both by ANOVA). Summary of all animals showed the following NHE-3 signals: SHM, 100 ± 25%; ADX, 54 ± 51%; SHM + DEX, 345 ± 23%; ADX + DEX, 367 ± 26%.

NHE-3 protein was expressed in the brush border of S1 and S2 segments of proximal tubules in the cortical labyrinth (Figure 2B). NHE-3 was localized predominantly in the base of the brush border, as demonstrated by costaining of F-actin (Figure 2C). In medullary rays, there was an axial decrease of NHE-3 expression along the S2 segments. In S3 segments of medullary rays and the outer stripe, no immunoreactivity for NHE-3 was observed. Intracellular staining was not evident in these sections. NaP1 staining was restricted to cortex and was evident along the entire length of the brush border (Figure 2A).

ADX caused a very small decrease in NHE-3 expression in proximal tubules (Figure 3B) compared with sham-operated rats (Figure 3A). In ADX + DEX animals, NHE-3 abundance was profoundly increased compared to both ADX and SHM animals (Figure 3, A through C). These findings are similar to those of the immunoblots shown above. The effect of both glucocorticoid depletion or excess seemed to affect S1 and S2 proximal tubules in a similar way. The pattern described above was found consistently, with slight differences in the magnitude of the changes in all animals of the corresponding groups.

In the apical membrane of the thick ascending limb (TAL), ADX had modest effects on NHE-3 expression on immunohistochemistry (Figure 4, A and B). In SHM rats, TAL NHE-3 expression was similar in all animals, whereas much larger variations were seen among the individual animals of the ADX groups. This variable effect of ADX on TAL NHE-3 was also evident in the immunoblots performed on medullary membrane shown above. However, TAL NHE-3 expression was dramat-
GLUCOCORTICOID AND RENAL EXPRESSION OF NHE-3 AND NaP-2

Figure 3. Detection by immunofluorescence of NHE-3 in rat renal cortex of a sham-operated rat (A), an ADX rat (B), and an ADX + DEX rat (C). Immunostaining is seen in the brush border of proximal tubules. Adrenalectomy minimally reduces whereas administration of dexamethasone significantly increases NHE-3 abundance in the brush border of proximal tubules. Bar, 100 μm. Number of experiments: four for each group.

Effect of Glucocorticoids on NaP-2

Figure 5 is a representative immunoblot quantifying NaP-2 abundance in apical membrane vesicles from the four experimental groups. ADX increased NaP-2 immunoexpression in three out of four sets of animals, with no change in one set. DEX dramatically suppressed NaP-2 expression in all ADX or SHM animals. A summary of all four sets of animals showed the following relative NaP-2 levels: SHM, 100 ± 23%; ADX, 217 ± 35%; SHM + DEX, 15 ± 45%; ADX + DEX, 18 ± 2% (P < 0.05 for all comparisons except between SHM + DEX and ADX + DEX; ANOVA). Immunoblots with cortical membranes showed a similar pattern (data not shown).

On kidney sections (Figure 6), ADX increased NaP-2 immunoexpression in the brush border of all nephrons examined compared with SHM (Figure 6, A and B). Intracellular staining in ADX animals was slightly more distinct, but did not appear to be more abundant than in SHM controls (Figure 6, A and B). ADX + DEX (Figure 6C) drastically reduced NaP-2 expression in the brush border of all nephrons. Intracellular staining for NaP-2 was more diffuse and abundant in ADX + DEX than in ADX or SHM animals.

Discussion

Increased proximal tubule apical membrane Na/H exchanger activity contributes to the increased NaHCO₃ absorption and
Figure 4. Detection by immunofluorescence of NHE-3 in the inner stripe of the rat renal medulla of a sham-operated rat (A), an ADX rat (B), and an ADX + DEX rat (C). Immunostaining is seen in the thick ascending limbs and the thin descending limbs. Adrenalectomy minimally decreases whereas administration of dexamethasone significantly increases NHE-3 abundance in the luminal membrane of thick ascending limb cells. The weak immunostaining of the luminal membrane of thin limbs is not affected by adrenalectomy or by dexamethasone treatment. Bar, 100 μm. Number of experiments: four for each group.

Figure 5. Immunoblot of renal cortical apical membranes for NaPi-2 and β-actin protein abundance. Mobility in kilodaltons is indicated on the right. ADX increased whereas DEX decreased NaPi-2 expression. Number of experiments: four for each group.

Increased NH₄ secretion in states of glucocorticoid excess (1,3,4). Kinsella and coworkers have demonstrated that adrenalectomy per se did not lower apical membrane Na/H activity, whereas dexamethasone administration increased the Vₘₐₓ of the Na/H exchanger by 40 to 90% without altering its affinity for Na or H (1,7). This increase was observed in the background of both adrenalectomized or sham-operated animals (1). Our findings of changes in NHE-3 antigen by immunoblot and immunohistochemistry are in agreement with previous studies on apical membrane Na/H exchanger activity. Adrenalectomy per se did not have a significant effect on NHE-3 protein abundance on immunoblots performed on total cortical or brush-border membranes, although it is possible that a small decrease in proximal apical NHE-3 expression may not be detectable by immunoblot and immunohistochemistry. The increase in NHE-3 protein abundance induced by dexamethasone was profound, as assayed by both immunoblots and immunohistochemistry. This increase was independent of whether the animals were adrenalectomized. This finding is expected because 60 μg/kg represents a pharmacologic dose of glucocorticoid. The agreement between Na/H exchange activity in the previous studies (1,7) and NHE-3 abundance in the present study further confirms that NHE-3 mediates a significant portion of proximal tubule apical membrane Na/H exchange.

In the medulla, adrenalectomy seemed to have a variable suppressive effect on TAL NHE-3 expression. The suppression was demonstrable on immunoblots despite the large variation due to the more quantitative nature of immunoblots compared with immunohistochemistry. The increased TAL NHE-3 expression in DEX animals was unequivocal in immunoblots and evident even in immunohistochemistry. Immunoblots could not distinguish changes originating from TDL or TAL in the medulla, whereas immunohistochemistry clearly showed that the increased medullary NHE-3 was all due to increased expression in TAL. The lack of response in the TDL suggests that...
there are nephron segment-specific factors mediating the effect of glucocorticoid on NHE-3 expression.

The increased NHE-3 protein abundance is compatible with the current model of activation of NHE-3 by glucocorticoids at the level of gene transcription. In OKP cells, the glucocorticoid-induced increase in NHE-3 activity can be accounted for by an increase in steady-state NHE-3 transcript levels due to activation of transcription of the NHE-3 gene (18–20). Parallel increases in NHE-3 mRNA, protein, Na/H exchange activity, and transepithelial NaHCO₃ flux have been described in the maturing nephron of the neonate (21,22). The decrease in TAL apical membrane NHE-3 expression in adrenalectomized rats is compatible with the finding that rat TAL NaHCO₃ absorption was decreased by 40% in an in vivo microperfusion study by adrenalectomy and restored to normal by low-dose dexamethasone (23). The adrenalectomy-induced decrease in TAL Na-K-ATPase (24) and TAL transepithelial NaCl absorption (25,26) can be restored by aldosterone but not by dexamethasone. It is conceivable that regulation of TAL NaHCO₃ transport by adrenal steroids may be mediated by effects of glucocorticoid on the apical membrane NHE-3 rather than the effect of mineralocorticoids on basolateral Na-K-ATPase. A commensurate increase in proximal tubule NHE-3 protein abundance and activity and TAL NHE-3 mRNA and protein abundance has been described in chronic metabolic acidosis in rats (27,28).

The effect of glucocorticoids on phosphate balance is complex and includes effects on gastrointestinal absorption, bone mineral balance, and renal phosphate handling. Phosphaturia and hypophosphatemia have been described in patients with
and in animal models of glucocorticoid excess (29,30). The principal regulatory step of renal phosphate excretion resides in the proximal tubule apical membrane Na-P\textsubscript{04} cotransport system. There are two points of disparity between the current data and those of earlier transport studies. First, although dexamethasone reduced the $V_{\text{max}}$ of the Na-P\textsubscript{1} cotransport by approximately 30%, adenectomy per se had little effect on apical membrane Na-P\textsubscript{1} cotransport (1,31). Present studies show a definite increase of NaP\textsubscript{1}-2 protein abundance in adrenalectomized animals by both immunoblot and immunohistochemistry. Second, the effect of dexamethasone administration on NaP\textsubscript{2} protein abundance in this study and in the report by Levi and coworkers (14) is far greater than the observed 30% decrease in apical Na-P\textsubscript{04} cotransport $V_{\text{max}}$ (1,31). The difference between the transport data and the protein abundance is likely due to the fact that Na-P\textsubscript{04} cotransport activity reflects more than one form of Na-P\textsubscript{} cotransporter. The relative contributions of NaP\textsubscript{1}-2 and other potential Na-P\textsubscript{04} cotransporter isoforms to the Na-coupled phosphate flux in vesicles is currently unknown. It has been shown that dexamethasone administration in adrenal-intact animals decreases NaP\textsubscript{2} but does not affect NaP\textsubscript{1}-1 protein abundance (14). The contribution of Na-P\textsubscript{04} cotransporter isoforms other than NaP\textsubscript{2} to apical membrane Na-P\textsubscript{04} cotransport can also account for the lack of detectable effect of adenectomy on apical membrane Na-P\textsubscript{04} cotransport.

The increase in intracellular staining of NaP\textsubscript{2} that accompanies the decrease in apical membrane staining in dexamethasone-treated animals suggests that decreased membrane insertion and/or increased internalization likely contribute in part to the decrease in apical membrane NaP\textsubscript{1}-2 protein. Protein trafficking is well described mechanism by which acute dietary phosphate or parathyroid hormone administration regulates apical membrane NaP\textsubscript{1}-2 protein abundance (15,16), and trafficking could well be playing a role in mediating the effect of glucocorticoids on NaP\textsubscript{2}. Because NaP\textsubscript{1}-2 abundance in total cortical membranes also varied with glucocorticoid status, changes in kinetics of insertion and internalization cannot be the sole mode of regulation of NaP\textsubscript{2} in response to chronic changes in glucocorticoid excess. At present, one cannot conclude on the relative roles of trafficking versus protein synthesis/degradation in mediating the effect of glucocorticoid on proximal tubule apical NaP\textsubscript{2} abundance. It is possible that glucocorticoids may first induce endocytosis followed by subsequent intracellular degradation of NaP\textsubscript{2}.

In summary, the present study demonstrates that the increase in Na/H exchange and suppressed Na-P\textsubscript{04} cotransport activity previously described in the proximal tubule apical membrane is due to changes in the protein abundance of NHE-3 and NaP\textsubscript{2}, respectively. In addition, NHE-3 protein expression in the apical membrane of the TAL is inversely proportional to the glucocorticoid status of the animal, whereas NHE-3 expression in the TDL is not regulated by glucocorticoids.

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