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Selective Suppression of Renal Na^+/H^+ Exchanger Isoform-3 by Prolonged Stimulation of Rats with Adrenocorticotropic Hormone

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Prolonged stimulation with adrenocorticotropic hormone (ACTH) causes hypertension and increases Na^+ intake and urine output in humans and animals. However, its biochemical basis remains to be established. Since renal Na^+/H^+ exchanger isoforms (NHE) and the sodium pump play an important role in this condition, their levels were examined in rats stimulated with ACTH. Male Wistar rats received daily sc injection of ACTH (30 µg/100 g of body wt) for 4 d. Half of the ACTH-stressed rats were kept for four additional days without injection of ACTH (poststimulation). In a third group, the animals were treated with dexamethasone (50 µg/100 g of body wt) daily for 4 d. A fourth group consisted of unstressed control animals. Levels of NHE proteins were measured by Western blot analysis. Sodium pump activity was assessed by the level of ouabain-sensitive K-stimulated *p*-nitrophenylphosphatase activity (PNP) in the renal cortex. ACTH caused a selective decrease in NHE-3, but not of NHE-1 or α-actin levels. Interestingly, this ACTH-induced change was not duplicated in the animals treated with dexamethasone. Immunofluorescence data demonstrated that NHE-3 is located in the renal proximal tubules. PNP activity, on the contrary, was increased in both the ACTH-stimulated and dexamethasone-treated animals. More important, these changes in NHE-3 and PNP activity returned to the control level poststimulation. In conclusion, while PNP upregulation may be mediated by adrenocortical glucocorticoid, a role for glucocorticoids in the suppression of NHE-3 is less clear. These changes might impair renal tubular Na^+ reabsorption and hence increase Na^+ and water excretion in ACTH stimulation, thus acting as a counterbalance to normalize blood pressure in ACTH-stimulated animals.

Key Words: NHE; sodium pump; stress; ACTH; dexamethasone; glucocorticoid.

Introduction

Prolonged stress conditions such as depression and anxiety are associated with elevated concentrations of glucocorticoids in the circulation *(1,2)*. Glucocorticoids are synthesized by the adrenal cortex in response to stimulation by adrenocorticotropic hormone (ACTH) released from the pituitary gland. Chronic stimulation with ACTH frequently leads to the development of hypertension in both humans and rats *(3)*. In addition, this is accompanied by increases in Na^+ and water intake and urine output, implicating a possible deregulation of ion transporter mechanisms *(4–9)*. Kidneys play an important role in the regulation of Na^+ and water via several Na^+-handling mechanisms. The tubular sodium hydrogen exchanger (NHE) reabsorbs Na^+ from renal filtrate from proximal tubule, and the sodium pump produces transcellular Na^+ transport *(10)*. NHE is encoded by a multigene family consisting of at least six isoforms *(10–12)*. Overexpression of these isoforms that causes volume expansion by increasing cardiac output and vascular resistance may contribute to the pathophysiology of hypertension *(13,14)*. In this context, the results of epidemiologic studies indicate a link between Na^+ imbalance and stress-induced hypertension *(5)*, although the mechanistic relationship between stress and ion transporters remains to be established.

The primary aim of the present study was to investigate the levels of renal cortical NHE-1 and NHE-3 proteins by Western blot analysis, and of sodium pump activity, obtained from ACTH-stimulated and unstimulated rats.

Results

Characterization of Stress Model

In response to ACTH stimulation, the 24-h urinary-free corticosterone content of the stimulated rats significantly increased compared with that of the control animals (Fig. 1). Animal weights generally decreased by 10–20%. By contrast, the adrenal wet weights increased by 82–193%, indicating glandular hypertrophy (not shown). The enlargement of adrenal glands was accompanied by a reduction in glandular cholesteryl esters, as measured by a specific reverse-phase high-performance liquid chromatography method *(15)*. All these data verify that the animals were stimulated by the hormone.

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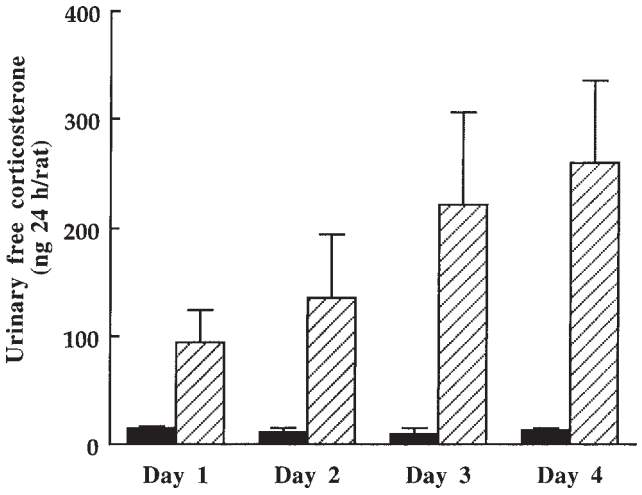

Fig. 1. Release of urinary free corticosterone by rats stimulated with ACTH daily (▨) with 30 μg/100 g of body wt and unstimulated control (■) rats. Data are mean ± SD (n = 8) of duplicate determinations.

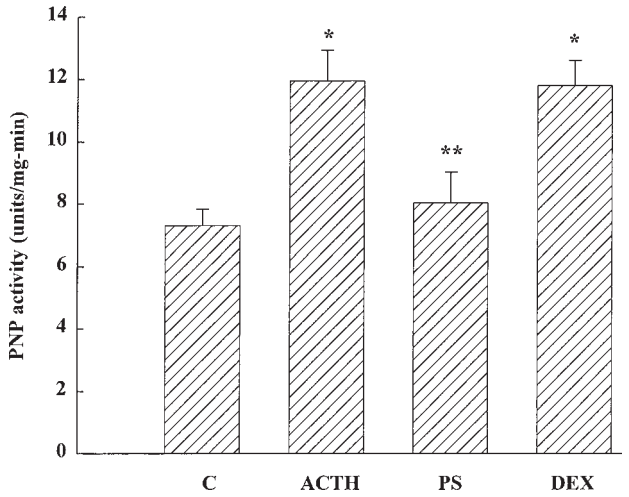

Fig. 3. PNP activity in renal cortex from control (C), ACTH-stimulated (ACTH), poststimulation (PS), and dexamethasone-treated (DEX) rats. Data are mean + SEM (n = 8). *$p < 0.05$ with respect to controls; **$p < 0.05$ with respect to ACTH-stimulated or dexamethasone-treated rats.

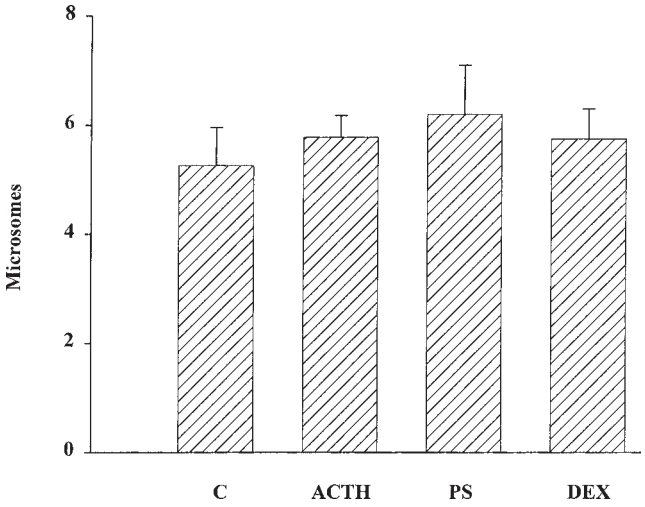

Fig. 2. Microsomal yield fron renal cortex (mg/g tissue) from control (C), ACTH-stimulated (ACTH), poststimulation (PS), and dexamethasone-treated (DEX) rats. Data are mean ± SEM of duplicate determinations (n = 8).

Crude Microsomes

The yields of crude microsomes (mg of protein/g of renal cortex) in renal cortex of ACTH-stimulated (5.8 ± 0.4), poststimulation (6.2 ± 0.9), and dexamethasone-treated (5.8 ± 0.6) rats were not significantly different from that of the control unstressed (5.3 ± 0.7) animals (Fig. 2).

Ouabain-Sensitive K^+-Stimulated p-Nitrophenylphosphatase Activity

p-Nitrophenylphosphatase (PNP) activity as measured in our study represents adenosine triphosphatase (ATPase) activity of the sodium pump. However, because K-stimulated and ouabain-inhibitable activity were calculated, this PNP activity does not represent the general ATPase activity. The level of PNP activity was increased in ACTH-stimulated and dexamethasone-treated animals over unstimulated control rats or the animals poststimulation (n = 8; $p < 0.05$) (Fig. 3).

Specificity of Polyclonal Antibodies

Polyclonal antibodies against rat NHE-1 and NHE-3-GST fusion protein have recently been characterized *(10)*. In the present study, NHE-3 polyclonal antibodies were raised using the NHE-3-GST fusion protein antigen in chickens. The NHE-3 polyclonal antibody reacted with a protein of 80- to 85-kDa molecular mass with no crossreaction against any other protein in rat colonic crude microsomes (Fig. 4 left, lane 1). The reaction was blocked by the NHE-3-GST fusion antigen (Fig. 4 left, lane 2), but not by the GST fusion protein alone (Fig. 4 left, lane 3). The anti-rat NHE-1 polyclonal antibodies reacted with a protein of 110 kDa on electrochemiluminescence (ECL) Western blot analysis in both the colon (Fig. 4 right, lane 1) and crude renal microsomes (Fig. 4 right, lane 2).

NHE Expression

The level of NHE-3 protein was selectively suppressed in ACTH-stimulated animals compared with control (n = 8, $p < 0.05$), dexamethasone-treated animals or in the animals poststimulation (Fig. 5A–C). However, the level of NHE-1 isoform (Figs. 5A, 6) and the internal control α-actin (Figs. 5A, 7) did not change under these conditions. Furthermore, the level of NHE-3 normalized with α-actin also exhibited the same pattern of changes (Fig. 5C).

Dose Dependence

The effect of different doses of ACTH (5, 10, and 30 μg/100 g of body wt) on the expression of NHE-3 in the rat

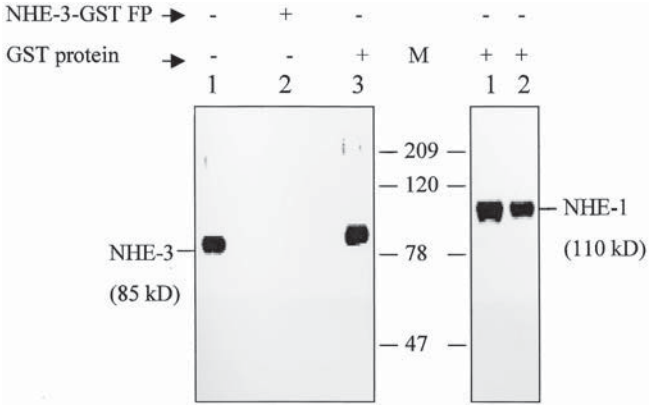

Fig. 4. Representative gel showing characterization of antirat NHE-3 antibodies using ECL Western blot analysis. Antibodies were treated as indicated with (+) or without (–) NHE-3-GST fusion protein (NHE-3-GST FP) or GST protein. (**Left**) Reaction of antirat NHE-3 with rat colonic microsomes (lanes 1–3, 30 µg/lane); (**right**) reaction of antirat NHE-1 antibody with rat colonic crude microsomes (lane 1, 30 µg/lane) and kidney microsomes (lane 2, 80 µg/lane). Arrows indicate the molecular mass of the indicated NHE isoforms.

renal cortex was examined over a period of 4 d. There was a significant decrease in the level of NHE-3 protein in rats stimulated with 10 and 30 µg of ACTH, whereas the level did not change in the control rats and in animals stimulated with 5 µg of ACTH (Fig. 8). In addition, the level of the α-actin internal control remained unchanged under these conditions (Fig. 8).

Immunofluorescence Localization of NHE-3 in Rat Kidney

Since the changes were observed in only NHE-3, we studied the localization of NHE-3 in the renal transverse sections (5 µm). The polyclonal antibodies reacted with the renal tubules in the cortex region (Fig. 9). There was no reaction in the renal sections stained with 2ºAbs alone (data not shown).

Discussion

ACTH Stimulation, NHE Expression, and Sodium Pump Activity

Our study was designed to investigate specifically the role of NHE isoforms 1 and 3 and sodium pump in ACTH-stimulated rats. This issue was addressed by examining the changes in the expression of NHE-1 and NHE-3 and sodium pump activity in the present model of stress. Selective suppression of the NHE-3 isoform and stimulation of sodium pump activity in the ACTH-stimulated rat renal cortex without a change in the NHE-1 isoform or in the internal control α-actin suggest hormone-specific alterations in these transport mechanisms. In view of a prominent role of NHE-3 in the reabsorption of Na^+ and water from the renal filtrate, its selective suppression will leave more Na^+ and water in the renal filtrate to be excreted through urine. In this context, the observed changes may explain the mechanism of

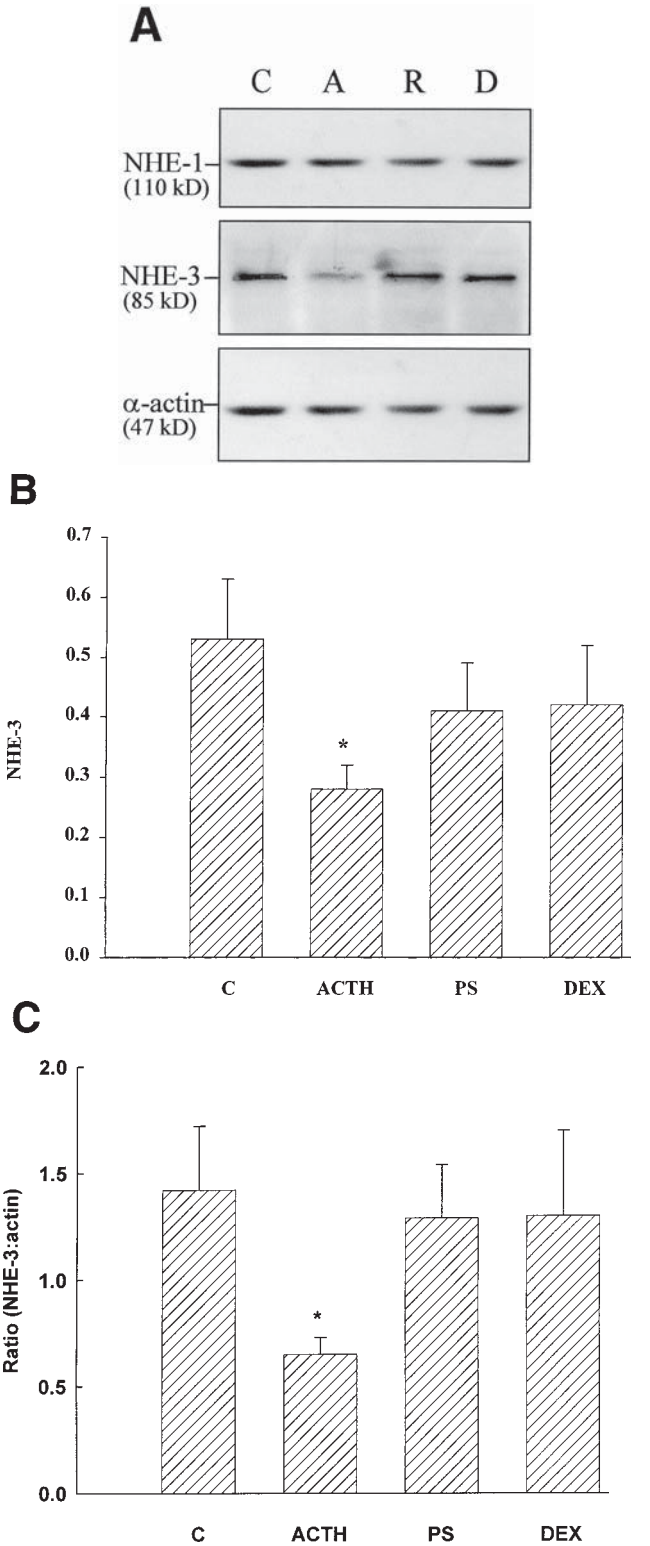

Fig. 5. (**A**) Representative (n = 8) ECL Western blot analysis showing renal cortical levels of indicated isoforms and their molecular mass in control (C), ACTH-stimulated (A), poststimulation (R), and dexamethasone (D)-treated rats; (**B**) changes in renal cortex levels of NHE-3 protein in control (C), ACTH-stimulated (ACTH), poststimulation (PS), and dexamethasone-treated (DEX) rats. (**C**) Bar diagram showing ratios of the indicated proteins in control (C), ACTH-stimulated (ACTH), poststimulation (PS), and dexamethasone (DEX) treated rats. Data are mean ± SEM. *$p < 0.05$ versus controls.

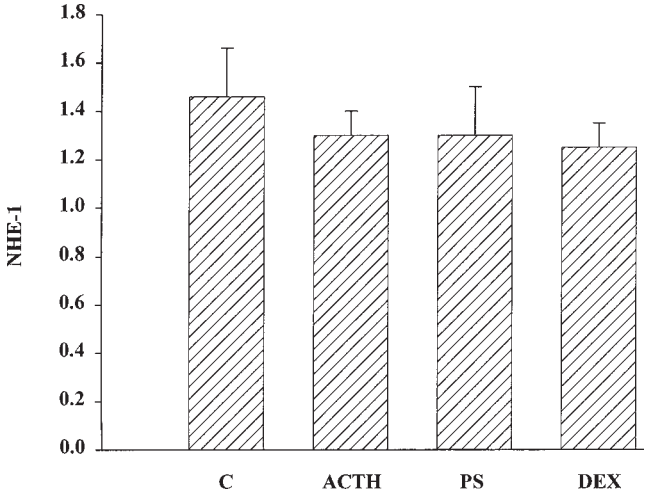

Fig. 6. Kidney cortex levels of NHE-1 protein in control (C), ACTH-stimulated (ACTH), poststimulation (PS), and dexamethasone-treated (DEX) rats. Data are mean ± SEM (n = 8) of duplicate determinations.

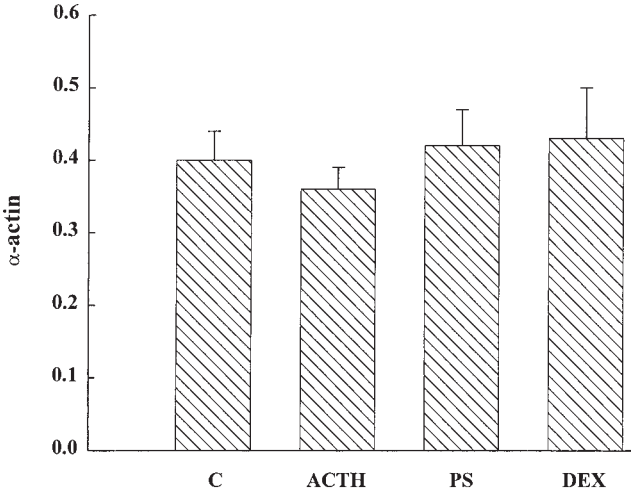

Fig. 7. Renal cortex levels of α-actin protein in control (C), ACTH-stimulated (ACTH), poststimulation (PS), and dexamethasone-treated (DEX) rats. Data are mean ± SEM (n = 8) of duplicate determinations. *$p < 0.05$ with respect to controls.

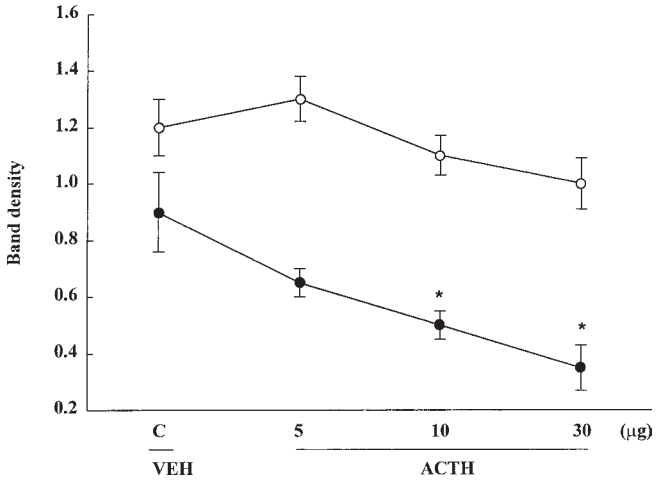

Fig. 8. Dose-dependent effect of ACTH stimulation on expression of NHE-3 (●) and α-actin (○). Quantitative changes in the renal cortex levels of NHE-3 protein in the control rats (C) and those stimulated with the indicated amounts of ACTH (ACTH) for 4 d as shown. Data are mean ± SEM (n = 5). *$p < 0.05$ with respect to controls. VEH, vehicle.

increased Na^+ and water intake and urine output reported previously *(4–9)* in this condition. Enhanced expression of NHE-3 has been reported in several forms of hypertension *(16)*, which owing to absorption of more Na^+ and water leads to volume expansion, vascular resistance, arrhythmia, and hypertension *(13,17,18)*. Although development of hypertension has been associated with ACTH stimulation, under our experimental conditions, the blood pressure (BP) of ACTH-stimulated animals was not different from that of the controls (not shown). Therefore, NHE-3 suppression might be viewed as a compensatory mechanism to normalize BP under the present experimental conditions.

The sodium pump, located on the basolateral domain in the renal tubular epithelial cells, provides paracellular Na^+ transport and maintains a necessary gradient, which acts as a driving force for the NHE-3 locating on the apical domain. An increase in the sodium pump activity might suggest an enhanced driving force for NHE- 3 in renal tubules. Alternatively, elevation of sodium pump activity can affect the excitability of the renal vasculature as well. The sodium pump in the excitable cells such as smooth muscle and neuronal cells maintains membrane potential. Thus, elevation of the sodium pump activity in the renal cortex of the ACTH-stimulated rats might decrease the vascular tone, which would be important in the regulation of adequate renal blood flow and glomerular filtration during stress conditions.

Mechanism of Regulation

ACTH is known to stimulate adrenal glucocorticoid synthesis in stressed animals *(3)*. Cessation of ACTH stimulation accompanied by a reversal of changes in both NHE-3 level and sodium pump activity in ACTH-stimulated rats suggests that the changes are ACTH-specific phenomena. Interestingly, dexamethasone treatment of rats was unable to duplicate the changes in the NHE-3 expression observed in ACTH-stimulated animals, whereas it did increase the sodium pump activity similar to ACTH stimulation. These findings collectively suggest that sodium pump activity is regulated by glucocorticoid in the ACTH-stimulated animals, consistent with earlier findings *(19–23)*. However, the present findings do not support the role of this steroid in the regulation of NHE-3 expression under our experimental conditions. Of interest, dexamethasone increases Na^+ uptake and NHE-3 expression in a region-specific manner in the gastrointestinal tract *(24)*. The present discrepancy could be owing to differences in the dose and duration of dexamethasone, or the type of tissue being investigated.

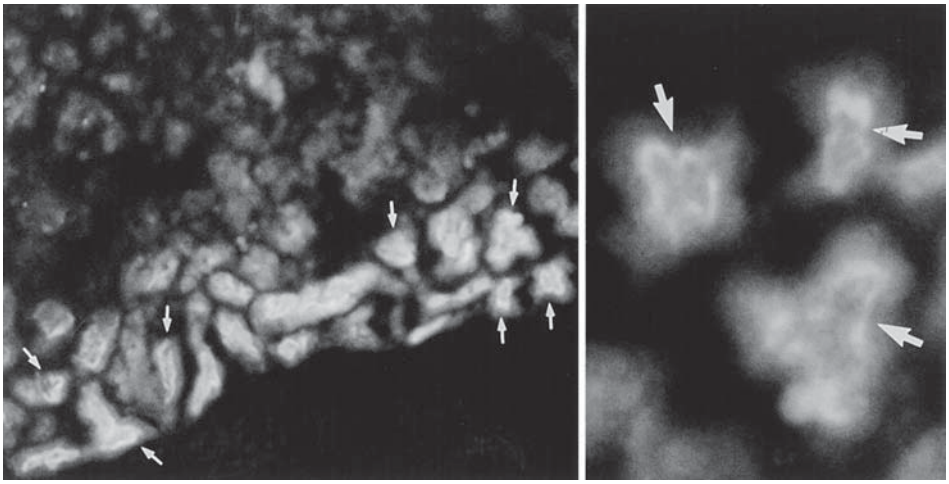

Fig. 9. Representative immunofluorescence localization of NHE-3 in rat renal transverse sections at low (**left**) and high magnification (**right**). Rhodamine-labeled 2º-antibodies were used.

Summary

We have demonstrated that ACTH stimulation of rats causes a selective but reversible suppression of NHE-3 and stimulation of the sodium pump. Our findings further suggest that glucocorticoid regulates the sodium pump but not NHE-3 in this experimental model. Suppression of NHE-3 together with the elevation of the sodium pump activity might contribute to the maintenance of Na^+ and water homeostasis and help normalize the BP in the ACTH-stimulated animals.

Materials and Methods

Animals, Adrenal Gland, and Adrenal Cortex

Male Wistar rats with a mean body wt of 138 g were used as a model for studies. Animals were divided into four groups according to experimental treatments. Group 1 rats were daily injected with 5–30 µg of ACTH (Synatchen Depsito, Ciba-Geigy, Switzerland)/100 g of body wt. Group 3 rats were daily injected with 50 µg of dexamathasone (Sigma, UK)/100 g of body wt. Group 4 rats were daily injected with saline to serve as the control. All injections were given subcutaneously for four consecutive days *(25)*. Rats were sacrificed 24 h after the last injection to minimize acute effects. Group 2 rats consisted of a portion of ACTH-stimulated rats from group A that were kept for four additional days without further stimulation and then killed to examine the reversibility (poststimulation). The 24-h urinary-free corticosterone content that served as an index for ACTH stimulation was measured by a radioimmunoassay kit (ICN Biotrak, no. RPA 548). During the entire experimental period, rats were maintained on a standard laboratory diet and drinking water. Animals moved, ate, and drank freely; no abnormality was observed.

Preparation of Crude Microsomes

The animals were euthanized by guillotine and kidneys were removed and washed with cold phosphate-buffered saline (PBS). Renal cortex was separated and homogenized using a MOPS buffer, pH 7.4 (20 m*M* MOPS, 250 m*M* sucrose, 10 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, and 5 m*M* dithiothreitol) *(26–28)*. Briefly, tissue (1 g) was homogenized in 10 mL of MOPS buffer, and cell debris was cleared by centrifugation at 1000*g* for 10 min. The supernatants obtained were cleared by passing through cheesecloth. The resulting clear supernatant was centrifuged at 110,000*g* for 45 min at 4ºC (Beckman ultracentrifuge). The pellet was suspended in MOPS buffer and designated as the crude microsomes. Total protein contents were determined using a dye binding kit (Bio-Rad). Aliquots of crude microsomes were used to detect the NHE-1 protein in different tissues using Western blot analysis.

Ouabain-Sensitive K^+-Stimulated PNP Activity

K-stimulated ouabain-sensitive PNP represents the partial reaction of sodium pump and has been used as a marker of sodium pump *(29)*. PNP activity was estimated in the aliquots (20 µg) of crude membrane using a buffer containing 3 m*M* imidazole, pH 7.5; 5 m*M* KCl; 5 m*M* $MgCl_2$; 1 m*M* PNP (Sigma); and 1 m*M* ouabain (Sigma). Assay was performed with or without ouabain and KCl, and the difference in the activity was taken as a measure of sodium pump activity. The reaction was performed at 37ºC for 1 h, and optical density was obtained at 420 nm. Enzyme unit activity is defined as the micromoles of the product released/(minute)·milligrams of protein) at 37ºC and was calculated using a 1.32×10^4 molar extinction coefficient of *p*-nitrophenol.

Preparation of Antirat NHE-3 Antibodies Using GST Fusion Protein

We used antirat polyclonal antibodies against rat NHE-1 and NHE-3. Characterization of NHE-1 polyclonal antibodies and the NHE-3-GST fusion protein have been described recently *(10)*. However, for our work, we raised and characterized the antirat NHE-3 polyclonal antibody using this NHE-3-GST fusion protein as an antigen in chickens. The polyclonal antibodies (IgY) were isolated from egg yolk using a standard PEG precipitation method. The specificity of the antibodies was examined using colonic crude microsomes with antibodies that were blocked with NHE-3-GST fusion protein and GST fusion partner protein separately using ECL Western blot analysis.

ECL Western Blot Analysis

The polyclonal antibodies used were raised separately and were characterized using the Western blot analysis. Aliquots of renal crude microsomes were separated electrophoretically on an 8% polyacrylamide gel *(28,30)* and transferred onto a nitrocellulose filter paper electrophoretically (Bio-Rad) overnight at 4ºC. The filters were then blocked with 5% nonfat milk in PBS for 1 h at room temperature. The filters were incubated with the polyclonal rabbit antirat NHE-1, chicken antirat NHE-3, or monoclonal α-actin (Sigma) using a dilution of 1:2000. The filters were washed with PBS and incubated for 1 h at ambient temperature with antirabbit (NHE-1), antichicken (NHE-3), or antimouse (α-actin) secondary antibodies horseradish peroxidase conjugates (Sigma) using a 1:2000 dilution. The filters were washed thoroughly and specific bands were detected using an ECL kit (Amersham). The size of NHE-1-reactive protein was determined using a prestained protein size marker (Bio-Rad).

Quantitation

The positive bands were scanned on a densitometer (UltroScan; Beckman) to obtain the band density and area. The level of each message was compared with the level in the unstressed control kidney cortex.

Statistical Analyses

Data are presented as the mean ± SEM of duplicate determinations ($n = 8$) unless specified otherwise. For each message, the level was compared with the unstressed control rat kidney cortex. Unpaired student's *t*-test was calculated, and a value of $p < 0.05$ was considered statistically significant.

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