Regulation of NHE3, NKCC2, and NCC abundance in kidney during aldosterone escape phenomenon: role of NO

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Turban, Sharon, Xiao-Yan Wang, and Mark A. Knepper. Regulation of NHE3, NKCC2, and NCC abundance in kidney during aldosterone escape phenomenon: role of NO. Am J Physiol Renal Physiol 285: F843–F851, 2003. First published July 1, 2003; 10.1152/ajprenal.00110.2003.—Escape from aldosterone-induced renal NaCl retention is an important homeostatic mechanism in pathophysiological states in which plasma aldosterone levels are inappropriately elevated, e.g., in primary aldosteronism. Our previous studies demonstrated that the escape process occurs largely as a result of a marked suppression of the abundance of the thiazide-sensitive Na-Cl cotransporter (NCC) of the distal convoluted tubule but have also demonstrated a paradoxical increase in the protein abundance of the apical Na/H exchanger of the proximal tubule (NHE3). In the present study, we confirmed the increase in NHE3 and also showed that a similar increase in NHE3 protein abundance occurs in escape from ANG II-mediated NaCl retention. To investigate the potential role of nitric oxide (NO) in the observed upregulation of NHE3, we repeated the aldosterone escape experiment with a superimposed infusion of a NO synthase inhibitor, Nω-nitro-l-arginine methyl ester (l-NAME). l-NAME infusion abolished the increase in NHE3 protein abundance. Furthermore, in a different experiment, NO synthase inhibition uncovered an associated decrease in the abundance of the Na-K-2Cl cotransporter (NKCC2) of the thick ascending limb, not seen with simple aldosterone escape. However, NO synthase inhibition did not block the decrease in NCC abundance normally seen with aldosterone escape. Furthermore, l-NAME infusion in aldosterone-treated rats markedly decreased both NHE3 and NKCC2 protein abundance, without changes in the corresponding mRNA levels. We conclude that NHE3 and NKCC2 protein abundances in kidney are positively regulated by NO and that the increase in NHE3 abundance seen in the aldosterone escape phenomenon is NO dependent.

pressure natriuresis; Na/H exchanger 3; Na-K-2Cl cotransporter; Na-Cl cotransporter

ALDOSTERONE IS AN important element of the overall regulatory mechanism that allows the kidney to increase sodium chloride and fluid absorption in response to decreases in extracellular fluid volume. When aldosterone levels are elevated under inappropriate circumstances, i.e., under conditions of normo- or hypervolemia, the kidney can overcome aldosterone’s sodium chloride-retaining action by activation of the so-called “aldosterone escape” mechanism, in which renal tubule NaCl reabsorption decreases despite the continued presence of aldosterone (23). Such a process is likely to be important in limiting the hypertension that occurs in primary aldosteronism, for example. On the basis of experiments by Hall and colleagues (14) in which renal perfusion pressure was prevented from increasing by a servo-null system, the aldosterone escape process appears to depend on an increase in renal perfusion pressure. Thus aldosterone escape is generally viewed as a manifestation of the pressure-natriuresis phenomenon (11).

In our previous study, we found that aldosterone escape in rats is associated with a marked decrease in the renal abundance of the thiazide-sensitive Na-Cl cotransporter (NCC) of the distal convoluted tubule (35). However, we were surprised to find an apparent increase in the abundance of Na/H exchanger 3 (NHE3) in aldosterone escape (35). The purpose of this paper is to confirm the increase in renal NHE3 abundance in aldosterone escape and to investigate the mechanisms involved. Because of previous studies that demonstrated a seemingly paradoxical effect of nitric oxide (NO) to increase proximal tubule salt and water reabsorption (24), we focus on the role of NO to regulate NHE3 abundance in the setting of aldosterone escape.

METHODS

Animals. The experiments were conducted in accordance with an animal protocol (9KE-5) approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute. Experiments were carried out in male Sprague-Dawley rats (185–250 g) from Taconic Farms (Germantown, NY). Rats were kept in metabolic cages in a temperature-controlled room regulated on a 12:12-h light-dark cycle for the duration of the experiments.

Escape models. The protocols for escape experiments are diagrammed in Fig. 1. On day 1, all rats were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and subcutaneously implanted with osmotic minipumps (models 2ML1 and/or 2001, Alzet, Palo Alto, CA). Depending on the experiment, the pumps delivered either ANG II (Calbiochem,
The l-NAME dose was chosen on the basis of preliminary measurements of nitrate/nitrite excretion in normal rats receiving minipump infusions of l-NAME or vehicle. In these studies, an l-NAME dose of 30 mg·kg body wt−1·day−1 for 4 days decreased urinary nitrate/nitrite excretion to 63 ± 3% of control, whereas a dose of 60 mg·kg body wt−1·day−1 decreased nitrate/nitrite excretion by a similar degree (to 58 ± 2% of control). Twenty-four-hour urinary nitrate/nitrite was measured using a commercial kit (780001, Cayman, Ann Arbor, MI).

Ration feeding protocol. The amount of sodium and water and number of calories that were provided to the rats were controlled by ration feeding of a fixed daily amount of gelled food. The NaCl-restricted diet consisted of a mixture of a synthetic low-NaCl diet (Formula 53140000, Ziegler Brothers, Gardner, PA), deionized water (25 ml/15 g of food), and agar (0.125 g/25 ml of water). The NaCl-replete diet was the same except for the addition of NaCl at 2.0 meq per 15 g of dry food. All rats received 40 g·200 g body wt−1·day−1 of the gelled concoctions, providing −0.02 and 2.0 meq·200 g body wt−1·day−1 for the NaCl-restricted and NaCl-replete diets, respectively. This ration was eaten in its entirety by the rats.

Antibodies. For both immunoblotting and immunocytochemistry, affinity-purified rabbit polyclonal antibodies to the following renal sodium transporters were used: NHE3 of the proximal tubule (16), the Na-K-Cl cotransporter (BSCI/NKCC2) of the thick ascending limb (17), the thiazide-sensitive cotransporter (TSC/NCC) of the distal convoluted tubule (18), and all three subunits of the epithelial Na channel (ENaC) of the collecting duct (25). In addition, we carried out immunoblotting with antibodies to NO synthase (NOS)1 (neuronal NOS (nNOS; Santa Cruz Biotechnology, no. SC-648), NOS2 or inducible NOS (iNOS; Santa Cruz, SC-650-G), NOS3 or endothelial NOS (eNOS; Santa Cruz, SC-653), aquaporin-1 (31) and β-actin (Sigma, A-2066).

Semiquantitative immunoblotting. Both kidneys were rapidly removed after decapitation, and immunoblotting was performed as described in detail previously (17, 31). Initial loading gels were run and stained with Coomassie blue to verify equal loading among samples, as previously described (17, 31).

Immunocytochemistry. As previously described (26), kidneys were perfusion-fixed with a paraformaldehyde-based fixative, followed by preparation of 2-μm paraffin sections. Sections were then labeled using the immunoperoxidase method described by Hager et al. (12). We used an antibody to NHE3 kindly provided by Dr. A. McDonough, which has been demonstrated to be capable of showing differences in NHE3 distribution in proximal tubule cells in response to hypertension (36).

Effect of l-NAME infusion in aldosterone-infused and NaCl-replete volume-expanded rats. In another set of experiments, all rats received a 7-day infusion of aldosterone (200 μg/day) via minipump, whereas the experimental rats received an infusion of l-NAME (30 mg·kg body wt−1·day−1) in addition to aldosterone. Control rats received the vehicle for l-NAME (deionized water). All rats were initially placed on a NaCl-restricted diet (as described above). After 3 days, all of the rats were switched to the NaCl-replete diet. Blood and urine were collected as described above.
t-test (INSTAT; Graphpad Software, San Diego, CA). The criterion for statistical significance was a P value of <0.05.

RESULTS

NHE3 and NCC abundances in aldosterone escape. Our previous study of the aldosterone escape phenomenon produced the seemingly paradoxical finding that the escape process is associated with an increase in NHE3 protein abundance in the renal cortex (35). To confirm this finding, we repeated the aldosterone escape experiment (Fig. 1A) and compared NHE3 abundance in renal homogenates from rats undergoing aldosterone escape (aldosterone plus NaCl-replete diet) vs. control rats that received aldosterone but did not undergo escape (aldosterone plus NaCl-restricted diet) (Fig. 2). There was a statistically significant increase in NHE3 band density in association with aldosterone escape to $153 \pm 8\%$ of control. Figure 2 also shows an immunoblot of another proximal tubule protein, aquaporin-1, the water channel that mediates water transport across the apical and basolateral plasma membranes of the proximal tubule (27). In contrast to NHE3, aquaporin-1 abundance did not change significantly. An immunoblot for NCC (Fig. 2, bottom) confirmed the previous observation that NCC abundance is markedly decreased in kidneys from aldosterone escape rats (35), providing an explanation for decreased NaCl reabsorption. The total protein content of the kidneys was unchanged in the aldosterone escape animals (aldosterone with NaCl-replete diet $135 \pm 7$ mg/kidney; aldosterone with NaCl-restricted diet $147 \pm 11$ mg/kidney; not significant). Figure 3 shows an example of NHE3 immunocytochemistry in aldosterone escape vs. control rats. Over 100 proximal tubules were evaluated in sections from five different animals (3 control rats and 2 escape rats). In general, among all sections examined, there was no consistent difference in the distribution of NHE3 labeling in proximal tubule cells between escape and control rats and no evidence for an intracellular redistribution of NHE3.

NHE3 and NCC abundances in ANG II escape. To determine whether the increase in NHE3 abundance seen in aldosterone escape is unique to this model, we set up another escape model, ANG II escape (Fig. 1B). In this experiment, the experimental design was analogous to that of the aldosterone escape experiment. Both groups received ANG II by osmotic minipump at a rate of 80 ng/min. The control group received a sodium-restricted diet ($<0.1$ meq Na$^{+}$/200 g body wt$^{-1}$ day$^{-1}$), whereas the experimental group received a sodium-replete diet (2.0 meq Na$^{+}$/200 g body wt$^{-1}$ day$^{-1}$) for the last 4 days. With this ANG II escape model, renal NHE3 abundance increased as it did for aldosterone escape (Fig. 4). Thus the increase in NHE3 is not specific to the agent used to induce sodium retention in the escape models. Moreover, because circulating ANG II was clamped at a high level in this experiment, it appears unlikely that changes in circulating ANG II concentrations are responsible for the increase in NHE3 levels in the aldosterone escape phenomenon. Figure 4 also shows the levels of NCC and $\alpha$-ENaC in this ANG II escape model. As seen for aldosterone escape, NCC decreased in response to the
increase in salt intake, whereas α-ENaC was unchanged. α-ENaC abundance in the collecting duct is strongly regulated by aldosterone and the lack of a change argues against major changes in effective aldosterone levels between the two conditions. Indeed, direct measurements revealed that, for both groups, serum aldosterone concentrations were much higher than the $K_d$ of the mineralocorticoid receptor [1.3 nM (2)]: 28 ± 5 nM in the NaCl-restricted, ANG II-infused rats and 2.4 ± 0.4 nM in the NaCl-replete, ANG II-infused rats.

**Escape from sodium retention induced by aldosterone in presence of NOS inhibition.** To determine whether the rise in NHE3 abundance in aldosterone escape is dependent on NO, we repeated the escape study with superimposed administration of the nonselective NOS blocker L-NAME at a rate of 30 mg kg$^{-1}$ day$^{-1}$. [This dose was found to decrease urinary NO excretion in normal rats in dose-response studies (see METHODS)]. Thus both L-NAME and aldosterone were given to all rats (Fig. 1C). Control rats received a NaCl-restricted diet (<0.1 meq Na·200 g body wt$^{-1}$·day$^{-1}$), whereas escape rats received a NaCl-replete diet (2.0 meq Na·200 g body wt$^{-1}$·day$^{-1}$) for 4 days. Figure 5 shows the results. In contrast to escape from aldosterone alone, escape from aldosterone plus L-NAME was not associated with an increase in NHE3 protein abundance. In fact, whole kidney NHE3 abundance was moderately decreased in the Na-replete rats. [This observation was confirmed in a separate set of rats studied under identical conditions (normalized band densities: NaCl restricted 100 ± 7, n = 4; NaCl replete 63 ± 6, n = 4; $P < 0.01$).] Furthermore, Fig. 5 shows that NKCC2 abundance was decreased in response to the increase in NaCl intake. In addition, NCC abundance was decreased to 48% of the baseline level, indicating that L-NAME did not block the fall in NCC seen in aldosterone escape (compare with Fig. 2). In contrast, there was no significant change in the abundance of β-actin and there were no demonstrable changes in the general abundance of proteins seen on a Coomassie blue-stained gel (Fig. 5, bottom).

Body weights, serum solute concentrations, and urinary excretion rates for this experiment are summarized in Table 1. There were no significant differences in body weight, water excretion, serum aldosterone concentration, or creatinine clearance between the two groups. The group receiving the sodium-replete diet had a significant increase in Na and K excretion. In addition, these rats were found to have a significantly lower serum potassium and chloride concentration than the sodium-restricted rats. The serum bicarbonate was significantly higher in the sodium-replete rats, consistent with a metabolic alkalosis.

Table 2 reports CT values for real-time RT-PCR measurements of mRNA levels for NHE3, NKCC2, and β-actin for the opposite kidneys from the same experiment. There were no changes in NHE3, NKCC2, or actin CT values, suggesting that NHE3 and NKCC2 protein levels changed by mechanisms independent of changes in mRNA abundance.

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**Fig. 4.** Immunoblots assessing protein abundances of NHE3, NCC, and α-epithelial Na channel (αENaC) in homogenates from whole kidney in control (ANG II infused/NaCl restricted; left) and escape (ANG II infused/NaCl replete; right) rats. NHE3 protein abundance was significantly increased, whereas NCC protein abundance was significantly decreased (*).

**Fig. 5.** Immunoblots assessing protein abundances of NHE3, NCC, and β-ENaC in homogenates from whole kidney in control (aldosterone and L-NAME infused/NaCl restricted; left) and escape (aldosterone and L-NAME infused/NaCl replete; right) rats. NHE3 protein abundance was significantly increased, whereas NCC protein abundance was significantly decreased (*).

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[Table 1 and Table 2 are not included in this text, but can be found in the original article.]
Our previous study of aldosterone escape demonstrated that the increase in NHE3 abundance associated with the escape process is seen in the cortex but not the outer medulla, indicating that it occurs in the proximal tubule (35). We repeated the aldosterone/L-NAME escape study diagrammed in Fig. 1 with measurements of NHE3 and NKCC2 specifically in the cortex (Fig. 6). As can be seen, L-NAME blocked the rise in NHE3 normally seen in aldosterone escape and uncovered a decrease in NKCC2 abundance, confirming the whole kidney results reported in Fig. 5.

**Effect of L-NAME infusion in aldosterone/NaCl-replete condition.** The ability of the NOS inhibitor L-NAME to block the increase in NHE3 abundance normally seen in aldosterone escape suggests that NO regulates NHE3 abundance in extracellular fluid (ECF) volume-expanded states. To test this, we infused L-NAME at a rate of 30 mg·kg body wt<sup>-1</sup> day<sup>-1</sup> for 4 days into aldosterone-infused, NaCl-replete rats. As hypothesized, L-NAME administration significantly decreased the abundance of NHE3 in kidney homogenates from aldosterone/NaCl-replete rats (Fig. 7). In addition, there was a marked decrease in the renal abundance of NKCC2 in response to L-NAME administration. In contrast, L-NAME infusion did not significantly alter NCC abundance in this setting.

Table 3 reports C<sub>T</sub> values for real-time RT-PCR measurements of mRNA levels for NHE3, NKCC2, and β-actin for the opposite kidneys from the same experimental group. Aldosterone/NaCl-replete/vehicle; left) vs. L-NAME-infused (aldosterone/NaCl replete/L-NAME; right) rats. Band densities were assessed by laser densitometry. *Significant differences. Bottom: preliminary 12% SDS polyacrylamide gels were run and were stained with Coomassie blue to confirm equality of loading in each lane.

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### Table 1. Body weight, serum concentrations, creatinine clearance, and urinary excretion rates

<table>
<thead>
<tr>
<th></th>
<th>Aldosterone/NaCl Restricted</th>
<th>Aldosterone/L-NAME/NaCl Replete</th>
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<tbody>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Day -3</strong></td>
<td>221 ± 4</td>
<td>221 ± 3</td>
</tr>
<tr>
<td><strong>Day 4</strong></td>
<td>245 ± 2</td>
<td>236 ± 5</td>
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<tr>
<td><strong>Serum concentrations</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Sodium, mM</strong></td>
<td>145.8 ± 0.9</td>
<td>144.3 ± 0.3</td>
</tr>
<tr>
<td><strong>Potassium, mM</strong></td>
<td>6.5 ± 0.4</td>
<td>4.7 ± 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Chloride, mM</strong></td>
<td>106.5 ± 1.0</td>
<td>98.0 ± 0.6&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td><strong>Bicarbonate, mM</strong></td>
<td>27.0 ± 0.4</td>
<td>33.2 ± 0.5&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td><strong>Urea, mM</strong></td>
<td>5.2 ± 1.0</td>
<td>4.5 ± 0.2</td>
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<tr>
<td><strong>Creatinine, µM</strong></td>
<td>42 ± 2</td>
<td>43 ± 2</td>
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<tr>
<td><strong>Aldosterone, nM</strong></td>
<td>49 ± 3</td>
<td>60 ± 8</td>
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<tr>
<td><strong>Creatinine clearance, ml/h</strong></td>
<td>72 ± 2</td>
<td>71 ± 4</td>
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<tr>
<td><strong>Urinary excretion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Water, ml/day</strong></td>
<td>12 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td><strong>Sodium, meq/day</strong></td>
<td>&lt;0.06&lt;sup&gt;†&lt;/sup&gt;</td>
<td>1.7 ± 0.1&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Potassium, meq/day</strong></td>
<td>0.8 ± 0.0</td>
<td>1.4 ± 0.0&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Urea, mmol/day</strong></td>
<td>8.0 ± 0.4</td>
<td>7.1 ± 1.0</td>
</tr>
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</table>

Values are means ± SE. L-NAME, nitro-L-arginine methyl ester. *P < 0.05; †urine Na concentration reported as <5 meq/l.

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### Table 2. C<sub>T</sub> values for RT-PCR in aldosterone-L-NAME escape

<table>
<thead>
<tr>
<th></th>
<th>Aldosterone/NaCl Restricted</th>
<th>Aldosterone/L-NAME/NaCl Replete</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>NHE3</strong></td>
<td>23.39 ± 0.1</td>
<td>23.55 ± 0.15</td>
</tr>
<tr>
<td><strong>NKCC2</strong></td>
<td>19.98 ± 0.09</td>
<td>20.05 ± 0.09</td>
</tr>
<tr>
<td><strong>β-Actin</strong></td>
<td>18.46 ± 0.06</td>
<td>18.42 ± 0.08</td>
</tr>
</tbody>
</table>

Values are means ± SE. NHE3, Na/H exchanger 3; NKCC2, Na-K-2Cl cotransporter.

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### Table 3. CT values for real-time RT-PCR

<table>
<thead>
<tr>
<th></th>
<th>Aldosterone/NaCl Replete</th>
<th>Aldosterone/L-NAME/NaCl Replete</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>NHE3</strong></td>
<td>100 ± 18</td>
<td>82 ± 11</td>
</tr>
<tr>
<td><strong>NKCC2</strong></td>
<td>100 ± 6</td>
<td>69 ± 11&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>β-Actin</strong></td>
<td></td>
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</table>

Fig. 6. Immunoblots assessing protein abundances of NHE3 and NKCC2 in homogenates from kidney cortex in control (aldosterone and L-NAME infused/NaCl restricted; left) and escape (aldosterone and L-NAME infused/NaCl replete; right) rats. Band densities were assessed by laser densitometry. *Significant difference.
higher than the groups. Serum aldosterone concentrations were much

Body weight and serum concentrations of small

Table 3. $C_T$ values for RT-PCR in aldosterone-infused/Na-replete rats with and without L-NAME

<table>
<thead>
<tr>
<th>Aldosterone/ NaCl Replete/Vehicle</th>
<th>Aldosterone/ NaCl Replete/L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>$n$</td>
<td>5</td>
</tr>
<tr>
<td>NHE3</td>
<td>24.26 ± 0.81</td>
</tr>
<tr>
<td>NKCC2</td>
<td>20.02 ± 0.09</td>
</tr>
<tr>
<td>β-Actin</td>
<td>18.31 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE.

In addition, we tested the effect of increasing NaCl intake from <0.1 to 2.0 meq/day in rats infused with L-NAME at 30 mg·kg body wt$^{-1}$·day$^{-1}$, mimicking the escape protocols described in Fig. 1 except that no aldosterone was used to stimulate NaCl retention. In this experiment, there were no significant changes in either NHE3 [NaCl restricted 100 ± 14 ($n = 6$); NaCl replete 110 ± 23 ($n = 6$), not significant] or NKCC2 [NaCl restricted 100 ± 13 ($n = 6$); NaCl replete 83 ± 11 ($n = 6$), not significant] in response to the increase in NaCl intake. Thus changes in salt intake in the presence of NOS inhibition appear to have little effect on NHE3 and NKCC2 in the absence of ECF volume expansion.

**DISCUSSION**

*NCC in aldosterone escape.* The kidney possesses escape mechanisms that resolve homeostatic conflicts occurring when hormones that regulate renal transport processes are secreted inappropriately. Examples include escape from the antidiuretic action of vasopres-
sin and escape from the antinatriuretic action of aldosterone. In vasopressin escape, the kidney can excrete large amounts of water despite high levels of circulating vasopressin through a marked suppression of collecting duct aquaporin-2 levels (9). In aldosterone escape, the kidney can excrete large amounts of NaCl despite high levels of aldosterone through a marked suppression of the expression level of NCC, the thiazide-sensitive NaCl cotransporter of the distal convoluted tubule (35). In the present study, we investigated mechanisms involved in aldosterone escape.

The approach used was to observe the response to an increase in NaCl intake in the presence of aldosterone infusion in rats (Fig. 1A). Despite high levels of circulating aldosterone, the rats were able to excrete the excess NaCl load (aldosterone escape). In this study, we confirmed that the aldosterone escape process was associated with a marked decrease in renal NCC protein abundance, which is presumably responsible for the increase in NaCl excretion. Furthermore, we demonstrated that a similar decrease in NCC abundance can be elicited in response to increased NaCl intake when an ANG II infusion is substituted for the aldosterone infusion (ANG II escape) (Fig. 4). The absence of a decrease in renal α-ENaC abundance in this ANG II escape experiment points to the conclusion that the decrease in NCC was not simply due to antagonism of aldosterone’s action or a reduction in renal interstitial aldosterone levels. Thus the escape process is not specific to aldosterone as the mediator of NaCl retention. Furthermore, we can conclude that the escape-associated suppression of renal NCC abundance is not mediated by a fall in ANG II levels because it occurred in the presence of an ANG II clamp, achieved through ANG II infusion. In addition, NOS inhibition via L-NAME infusion did not block the escape-associated suppression of NCC abundance (Fig. 5), leading to the conclusion that the suppression of NCC in aldosterone escape is not likely to be mediated by NO. In previous studies, Hall and colleagues (13, 14) demonstrated that both aldosterone escape and ANG II escape are responses to increased renal perfusion pressure (pressure natriuresis), although the specific mediator of the response has not been defined.

NHE3 in aldosterone escape. The main objective of this study was to investigate the mechanism of the increase in renal NHE3 abundance seen in aldosterone escape. NHE3 is an 83-kDa integral membrane protein that functions as a Na/H exchanger in the apical plasma membrane of a variety of transporting epithelia including the proximal tubule of the kidney (3). In the proximal tubule, it pairs with a chloride/formate exchanger (SLC26A6) (19) to mediate a large fraction of the total proximal tubule NaCl reabsorption. Working in a chloride/formate exchange-independent mode, NHE3 absorbs sodium bicarbonate from the lumen. Previous studies demonstrated that the escape-associated increase in NHE3 abundance is seen in the renal cortex, but not in the medulla, establishing that it occurs in the renal proximal tubule (35). Previous studies in rabbits demonstrated that mineralocorticoid escape induced with deoxycorticosterone is associated with an increase in fluid absorption (a measure of NaCl transport) in the S2 portion of the proximal tubule (22). Because the escape-associated increase in NHE3 abundance was also seen in ANG II escape (with clamped ANG II levels), it appears unlikely that ANG II is the mediator of the increase in NHE3. However, we found that administration of the NOS blocker L-NAME reproducibly blocked the escape-associated increase in renal NHE3 abundance, as evidenced from the analysis of both whole kidney (Fig. 5) and renal cortical (Fig. 6) samples. Separate experiments established that NOS inhibition with L-NAME significantly decreased the renal abundance of NHE3 in aldosterone-treated, NaCl-replete rats. Thus we conclude that the aldosterone escape-associated increase in NHE3 in the proximal tubule is likely mediated by NO and that NO has a long-term effect to increase the renal abundance of NHE3.

The latter conclusion is consistent with findings in prior studies. Previous studies established a role for NO in both short- and long-term regulation of proximal tubule sodium and bicarbonate absorption. For example, papers from Blantz and colleagues (8, 10) and Wang (32) showed by micropuncture that acute administration of NOS blockers in rats decreases proximal fluid and NaCl reabsorption. Long-term effects of NO in proximal tubule fluid and NaCl reabsorption have been established by Wang’s studies of mice in which specific isoforms of NOS have been knocked out (33, 34). These studies demonstrated that ablation of the iNOS gene or the nNOS gene, but not the eNOS gene, resulted in a marked decrease in proximal fluid and NaCl reabsorption. Our results showing a suppression of NHE3 expression after long-term NOS blockade with L-NAME are consistent with the observations in knockout mice. The lack of a similar effect of L-NAME in the absence of ECF volume expansion suggests that NO levels in the proximal tubule cells of rats may be too low in the absence of ECF volume expansion to realize a difference when L-NAME is administered. Interestingly, there were no changes in nNOS (NOS1) or iNOS (NOS2) protein abundance in aldosterone escape, although there was a significant increase in eNOS (NOS3) abundance in the cortex of rats undergoing aldosterone escape (Fig. 8).

NKCC2 in aldosterone escape. Aside from its ability to block the escape-associated increase in renal NHE3 abundance, L-NAME administration was found to uncover an escape-associated reduction in renal NKCC2 abundance (Figs. 5 and 6). NKCC2 mediates Na and Cl entry into thick ascending limb cells and is important to the process that concentrates the urine through countercurrent multiplication. Separate experiments established that NOS inhibition with L-NAME significantly decreased the renal abundance of NKCC2 in aldosterone-treated, NaCl-replete rats (Fig. 7). Previous evidence for an important role for NO in regulation of thick ascending limb transport has been obtained (28). These studies demonstrated that NO has a short-term effect to decrease thick ascending limb NaCl...
as absorption, but to our knowledge, studies of long-term effects of NO on thick ascending limb NKCC2 expression have not previously been reported.

**mRNA measurements.** The observed L-NAME-induced changes in NHE3 and NKCC2 protein abundance occurred in the absence of demonstrable changes in the corresponding mRNA levels. This points to a posttranscriptional mechanism of protein abundance regulation. Recent studies that combined oligonucleotide or cDNA arrays with proteomics methods in yeast (15) and in mammalian kidney (5) established that posttranscriptional regulation of protein abundance is a common event in eukaryotic cells. Two general mechanisms exist for posttranscriptional regulation of protein abundance, viz. translational regulation and regulation of protein half-life (20). The mechanism involved in posttranscriptional regulation of NHE3 and NKCC2 protein abundance in response to chronic L-NAME administration in this study remains unknown. In general, little information is available regarding the role of posttranscriptional regulatory processes in the control of NHE3 and NKCC2 activity. However, previous studies by Ambuhl et al. (1) demonstrated that glucocorticoids increase NHE3 abundance in a membrane fraction from cultured opossum kidney cells without a change in mRNA, possibly by translational regulation. Whether NO plays a role in this response has not been investigated.

**Sodium balance in L-NAME vs. vehicle infusion experiments.** L-NAME infusion markedly decreased NHE3 and NKCC2, effects that would predict increases in sodium excretion (Fig. 7). Therefore, at first glance, it may seem surprising that sodium excretion rate was not increased in these experiments (Table 4). Rats were maintained on ration feeding protocols, which ensure equal intake of NaCl and water in control vs. experimental groups. These studies were carried out for a long enough period to allow a steady state to be reached at which sodium output equals sodium intake. Presumably, compensatory changes occurred that would counter the effects of decreased NHE3 and NKCC2 abundance seen in response to L-NAME infusion. The implication is that L-NAME infusion may increase NaCl reabsorption in the distal nephron or collecting ducts, presumably by inhibiting NO production. Indeed, there is direct evidence from isolated, perfused tubule studies that NO has rapid effects to decrease Na absorption in isolated, perfused thick ascending limbs (28) and cortical collecting ducts (30), suggesting that L-NAME would increase sodium transport by inhibiting these responses. These considerations suggest that NO shifts NaCl absorption in a proximal direction along the nephron but does not necessarily alter NaCl excretion.

**Integrative interpretation.** In the normal kidney, sodium reabsorption in each nephron segment plays a different role (21), each of which contributes to homeostatic regulation of the balances of various solutes and water. For example, sodium reabsorption in the proximal tubule is important for acid-base balance through its role in bicarbonate absorption, is important in the retrieval of a variety of filtered substrates including glucose and amino acids, and is important in phosphate balance through sodium-coupled phosphate reabsorption. Sodium transport in the thick ascending limb of Henle is part of the urinary concentrating and diluting mechanism, and its regulation is geared to regulation of water excretion. Sodium transport in the distal convoluted tubule and collecting duct is regulated largely as a means of achieving fine regulation of sodium and potassium excretion under the control of aldosterone and vasopressin, enabling precise adjustments needed for day-to-day regulation of ECF volume. These distal regulatory mechanisms require a fairly invariant delivery of NaCl to the distal convoluted tubule, a process that is dependent on the tubuloglomerular feedback (TGF) mechanism at the macula densa (29) and on the ability of the thick ascending limb to adjust its NaCl absorption in response to variations of sodium delivery to it (6). In day-to-day renal function, when the kidney responds to mild perturbations of ECF fluid volume and blood pressure, these two powerful mechanisms isolate the proximal and distal sodium reabsorptive systems, allowing them to carry out their separate functions independently. Hence, regulation of sodium excretion is not normally a function of the proximal tubule. However, with more severe perturbations, the TGF and thick ascending limb buffer mechanisms can be overcome, allowing suppression of sodium transport in the proximal tubule to increase sodium excretion.

On the basis of the results of the present study, we propose the following scheme for escape from Na-retaining factors, which are presumably manifestations of general pressure-natriuresis mechanisms. We propose that pressure-natriuresis is a multifactorial process that involves all sodium-transporting segments: proximal tubule, thick ascending limb, distal convoluted tubule, and collecting duct. Intrinsic processes evoked during aldosterone escape cause decreases in NHE3 abundance (this paper; in the presence of NOS inhibition), NKCC2 abundance (this paper; in the presence of NOS inhibition), NCC abundance (Ref. 35 and this paper), and 70-kDa γ-ENaC form abundance (35), corresponding to downregulatory processes in each segment. However, with mild chronic changes in renal interstitial pressure in which distal mechanisms may suffice to lower systemic blood pressure, mechanisms may exist to counter the macula densa natriuretic processes, restoring the sodium-dependent transport rates to their normal state and maintaining glomerular filtration rate. We suggest, based on the findings of the present study, that NO-mediated increases in NHE3 and NKCC2 abundance serve this purpose. Thus we propose that increased intrarenal NO production maintains NaCl reabsorption in macula densa segments through increases in NHE3 and NKCC2 abundances, thereby maintaining the transport functions of the proximal tubule and thick ascending limb that are unrelated to regulation of NaCl balance.
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DISCLOSURES

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