Targeted Proteomic Profiling of Renal Na\(^+\) Transporter and Channel Abundances in Angiotensin II Type 1a Receptor Knockout Mice

Heddwen L. Brooks, Alicia J. Allred, Kathleen T. Beutler, Thomas M. Coffman, Mark A. Knepper

Abstract—The renal tubule transporters responsible for Na\(^+\) and water transport along the nephron have been identified and cloned, permitting comprehensive analysis of transporter protein abundance changes in complex physiological models by using a “targeted proteomics” approach. Here, we apply this approach to screen renal homogenates from mice in which the gene for the angiotensin II type 1a (AT\(_{1a}\)) receptor has been deleted (versus wild-type mice) to determine which sodium transporters and channels are regulated by the AT\(_{1a}\) receptor at the protein abundance level. In mice maintained on a low NaCl diet (<0.02% NaCl), (1) the abundances of 2 aldosterone-regulated transporters were markedly decreased in knockout versus wild-type mice, namely, the thiazide-sensitive cotransporter and the \(\alpha\)-subunit of the amiloride-sensitive Na\(^+\) channel (\(\alpha\)-ENaC); (2) the abundances of \(\beta\)-ENaC and \(\gamma\)-ENaC were markedly increased; and (3) there were no significant changes in the abundances of the proximal tubule Na\(^+\)+H\(^+\) exchanger or the Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter of the thick ascending limb. When the experiment was repeated on higher NaCl diets (0.4% or 6% NaCl), the decrease in \(\alpha\)-ENaC abundance persisted, whereas the other changes were abolished. Analysis of serum aldosterone concentration in AT\(_{1a}\) knockout mice and wild-type mice on the low NaCl diet revealed the absence of a decrease with AT\(_{1a}\) gene deletion (11.8±2.3 nmol/L for knockout mice and 5.7±0.8 nmol/L for wild-type mice [significantly increased]). These results reveal that the AT\(_{1a}\) receptor plays an important role in regulation of Na\(^+\) transporter and channel proteins in the “post–macula densa” region of the renal tubule via a mechanism that is not dependent on altered circulating aldosterone concentrations. (Hypertension. 2002;39[part 2]:470-473.)

Key Words: sodium ■ angiotensin II ■ receptors, angiotensin II ■ kidney ■ mice

The Na\(^+\) transporters and channels that mediate Na\(^+\) reabsorption in each renal tubule segment were initially identified via micropuncture and isolated perfused tubule experiments during the second half of the 20th century. Over the past 10 years, complementary DNAs for all of the major Na\(^+\) transporters and channels expressed along the renal tubule have been cloned and sequenced (Table). That work has allowed us to produce a comprehensive set of polyclonal antibodies, which are now being used in multiplex fashion (targeted proteomics) for analysis of renal adaptation of Na\(^+\) transport in complex physiological and pathophysiological models in rats and mice.\(^{1-6}\) The approach used assesses abundance changes for each renal tubule transporter protein in response to a systemic physiological perturbation. For example, we have been able to profile Na\(^+\) transporter changes along the renal tubule in response to increases in circulating levels of aldosterone\(^{2,3}\) and vasopressin,\(^{5,7}\) circulating substances that are generally recognized as important regulators of Na\(^+\) and water balance. Another key element of the regulation of Na\(^+\) and water balance has not yet been investigated with this technique, namely, angiotensin II. It is generally recognized that angiotensin II plays a central role in arterial blood pressure regulation through a variety of actions at the level of the vascular smooth muscle, the brain, the renal glomerulus, and the renal tubule. Micropuncture and isolated perfused tubule studies have established a clear role for angiotensin II in the regulation of NaCl absorption in the proximal tubule\(^{8,9}\) and the thick ascending limb,\(^{10-12}\) but a role in the distal renal tubule segments located beyond the site of tubuloglomerular feedback (the macula densa) is less clear. Most renal tubular effects of angiotensin II are believed to be mediated by the angiotensin II type 1 (AT\(_1\)) receptor. In the present study, we use a well-characterized gene-deletion model, the angiotensin II type 1a (AT\(_{1a}\)) receptor knockout mouse\(^{13}\) to identify the sites of long-term action of angiotensin II to regulate Na\(^+\) transporter and Na\(^+\) channel protein abundance in the kidney.

Methods

Animals

Mice lacking AT\(_{1a}\), receptors for angiotensin II were generated by homologous recombination in embryonic stem cells, as previously
Major Na\textsuperscript{+} Transporter and Na\textsuperscript{+} Channel Proteins in Kidney: Quantitative Summary of Results

<table>
<thead>
<tr>
<th>Name</th>
<th>Identification</th>
<th>Location</th>
<th>Low Na\textsuperscript{+} Diet†</th>
<th>Intermediate Na\textsuperscript{+} Diet†</th>
<th>High Na\textsuperscript{+} Diet†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE3</td>
<td>Type 3 Na\textsuperscript{+}-H\textsuperscript{-} exchanger</td>
<td>PT (apical)</td>
<td>79%\textpm%13</td>
<td>128%\textpm%8</td>
<td>80%\textpm%20</td>
</tr>
<tr>
<td>NKCC2</td>
<td>Type 2 Na\textsuperscript{+}-K\textsuperscript{-}-2Cl\textsuperscript{-} cotransporter</td>
<td>TAL (apical)</td>
<td>80%\textpm%10</td>
<td>80%\textpm%4</td>
<td>116%\textpm%29</td>
</tr>
<tr>
<td>NCC</td>
<td>Na\textsuperscript{+}-Cl\textsuperscript{-} cotransporter</td>
<td>DCT (apical)</td>
<td>56%\textpm%6†</td>
<td>112%\textpm%11</td>
<td>89%\textpm%5</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>α-Subunit of epithelial Na\textsuperscript{+} channel</td>
<td>CNT, CD (apical)</td>
<td>36%\textpm%3‡</td>
<td>54%\textpm%19†</td>
<td>24%\textpm%6‡</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>β-Subunit of epithelial Na\textsuperscript{+} channel</td>
<td>CNT, CD (apical)</td>
<td>170%\textpm%28†</td>
<td>136%\textpm%21</td>
<td>84%\textpm%11</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>γ-Subunit of epithelial Na\textsuperscript{+} channel</td>
<td>CNT, CD (apical)</td>
<td>217%\textpm%42‡</td>
<td>136%\textpm%20</td>
<td>87%\textpm%9</td>
</tr>
<tr>
<td>Na\textsuperscript{+},K\textsuperscript{-}-ATPase</td>
<td>Na\textsuperscript{+},K\textsuperscript{-}-ATPase, α\textsubscript{1}-subunit All segments (basolateral)</td>
<td></td>
<td>85%\textpm%13</td>
<td>119%\textpm%10</td>
<td>71%\textpm%12</td>
</tr>
</tbody>
</table>

†Band density for Agtr1a\textsuperscript{a/-} mice, expressed as percentage of Agtr1a\textsuperscript{+/+} control; for mice on 0.02%, 0.4%, and 6% Na\textsuperscript{+} diets, indicating low, intermediate, and high Na\textsuperscript{+}, respectively.

‡P<0.05 vs no change (100\% of Agtr1a\textsuperscript{+/+} control).

Sample Preparation, SDS-PAGE, and Immunoblotting

Full details of sample preparation, SDS-PAGE, and immunoblotting procedures are given in prior studies.\textsuperscript{1–4} In brief, whole kidneys were homogenized in 1 mL ice-cold isolation solution (250 mmol/L sucrose and 10 mmol/L Triethanolamine [pH 7.6] containing 1 μmol/L leupeptin and 0.1 mg/mL phenylmethylsulfonyl fluoride) by use of a tissue homogenizer (Omni 1000 fitted with a micro-sawtooth generator) at maximum speed for three 15-second intervals. Total protein concentrations were measured (BCA kit, Pierce Chemical Co), and the samples were solubilized in Laemml sample buffer at 60°C for 15 minutes.

Semi-quantitative immunoblotting was carried out as described in a previous study\textsuperscript{1} to assess the relative abundances of the proteins of interest. To confirm that protein loading of the gels was equal, preliminary 12% polyacrylamide gels were stained with Coomassie blue, as previously described.\textsuperscript{1} Densitometry (Personal Densitometer SI, Molecular Dynamics) was performed on representative bands to ensure equal loading (generally, <5\% variation relative to the mean). For immunoblots, protein loading amounts were as follows (μg): NHE3 5, NKCC2 10, NCC 20, all ENaC subunits 25, and Na\textsuperscript{+},K\textsuperscript{-}-ATPase 2. Proteins were separated on 7.5, 10, or 12% polyacrylamide gels by SDS-PAGE, and the proteins were transferred to nitrocellulose membranes electrophoretically (Bio-Rad Mini Trans-Blot Cell). Membranes were blocked for 1 hour at room temperature with 5% non-fat dry milk and probed overnight at 4°C with the respective primary antibodies. Membranes were washed and exposed to secondary antibodies (goat anti-rabbit IgG conjugated to horseradish peroxidase, Pierce No. 31453; rabbit anti-mouse IgG conjugated to horseradish peroxidase, Pierce No. 31450; both were diluted either 1:5000 or 1:5000) for 1 hour at room temperature. After they were washed, the bands were visualized by using a luminol-based enhanced chemiluminescence substrate (LumiGLO, Kirkegaard and Perry Laboratories). Band densities were determined by laser densitometry (Personal Densitometer SI, Molecular Dynamics).

Statistical Analysis

Quantitative data are presented as mean±SEM. Densitometric data (expressed as a ratio of experimental to control values) were subjected to a logarithmic transformation before statistical analysis. Statistical comparisons were accomplished by unpaired t test. A value of P<0.05 was considered to be statistically significant.

Results

Many of the physiological characteristics of AT\textsubscript{1a} knockout mice on the diets used in the present study have been previously published (eg, see previous studies\textsuperscript{13–15}). On a low NaCl diet (<0.02% NaCl) or an intermediate NaCl diet (0.4% NaCl), Agtr1a\textsuperscript{a/-} mice exhibited lower blood pressures than
did Agtr1a+/+ mice, whereas a high NaCl diet (6% NaCl) diminished the differences in blood pressure between Agtr1a−/− and Agtr1a+/+ mice without fully abolishing it. Measurements of Na+ balance revealed a negative balance after shifting to the low NaCl diet in Agtr1a−/− mice that was not seen in the Agtr1a+/+ mice. In the present study, using a previously described nephron-profiling method, we investigated whether the decreased blood pressure in Agtr1a−/− mice on the low NaCl diet was associated with a decrease in the abundance of any of the proteins that mediate Na+ transport along the renal tubule. Figure 1 shows results in Agtr1a−/− mice versus Agtr1a+/+ mice fed the low NaCl diet (0.02% NaCl) for 7 days, and the Table shows densitometric analysis of these data. Statistically significant decreases in band density were seen for 2 transporter proteins, both of which are expressed in segments beyond the macula densa: the thiazide-sensitive Na+/Cl− cotransporter, NCC, and the α-subunit of the amiloride-sensitive Na+ channel, ENaC. The former is expressed in the distal convoluted tubule, and the latter is expressed in the collecting duct. These 2 segments reabsorb 7% to 10% of the filtered load of Na+, and decreased transport in these segments in Agtr1a−/− mice could potentially account in part for the tendency for these mice to waste NaCl. In addition, there was a significant increase in the abundances of the β- and γ-subunits of ENaC in Agtr1a−/− mice. These changes in β- and γ-ENaC cannot account for salt losing in the Agtr1a−/− mice but are consistent with previously observed tendencies toward reciprocal regulation of the ENaC subunits (M.A.K., unpublished data, 2001). As previously seen in rats, γ-ENaC was seen as 2 bands (85 and 70 kDa), the lower of which is thought to be a consequence of a physiological proteolytic cleavage of an extracellular portion of the molecule induced by aldosterone. As can be seen, the amount of the 70-kDa protein is variable and seemingly unrelated to the gene deletion. As expected, the amount of the 70-kDa form of the protein was decreased with higher NaCl intakes (compare Figures 2 and 3). Two other transporters, namely, NHE3 and NKCC2, which are likely pre-macula densa sites of regulation by angiotensin II, did not show differences in absolute abundance, indicating that regulation of these transporters by angiotensin II may involve other mechanisms, such as protein trafficking. Finally, there...
was no difference in the total abundance of the α1-subunit of the Na\(^+\),K\(^+\)-ATPase between Agtr1a\(^{-/-}\) and Agtr1a\(^{+/+}\) mice. This isoform is expressed in all renal tubule segments.

Figure 2 shows the Na\(^+\) transporter profiling results for mice on a diet with an intermediate level of NaCl (0.4% NaCl diet), and Figure 3 shows data for mice on a high level of NaCl (6% NaCl diet). The renin-angiotensin system can be expected to play a lesser role in the regulation of renal tubule Na\(^+\) reabsorption with these higher levels of NaCl intake than with the very low levels of NaCl intake. In line with that expectation (Table), the abundance of distal convoluted tubule transporter NCC was unchanged in Agtr1a\(^{-/-}\) mice versus Agtr1a\(^{+/+}\) mice on these diets. Furthermore, the increases in β- and γ-ENaC seen in the low NaCl diet experiment were not seen with higher NaCl intakes. In contrast, the decrease in the abundance of α-ENaC in the low NaCl diet experiment was observed in mice receiving higher NaCl intakes (Table). Previous studies have established that Agtr1a\(^{-/-}\) mice fed these higher levels of NaCl exhibit higher blood pressures than when they are fed the low NaCl diet, although blood pressure did not reach the level seen in Agtr1a\(^{+/+}\) mice on any of the 3 diets. These results are compatible with the results of previous studies showing that ENaC is an important determinant of blood pressure and that α-ENaC abundance is rate limiting for the assembly of functional ENaC complexes. The difference in salt sensitivity of the regulation of NCC abundance versus α-ENaC abundance in Agtr1a\(^{-/-}\) mice suggests that factors other than the expression of the AT\(_2\) receptor play differential roles in the regulation of the two Na\(^+\) transport proteins.

One potential explanation for the effect of Ang II gene deletion on NCC and ENaC subunit abundance with dietary NaCl restriction is that circulating aldosterone levels may be affected. Aldosterone has been demonstrated to increase the abundance of Na\(^+\)–K\(^+\)–2Cl\(^-\) cotransporter expression in thick ascending limb of Henle’s loop. Ang II decreases in NCC and ENaC subunit abundances seen in Agtr1a\(^{-/-}\) mice are due to loss of the actions of aldosterone normally seen in the setting of dietary NaCl restriction. However, previous measurements had established that aldosterone excretion was not decreased but rather tended to be increased in Agtr1a\(^{-/-}\) mice versus Agtr1a\(^{+/+}\) mice when they were fed the low NaCl diet. To readdress the role of aldosterone, we studied an additional set of Agtr1a\(^{-/-}\) mice and Agtr1a\(^{+/+}\) mice maintained on the very low NaCl diet (<0.02% NaCl). In line with the previous measurements of aldosterone excretion, we found that the plasma aldosterone levels in the Agtr1a\(^{-/-}\) mice were increased rather than decreased relative to values in Agtr1a\(^{+/+}\) mice (plasma aldosterone values were as follows: for Agtr1a\(^{-/-}\) mice, 11.8 ± 2.3 nmol/L; for Agtr1a\(^{+/+}\) mice, 5.7 ± 0.8 nmol/L; \(P<0.05\)). Thus, the decreases in NCC and ENaC subunit abundances seen in the Agtr1a\(^{-/-}\) mice versus the Agtr1a\(^{+/+}\) mice cannot be attributed to a decrease in circulating aldosterone levels. It appears possible that angiotensin II has a direct effect on distal convoluted tubule cells and collecting duct principal cells to regulate these transporters. One other possible explanation not explored in the present study is that the expression or activity of type 2 11β-hydroxysteroid dehydrogenase in distal convoluted tubule and collecting duct principal cells may be increased in the Agtr1a\(^{-/-}\) mice. This enzyme normally degrades glucocorticoids (corticosterone in mouse) in post–macula densa regions of the renal tubule, limiting the ability of glucocorticoid to bind to and activate mineralocorticoid receptors in these cells. Thus, changing 11β-hydroxysteroid dehydrogenase activity in these cells would be expected to mimic the effect of changing aldosterone levels.

References
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