Kidney function in mice lacking aldosterone

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Makhanova, Natalia, Gene Lee, Nobuyuki Takahashi, Maria L. Sequeira Lopez, R. Ariel Gomez, Hyung-Suk Kim, and Oliver Smithies. Kidney function in mice lacking aldosterone. Am J Physiol Renal Physiol 290: F61–F69, 2006. First published August 23, 2005; doi:10.1152/ajprenal.00257.2005.—To explore the effects of decreased amounts or absence of aldosterone, we have disrupted the gene coding for aldosterone synthase (AS) in mice and investigated blood pressure and kidney function in AS+/+, AS−/−, and AS−/− mice. AS−/− mice have normal blood pressures and show no abnormalities in electrolytes or kidney gene expression, but they have significantly higher than normal urine volume and lower urine osmolality. In contrast, the AS−/− mice have low blood pressure, abnormal electrolyte homeostasis (increased plasma concentrations of K+, Ca2+, and Mg2+ and decreased concentrations of HCO3− and Cl− but no difference in the plasma Na+ level), and disturbances in water metabolism (higher urine output, decreased urine osmolality, and impaired urine concentrating and diluting ability). Absence of aldosterone in the AS−/− mice induced several compensatory changes: an increased food intake-to-body weight ratio, an elevated plasma concentration of glucocorticoids, and strong activation of the renin-angiotensin system. Parallel with the markedly increased synthesis and release of renin, the AS−/− mice showed increased expression of cyclooxygenase-2 (COX-2) in macula densa. On salt supplementation, plasma electrolyte concentrations and kidney renin and COX-2 levels became similar to those of wild-type mice, but the lower blood pressure of the AS−/− mice was not corrected. Thus absence of aldosterone in AS−/− mice results in impairment of Na+ reabsorption in the distal nephron, decreased blood pressure, and strong renin-angiotensin system activation. Our data show the substantial correction of these abnormalities, except the low blood pressure, by high dietary salt does not depend on aldosterone.

aldosterone synthase; blood pressure; electrolytes; renin; cyclooxygenase-2

THE MOST IMPORTANT FUNCTION of aldosterone is in control of fluid and electrolyte homeostasis. In the kidney, aldosterone-dependent regulation of Na+ reabsorption and K+ secretion takes place in the aldosterone-sensitive distal nephron, which includes the second half of the distal convoluted tubule (DCT), the connecting tubule (CNT), and the cortical and outer and inner medullary collecting ducts (22). The effectors of the response to aldosterone are the amiloride-sensitive epithelial Na+ channel (ENaC), the K+ channel (ROMK), and the serosal Na+–K+–ATPase, with the Na+–K+–ATPase pump providing the electrochemical driving force necessary for the luminal entry of Na+ and exit of K+. The limiting step in Na+ reabsorption appears to be the amiloride-sensitive ENaC. However, the luminal thiazide-sensitive Na+–Cl− cotransporter (NCC) is also an important effector (14). ENaC, the mineralocorticoid receptor (MR), and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), a key enzyme for aldosterone selectivity of MR, are localized in the terminal part of the DCT, the CNT, and the collecting duct in mice. The thiazide-sensitive NCC is localized in the proximal and distal segments of the DCT.

Several aldosterone-sensitive genes have recently been identified, including a serum and glucocorticoid-inducible kinase (Skg), the small monomeric Kirsten Ras GTP-binding protein, and the corticosteroid hormone-induced factor. Skg has received the most attention (36); it appears to phosphorylate other proteins involved in trafficking of ENaC into and retrieval from the cell membrane. Recently, binding of ubiquitin to specific membrane proteins has been shown to lead to their internalization and degradation by proteosomes. For example, ubiquitin ligase Nedd4–2 binds specifically to the “PY domain” at the COOH-terminal region of all three ENaC subunits, and Snyder et al. (34) have shown that Skg binds to and phosphorylates Nedd4–2, which reduces its activity.

The actions of aldosterone are mediated through the MR, which is a nuclear receptor. MR knockout mice die between 8 and 13 days after birth, with reduced weight and severe dehydration due to failure to reabsorb Na+. At day 8 they show hypotremia, hyperkalemia, high renal salt wasting, and a strongly activated renin-angiotensin system (RAS; see Ref. 2), thus demonstrating the central role of MR in aldosterone action.

Aldosterone synthase (AS) deficiency in humans is a rare autosomal recessively inherited disorder caused by mutations in the AS gene (CYP11B2). AS catalyzes the last step in the synthesis of aldosterone, which involves 11β-hydroxylation of 11-DOC to form corticosterone, 18-hydroxylation of corticosterone to form 18-hydroxyxycorticosterone, and oxidation at position C18 to give aldosterone. There are two forms of AS deficiency: corticosterone methylxidase AS deficiency types I and II (CMO I and CMO II; see Ref. 39). CMO I is characterized by decreased levels of 18-hydroxyxycorticosterone, whereas CMO II is characterized by increased levels of this steroid. In both syndromes, aldosterone biosynthesis is impaired and may result in Na+ loss, but the symptoms decline with age (29, 40). Patients with CMO I and CMO II deficiency are most often diagnosed because of hyperkalemia, metabolic acidosis, and markedly elevated plasma renin activity (29).

To study the effects of decreased amounts or absence of aldosterone, we have disrupted the gene coding for AS in mice.

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In a previous paper (21), we described the generation of mice lacking AS and the homeostatic responses that resulted in their adrenal glands. Here we describe the regulation of blood pressure, electrolyte homeostasis, and kidney function in AS\(^{+/+}\), AS\(^{+-}\), and AS\(^{-/-}\) mice.

**MATERIALS AND METHODS**

**Mice.** The AS null mice were generated by gene targeting in strain 129/ES cells, as described previously (21). Male chimeras were mated with 129/SvEv females to derive coisogenic offspring. Wild-type mice (AS\(^{+/+}\)) and heterozygous (AS\(^{+-}\)) and homozygous (AS\(^{-/-}\)) littersmates were used in this study. Mice were fed normal mouse chow (0.8% NaCl) or a high-salt diet (8% NaCl, Harlan Teklad, Madison, WI). All animals had free access to food and water. All experiments were conducted with female and male mice \(\sim 3–4\) mo old and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina, Chapel Hill.

**Blood pressure measurement.** Blood pressures were measured in conscious mice with a computerized tail-cuff system (Visitech Systems, Cary, NC; see Ref. 19).

**Blood analysis.** Blood was drawn under anesthesia from the retro-orbital sinus, and plasma electrolyte concentrations were measured using a VT250 Chemical Analyzer (Orthodiagnostic Clinical). Plasma aldosterone was measured with the Coat-A-Count RIA procedure (Diagnostic Products, Los Angeles, CA). Plasma corticosterone was measured with an RIA kit (ICN Biomedicals, Palo Alto, CA).

**Histological analysis.** Organs were fixed in 4% buffered paraformaldehyde overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, periodic acid-Schiff, or Masson-Trichrome for light microscopy.

**Renin and COX-2 immunostaining in kidney.** The immunohistochemical detection of renin was performed as described previously (17) by incubating deparaffinized kidney sections with a polyclonal renin antibody (1:10,000; gift from Dr. Tadashi Inagami, Vanderbilt University, Nashville, TN). For cyclooxygenase (COX)-2 immunostaining, frozen sections (5 \(\mu\)m) of kidney were incubated with primary antibody (COX-2; Cayman Chemicals) at 4°C overnight. Sections were then washed, and biotinylated secondary antibody was applied, followed by avidin-biotin complex (Vector Laboratories) with diaminobenzidine as the chromogen.

**Real-Time RT-PCR.** Expression of genes in the kidney was determined by quantitative real-time PCR with an Applied Biosystems 7700 Sequence Detection System (Perkin-Elmer), as described (16). RNA was isolated from tissue with ABI Prism 6700 automated nucleic acid work station. Relative levels of gene expression are expressed as percentage of wild type.

**Analyses of urine and kidney function.** Mice were maintained on a 12:12-h light-dark cycle with water and food ad libitum. To estimate 24-h water and food intake, urine volume, and excretion of electrolyte, mice were housed in metabolic cages for 3 days. Urine osmolality was determined by freeze-drying the urine. Urine electrolytes and creatinine were measured using a VT250 Chemical Analyzer. To determine the ability of mice to concentrate urine, they were deprived of water for 20 h. To test the ability of the kidney to dilute urine, mice were given water (4% body wt) by gavage (25). Creatinine clearance was used as a measure of glomerular filtration rate (13). To detect ketones in urine, we used Boehringer Mannheim Chemstrip (Boehringer Mannheim Diagnostics, Indianapolis, IN).

**Statistical analysis.** All statistical analyses were performed using JMP statistical Software (SAS Institute, Cary, NC), and are presented as means \(\pm\) SE. Statistical significances were assessed with ANOVA, and post hoc analyses were performed using the unpaired \(t\)-test.

**RESULTS**

**Body and kidney weights and blood data in AS\(^{+/+}\), AS\(^{+-}\), and AS\(^{-/-}\) mice.** The body weights of the AS\(^{-/-}\) homozygous mice were significantly lower than their heterozygous and wild-type littermates (Table 1). The gross weights of the kidneys were significantly reduced in the AS\(^{-/-}\) mice compared with AS\(^{+/+}\) and AS\(^{+-}\) mice, but the ratio of kidney weight to body weight was not significantly different in mice of the three genotypes, indicating that the absence of aldosterone causes a general rather than organ-related impairment of growth. Food intake per 20 g body wt was increased in the homozygous AS null mice compared with wild-type mice (\(P < 0.001\)) and heterozygous (\(P < 0.05\)) mice. However, the heterozygous mice also consumed more food per 20 g body wt than wild-type mice (Table 1).

Blood data showed that the heterozygotes and the homozygous AS null mice did not differ significantly from the wild type in their hematocrits, but the homozygous null animals had increased total plasma protein concentrations (Table 1). There were no differences in the plasma glucose concentration among the three genotypes. Plasma aldosterone was not detectable in the AS\(^{-/-}\) mice. The concentration of aldosterone in the plasma of the heterozygotes was not significantly different from the wild type. Absence of aldosterone in the AS\(^{-/-}\) mice resulted in an increased level of corticosterone compared with the wild type (\(P < 0.05\); Table 1). The concentration of corticosterone in the heterozygous mice was intermediate be-

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**Table 1. Body and kidney weights and blood data in AS\(^{+/+}\), AS\(^{+-}\), and AS\(^{-/-}\) mice on a normal-salt diet**

<table>
<thead>
<tr>
<th></th>
<th>AS(^{+/+})</th>
<th>AS(^{+-})</th>
<th>AS(^{-/-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>25.1 ± 0.7</td>
<td>24.6 ± 1.1</td>
<td>20.3 ± 1.1</td>
</tr>
<tr>
<td>Kidney weight, g</td>
<td>0.35 ± 0.03</td>
<td>0.32 ± 0.02</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Kidney weight, g/20 g body wt</td>
<td>0.27 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>Food intake, g/20 g body wt</td>
<td>3.01 ± 0.14</td>
<td>3.43 ± 0.12</td>
<td>3.90 ± 0.19</td>
</tr>
<tr>
<td>Blood chemistry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>53.0 ± 2.2</td>
<td>51.5 ± 3.8</td>
<td>54.9 ± 1.9</td>
</tr>
<tr>
<td>Protein, g/dl</td>
<td>5.8 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>212 ± 19</td>
<td>214 ± 12</td>
<td>223 ± 8</td>
</tr>
<tr>
<td>Aldosterone, pg/ml</td>
<td>604 ± 130</td>
<td>515 ± 70</td>
<td>ND</td>
</tr>
<tr>
<td>Corticosterone, ng/ml</td>
<td>51 ± 11</td>
<td>67 ± 16</td>
<td>79 ± 6</td>
</tr>
</tbody>
</table>

Values are means \(\pm\) SE; no. of animals is in parentheses. AS, aldosterone synthase; ND, not detectable. *\(P < 0.05\) vs. AS\(^{+/+}\) mice. #\(P < 0.01\) vs. AS\(^{+/+}\) mice. \#\(P < 0.05\) vs. AS\(^{+-}\) mice. \#\(P < 0.001\) vs. AS\(^{+/+}\) mice.
between those of the wild-type and homozygous null mice, although the difference did not reach significance.

**Abnormal electrolyte levels in AS−/− mice.** To determine the effects of absence of aldosterone on electrolyte homeostasis, we measured plasma and urine electrolytes in the AS+/+, AS−/−, and AS−/− mice. There were no differences in the plasma Na⁺ concentration and urinary excretion of Na⁺ among the three genotypes on a normal salt diet (Table 2). However, there were significant differences in the concentrations of K⁺, Ca²⁺, Mg²⁺, and Cl⁻ in plasma in the homozygous null mice compared with wild-type and heterozygous mice. The heterozygous mice showed no significant differences from the wild type.

The AS−/− mice had a significantly higher plasma K⁺ concentration than the heterozygous (P < 0.05) and wild-type mice (P < 0.01). However, urinary excretion of K⁺ was similar in three genotypes (Table 2). The concentration of Cl⁻ in plasma in the AS−/− mice was lower than in AS+/+ and AS−/− mice (P < 0.05; Table 2). The AS−/− mice also had higher concentrations of Ca²⁺ and Mg²⁺ in plasma than AS+/+ and AS−/− mice. There were no differences in the plasma lactate and phosphate concentrations among the three genotypes on a normal salt diet. The pH of plasma and the plasma concentration of HCO₃⁻ were both decreased in AS null mice compared with AS+/+ mice (Table 2). Tests for ketones in urine were negative in all genotypes (Table 2).

In comparison, plasma electrolytes of AS+/− mice were not different from wild-type mice.

**Blood pressure.** The blood pressure of the AS−/− mice (96 ± 2 mmHg) was significantly lower than that of wild type (110 ± 2 mmHg, P < 0.0001) and AS−/− (107 ± 2 mmHg) mice (Fig. 1A).

**RAS.** The AS null mice had extremely strong activation of the RAS, as judged by their ~40 times normal plasma renin concentration (1.29 ± 0.24 ng ANG I·ml⁻¹·h⁻¹) compared with AS+/+ mice, 33 ± 8 ng ANG I·ml⁻¹·h⁻¹, P < 0.001; Fig. 1B). The concentration of ANG II in the plasma of the AS null mice (135 ± 37 pg/ml) was approximately four times higher than in wild-type mice (38 ± 13 pg/ml, P < 0.05; Fig. 1C). AS−/− heterozygous mice, on the other hand, had normal plasma concentrations of renin (40 ± 8 ng ANG I·ml⁻¹·h⁻¹) and ANG II (40 ± 11 pg/ml; Fig. 1, B and C).

**Kidney function.** To further characterize the impact of absence of aldosterone on kidney function, we used creatinine clearance as a measure of glomerular filtration rate and found no significant differences in creatinine clearance between the genotypes (Table 2). However, as shown in Fig 2A, the 24-h urine volume per 20 g body wt of the AS−/− mice was approximately two times more than that of wild-type mice (P < 0.0001), with the heterozygotes being intermediate at ~1.3 times the wild type (P < 0.001). Correspondingly, the AS null mice consumed significantly more water per 20 g body weight.
urine osmolality was increased in all three genotypes, although significantly different from the wild type (P < 0.0001, Fig. 2B). Urine osmolality in the homozygous null mice on a normal salt intake was significantly less than in wild-type mice (P < 0.0001), with the heterozygotes again being intermediate although not significantly different from the wild type (P = 0.057; Fig. 2C).

To determine the ability of mice to concentrate urine, they were deprived of water for 20 h. After this water deprivation, urine osmolality was increased in all three genotypes, although osmolalities in the AS−/− mice (P < 0.0001 vs. wild type) and the AS+/− mice (P < 0.05 vs. wild type) were still less than in the wild type. The ability of AS−/− mice to dilute water was also impaired (P < 0.05 vs. wild type; Fig. 2D).

RNA studies in the kidney. To characterize the kidney molecular phenotype in the AS+/+, AS+/−, and AS−/− mice, we used quantitative RT-PCR to determine gene expression. In agreement with the strong RAS activation detected in plasma, the renin mRNA level in the AS−/− kidney was ~12 times the level in wild-type mice (P < 0.0001) and ~7 times the level in heterozygotes (P < 0.0001). Renin mRNA in the heterozygotes was ~1.5 times that in wild-type mice, although the difference did not reach significance (P = 0.12; Table 3).

Kidney MR mRNA in the AS−/− mice on a normal salt diet was not different from AS+/+ mice. However, the increased level of glucocorticoid observed in AS−/− mice (Table 1) was accompanied by a modest decrease in the mRNA of glucocorticoid receptors to ~2/5 of that in AS+/+ and AS+/− mice. Glucocorticoids are selectively inactivated by 11β-HSD2 in aldosterone-sensitive tissues, and we found that the level of 11β-HSD2 mRNA in the AS−/− mice was increased on a normal salt diet to ~1.5 that of the wild type. The level of 11β-HSD2 mRNA, which converts inactive glucocorticoids to their active form, was not significantly decreased in the AS−/− and AS+/− mice, although there was a downward trend.

The abundance of mRNA for the aldosterone-sensitive gene Sgk, the α-, β-, and γ-subunits of ENaC, Na+/K+−2Cl− cotransporter, and Na+/H+ exchanger type 3 in the kidney were unaffected by the absence of aldosterone. However, the mRNA level of NCC was decreased in AS null mice to ~75% of the wild type. In agreement with the observations by Wald et al. (41) that cortical ROMK expression is regulated by aldosterone and K+, the level of ROMK mRNA in the kidney

| Table 3. mRNA levels in the kidney of AS+/+, AS+/−, and AS−/− mice on normal salt diet |
|------------------------------------------|------------------|------------------|
| Renin | 100±7 (19) | 157±36 (18) | 1,171±109* (20) |
| MR | 100±7 (19) | 95±6 (18) | 83±7 (20) |
| GR | 100±8 (19) | 97±10 (18) | 66±5* (20) |
| 11β-HSD1 | 100±7 (19) | 98±8 (18) | 82±12 (20) |
| 11β-HSD2 | 100±8 (19) | 120±12 (18) | 145±6* (20) |
| Sgk | 100±8 (19) | 114±16 (18) | 97±10 (20) |
| α-ENaC | 100±9 (19) | 99±4 (17) | 111±8 (20) |
| β-ENaC | 100±6 (19) | 108±11 (18) | 113±9 (20) |
| γ-ENaC | 100±7 (19) | 93±8 (18) | 117±8 (20) |
| NCC | 100±5 (19) | 91±8 (18) | 76±5 (20) |
| NKC2 | 100±7 (20) | 105±7 (17) | 104±6 (20) |
| NHE3 | 100±6 (20) | 98±7 (16) | 97±5 (20) |
| ROMK (cortex) | 100±15 (9) | 82±13 (9) | 59±10 (8) |
| COX-1 (cortex) | 100±16 (9) | 102±12 (9) | 105±16 (9) |
| COX-2 (cortex) | 100±15 (9) | 122±19 (9) | 539±91* (9) |

Values (wild type as 100%) are means ± SE; no. in parentheses is no. of animals. MR, mineralocorticoid receptor; GR, glucocorticoid receptor; 11β-HSD, 11β-hydroxysteroid dehydrogenase; Sgk, serine- and glucocorticoid-inducible kinase; ENaC, epithelial Na+ channel; NCC, Na+/Cl− cotransporter; NKCC, Na+/K+−2Cl− cotransporter; NHE, Na+/H+ exchanger; COX, cyclooxygenase. *P < 0.05 vs. AS+/+ mice. **P < 0.01 vs. AS+/+ mice. ***P < 0.001 vs. AS+/+ mice. "P < 0.0001 vs. AS+/+ mice. aP < 0.0001 vs. AS+/− mice. bP < 0.0001 vs. AS+/− mice. cP < 0.05 vs. AS+/− mice. dP < 0.0001 vs. AS+/− mice. eP < 0.0001 vs. AS−/− mice. fP < 0.0001 vs. AS−/− mice.
cortex of AS null mice was 59% of the wild type ($P < 0.05$, Table 3).

COX-2-derived prostaglandins in the renal cortex have recently been shown to play a role in regulating synthesis and/or secretion of renin (7, 9, 31). We therefore investigated the mRNA level of COX-2 and COX-1 in our mice. The COX-1 mRNA levels in the renal cortex did not differ between the genotypes, but the COX-2 mRNA level in the renal cortex in the AS null mice was approximately five times that of wild-type mice (Table 3).

Expression of genes in the kidneys of AS$^{+/−}$ heterozygotes was not significantly different from wild-type mice.

Abnormalities in kidney structure. Histological examinations demonstrated no notable differences between the kidneys of the AS$^{+/+}$ and AS$^{+/−}$ mice.

In contrast, the kidneys of the AS$^{−/−}$ mice exhibited several abnormalities. In five out of nine AS null mice, the kidneys showed hydropnephrosis, varying in severity from mild to moderate. Figure 3B shows the appearance of a kidney from an AS null mouse with moderate hydropnephrosis.

Immunostaining for renin in wild-type (Fig. 3C) and heterozygous mice (data not shown) showed the classic adult pattern, with renin staining confined to the juxtaglomerular apparatus and with the glomeruli showing no renin staining. However, the AS null mice showed a marked increase in the number of renin-expressing cells in the juxtaglomerular areas and along the preglomerular arterioles (Fig. 3D). In addition, there was an increase in size of individual renin-producing cells in the AS$^{−/−}$ mice. The AS null mice also showed hypertrophy of the juxtaglomerular apparatus and extensive enlargement of the macula densa (Fig. 3F) compared with wild-type mice (Fig. 3E).

COX-2 is normally expressed at low levels in the tubular cells of the thick ascending limb (TAL) of Henle, including the macula densa region. No immunoreactive COX-2 was detectable in the macula densa region of the wild type (Fig. 3G) and heterozygous (data not shown) mice. However, a strongly positive immunohistochemical reaction for COX-2 was observed in the macula densa cells of the homozygous null mice (Fig. 3H), in agreement with the increased COX-2 mRNA in these mice (Table 3).

Salt supplement in the AS$^{−/−}$ mice. To investigate whether dietary salt supplement could correct any of the abnormalities in the AS null mice, wild-type and AS$^{−/−}$ mice were fed a high-salt diet. Surprisingly, as shown in Fig. 4A, the high-salt diet had no effect on the blood pressure of the AS$^{−/−}$ mice (96 mmHg in the normal salt diet and 95 mmHg in the high-salt diet). Nevertheless, there was clear recovery in the plasma concentration of K$^+$ in the AS$^{−/−}$ mice (5.8 in $^{+/+}$ and $^{−/−}$ mice; Fig. 4B) and of other electrolytes (Ca$^{2+}$, Mg$^{2+}$, and Cl$^-$; data not shown). However, the most striking effect of the high salt was an almost complete return to normal of the synthesis and secretion of renin and of COX-2 expression in the AS null mice (Fig. 4, C–E). Thus plasma renin concentration was not significantly different between the AS$^{+/+}$ and AS$^{−/−}$ mice on the high-salt diet, and the level of renin mRNA in the kidney decreased from ~40 times in the wild type to ~2 times on the high-salt diet (Fig. 4C). Parallel with the renin decrease, the mRNA level of COX-2 in the kidney cortex decreased from ~6 times in the wild type to only ~1.3 times (Fig. 4E). Immunohistochemical signals for COX-2 were no longer detectable in the macula densa cells of AS$^{−/−}$ kidneys on the high-salt diet (data not shown).

DISCUSSION

We have previously reported the use of gene targeting to generate mice homozygous (AS$^{−/−}$) for a disrupted allele of the gene coding for AS and have described their general characteristics and the homeostatic changes that occur in their adrenal glands in response to their inability to synthesize aldosterone (21). The present study focuses on changes that occur in the kidney function of the AS$^{−/−}$ and AS$^{+/−}$ mice, and on how these changes are modified by feeding a high-salt diet. Our data demonstrate that adult AS$^{−/−}$ mice have low blood pressure and abnormal electrolyte homeostasis and water handling. The water-handling ability of the AS$^{+/−}$ heterozygotes is also affected.

Because aldosterone is a key factor in the regulation of electrolytes, abnormalities in electrolyte homeostasis were expected in the AS null mice. Thus they exhibited modest but significant increases in plasma concentration of K$^+$ and decreases in plasma concentration of Cl$^-$, HCO$_3^-$, and pH. These abnormalities agree with the presumption that Na$^+$ reabsorption in the aldosterone-sensitive distal nephron is impaired, since the diminished activity of Na$^+$ channels results in a lumen negative voltage, which in turn decreased Cl$^-$ reabsorption and K$^+$ and H$^+$ secretion (30). Also in agreement with previous clinical and experimental data indicating profound interaction between Na$^+$, Ca$^{2+}$, and Mg$^{2+}$ transport within the DCT, the AS null mice have increased Ca$^{2+}$ and Mg$^{2+}$ in their plasma.

Absence of aldosterone in the AS$^{−/−}$ mice can cause metabolic acidosis by at least two mechanisms. The impaired reabsorption of Na$^+$ in the CNT and the collecting duct will make the tubular lumen less negative and cause a decrease in proton secretion (30). Additionally, because aldosterone directly increases the activity of the H$^+$-ATPase in the collecting duct (38), its absence will result in decreased proton secretion.

Because aldosterone normally stimulates Na$^+$ reabsorption by activating ENaC and NCC in the aldosterone-sensitive distal nephron, its absence was expected to lead to Na loss. However, the AS$^{−/−}$ mice have a concentration of Na$^+$ in plasma not significantly different from wild-type mice. Evidently, compensation of some type occurs in the AS$^{−/−}$ mice. Compensatory enhancement of Na$^+$ reabsorption in the proximal tubule can be stimulated by glucocorticoids (32, 44) and/or by ANG II (4, 6, 27). Because AS$^{−/−}$ mice on a normal-salt diet have ~1.6 times the wild-type concentration of plasma glucocorticoids and ~4 times the wild-type plasma level of ANG II, it is probable that the compensation is at least in part in the proximal tubule.

Although previous studies have shown that chronic administration of aldosterone increases the abundance of α-ENaC at both the protein (23) and mRNA levels (37), the levels of mRNA of all subunits ($\alpha$, $\beta$, and $\gamma$) of the amiloride-sensitive channels (ENaC) did not significantly differ in the AS$^{−/−}$, heterozygous AS$^{+/−}$, and wild-type mice. Similarly, Berger et al. (2) found no changes in mRNA levels for the ENaC subunits in MR knockout mice. The absence of changes in the mRNA of the ENaC subunits in the AS$^{−/−}$ and MR$^{−/−}$ mice suggests that either compensatory changes induced in these
animals are not at transcriptional levels or that compensation is essentially complete, possibly as a result of increased levels of glucocorticoids and of ANG II. The latter may be the case, since glucocorticoid treatment in MR knockout mice increases the abundance of α-ENaC mRNA (33), and Beutler et al. (3) have also demonstrated that ANG II administration increases the expression of α-ENaC at both the mRNA and protein levels in AT1R+/− heterozygous mice. In addition, the absence of aldosterone in AS−/− mice could activate RAS in the central nervous system, leading to a stimulation of thirst and to vasopressin secretion. Studies by Ecelbarger et al. (5) have shown that chronic exposure to vasopressin in rats results in a

Fig. 3. Histological and immunocytochemical analysis of kidneys of AS null mice. A: kidney of AS+/+ mouse; B: AS null mice had hydronephrosis of varying severity; C: immunostaining for renin in wild-type mice showed classic adult pattern with renin staining conferred to juxtaglomerular apparatus; D: AS null mice had a marked increase in the no. of renin-expressing cells in juxtaglomerular areas and along the preglomerular arterioles; E: macula densa in kidney in AS+/+ mouse; F: extensive enlargement of the macula densa in AS null mice; G: absence of COX-2 immunostaining in AS−/− mice; H: COX-2 immunostaining in the macula densa in AS null mice.
marked increase in abundances of β- and γ-ENaC in whole kidney.

The most significant changes in the expression of genes in the AS−/− kidneys were increases in renin mRNA to ~12 times the wild type and in COX-2 (renal cortex) to ~5 times the wild type. Both of these increases were readily documented by histochemistry. They were accompanied with ~40 times wild-type plasma renin concentration and ~4 times plasma ANG II concentration. Significant although less marked changes include an increase in the expression of 11β-HSD2 and a decrease in NCC and ROMK (renal cortex). The AS−/− mice also showed histological abnormalities in renal structure, including hypertrophy of the juxtaglomerular apparatus and in some animals hydronephrosis. Several factors may be involved in the very high expression and release of renin in the AS null mice. Previous studies have shown that alterations in luminal Cl− change the rate of Na+–K+–2Cl− cotransport in the macula densa (26, 31), which stimulates COX-2, the enzyme responsible for prostaglandin synthesis (1). Although the role of COX-2 in the macula densa is not fully understood, it has been suggested that COX-2-derived prostaglandins from macula densa may regulate renin expression and release (7, 12). Several studies have also demonstrated that COX-2 expression in macula densa/cTALH-cortical thick ascending limb of the loop Henle increases in high-renin states (salt restriction, angiotensin-converting enzyme inhibition, renovascular hypertension) at both mRNA and immunoreactive protein levels (8, 10, 11, 42). Furthermore, Zhang et al. (45) demonstrated that COX-2 expression in the renal cortex of mature rats is negatively regulated by aldosterone and to some extent by glucocorticoids. Our finding that the levels of COX-2 mRNA in the renal cortex of the AS−/− mice were increased to ~5 times and ~1.3 times the wild type is consistent with their observations.

Our study of the AS−/− and AS+/− mice demonstrates a role of aldosterone in regulation of water balance, as might be
expected from previous work of others (20, 24, 28). On normal chow, both have higher urine output, decreased urine osmolality, and an impaired ability to concentrate and dilute urine. The absence of aldosterone impairs Na\(^+\) reabsorption in the CNT and collecting duct, which leads to less water reabsorption in the collecting ducts. Changes in the expression of aquaporins have also been described in adrenalectomized rats maintained with glucocorticoids but not aldosterone (20, 24). This could at least partly explain the impaired ability of AS\(^{-/-}\) and AS\(^{+/–}\) mice to concentrate urine. Aldosterone deficiency also alters fluid balance through effects on renal hemodynamics. Klar et al. (18) have shown that renal afferent arterioles, including their juxtaglomerular portion, express MR. There is also evidence that aldosterone is required for normal function of TAL. Thus in vivo TAL microperfusion studies by Stanton (35) showed a 33% reduction in Na\(^+\) reabsorption in adrenalectomized rats, which was restored by aldosterone, but not by glucocorticoids. Experiments by Work and Jamison (43) have confirmed these findings in vitro. Absence of aldosterone is therefore likely to decrease salt reabsorption in TAL and consequently the maintenance of an axial osmolar gradient, thereby impairing the ability of the kidney to concentrate urine.

Although hydronephrosis is present in about half of the AS null adults, it is unlikely to be the cause of the abnormalities in water handling for the following reasons: we observed impaired ability to concentrate urine in animals without hydronephrosis, it is never more than moderate, and because the kidney-to-body weights of the AS\(^{-/-}\) mice are not different from wild-type mice. In agreement with this reasoning, urine volumes in the heterozygotes on water ad libitum and osmolalities achieved during water deprivation are both significantly different from wild-type mice even though none of the heterozygotes have hydronephrosis. The death of 30% of the AS\(^{-/-}\) pups before weaning, apparently from dehydration, and the occurrence of hydronephrosis in one-half the surviving adults suggest that the balance in neonatal mice is precarious between a failure to compete with siblings for milk, leading to dehydration, and salt/water wasting leading to hydropnephrosis. The normal hematocrit and creatinine clearance of the AS\(^{-/-}\) mice that survive to adulthood indicates that they can achieve an almost normal balance between water intake and water excretion.

Perhaps the most important finding of the current study is that high dietary salt is able to normalize the plasma electrolyte concentrations in AS\(^{-/-}\) mice, even though they remain hypertensive and that increased expression of renin and COX-2 in AS null mice is minimized by the high salt. This suggests that electrolyte disturbances in the AS\(^{-/-}\) mice may change the activity of the macula densa Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter, leading to an increase in COX-2 activity. COX-2-derived prostaglandins may then increase the synthesis and release of renin. Because the AS null mice have reduced blood pressure, it is likely that renin expression and release are also stimulated by an increase in renal sympathetic nerve activity. However, despite the restoration of electrolyte homeostasis, and the near normalization of renin and COX-2 synthesis, their blood pressure remained low.

Finally, we comment on the phenotype of AS\(^{+/–}\) heterozygotes. It is a common experience with mice in which genes have been disrupted that the effects of homozygous absence of gene function are easy to demonstrate while the effects of heterozygosity may be detectable but not reach significance. Yet the heterozygous effects are important indicators of the likelihood that small differences in expression of the gene of interest will have effects in humans. In the present case, in 16 out of 20 variables for which homozygous null animals significantly differed from the wild type, the value for the heterozygote differed from the wild type in the same direction as did the homozygote, although not reaching significance. In three additional variables related to water handling, the heterozygous mice differed significantly from the wild type. Thus it is likely that polymorphisms that affect the levels of AS gene expression in the human population will have effects, albeit small, on water balance and possibly blood pressure.

In summary, our data show that the absence of aldosterone results in impairment of Na\(^+\) reabsorption in the distal nephron and low blood pressure, which activates several compensatory mechanisms, including increased Na\(^+\) uptake, a huge stimulation of the RAS, and an elevated level of glucocorticoids. They also show that a high intake of dietary NaCl largely normalizes these impairments, with the exception of the low blood pressure.

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