Postnatal adrenalectomy impairs urinary concentrating ability by increased COX-2 and leads to renal medullary injury

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Stubb J., Madsen K., Nielsen FT, Bonde RK, Skøtt O., Jensen BL. Postnatal adrenalectomy impairs urinary concentrating ability by increased COX-2 and leads to renal medullary injury. Am. J. Physiol. Renal Physiol. 293:F780–F789, 2007. First published June 20, 2007; doi:10.1152/ajprenal.00193.2007.—We hypothesized that aldosterone promotes development of the renal medulla in the postnatal period and that cyclooxygenase-2 (COX-2) activity contributes to renal dysfunction after impaired aldosterone signaling. To test these hypotheses, rat pups underwent either sham operation or adrenalectomy at postnatal day 10. Adrenalectomized rats were divided into no steroid substitution (ADX), corticosterone replacement (ADX-C), and corticosterone and DOCA substitution (ADX-CD) groups that received subcutaneous pellets with steroids. Without replacement, pups failed to thrive and exhibited impaired urinary-concentrating ability. The renal medulla was significantly smaller, and the medullary interstitial osmolality was lower in the ADX group, whereas COX-2 and PGE2 tissue levels were significantly elevated compared with levels shown in sham animals. Substitution with DOCA and corticosterone corrected these changes, whereas corticosterone replacement alone improved survival but not weight gain and urinary-concentrating ability. Administration of a COX-2 inhibitor to ADX rats (parecoxib, 5 mg kg−1 day−1, days 17–20) increased weight gain, urinary-concentrating ability, and papillary osmolality. After fluid deprivation, parecoxib attenuated weight loss and the increase in plasma Na+ concentration and osmolality. It is concluded that mineralocorticoid is required for normal postnatal development of the renal medulla. COX-2 contributes to impaired urine-concentrating ability, NaCl loss, and extracellular volume depletion in postnatal mineralocorticoid deficiency.

Disturbed formation of nephrons during critical “windows” of kidney development may be a predisposing factor for later development of hypertension (3, 25). Nephron formation ceases in the 36th wk of gestation in humans; however, in mice and rats, induction of new nephrons proceeds 6–7 days after birth (24). After nephron formation is complete, the renal medulla is structurally and functionally immature. Loops of Henle undergo considerable elongation to achieve the adult conformation during the second and third postnatal weeks (in rats) and during the first year of life (in humans), which coincides with a marked increase of urinary-concentrating ability (23). Loop growth takes place through mitotic activity in thick ascending limbs and apoptotic remodeling in thin limbs (4, 10). Subtle changes in medullary differentiation in late stages of kidney development might alter long-term blood pressure homeostasis despite a normal number of nephrons.

In rats, plasma glucocorticoid exhibits a developmental nadir in the second postnatal week (9). Exposure to elevated glucocorticoid in this period leads to hypertension in the adult rat (21) and inhibits proliferation of the loop of Henle and the kidney outer medulla (27). Little is known about factors that initiate and maintain proliferation of loop cells at the outer medulla-cortex junction. Pharmacological and genetic approaches employed to inhibit or delete components of the renin-angiotensin-aldosterone system in the postnatal period have consistently shown a range of abnormalities with diminished kidney growth, decreased ability to concentrate urine, and pathologic changes of the kidney medulla (6, 7, 14, 18, 20, 29, 30). In some of these experimental models, plasma aldosterone is suppressed (29) and mice deficient in aldosterone synthase (CYP11B2−/−) share several phenotypic characteristics with kidneys from animals with interrupted ANG II signaling (14). These observations imply a point of convergence at the aldosterone-mineralocorticoid receptor (MR) pathway for renal phenotypic alterations. It is not known whether aldosterone is necessary for proliferation or apoptotic remodeling within the renal medulla and at which time during postnatal development such sensitivity is present. Mineralocorticoid might exert direct effects on the loop epithelium or influence proliferation and growth indirectly through effects on Na+ homeostasis. In this context, renal prostanoids appear particularly relevant. Mineralocorticoid suppresses cortical cyclooxygenase-2 (COX-2) expression in developing and adult kidney, whereas administration of AT1-receptor blockers and MR antagonists stimulate renal COX-2 in the postnatal period (26, 31, 32). Intrarenal COX-2 is elevated and promotes NaCl wasting in NaCl-loosing tubulopathies (11, 19, 28). Whether enhanced COX-2 activity contributes to renal dysfunction in conditions with impaired postnatal mineralocorticoid signaling has not previously been analyzed. We hypothesized that 1) aldosterone promotes renal medullary development in the late postnatal phase after nephrogenesis by stimulating tubular cell proliferation and remodeling and that 2) aldosterone promotes development of urinary-concentrating ability by indirect effects on COX-2 activity.

These hypotheses were tested in rat pups subjected to adrenalectomy in the postnatal developmental “window,” during which medullary proliferation peaks and nephron formation is complete [between postnatal days 10 and 20 (P10–P20)]. Pups were given differential replacement therapy by chronic implantation of pellets that contained glucocorticoid and mineralocorticoid. In addition, short-term intervention with a COX-2 blocker was used to elucidate the role of COX-2 for impaired concentrating ability in adrenalectomized rats.
**Materials and Methods**

**Animal Surgery and Protocols**

All procedures conformed to the Danish national guidelines for the care and handling of animals and the published guidelines from the National Institutes of Health. The Danish Animal Experiments Inspectorate under the Department of Justice approved all experimental procedures that involved the use of animals (no. 2003/561-761).

Female Sprague-Dawley rats had free access to standard pathogen-free rat chow (Altromin-1310, Lage, Germany) (2 g/kg Na⁺, 5 g/kg Cl⁻) and tap water. In the first 24 h after birth, litters were reduced to 10 to ensure equal feeding with five males and females in each litter, if possible. The dams and pups were kept at a 12:12-h light-dark cycle. At P10, pups were anesthetized with ketamine (25 mg/kg) and xylazine (5 mg/kg), and bilateral adrenalectomy (ADX) was performed through two lumbar-dorsal incisions. After surgery, rats were divided into four groups: 1) sham group, which underwent a surgical protocol without removal of adrenals [a pellet (50 mg cholesterol) was inserted in the subcutis]; 2) ADX group, which did not have corticosteroid substitution and where a 50-mg pure cholesterol pellet was inserted. In pilot experiments, most pups in this group died before P10. Therefore, we adopted a protocol shown to sustain survival in MR⁻/⁻ mice where pups were injected subcutaneously with isotonic NaCl in an amount corresponding to urinary Na⁺ losses (20). The final two groups were as follows: 3) ADX with corticosterone replacement only (ADX-C), which had a pellet (50 mg) with 12.5% corticosterone inserted in the subcutis; and 4) ADX with corticosterone and mineralocorticoid (DOCA) substitution (ADX-CD), which had a pellet (50 mg) with 12.5% corticosterone and 12.5% DOCA inserted. Pellets were manufactured as described by Meyer et al. (15) for adult rats and reduced from a 100-mg to a 50-mg size to adjust for different body sizes and to maintain corticosterone at a level characteristic for the second postnatal week in the rat (9). Pellets were “sterilized” by heating for 2 h at 105°C. Pellets were stored at 4°C for a maximum of 2 wk.

**Experimental Series**

**Series 1.** Spot urine was collected at day 20 from the four experimental groups, and mixed trunk blood was sampled in EDTA-coated or heparin-coated vials after decapitation. Kidneys were dissected in outer medulla-cortex and inner medulla, snap frozen in liquid nitrogen and stored at −80°C.

**Series 2.** To determine urinary-concentrating ability, the dam was removed from the pups between P19 and P20 for 20 h. Spot urine was collected at decapitation, and mixed trunk blood was sampled. Kidneys were dissected in outer medulla-cortex and inner medulla and then frozen in liquid nitrogen and stored at −80°C.

**Series 3.** Pups from the four groups were anesthetized at P20 as described above. Kidneys were fixed by systemic perfusion through the left cardiac ventricle with 4% phosphate-buffered paraformaldehyde for 4 min. Coronal tissue blocks were processed for immunohistochemistry as described (27).

**Series 4.** Four litters were adrenalectomized at P10 and only supplemented with isotonic NaCl injections. Pups were given subcortaneous injections of a COX-2-selective antagonist (parecoxib; Dynastat; Merck), at 5 mg·kg⁻¹·day⁻¹ divided in two daily doses (2.5 μg/μl in 5 μl/g) from P17 to P20. The pups were then fluid deprived for 20 h. Spot urine was collected, and pups were decapitated. Plasma, urine, and kidneys were sampled. Inner medulla and cortex-outer medulla were dissected out and rapidly frozen.

**Plasma and Tissue Electrolyte Concentrations**

In initial experiments, papillae were homogenized in pure water and then centrifuged 10 min at 13,000 g, and supernatants were used for measurements. In subsequent experiments, osmolalities were quantitatively extracted as described (27). Osmolality was analyzed by freeze-point depression (Osmomat 030-D, Gonotec, Bie & Berntsen). Na⁺ and K⁺ concentrations were determined in plasma and renal medullary interstitial fluid by flame photometry (model IL 943; Instrumentation Laboratory, Lexington, MA). Urea was determined kinetically as the amount of NADH consumed over time measured spectrometrically (UV) after hydrolysis of urea by urease and the formation of L-glutamate by glutamate dehydrogenase (ABX Penta Urea CP, ABX Diagnostics).

**Hormone Analyses**

Plasma aldosterone was measured in all pups from series 1, 2, and 4 as a control of complete removal of all adrenal tissue with a commercial kit (COAT-A-COUNT; Diagnostic Products, Los Angeles, CA). The detection limit was 13.0 pg/ml, and the intra-assay coefficient of variation was <4%. Plasma total corticosterone was measured with the radioimmunoassay kit from Amersham’s Biomedical series, using ¹²⁵I-labeled corticosterone as a tracer. Plasma renin concentration was analyzed as described (22). For tissue PGE₂, samples were homogenized as described for Western blotting, and 250 μl of homogenate were acidified with 18 ml of 50% formic acid. Samples were extracted with 1 ml ethylacetate-hexane (70/30) by

| Table 1. Characteristics of adrenalectomized and sham-operated rat pups at P20 |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Body weight P20, g | 44.4±1 (n=15) | 35.9±1* (n=8) | 37.8±2.4† (n=8) | 44.2±3.7§* (n=11) |
| Kidney weight, mg | 456±16 (n=8) | 368±28* (n=4) | 365±39* (n=4) | 492±89* (n=6) |
| Kidney-to-body weight ratio, mg/g | 9.9±0.1 (n=8) | 9.9±0.4 (n=4) | 9.4±0.1 (n=4) | 10.5±0.2* (n=6) |
| Urine osmolality, mosmol/kgH₂O | 503±24 (n=8) | 668±123 (n=6) | 758±115 (n=8) |
| Plasma [Na⁺], mM | 134±1.5 (n=6) | 128±2.6 (n=4) | 118±5.8 (n=4) | 139±5.5 (n=6) |
| Plasma renin concentration, 10⁻⁶ GU/ml | 63±46 (n=13) | 1886±935*(n=6) | 1539±750* (n=5) | 2.9±0.4§ (n=7) |
| Plasma corticosterone, ng/ml | 137±30 (n=11) | 26±10*(n=5) | 29±9 (n=4) | 25±5§ (n=8) |

**Fluid deprivation**

| Plasma [Na⁺], mM | 139±1 (n=6) | 127±3* (n=6) | 129±2* (n=6) | 155±4§ (n=6) |
| Urinary osmolality, mosmol/kgH₂O | 1690±177 (n=7) | 474±45*(n=5) | 860±128* (n=5) | 1476±138§* (n=7) |
| Papillary osmolality, mosmol/kgH₂O | 562±26 (n=7) | 311±21*(n=8) | 392±29† (n=9) | 493±37§ (n=10) |
| Papillary [Na⁺], mM | 76±3 (n=7) | 44±3 (n=8) | 48±4† (n=9) | 77±6§ (n=10) |

Values are means ± SE. P20, postnatal day 20; [Na⁺], Na⁺ concentration. GU, Glublatt units. Adrenalectomy or sham surgery was performed at postnatal day 10 (P10), and rat pups were divided into the following groups: Sham (no steroid substitution, NaCl supplementation), ADX (adrenalectomy with no steroid substitution), ADX-C (adrenalectomy with corticosterone and DOCA substitution). In the fluid-deprivation protocol, pups were removed from the dam for 20–22 h. Data sets that were normally or log-normally distributed were evaluated statistically by ANOVA followed by Tukey-Kramer multiple comparisons test. Data that were not normally distributed were evaluated by Kruskal-Wallis test followed by Dunn’s multiple comparison test. P < 0.05: *Sham vs. ADX; †Sham vs. ADX-C; ‡Sham vs. ADX-CD; §ADX vs. ADX-CD; †ADX-C vs. ADX-CD.

**References**

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vortexing and subsequent centrifugation. The supernatants were vacuum dried and dissolved in 250 μl of assay buffer. PGE2 was determined by ELISA (Assay Designs).

**Messenger RNA Analyses**

Isolation of RNA and quantitative RT-PCR were performed as described previously (27). A standard curve was constructed by plotting threshold cycle against serial dilutions of linearized plasmids. A subset of quantitative RT-PCR data sets was validated by subsequent ribonuclease protection assay as previously described (27).

**Western Blotting**

Tissue samples were homogenized in buffer as previously described in detail (27). Protein concentration determination, gel electrophoresis, electroblotting, and chemiluminescence detection were done as previously described (27). Densitometry was evaluated by

![Images of kidney sections](image-url)
QuantityOne software (BioRad). Primary antibodies used were as follows: COX-2 (1:2,000, M-19, Santa Cruz); Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) (1:2,000, Upstate); proliferating cell nuclear antigen (PCNA; 1:2,000, Santa Cruz); and sodium-potassium-2-chloride cotransporter (NKCC2; 1:2,000, Chemicon).

**Morphometry and Immunohistochemistry**

Fixation and paraffin embedding for immunohistochemistry was as described previously (27). Primary antibodies for immunohistochemistry were as follows: COX-2 (1:1,000, M-19, Santa Cruz); NKCC2 (1:1,000, Chemicon); the potassium channel ROMK (1:500, Alomone Labs); Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\) (1:100, Upstate); PCNA (1:100, Santa Cruz); and Tamm-Horsfall glycoprotein (1:200, Biotrend). For morphometric analysis, coronal sections through the tip of papilla were labeled for Tamm-Horsfall glycoprotein to allow precise delineation of kidney outer medulla and inner medulla. Juxtamedullary glomeruli defined the cortical-outer medulla junction in morphometric analysis. Measurement of kidney dimensions was done with Image Tool version 3.0 (National Institutes of Health) as described (27).

**Statistics**

If data were not normally distributed, they were log-transformed and tested for normality. Statistical significance between several groups was evaluated by one-way ANOVA followed by the Tukey-Kramer multiple comparisons test. Unpaired Student’s t-test was used when two groups were evaluated. If data were not normally distributed or variance was not homogenous, a nonparametric Mann-Whitney test was used to test for significant differences between two groups, and differences between more than two groups were estimated by the Kruskal-Wallis test followed by Dunn’s multiple comparisons test. Unpaired Student’s t-test was used to detect significant differences between two groups and differences between more than two groups were estimated by the Kruskal-Wallis test followed by Dunn’s multiple comparisons test. *P* values < 0.05 were considered significant. Arithmetic means are shown as ± SE; for log-transformed data, geometric means are shown. All calculations were performed with GraphPad Prism InStat software (version 3.0; GraphPad, San Diego, CA).

**RESULTS**

**Effect of Adrenalectomy on Body and Kidney Growth and Plasma Hormone and Electrolyte Concentrations**

Plasma aldosterone concentration was 87 ± 22 pg/ml in sham-operated rats at P20 (n = 13) and was below the detection limit after adrenalectomy in all included rats (not shown; assay limit of 11 pg/ml, n = 7–9 animals in each group). In control pups not subjected to surgery, plasma corticosterone increased ~10 times between P10 and weaning at P20 (13.4 ± 1.3 ng/ml vs. 128.7 ± 28 ng/ml; n = 5 in both groups), which was not different from plasma corticosterone in sham-operated pups at P20 (137 ± 30 ng/ml; n = 13). In the ADX group that received no steroid replacement, corticosterone was detectable in plasma at P20, but the developmental surge was prevented (Table 1). In ADX-C pups, plasma corticosterone was not significantly different from sham animals at P20 (Table 1). In ADX-CD pups, plasma corticosterone was significantly lower than in sham animals (Table 1).

After adrenalectomy, pups exhibited a significantly reduced weight gain in the ADX and ADX-C groups compared with sham (Table 1). ADX-CD rats gained weight similarly to sham-operated rats (Table 1). Total kidney mass was significantly reduced in ADX and ADX-C rats, but kidney-to-body weight ratios were not different from sham animals (Table 1). ADX-CD rats had an increased kidney-to-body weight ratio compared with ADX-C rats (Table 1).

In ADX pups supplemented with NaCl and in ADX-C pups, plasma renin concentration was significantly elevated compared with sham and ADX-CD groups (Table 1). Kidney renin mRNA level was significantly elevated in the ADX group compared with sham and ADX-CD, whereas, in ADX-C pups, renin mRNA was not different from sham (Table 3).

Kidneys from the ADX group were smaller and exhibited medullary injury with dilated pelvis and a small papilla compared with sham and ADX-CD rats (Fig. 1A). Kidneys from the ADX-CD group were macroscopically indistinguishable from sham (Fig. 1A). Morphometric assessment showed that kidneys from ADX pups displayed a significantly reduced papillary area, a thinner outer medulla, and a significant reduction in cortical surface-papillary tip (“axis”) length (Fig. 1B and Table 2). Corticosterone replacement corrected the change in outer medulla thickness and axis length, but not papillary area (Table 2). ADX-CD kidneys displayed no significant difference of any measured parameter compared with sham (Table 2). To address whether proliferation was affected in the four groups, PCNA was used as a mitotic marker. Immunostaining of kidney sections for PCNA at P20 did not reveal any marked difference in tissue distribution and labeling intensity between kidneys from the various groups (Fig. 1C). Western blotting on whole kidney protein showed that PCNA abundance was not different between ADX and sham rats at P20 (Fig. 1D).

Renal COX-2 Expression in Response to Adrenalectomy

In the ADX group, COX-2 mRNA and protein abundances in kidney cortex-outer medulla fraction were significantly higher than those shown in sham animals (Fig. 2A). Corticosterone replacement did not decrease kidney COX-2 mRNA or protein levels compared with ADX animals. For the ADX-CD

<table>
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<tr>
<th>Table 2. Dimensions of kidney regions determined in histological sections at P20 in sham, ADX, ADX-C, and ADX-CD rats</th>
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<tbody>
<tr>
<td><strong>Sham</strong></td>
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<tr>
<td>----------</td>
</tr>
<tr>
<td>Cortex thickness, (\mu m)</td>
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<tr>
<td>Outer medulla thickness, (\mu m)</td>
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<tr>
<td>Papilla length, (\mu m)</td>
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<tr>
<td>Axis length, (\mu m)</td>
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<tr>
<td>Papillary area, (m^2)</td>
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Values are means ± SE; \(n = 5\) separate rats in each group. All data sets were normally distributed. *P < 0.05 as estimated by ANOVA followed by Dunnet’s post hoc test where the sham group was control.
group, renal COX-2 mRNA and protein levels were similar to those for sham animals (Fig. 2A). Tissue concentration of PGE₂ in cortex-outer medulla tissue from ADX-CD rats was about four times lower than that shown in ADX pups [20.7 ± 8.5 pg/mg protein (n = 6) vs. 84.7 ± 9.1 pg/mg protein (P < 0.05) (n = 6)]. Immunohistochemical labeling of kidney tissue showed that COX-2 signal was localized predominantly in cortex in all four experimental groups (Fig. 2B). COX-2 was not readily detectable in the medulla, and visualization required amplification. COX-2 was associated with inner medullary interstitial cells (not shown). Labeling of kidney tissue from ADX rats for COX-2 yielded more intense immunoreactivity and more widely distributed signal than shown for sham and ADX-CD kidneys (Fig. 2B). Kidney sections from ADX-C rats also displayed more widely distributed COX-2-immunoreactive protein than shown in sham and ADX-CD animals (Fig. 2B). At high-power magnification, COX-2-positive signals were associated with the cytoplasm in the majority of loop of Henle cells in the kidney cortex of ADX rats (Fig. 2B, bottom). In contrast, COX-2-immunoreactive signal from ADX-CD rat kidney was more faint and detectable only in a minority of loop of Henle cells (Fig. 2B, bottom).

Fig. 2. A: cyclooxygenase-2 (COX-2) abundance in rat pup kidneys at P20 at the level of mRNA (left) and protein (right). Pups were sham operated (sham) or adrenalectomized at postnatal day 10 (P10) and substituted with cholesterol pellets with no steroid (sham and ADX), with 12.5% corticosterone (ADX-C), and with 12.5% corticosterone and 12.5% DOCA (ADX-CD); n = 5 or 6 in each group in the quantitative RT-PCR experiments and n = 4 in the Western blotting experiments. *P < 0.05. B, top: micrographs of histological sections of kidneys at P20 in sham or ADX groups at P10 that are reacted with a COX-2-specific antibody by histochemical reaction. Bars = 200 μm. B, bottom: high-power micrographs of kidney sections labeled for COX-2 from an ADX rat (left) and ADX-CD rat (right). Bars = 50 μm.
Urinary-Concentrating Ability in Response to Adrenalectomy

In normohydrated pups, there were no significant differences in urine osmolality between the four experimental groups at P20 (Table 1). There was a tendency for lower plasma Na\(^+\) concentration ([Na\(^+\)]) in ADX and ADX-C groups, but this was not statistically significant; however, plasma [Na\(^+\)] in ADX-CD rats was significantly elevated compared with that shown for ADX-C rats (Table 1). In response to fluid deprivation, plasma [Na\(^+\)] was significantly lower in ADX-C and ADX pups than in sham rats (Table 1). In the ADX-CD group, plasma [Na\(^+\)] was significantly elevated compared with that shown for all other groups (Table 1). Fluid deprivation increased urine osmolality in sham-operated rats compared with normohydrated sham-operated pups (Table 1). In ADX and ADX-C groups, urine-concentrating ability was significantly lower than that shown for sham rats (Table 1). In response to fluid deprivation, urine osmolality in ADX and ADX-C rats did not increase significantly compared with normohydrated counterparts (Table 1). In the ADX-CD group, urinary-concentrating ability was not different from that shown in sham animals and significantly higher than that shown in ADX and ADX-C groups (Table 1). Osmolality and [Na\(^+\)] in papillary tissue interstitial fluid changed similarly to urine-concentrating ability; these levels were significantly reduced in ADX and ADX-C groups compared with sham, whereas levels in ADX-CD rats were not significantly different from those in sham animals (Table 1). NaCl transport molecule expression was determined in cortex-outer medulla tissue fraction. Adrenalectomy with or without glucoc- and mineralocorticosteroid substitution did not significantly alter mRNA abundances of the investigated NaCl transport molecules [NKCC2, ROMK, Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit, renal Na\(^+\)/H\(^+\) exchanger (NHE3), and \(\alpha\)-epithelial Na\(^+\) channel] and 11\(\beta\)-hydroxysteroid dehydrogenase-2 as determined by quantitative RT-PCR (Table 3). Ribonuclease protection assays for NKCC2 and Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit mRNAs corroborated the results obtained by quantitative RT-PCR (Fig. 3A). Western blotting experiments showed a lower abundance of Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit protein in kidney tissue from ADX group (Fig. 3B), whereas NKCC2 was not changed (not shown). Immunohistochemical staining for NKCC2 (Fig. 3C) and ROMK (not shown) confirmed the pathological change with a thinner outer medulla but did not reveal any appreciable differences in tissue distribution or labeling intensity of immunoreactive proteins. NKCC2 was associated with the loops of Henle in outer medulla and in cortical medullary rays (Fig. 3C). Labeling intensity was reduced for Na\(^+\)-K\(^+\)-ATPase \(\alpha_1\)-subunit in ADX rats compared with the other groups (Fig. 3C). Immunoreactive signal for Na\(^+\)-K\(^+\)-ATPase was associated with the basolateral aspect of epithelial cells in the distal convoluted and connecting tubules in cortex and loop of Henle cells in the outer medulla (Fig. 3C). There were no obvious differences in the distribution pattern and staining intensity between control, ADX-C, and ADX-CD groups (Fig. 3C).

Role of COX-2 for Impaired Concentrating Ability and Weight Gain in ADX Rats

At P17, ADX rats were divided into two groups: vehicle (weight: 33.5 ± 0.7 g, mean ± SE, n = 13) and parecoxib (weight: 34.1 ± 1 g, n = 14). Subcutaneous injection of the COX-2-selective antagonist parecoxib (5 mg·kg\(^{-1}\)·day\(^{-1}\), P17-P20) enhanced weight gain significantly (Fig. 4A). The COX-2 inhibitor reduced weight loss after fluid deprivation for 20 h (Fig. 4A). In response to fluid deprivation, plasma osmolality and hematocrit increased significantly less in parecoxib-treated rats than in vehicle-treated ADX pups (Table 4). Parecoxib treatment significantly improved urinary-concentrating ability and augmented osmolality of papillary interstitial fluid (Fig. 4, B and C). The increase in osmolality of papillary interstitial fluid after COX-2 inhibition was achieved through significantly increased accumulation of Na\(^+\) and urea but not K\(^+\) (Table 4). The COX-2 inhibitor lowered plasma renin concentration significantly to a level ~1/30 of that in vehicle-injected fluid-deprived pups (Fig. 4D).

DISCUSSION

The present data show that absence of aldosterone for 10 days before weaning in rat pups leads to renal medullary injury and impaired urinary-concentrating ability associated with somatic growth inhibition and extracellular volume depletion. The lesions were most prominent in the outer medulla and the papilla. The injury was elicited late in the postnatal period (between P10 and P20) despite NaCl supplementation and at a time when formation of new nephrons has ceased. This postnatal “window” coincides with intense proliferation of the loops of Henle (4, 27) and expansion of the renal medulla. The data indicate that mineralocorticoid supports renal and somatic growth equally with no detectable effect on renal cell proliferation. In the ADX group, corticosterone was detectable in plasma (8), but the physiological surge at weaning (P20) was prevented. Despite the fact that plasma corticosterone was not significantly elevated in the ADX-C group compared with that shown in ADX animals 10 days after pellet implantation, the replacement therapy improved some ADX-induced pathologi-

### Table 3. Messenger RNA levels at P20 in the kidneys of rats that were either sham-operated or adrenalectomized at P10

<table>
<thead>
<tr>
<th></th>
<th>Sham (n = 9)</th>
<th>ADX (n = 7)</th>
<th>ADX-C (n = 5)</th>
<th>ADX-CD (n = 9)</th>
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<tr>
<td>NKCC2</td>
<td>2.3 ± 0.4</td>
<td>3.5 ± 0.5</td>
<td>2.6 ± 0.6</td>
<td>2.4 ± 0.6</td>
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<tr>
<td>ROMK</td>
<td>2.8 ± 0.3</td>
<td>2.5 ± 0.3</td>
<td>2.1 ± 0.4</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Na(^+)-K(^+)-ATPase (\alpha_1)-subunit</td>
<td>4.3 ± 0.6</td>
<td>5.4 ± 0.6</td>
<td>4.4 ± 0.7</td>
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<tr>
<td>NHE3</td>
<td>0.22 ± 0.04</td>
<td>0.21 ± 0.03</td>
<td>0.27 ± 0.05</td>
<td>0.24 ± 0.04</td>
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<tr>
<td>ENaC</td>
<td>0.98 ± 0.2</td>
<td>0.96 ± 0.3</td>
<td>0.81 ± 0.2</td>
<td>1.1 ± 0.2</td>
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<tr>
<td>11(\beta)HSD-2</td>
<td>0.70 ± 0.08</td>
<td>1.02 ± 0.14</td>
<td>0.76 ± 0.15</td>
<td>0.76 ± 0.10</td>
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<tr>
<td>Renin</td>
<td>0.43 ± 0.06</td>
<td>3.20 ± 0.12*</td>
<td>2.64 ± 0.58</td>
<td>0.29 ± 0.05†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of animals. 11\(\beta\)HSD-2, 11\(\beta\)-hydroxysteroid dehydrogenase-2. Values are starting quantity in ng ×10\(^3\), and data sets were normally distributed. NHE3, renal Na\(^+\)/H\(^+\) exchanger; ENaC, a-endothelial Na\(^+\) channel. Statistical evaluation was performed by ANOVA followed by Tukey-Kramer multiple comparisons test. P < 0.05: *sham vs. ADX and †ADX vs. ADX-CD.
cal features (survival, thickness of outer medulla). However, glucocorticoid at plasma levels that are normal in the postnatal period did not compensate for the lack of mineralocorticoid judged from reduced somatic growth rate, elevated plasma renin concentration, decreased urine concentrating ability, and more marked effect of dehydration on intravascular volume. This notion is consistent with data that show that betamethasone cannot substitute for the lack of aldosterone signaling in MR knockout mice (1), but it improves urinary-concentrating ability in rats when administered before weaning (23). Addition of DOCA to corticosterone pellets normalized weight gain, led to kidney hypertrophy and hypernatremia, and restored urine-concentrating ability and papillary osmolality in ADX rats. The ADX and ADX-C groups resemble mouse models with targeted disruption of aldosterone receptor (MR) and aldosterone synthase that display severe NaCl wasting, urinary-concentrating defect, and variable degrees of hydronephrosis despite intact glucocorticoid synthesis (1, 14). The present study indicates a distinct role of impaired aldosterone signaling for the renal lesions observed after deletion of upstream renin-angiotensin system components (6, 7, 18, 20, 29, 30).

The observations in the ADX and ADX-C groups that plasma [Na⁺] is not elevated and does not increase significantly after fluid deprivation indicate that rats suffer primarily from NaCl losses and subsequent water losses. The level of Na⁺-K⁺-ATPase α₁-subunit was lower in the ADX group, whereas levels of other NaCl transport proteins were not

Fig. 3. A, top: result of ribonuclease protection assays for NKCC2, Na⁺-K⁺-ATPase α₁-subunit (NaK-α₁), and β-actin. Total RNA from kidneys of sham and nonsubstituted ADX pups at P20 was used for hybridization with specific radiolabeled probes. A, bottom: quantitative evaluation of the assays; n = 5 in each group. B, top: Western blotting experiment of whole kidney homogenate for Na⁺-K⁺-ATPase α₁-subunit from sham, ADX, ADX-C, and ADX-CD rat pups. B, bottom: densitometric values (OD); n = 4 in each group. *P < 0.05. C: micrographs of histological sections of kidneys at P20 in sham and ADX groups (ADX, ADX-C, and ADX-CD) at P10 that are reacted with a NKCC2-specific antibody (top) and an Na⁺-K⁺-ATPase α₁-subunit antibody (bottom) by histochemical reaction. Scale bars = 500 μm (top) and 50 μm (bottom).
changed (ROMK, NHE3, NKCC2, and epithelial Na\(^+\) channel) in outer medulla-cortex tissue, consistent with findings in aldosterone synthase- and MR-deficient mice (1, 14). The data imply that the effect of corticosteroids on Na\(^+\) transport are not mediated predominantly at the level of transport protein abundance.

COX-2 activity contributed significantly to the NaCl-wasting phenotype elicited by absence of aldosterone in the postnatal period. Administration of a COX-2 inhibitor, parecoxib, increased body weight gain abruptly, which implies an effect through renal Na\(^+\) handling with subsequent expansion of extracellular volume. Moreover, in response to fluid deprivation, parecoxib attenuated the increases in hematocrit, plasma osmolality, plasma [Na\(^+\)], and renin and improved the accumulation of Na\(^+\) and urea in the medullary interstitium. The improved interstitial hypertonicity achieved by COX-2 inhibition in the renal medulla is the most likely explanation for the improved urine-concentrating ability. This effect could be accomplished by inhibition of prostaglandin effects on the tubular epithelium and, indirectly, by an improved action of vasopressin and/or by decreased washout of solutes through a decrease in medullary perfusion. The primary deficiency in mineralocorticoid and the negative Na\(^+\) balance augments renal COX-2 activity, which further enhances renal Na\(^+\) loss. A similar mechanism is likely to be involved in renal dysfunction in aldosterone synthase-deficient mice, where intrarenal COX-2 expression is increased fivefold (14). Concentrating ability was improved significantly by short-term COX-2 inhibition (Fig. 4) but not reestablished to the level normally observed at weaning in adrenal-intact rats (~2,000 mosmol/kg\(H_2O\)). Whether intervention with COX inhibitors beyond 3 days can rescue the medullary phenotype induced by absence of mineralocorticoid is not resolved by the present experiments. We chose a short period of intervention because COX-2 inhibition or gene deletion from birth by itself causes renal injury in rodents, primarily in the renal cortex (5, 12, 17).

As noted by Makhanova et al. (14), the medullary injury as noted by Makhanova et al. (14), the medullary injury

Table 4. Effect of a COX-2 selective inhibitor parecoxib (5 mg·kg\(^{-1}\)·day\(^{-1}\) from P17 to P20) on plasma parameters and urinary-concentrating ability in adrenalectomized, nonsteroid-substituted rats that were fluid deprived for 20 h

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Parecoxib (5 mg/kg)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma [Na(^+)], mM</td>
<td>129±1.2 (13)</td>
<td>133.7±1.4 (14)*</td>
<td>0.02</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>40.9±1.2 (10)</td>
<td>35.7±1.2 (9)*</td>
<td>0.02</td>
</tr>
<tr>
<td>Plasma osmolality, mosmol/kg(H_2O)</td>
<td>324±4 (13)</td>
<td>301±1 (14)*</td>
<td>0.0001</td>
</tr>
<tr>
<td>Papillary urea, mM</td>
<td>92.4±12.6 (12)</td>
<td>178.7±14.2 (14)*</td>
<td>0.0002</td>
</tr>
<tr>
<td>Papillary [Na(^+)], mM</td>
<td>58.2±4.1 (12)</td>
<td>93.1±5.5 (14)*</td>
<td>0.0001</td>
</tr>
<tr>
<td>Papillary [K(^+)], mM</td>
<td>78.4 (12)</td>
<td>86.9 (14)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Values are means ± SE, except for papillary [K\(^+\)], which are medians; no. in parentheses are number of separate rats. All pups were adrenalectomized on P10 and given NaCl supplementation but no steroid replacement. COX-2, cyclooxygenase-2. Statistical evaluation was performed by unpaired Student’s t-test. Papillary [K\(^+\)] data were tested by Mann-Whitney test. *Significant at P < 0.05.
ment. In accord, administration of angiotensin-converting enzyme inhibitors from birth leads to dilatation of tubules in the rat kidney medulla (13), and deletion of AT1 receptors leads to increased intrapelvic pressure and impaired peristaltic propagation of urine in mice (16). In the present study, ANG II signaling was not impaired, but medullary damage was evident, suggesting a distinct role for aldosterone. The ADX pups are likely to exhibit polyuria similar to other situations with impaired renin-angiotensin signaling. This may directly lead to increased pelvic pressure as suggested to be the case also in RenIC-deficient mice, where aldosterone concentration is strongly decreased (29). Extreme polyuria in the neonatal period as induced by furosemide or NKCC2 gene deletion causes more pronounced but basically similar pathological changes in the medulla (28). Supplementation with isotonic NaCl after adrenalectomy was necessary for survival of pups in the present study, but it further augments urine flow rate. The difference in fluid load presented to the kidney and urinary tract may explain why hydropnephrosis was observed less consistently in kidneys from aldosterone synthase- and RenIC-deficient mice (14, 29) compared with the present study and to NKCC2-deficient mice (28). Thus, to survive mineralocorticoid deficiency with a defect in urine-concentrating mechanism, pups must drink a volume that is likely to damage the kidney.

We conclude that mineralocorticoid is required for normal postnatal development of the renal medulla. Renal COX-2 activity contributes significantly to NaCl loss, extracellular volume depletion, and decreased urine-concentrating ability in postnatal mineralocorticoid deficiency. COX inhibitor administration seems an attractive approach to reduce renal Na+ and water losses in such conditions.

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