Low endogenous glucocorticoid allows induction of kidney cortical cyclooxygenase-2 during postnatal rat development

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Madsen, Kirsten, Jane Stubbe, Tianxin Yang, Ole Skott, Sebastian Bachmann, and Boye L. Jensen. Low endogenous glucocorticoid allows induction of kidney cortical cyclooxygenase-2 during postnatal rat development. Am J Physiol Renal Physiol 286: F26–F37, 2004.—In postnatal weeks 2–4, cyclooxygenase-2 (COX-2) is induced in the rat kidney cortex where it is critically involved in final stages of kidney development. We examined changes in circulating glucocorticoids and mineralocorticoids or in their renal receptors in the suckling period (11). In the suckling period, plasma glucocorticoid concentrations peaked at birth, decreased to low levels at days 3–13, and increased to adult levels from day 22. Aldosterone peaked at birth and then stabilized at adult levels. Gluco- and mineralocorticoid receptor (GR and MR) mRNAs were expressed stably in kidney before, during, and after COX-2 induction. 11β-Hydroxysteroid dehydrogenase 2 was induced shortly after birth and was widely distributed in the whole collecting duct system in the suckling period and then returned to an adult pattern. Supplementation with corticosterone (20 mg·kg⁻¹·day⁻¹) or GR-specific dexamethasone (1 mg·kg⁻¹·day⁻¹) during low endogenous corticosterone suppressed renal COX-2 mRNA and protein and led to a restricted distribution of COX-2 immunolabeling. The ability of glucocorticoids to affect COX-2 was reflected in colocalization of GR-α and COX-2 immunoreactivity and mRNAs in thick ascending limb of Henle’s loop. The MR antagonist potassium canrenoate (20 mg·kg⁻¹·day⁻¹) enhanced COX-2 expression from days 5 to 10, but low MR-specific concentrations of DOCA (1 mg·kg⁻¹·day⁻¹) had no effect on COX-2. Renomedullary interstitial cells expressed GR-α and COX-2. Dexamethasone suppressed COX-2 in these cells. Thus low plasma concentrations of corticosterone allowed for cortical and medullary COX-2 induction during postnatal kidney development. Increased circulating glucocorticoid in the postnatal period may damage late renal development through inhibition of COX-2.

mineralocorticoid; aldosterone; 11β-hydroxysteroid dehydrogenase 2; nephrogenesis; prostaglandin

A rate-limiting step in prostaglandin synthesis is catalyzed by prostaglandin H synthase or cyclooxygenase. The inducible enzyme variant cyclooxygenase-2 (COX-2) is constitutively expressed in a subset of loop of Henle cells in the adult kidney cortex of several species and is strongly induced at this site in the early postnatal period (11, 28, 31, 35, 36, 40, 41). COX-2 expression has also been shown in human fetal kidney at gestational age 15–24 wk, a time of active nephrogenesis (18, 20), but data are lacking for later developmental stages. In the rat kidney, formation of nephrons proceeds for an additional 6–8 days after birth through induction of undifferentiated mesenchyme in the outermost part of the cortex, the nephrogenic zone (8, 30). Several lines of evidence suggest a critical role for COX-2 in late, extrauterine, stages of rodent kidney development. Gene targeting of COX-2 in mice leads to pathological changes in kidney development, which are similar to those observed after pharmacological inhibition of COX-2 from birth in both mice and rats (7, 21, 23, 26). These kidneys display a progressive loss of cortical architecture and functional disturbances (21, 26). Use of nonselective COX inhibitors in human pregnancy can induce a range of functional and morphological abnormalities in the neonatal kidney, which suggests a critical role for prostaglandins also in developing human kidneys (33, 34). The significance of intact COX-2 signaling for kidney development raises the important question of which factors are responsible for physiological regulation of renal COX-2 expression in perinatal life. In the present study, we focused on the role of adrenal corticosteroids, because data showed that steroids can damage kidney development (4, 22, 29) and, in adult and young rats, gluco- and mineralocorticosteroids were shown to suppress renocortical COX-2 expression (34, 40, 41). Postnatal developmental changes in plasma concentration of adrenal steroids have been reported in rodents previously (6, 12, 41), and differences in glucocorticoid receptor (GR) expression level have been shown to account for differential effects of dexamethasone on COX-2 expression in various cell types (14). Developmental changes in circulating gluco- or mineralocorticosteroids and/or in renal receptor expression could potentially have a regulatory function on renal COX-2 induction. Moreover, the microsomal enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD-2) metabolizes glucocorticoids to inactive metabolites and thereby reduces glucocorticoid sensitivity in a cell-specific way (9). 11βHSD-2 is expressed at high levels in placenta and in mineralocorticoid target cells, also during human fetal development (5). 11βHSD-2 protects the mineralocorticoid receptor (MR) from illicit activation by glucocorticoids (9), which bind to MR with the same affinity as to GR. Developmental changes in 11βHSD-2 in the kidney could significantly modify steroid sensitivity in COX-2-expressing cells. In the present study, we explored these possibilities by determination of plasma aldosterone and corticosterone concentrations and by mRNA and localization studies of steroid receptors, 11βHSD-2, and COX-2 during kidney development in rats. Moreover, we examined the effect of GR and MR agonists and antagonists on COX-2 expression during kidney development.

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Materials and Methods

In vivo protocols. All procedures conformed with the Danish national guidelines for the care and handling of animals and with the published guidelines from the National Institutes of Health. Female Sprague-Dawley rats had free access to standard pathogen-free rat chow (Altromin-1310, Lage, Germany; Na⁺: 2 g/kg, Cl⁻: 5 g/kg) and tap water. The dams and pups were kept on a 12:12-h light-dark cycle. The pups remained in the nest and were decapitated within 1 min by at least two persons. Mixed trunk blood was sampled in EDTA-coated vials, and organs were rapidly removed and frozen in liquid nitrogen and stored at −80°C. At embryonal (E) day 19, the pregnant rats were killed by decapitation of embryos were removed, and kidneys from one litter were pooled. Plasma was not obtained from the embryos for steroid measurements. All other organ and plasma samples were obtained separately from single rats. The GR-specific agonist dexamethasone (Decadron, MSD; 4 mg/ml) was injected twice daily in the neck fold from postnatal (P) days 5 to 10 (total 1 mg/kg−1·day−1). The endogenous hormone corticosterone (Sigma), which is not GR selective, was dissolved in sesame oil and injected twice daily into the neck fold of pups from P5 to P10 (total 20 mg/kg−1·day−1). The GR antagonist mifepristone (RU-486, Sigma) was dissolved in sesame oil and injected once daily into the neck fold of pups from P5 to P10 at a total of 5 mg/kg. The MR antagonist potassium canrenoate (Soldactone, Searle) was dissolved according to instructions and injected into the neck fold at 20 mg/kg−1·day−1 from P1 to P5 in one litter and from days 5 to 10 in a second litter. The MR agonist DOCA (Sigma) was dissolved in sesame oil and injected once daily into the neck fold from days 5 to 10 (1 mg/kg). Control littersmates received the same volume of respective vehicle.

Radioimmunoassay for plasma corticosterone and aldosterone concentrations. Plasma aldosterone was measured using 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 and using 125I-corticosterone as a tracer.
RT-PCR and cDNA cloning. PCR, cloning, and sequencing of cDNAs for rat 11βHSD-2, rat MR, and GR have been previously described (25). Primers and PCR for β-actin and COX-2 were as previously published (2, 15). To amplify aquaporin-1 (AQP1), the following primers were used: AQP1 sense 5′-CCA GCG AAA TCA AGA AGG CT-3′; antisense 5′-CTA TTT GGG CTT CAT CTC CAC C-3′, covering the translated region, 806 bp (GenBank acc. no. L07268).

Solution hybridization and ribonuclease protection assays. Frozen tissue samples (150–200 mg) were homogenized (Polytron PT300, Kinematica, Switzerland), and total RNA was isolated with the RNeasy midi kit (Qiagen, Albertslund, Denmark). RNA was eluted in pure water, and the yield was quantified by measuring OD at 260 nM (GeneQuant II, AmershamPharmacia, Hørsholm, Denmark). Messenger RNA levels were estimated by solution hybridization followed by autoradiography displaying the result of ribonuclease protection assay for 11βHSD-2 mRNA in rat kidneys at different embryonal (E) and postnatal (P) stages. tRNA is a negative control, where the probe was hybridized to 20 μg of yeast tRNA. Bottom: quantification of 11βHSD-2 mRNA in rat kidney during postnatal development. Hybrids were cut out of the gel, and the radioactivity was assayed by counting in a β-counter. Columns represent mean values ± SE of n = 4 determinations at each stage. *Significantly different from P3, P7, and P28 as estimated by 95% confidence intervals.

B: Western immunoblotting of 11βHSD-2 protein in rat kidneys during postnatal developmental stages. Proteins were separated by PAGE and blotted onto membranes that were reacted with anti-rat 11βHSD-2 antibody. Bottom: densitometric evaluation of the gel. Columns represent the mean of 2 separate determinations in a single experiment. Three independent experiments were performed.

C: Immunolabeling of rat kidney sections for 11βHSD-2 at postnatal days indicated. Top: cortex; bottom: medulla. In early postnatal stages, immunolabeling was associated with cortical distal convoluted tubules, connecting tubules, and the collecting duct system extending to the tip of the immature papilla (P2 and P7). After postnatal week 4 (P29), 11βHSD-2 immunoreactivity was redistributed and gradually disappeared from the outer and inner medulla, resembling the typical adult pattern. Bottom micrograph at P29 shows the transition zone from outer to inner medulla.
by A/T1 ribonuclease protection assay using plasmids and protocols as described (2, 15). mRNA-cRNA hybrids were separated by denaturing PAGE. Autoradiography (Biomax, Kodak) was performed at –80°C for 6 h to 3 days. Subsequently, protected probes were excised and radioactivity was quantitated in a β-counter.

**Immunohistochemical and immunofluorescence analysis of kidney sections.** For immunolabeling, rat pup kidneys were fixed by systemic perfusion through the left cardiac ventricle with 4% paraformaldehyde solution in PBS (pH 7.35) for 5 min. Processing of tissue for immunohistochemical analysis was essentially as described in detail previously (8, 30, 31).

Primary antibodies used were goat polyclonal anti-rat COX-2 directed against the COOH terminus of rat COX-2 (1:500, Santa Cruz Biotechnology, sc-1747); rabbit polyclonal anti-rat GR (1:100, Santa Cruz, SC-1002) directed against the COOH terminus of the hormone binding GR-α isofrom; and rabbit polyclonal anti-rat 11βHSD-2 (1:1,000, Chemicon, AB 1296) polyclonal rabbit anti-rat Tamm-Horsfall glycoprotein (THP) antibody (1:100, a gift from Dr. J. Hoyer, Philadelphia, PA). For immunoperoxidase labeling, the sections were incubated for 1 h with horseradish peroxidase (HRP)-conjugated rabbit secondary antibody directed against the relevant species (DAKO). Signals were visualized by incubation for 2–30 min with 0.01% diaminobenzidine and 0.02% H2O2. Negative controls included incubation without primary antibodies and preabsorption where COX-2 and GR-α antibodies were incubated with the peptides used for immunization (10 μg/ml for 1.5 h). Double immunofluorescence labeling for GR-α and COX-2 was performed on paraffin-embedded rat kidney sections from P10. Sections were incubated with anti-COX-2 (1:500), anti-GR (1:100), anti-THP (1:100), and anti-11βHSD-2 (1:1,000) antibodies for 16 h at 4°C in 5% dry milk in TTBS. After being washed several times, the sections were incubated with donkey anti-goat IgG coupled to Alexa 594 (1:250; Molecular Probes) for 1 h and, after several washes, with goat anti-rabbit IgG (1:100) coupled to Alexa 488 (Molecular Probes). In other series, we used secondary antibodies from Dianova for THP visualization coupled to Cy2 (1:100) and for 11β-visualization coupled to Cy3 (1:250). Next, the sections were washed three times and then mounted with fluorescence mounting medium (DAKO). Sections were inspected with an Olympus BX51 microscope (Olympus Denmark) equipped with an HBO fluorescence lamp. Photos were captured with a digital camera (Olympus DP50) with DP-Soft (Olympus) software and processed with Adobe Photoshop/Corell draw.

**Cell culture experiments.** Rat renal medullary interstitial cells (RMIC) were kindly provided by Dr. T. Maack (19). The cells were routinely propagated in RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Confluent RMIC were treated for 16 h with vehicle or dexamethasone (RMIC) were kindly provided by Dr. T. Maack (19). The cells were embedded in buffer as previously described in detail (25, 31). Protein concentration was determined spectrophotometrically (Bio-Rad protein assay reagent) using serial dilution of BSA as a standard. The samples were separated by SDS-PAGE (7–10% gel) at 150–200 V for 30–40 min. Proteins were electroblotted (Bio-Rad) onto PVDF immobilon membranes (Milipore), and the membranes were reacted with primary and secondary antibodies as described (25, 31). Proteins from whole interstitial cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline, followed by incubation for 1 h with rabbit anti-murine polyclonal antiserum to COX-2 (Cayman) at a dilution of 1:500. After being washed with Tris-buffered saline, blots were incubated with a goat-anti-rabbit HRP-conjugated secondary antibody and visualized with ECL kits (Amersham). The band densities were evaluated by QuantityOne software from Bio-Rad and background subtracted. Data are presented as means ± SE densitometric units per square millimeter.

**Microdissection of cortical thick ascending limb segments and RT-PCR analysis.** Microdissection was performed basically as described previously (2). Rat pups were decapitated, and kidneys were cut in coronal slices that were digested with collagenase (Roche, Hvidovre, Denmark) for 25 min. The cortical thick ascending limb (cTAL) segments were identified by their localization in cortex, often closely associated with glomeruli, by their straight unbranched course and their relatively thin appearance. The isolated segments were transferred in 3–5 μl medium to 500 μl fresh DMEM in a 24-well cell culture plate on ice. Segments from one rat kidney were pooled, and the total length of loops was assessed by using a calibrated micrometer scale built into the ocular. We obtained between 8- and 17-mm cTAL in each dissection session. The segments were transferred to 400 μl guanidinium thiocyanate (4 M) solution, and 12 μg yeast tRNA (Roche) were added as carrier. Samples were stored at –80°C until extraction of RNA. RNA was isolated by a modified phenol-chloroform extraction protocol (2). Part of the RNA was reverse transcribed as described (2), and PCR was performed on cDNA corresponding to 1 mm of cTAL length.

**Statistics.** All values are given as means ± SE. When several sets of data were compared at the same time (e.g., data from different developmental stages), a one-way ANOVA was used to indicate statistical significant differences within the set of data. If the ANOVA was significant at the 5% level, differences between groups were established using 95% confidence intervals. An unpaired Student’s t-test was used to compare a control with an intervention group. A value of P ≤ 0.05 was considered significant.

### RESULTS

**Developmental changes in plasma aldosterone and corticosterone concentration in the postnatal period.** Plasma corticosterone and aldosterone concentrations were determined in the first 4 postnatal wk and in adult rats, thus covering the period before (P0-P3), during (P5-P22), and after (P27, adult) kidney COX-2 induction (28, 31, 36, 40, 41). Plasma total corticosterone changed markedly at birth and during postnatal development, as reported previously (12) (Fig. 1A). Relatively high levels were found a few hours after birth (P0), which was followed by a rapid decrease on P1 to P3, and a stable, low level was observed until day 13. Between P13 and P22, plasma

### Table 1. Effect of adrenal steroid receptor agonists and antagonists on rat pup whole body growth and kidney growth when administered through postnatal days 5–10

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 16)</th>
<th>Corticosterone (n = 5)</th>
<th>Dexamethasone (n = 5)</th>
<th>Mifepristone (n = 5)</th>
<th>DOCA (n = 5)</th>
<th>K+Canrenone (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>24.2 ± 0.5</td>
<td>21.4 ± 0.7*</td>
<td>12.6 ± 0.4*</td>
<td>24.1 ± 1.1</td>
<td>25.1 ± 1.8</td>
<td>22.8 ± 2.1</td>
</tr>
<tr>
<td>Total kidney weight, mg</td>
<td>278 ± 8.3</td>
<td>271.6 ± 7.8</td>
<td>203.6 ± 12.2*</td>
<td>267.8 ± 13.2</td>
<td>274.6 ± 24.5</td>
<td>263.4 ± 16</td>
</tr>
<tr>
<td>Kidney/body weight ratio, mg/g</td>
<td>11.5 ± 0.3</td>
<td>12.7 ± 0.4</td>
<td>16.1 ± 0.5*</td>
<td>11.1 ± 0.3</td>
<td>10.9 ± 0.3</td>
<td>11.5 ± 0.5</td>
</tr>
</tbody>
</table>

Values are means ± SE, n. No. of animals. *Significantly different from control as estimated by unpaired student’s t-test, P = 0.05.
corticosterone increased to levels found in adult rats (114 ± 18.7 ng/ml, n = 10). In the period P3-P13, plasma corticosterone was significantly lower compared with early postnatal (P0-P2), late (P22, P27), and adult developmental stages. Plasma aldosterone concentration was significantly lower than corticosterone concentration at all developmental stages (e.g., at P0, plasma corticosterone was 69.4 ± 15 ng/ml, =200 nmol/l and aldosterone was 414.3 ± 160.1 pg/ml, =1.4 nmol/l). Aldosterone peaked on the day of birth and then decreased on P1 and remained stable during suckling. In unstrained adult rats (P80), plasma aldosterone concentration was 82.4 ± 21.8 pg/ml (n = 5), which was not different from levels measured in the suckling period (P1-P23). Thus both plasma aldosterone and corticosterone displayed a birth peak and then rapidly decreased to stable levels around P3. Whereas plasma aldosterone remained at this level through development and maturity, corticosterone increased 10 times to adult levels in late postnatal week 3 and early postnatal week 4 as renal COX-2 decreased.

Developmental changes in renal GR, MR, and 11βHSD-2 mRNA expression in the perinatal period. The developmental pattern of MR and GR and of 11βHSD-2 mRNA expression in postnatal rat kidney was characterized with a focus on the stages before renal COX-2 induction (E19, P0, P3), during COX-2 induction (P7), and after the developmental decrease of renal COX-2 (P28, P56). MR and GR mRNAs were expressed at all stages investigated, ranging from E19 to P56. GR mRNA abundance increased significantly after birth, as previously reported (Ref. 17; Fig. 2). MR was stably expressed in the suckling period and decreased significantly after weaning. Of note, in the period of COX-2 changes (P3, P7, P28), there were no statistical significant differences in either GR or MR expression levels in the kidney. β-Actin expression was determined as an RNA quality control; however, β-actin expression was also developmentally regulated, with higher levels in embryonal and early postnatal kidneys than at later stages. Thus β-actin levels were decreasing as GR and MR were stable or increasing, which implies that RNA quality differences were not the cause of the observed changes in mRNA levels.

Next, we investigated renal 11βHSD-2 expression. 11βHSD-2 mRNA increased between birth and P3 and then stabilized (Fig. 3A). Notably, between P3 and P28, when renal COX-2 expression changes dramatically, there were no significant changes in 11βHSD-2 mRNA in the kidney. Similar to MR, 11βHSD-2 mRNA decreased in weanling rat kidney between days 28 and 56. Changes in whole kidney 11βHSD-2 protein abundance were less pronounced compared with mRNA (Fig. 3B), and stable levels were encountered from P7 (Fig. 3B). With respect to the potential significance for regulation of COX-2, it was of particular interest to determine the localization of 11βHSD-2 during kidney development. Immunoreactivity for 11βHSD-2 was associated with distal nephron segments and the whole, immature, collecting duct system in early postnatal stages (P2 and P7; Fig. 3C), as previously reported (30). Labeling reached from collecting ducts in the superficial most recently differentiated cortex to the tip of the immature papilla. Between postnatal weeks 3 and 4, there was a significant redistribution of 11βHSD-2 immunoreactivity that corresponded to the lower level of mRNA expression. Thus, at P29, immunolabeling had disappeared in the inner medullary collecting ducts and diminished in intensity along

![Fig. 4. A: effect of dexamethasone (dexam; 1 mg/kg) and corticosterone (CS; 20 mg/kg) supplementation during postnatal days 5 to 10 on whole kidney cyclooxygenase-2 (COX-2) mRNA abundance as determined by ribonuclease protection assay. Top: results of ribonuclease protection assays in which total RNA was hybridized with COX-2 antisense probe. Radioactivity in the protected probe was assayed by cutting the hybrids out of the dry gel and counting them in a β-counter. Histograms show the means of β-actin-normalized COX-2 mRNA level. *P ≤ 0.05. B: effect of dexamethasone and corticosterone treatment during postnatal days 5 to 10 on rat renal COX-2 protein levels. Top: result of immunoblotting experiments where kidney proteins were separated by PAGE and blotted onto membranes that were reacted with anti-rat COX-2-specific antibody. Bottom: result of densitometric evaluation of the above bands. C, control. *P ≤ 0.05.](http://ajprenal.physiology.org/)
the axis from cortex to outer medulla (Fig. 3C, P29, bottom). Staining for 11βHSD-2 was absent in the vasculature, proximal tubules, and loops of Henle and was not seen in the absence of primary antibody (not shown).

Effect of GR pathway on renal COX-2 expression in postnatal period. Administration of GR-selective and 11βHSD-2-resistant dexamethasone (1 mg/kg) through the period of low plasma corticosterone level and high renal COX-2 (P5-P10) essentially stopped growth, whereas corticosterone (20 mg/kg) had less marked effects on body weight (Table 1). Total kidney mass was reduced significantly only by dexamethasone, but, when normalized to body weight, the kidney-to-body weight ratios were increased by dexamethasone treatment compared with control rats (Table 1). This suggests less-pronounced catabolic effects or even stimulatory effects of glucocorticoids on kidney growth. Mifepristone had no effect on any of the parameters when given from P5 to P10 (Table 1). Dexamethasone reduced renal COX-2 mRNA to one-fourth the level in vehicle-injected rats (Fig. 4A). Corticosterone had a less-marked effect and reduced COX-2 mRNA level by one-half compared with vehicle-injected rats (Fig. 4A). β-Actin expression was not changed by glucocorticoid treatment, and COX-2 values were normalized with respect to β-actin. The GR antagonist mifepristone did not significantly alter kidney COX-2 expression when administered from P5 to P10, which is the period with COX-2 induction and low endogenous corticosterone (control 547 ± 51 cpm/20 μg RNA, n = 6, mifepristone 517 ± 31 cpm/20 μg RNA, n = 5, not shown). COX-2 protein level was significantly lowered in response to both dexamethasone and corticosterone administration, as evidenced by densitometric evaluation of Western immunoblots (Fig. 4B).

Microscopic inspection of kidney sections did not reveal obvious changes in kidney morphology (e.g., cysts or necrotic

![COX-2](image1.png)

**Fig. 5.** Immunohistochemical analyses of glucocorticoid-induced effects on cellular localization of COX-2 in postnatal rat kidneys. **Top:** in control kidney cortex at postnatal day 10, scattered foci of intensely COX-2-positive cells were observed in cortical loops of Henle. **Middle:** in response to corticosterone and dexamethasone, COX-2 immunoreactivity was less widely distributed and decreased in intensity. **Bottom:** immunolabeling of a control P10 kidney section was prevented when the COX-2 antibody was preincubated with a surplus of peptide used for immunization. Ab, COX-2 antibody. Bar = 200 and 50 μm at left and right, respectively.
areas) after 5 days of steroid treatment. Immunohistochemical labeling of paraffin-embedded kidney sections with a specific COX-2 antibody showed that COX-2 immunoreactivity was associated with cTAL of Henle’s loop in control kidneys at P10, as shown previously (Refs. 31, 36, 40; Fig. 5). Sections from dexamethasone- and corticosterone-treated kidneys displayed a very faint immunoreactivity for COX-2 that was restricted to a few cells in the loops of Henle (Fig. 5). When the COX-2 antibody was omitted, or preabsorbed with the peptide used for immunization, no labeling was observed (Fig. 5). Thus both the level of mRNA and protein and the distribution of COX-2 in cTAL are markedly reduced by glucocorticoid supplementation in the early postnatal period.

Effect of the aldosterone-MR pathway on renal COX-2 in the postnatal period. Next, we examined whether the aldosterone-MR pathway had any effects on growth and COX-2 expression during postnatal development. The MR antagonist potassium canrenoate (20 mg·kg⁻¹·day⁻¹ soldactone) was administered before COX-2 induction from P1 to P5 and during COX-2 induction from P5 to P10. Potassium canrenoate did not change body growth or kidney growth (Table 1) but led to a significant increase in renal COX-2 expression from P5 to P10 (Fig. 6A). Immunohistochemical labeling of kidney sections from rat pups given potassium canrenoate from P5 to P10 did not display any obvious changes in labeling distribution or intensity compared with vehicle-treated pup kidneys (Fig. 6B). When given during early postnatal stages, from P1 to P5, potassium canrenoate had no effect on renal COX-2 mRNA abundance (control 427 ± 22, n = 12; potassium canrenoate 439 ± 5, n = 8, cpm normalized for β-actin). The effect of the MR agonist DOCA on renal COX-2 was examined from P5 to P10, when endogenous plasma aldosterone is stable and renal COX-2 is elevated. Treatment with low doses of DOCA (1 mg/kg) preferentially activates MR but did not alter kidney or body growth (Table 1). Renal COX-2 expression was not significantly altered by DOCA from P5 to P10, although there was a tendency toward lower COX-2 expression (control 547 ± 51, n = 6; DOCA 447 ± 51 cpm/20 μg RNA, n = 5).

Localization of GR in rat kidney cortex in the postnatal period. On the basis of the ability of GR activation to suppress renocortical COX-2, we hypothesized that GR was localized in the loop of Henle during postnatal development. Immunohistochemical analysis of kidney sections with a specific antibody directed against the COOH terminus of hormone binding subunit GR-α showed immunoreactive labeling at all stages examined (P2, P7, P10, and P28) in accordance with the mRNA data. GR-α immunoreactivity was associated preferentially with nuclei in some immunopositive structures (e.g., glomeruli, loops of Henle) and with cytoplasm in others (proximal tubules). GR immunoreactivity was absent in undifferentiated mesenchyme cells in P2 kidneys and was first observed in immature stage III-IV glomeruli, where nuclei were strongly labeled (Fig. 7; P2). In the mature deep cortex, intraglomerular cell nuclei were also immunopositive, whereas proximal tubules displayed cytoplasmic labeling. Immature loops of Henle with no lumen and the bend still in superficial parts of cortex were not labeled for GR. In more mature stage IV glomeruli, where the loops have lumen, labeling for GR was associated with nuclei along the tubules, including both the macula densa segment and outer medullary loops of Henle (Fig. 7). At postnatal day 2, the collecting ducts were negative for GR, whereas at later stages, single, dispersed cells along the cortical ducts were positive, most likely intercalated cells, since this labeling was absent in the inner medulla (Ref. 25; Figs. 7 and 8). Staining for GR was not observed in the absence of primary or secondary antibody or when the antibody had been blocked by the peptide used for immunization (Fig. 7).
Thus considering the expression pattern of 11βHSD-2, glucocorticoid and mineralocorticoid signaling pathways seem to be expressed at mutually exclusive sites through postnatal kidney development.

**Effect of glucocorticoids on COX-2 in the medulla.** It has previously been shown that COX-2 is expressed in RMIC (11, 31, 40). In the above experiments, we analyzed whole kidneys because at P10, medullary COX-2 levels are at least 10 times lower than cortical levels (31). However, to ensure that the effect of glucocorticoids on COX-2 in cortex was not obscured by opposite changes in the medulla, we examined whether glucocorticoids affected COX-2 expression in renal medullary cells. As shown in the kidney section in Fig. 8A, GR-α immunoreactivity was exclusively present in nuclei of medullary interstitial cells and not in inner medullary collecting duct cells. The interstitial cell expression of GR became more pronounced with development. We examined the effect of glucocorticoids on COX-2 expression in cultured RMIC from adult rats. RMIC were treated for 16 h with vehicle or GR-specific dexamethasone at 0.5–2 μmol/l, and COX-2 protein expression was determined by immunoblotting. RMIC expressed abundant COX-2 protein that was suppressed by dexamethasone (Fig. 8B). Immunolabeling of the inner medulla for COX-2 at P10 showed that immunoreactivity was associated with interstitial cells in particular with the perinuclear area and staining was less marked and seen in fewer cells in response to dexamethasone from P5 to P10 (Fig. 8C).

**Colocalization of GR, 11βHSD-2, and COX-2 in the loop of Henle.** In a second set of experiments, we addressed in further detail whether GR, MR, and 11βHSD-2 were expressed along with COX-2 in loops of Henle by RT-PCR analysis and double-immunofluorescence labeling. cTALs were microdissected from developmental stages with maximal COX-2 induction (P7–9, P11, and P18) (28, 31, 36, 40), and cDNA corresponding to 1-mm tubule length was used for PCR-amplification for 32 cycles. Only long segments reaching into the medulla were dissected. PCR for GR yielded amplification products at all stages examined. GR was amplified only in the presence of reverse transcriptase, confirming that the amplification products originated from mRNA (Fig. 9, top). MR and 11βHSD-2 were also detected in the microdissected samples (Fig. 9, bottom). COX-2 was readily amplified as a positive control from all samples. To ensure correct identification of TAL vs. descending limbs of loops of Henle, which can be mistaken during dissection, we amplified AQP1, which is a marker for the descending limb. AQP1 was not found in the dissected cTAL segments at any stage but was readily amplified from control cDNA from whole kidney (Fig. 9, bottom).

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**Fig. 7.** Immunohistochemical analysis of GR-α isoform distribution in paraffin-embedded kidney sections from various postnatal developmental stages. At postnatal day 2 (P2), GR-α-immunopositive cells were observed in glomeruli, proximal tubules, and mature loops of Henle with lumen, whereas immature growing loops and collecting ducts were negative. Immunolabeling was associated with cell nuclei in loops of Henle. At P7, glomeruli were labeled and one mature growing loop was GR positive, whereas 3 immature loops with their bend not far from the parent glomeruli were GR negative. At P10, growing loops, still in the outer medulla, displayed GR immunoreactivity in nuclei (no counterstaining). At P21, GR immunolabeling was associated with nuclei in glomerulus, the macula densa segment, intercalated cells, and interstitial cells. Bottom: preabsorption of the GR-α antibody (Ab) with peptide used for immunization abolished labeling. Bars = 50 μm.
We did not observe amplification products in the absence of added cDNA in any of the PCRs.

Subsequently, we performed double-immunofluorescence labeling experiments to specifically address whether cortical COX-2 immunoreactivity is colocalized with GR and 11βHSD-2-immunoreactive proteins in the early postnatal period. Kidney sections were labeled on P10, when COX-2 was maximally elevated and widely distributed. In the cortex, COX-2 immunofluorescence (Fig. 10, top and middle micrographs, red) was associated with loops of Henle only, whereas labeling for GR (middle, green) was associated with glomeruli, proximal convoluted tubules, and loops of Henle. All cells positive for COX-2 also expressed GR (double labeling, orange fluorescence), showing that both mRNA transcripts and proteins are present in cortical loop of Henle cells during postnatal development. In contrast to the colocalization of COX-2 and GR, 11βHSD-2 immunofluorescence (Fig. 10, top, green) was localized in the collecting ducts and there was no overlap with COX-2 (red fluorescence). This observation was confirmed by the TAL marker THP (Fig. 10, bottom, green fluorescence), which was not observed together with 11βHSD-2 (red fluorescence).

DISCUSSION

In the present paper, we confirm in the rat that following a marked birth peak, there was a developmental decrease in plasma total corticosterone concentration in the first postnatal weeks (12, 41), essentially the suckling period, that coincided with kidney COX-2 induction (28, 31, 36, 40). During low endogenous glucocorticoid and high intrarenal COX-2 (P5–P10), supplementation with GR-specific dexa-
methasone and nonselective corticosterone significantly decreased renocortical levels of COX-2 mRNA and protein and led to more restricted distribution of immunoreactive COX-2 protein. This sensitivity of COX-2 was reflected in colocalization of COX-2 and GR-α mRNA and immunoreactive protein in TAL of Henle’s loop (and in renomedullary interstitial cells). GR-α was expressed in developing loops of Henle at all stages of postnatal renal development, which is a prerequisite for the sensitivity of COX-2 to endogenous changes in circulating GR agonist. After a birth peak, plasma aldosterone rapidly stabilized at a level found in control adult rats and did not correlate with developmental changes in COX-2. Selective action of aldosterone depends on coexpression of 11βHSD-2. PCR amplification showed expression of 11βHSD-2 in microdissected loops of Henle in the first and second postnatal week, whereas immunohistochemical staining with an anti-11βHSD-2 antibody yielded weak or no labeling at all associated with this segment during postnatal development (Figs. 9 and 10), while collecting ducts were strongly labeled. The contrasting findings indicate that 11βHSD-2 is much less abundant in the loop of Henle compared with the collecting duct and suggest that an abrupt change in the ability to inactivate glucocorticoids in the loop of Henle cannot explain the induction of COX-2 during development. The sensitivity of COX-2 for corticosterone in the loop of Henle implies that 11βHSD-2 activity is insufficient for protection against GR activation, although high doses of exogenous corticosterone may have exceeded enzymatic capacity. However, the nuclear localization of GR immunoreactivity in loop of Henle cells during normal postnatal development also supports insufficient 11βHSD-2 activity. Nuclear translocation of activated GR is inhibited by the presence of active 11βHSD-2 (16, 27). On the other hand, blockade of MR by potassium canrenoate during P5–P10 increased cortical COX-2 expression, which is compatible with the notion that activation of MR, by either gluco- or mineralocorticoid, suppresses COX-2 expression in the loop of Henle, as observed previously in young and adult rats (39, 41). This effect could be mediated directly, because by PCR we found MR expression in the loop in the first 2 postnatal weeks. Previous reports showed very low expression of MR, or none at all, in the loop of Henle in fetal and adult kidney (5, 32). Supplementation with DOCA in low doses that are MR specific failed to change COX-2 levels, which argues against a direct, MR-mediated effect on COX-2. The stimulatory effect of potassium canrenoate on COX-2 could also be indirectly mediated through changes in NaCl and water homeostasis. Potassium canrenoate would be expected to block MR in the collecting duct system and increase sodium excretion in the pups. Renal COX-2 expression in the postnatal period is highly sensitive to changes in NaCl balance, and an increase in NaCl excretion would increase COX-2 expression, which is compatible with the notion that suppression of COX-2 is mediated predominantly through interaction with GR.

Fig. 10. Double-immunofluorescence analysis of COX-2 and 11βHSD-2 (top), COX-2 and GR-α (middle), and 11βHSD-2 and Tamm-Horsfall glycoprotein (THP) (bottom) localization at a stage with COX-2 induction in postnatal week 2. Top: COX-2 (red fluorescence) and 11βHSD-2 (green fluorescence) were mutually exclusive in kidney cortex. COX-2 (red fluorescence) colocalized (orange fluorescence) with GR (green fluorescence) in loops of Henle. Middle: GR was also detected in COX-2-negative structures, e.g., glomeruli. Bottom: labeling for the thick ascending limb marker THP (green fluorescence) confirmed the absence of 11βHSD-2 immunoreactivity (red fluorescence) in the cortical loops of Henle. Magnification: ×150.
COX-2 is expressed in medullary interstitial cells (11, 31, 40). Corticosterone has been shown to suppress COX-2 expression in cultured interstitial cells (38). Our data confirm that this effect is indeed GR mediated because it was mimicked by GR-specific dexamethasone in vitro and in vivo and reflected in coexpression of GR-α and COX-2 immunoreactivity in these cells in vivo. Overall, our data on GR and 11βHSD-2 localization indicate separate and mutually exclusive sites of glucocorticoid and mineralocorticoid action along the nephron, collecting ducts, and interstitial cells in the postnatal period. We found that GR-α was localized primarily in 11βHSD-2-negative structures in the developing kidney (e.g., glomeruli, proximal tubules, interstitial cells) similar to the reported distribution in adult rat kidney (25, 32) and in adult and fetal human kidney (5, 39). On the basis of the changes in 11βHSD-2 during development, aldosterone selectivity is expected to be enhanced during suckling along 11βHSD-2-positive segments, connecting tubule, and the whole collecting duct system. Aldosterone sensitivity is also likely to be enhanced, because MR expression was increased during suckling and decreased after weaning. The combination of normal circulating plasma aldosterone and low circulating corticosterone in the suckling period further adds to the selectivity of aldosterone over corticosterone in renal target cells and underlines the established, crucial role of intact aldosterone signaling for sodium conservation and survival in the first critical days and weeks of life (3). The high 11βHSD-2 activity in kidney shortly after birth might provide an explanation for the marked tissue-specific difference in glucocorticoid induction of aldosterone targets, e.g., ENaC, during early postnatal development, where the kidneys are relatively insensitive compared with lung (24).

Administration of glucocorticoid in the fetal or early postnatal period has profound effects on renal function at sites compatible with the present immunolocalization of GR-α. Thus, in fetal sheep, glucocorticoid increases glomerular filtration rate, renal blood flow, urinary flow rate, and tubular acidification (13), and expression of the proximal tubular Na-H exchanger-3 is augmented (10). In humans, glucocorticoid increases creatinine clearance and fractional sodium reabsorption when given to mothers before preterm delivery (1). Thus functional data, and the widespread expression of GR in the fetus (5), indicate that glucocorticoids regulate diverse organ functions, such as lung maturation, at birth, where glucocorticoid levels peak in many species. On the other hand, during intrauterine life the fetus is protected against glucocorticoids by high expression of 11βHSD-2 in both placenta and multiple fetal organs (5). The present set of data suggests that, apart from the birth peak, low levels of circulating corticosterone prevail in the first weeks of life in the rat, thus mimicking intrauterine conditions in other species. In this “window” of development, which is extraterine in the rat, circulating glucocorticoid spontaneously decreases and allows expression of glucocorticoid-suppressed pathways such as renal COX-2. Inhibition of COX-2-mediated signaling during late nephrogenesis could be one pathway by which excessive glucocorticoid exposure leads to aberrant development of the kidney. Glucocorticoid treatment in early postnatal life or an elevation of endogenous glucocorticoid leads to fewer glomeruli, sodium retention, and hypertension in adult rats (4, 22, 29). Similarly, COX-2 deficiency or COX-2 inhibition leads to cortical damage during development (7, 21, 23, 26). The window where renal COX-2 is elevated could define an interval where glucocorticoids are deleterious for nephrogenesis. This interval is likely to vary considerably between species but should be taken into account when glucocorticoids are administered in pregnancy or early postnatal life.

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