

# Cyclooxygenase-2–selective inhibitors impair glomerulogenesis and renal cortical development

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## Cyclooxygenase-2–selective inhibitors impair glomerulogenesis and renal cortical development.

**Background.** Antenatal exposure to nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with renal dysgenesis in humans.

**Methods.** These studies characterized cyclooxygenase-2 (COX-2) versus COX-1–selective inhibition on nephrogenesis in the rodent using histomorphometry, immunohistology, and *in situ* hybridization.

**Results.** Administration of a COX-2–selective inhibitor (SC58236), started during pregnancy until weaning, significantly impaired development of the renal cortex and reduced glomerular diameter in both mice and rats. An identical phenotype was demonstrated in COX-2  $-/-$  mice. In contrast to its effects on the developing kidney, a COX-2 inhibitor had no effect on glomerular volume in adult mice. This effect was specific for COX-2 because maternal administration of a COX-1–selective inhibitor (SC58560) did not affect renal development despite significantly inhibiting gastric mucosal prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis in pups. The expression of COX-2 immunoreactivity peaked in the first postnatal week and was localized to S-shaped bodies and the macula densa in the cortex. Treatment with a COX-2 inhibitor during this period (from postnatal day 0 to day 21) severely reduced glomerular diameter, whereas treatment limited to pregnancy did not affect glomerular size.

**Conclusion.** These data demonstrate an important role for COX-2 activity in nephrogenesis in the rodent, and define a specific time period of susceptibility to these effects.

Reports of renal dysgenesis in newborns of women treated with nonsteroidal antiinflammatory drugs (NSAIDs) during pregnancy suggest a role for prosta-

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**Key words:** cyclooxygenase, NSAIDs, nephrogenesis, prostaglandins, macula densa.

Received for publication July 7, 1999  
and in revised form September 1, 1999

Accepted for publication September 28, 1999

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glandins in renal development. These renal abnormalities range from oliguria to renal dysgenesis and have been seen particularly when NSAIDs were administered before the 32nd week of gestation [1, 2]. Similar findings were observed in offspring of rhesus monkeys treated with indomethacin during gestation [3]. Avner and co-workers showed that prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) is necessary for maximal growth and differentiation of metanephroi in culture [4]. Taken together, these data suggest an important role for cyclooxygenase (COX)-mediated prostaglandin formation in renal development.

Two isoforms of COX are known to exist. COX-1 is expressed constitutively and considered a housekeeping gene, having important roles in the maintenance of epithelial integrity. COX-2 was identified as an immediate early-response gene, up-regulated in fibroblasts treated with tumor-promoting phorbol esters [5]. COX-2 is not constitutively expressed in many tissues and is only observed after they are exposed to cytokines and growth factors. However, COX-2 is expressed constitutively in fetal and adult kidney in all species examined [6–9]. Gene knockout studies show that COX-1 disruption does not interfere with normal renal development [10]. In contrast, COX-2–deficient mice exhibit renal dysgenesis associated with hypoplastic glomeruli [11, 12]. These structural renal abnormalities in COX-2 null mice have not been quantitatively defined. Furthermore, the effect of COX-2 disruption on renal morphology in the adult could not be separated from the earlier effects on the neonate. Recently, COX-2–selective NSAIDs have become widely available for clinical use as antiinflammatory analgesics. It has been argued that these inhibitors might not cause the same renal changes seen in COX-2–deficient mice, which might rather be due to compensatory changes in other biologic pathways resulting from COX-2 gene disruption or interference with a single event at a critical time in development [13, 14]. The

purpose of these studies was to determine whether or not the administration of COX-2 isoform-selective NSAIDs cause renal abnormalities in developing and adult mice and rats, and compare these effects to those in COX-2<sup>-/-</sup> mice.

## METHODS

### Animals

C57 Bl/6 J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and Sprague Dawley rats (Harlan Bioproducts for Science Inc., Indianapolis, IN, USA) were used to produce timed pregnancies. Noon of the sperm-positive morning was designated as embryonic day 0.5 (E0.5); the day of birth was designated postnatal day 0 (P0). Offspring from C57 Bl/129 *Ptgs 2*<sup>-/+</sup> were genotyped as outlined previously [11]. The isozyme-specific COX inhibitors SC58560 (COX-1) and SC58236 (COX-2) were kindly provided by Drs. P.C. Isakson and K. Seibert (Monsanto Searle, St. Louis, MO, USA). For studies in mice, the compounds were diluted to a final concentration of 0.6 mg/L (SC58236) and 3 mg/L (SC58560) in tap water with 0.01% Tween and 0.2% polyethylene glycol 200 (PEG 200) as solvents. The administration of the drugs was initiated at E0.5 or P0. Blood levels of the drug were determined by high-performance liquid chromatography at Searle/Monsanto (kindly performed by Drs. Jerry Muhammed and Peter Isakson). For studies on pregnant rats, daily gavage of SC58236 in methylcellulose vehicle (10 mg/kg body wt/day) was begun on E10.5 and was continued through weaning.

### Quantitation of prostaglandin E<sub>2</sub> synthesis in gastric mucosa

Mucosal linings of stomachs from control and experimental animals were harvested and frozen in dry ice. Tissue was homogenized in 0.1 sodium phosphate buffer containing 15% methanol. Samples were centrifuged at 12,000 r.p.m. at 4°C in a tabletop centrifuge. Supernatants were passed through a C-18 column (Waters, Milford, MA, USA) preconditioned with 2 mL of methanol followed by 2 mL of water. C-18 columns were washed with H<sub>2</sub>O/methanol (97/3), H<sub>2</sub>O, and hexane. PGE<sub>2</sub> was eluted in 1 mL of ethylacetate. The solvent was evaporated under a stream of nitrogen and dissolved in 500 µL of extraction buffer. PGE<sub>2</sub> content in gastric mucosa was determined by enzyme immunoassay (EIA) (PGE<sub>2</sub> enzyme immunoassay kit; Oxford Biomedical Research Inc., Oxford, MI, USA).

### Reverse transcription-polymerase chain reaction

Total RNA was purified from mouse neonatal kidney using TRIZOL-REAGENT™ (GIBCO BRL, Grand Island, NY, USA) and reverse transcribed to single-

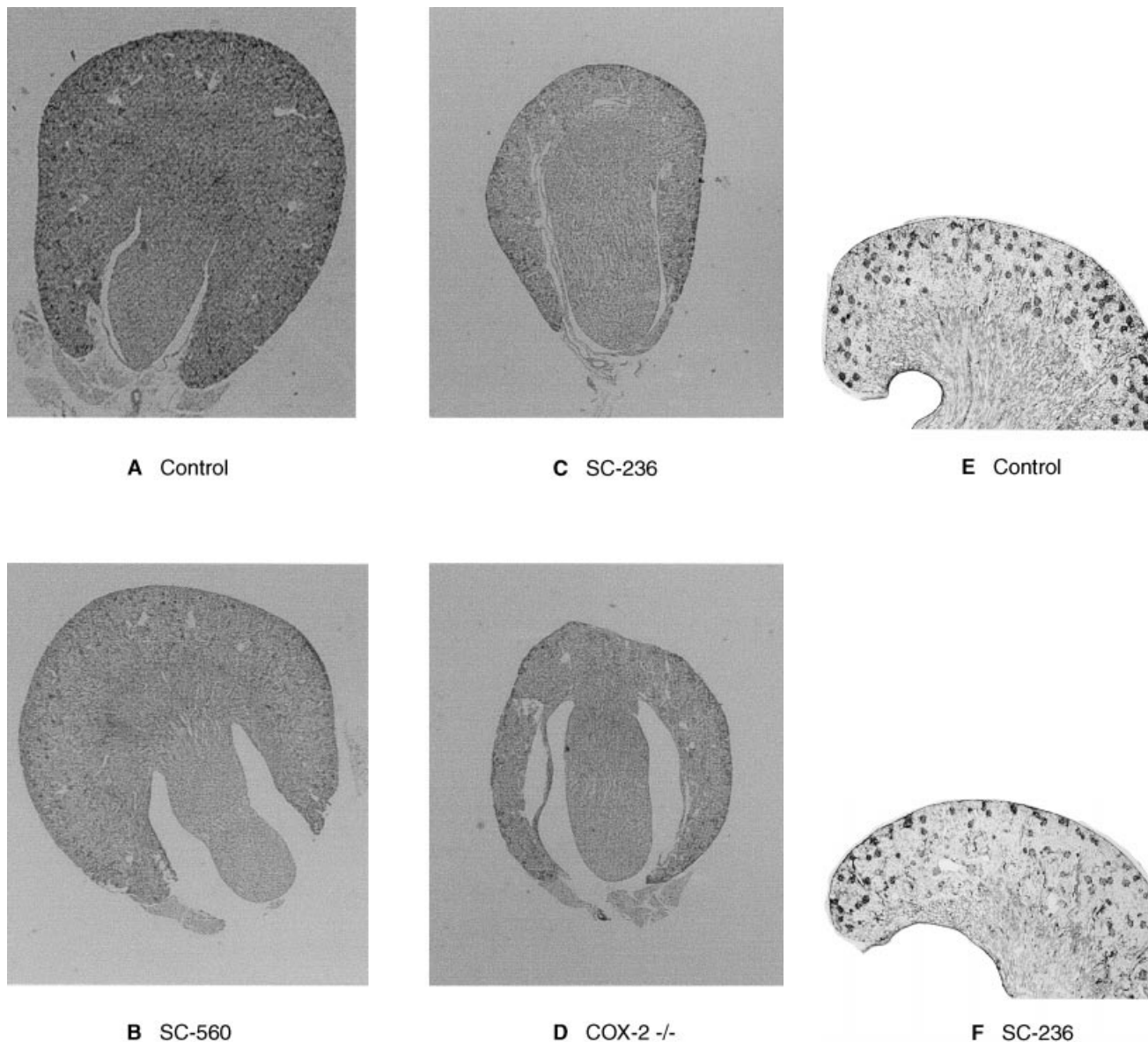
stranded cDNAs using Moloney murine leukemia virus reverse transcriptase and 2.5 µmol/L of random primers according to manufacturer's instructions (GeneAmp RNA PCR kit; Perkin Elmer Cetus, Norwalk, CT, USA). Polymerase chain reaction (PCR) was carried out using selective primers (10 pmol each primer), 2.5 U Taq DNA polymerase (Stratagene, La Jolla, CA, USA) in a 20 µL final reaction volume containing 2 mmol/L MgCl<sub>2</sub>, 60 mmol/L KCl, 12 mmol/L Tris-HCl, pH 8.3. The COX-2 upstream primer was 5' CAG AAG GCA ATG TTC TTG AAC 3', and the downstream primer was 5' CAA TCC CTG ACA TGG 3'. Amplification products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. Fragments were cloned into pBluescript (SK-; Stratagene) and identified by dideoxy sequencing. Sequence data were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>).

### Immunoblotting

Kidneys were homogenized in 30 mmol/L Tris (hydroxymethyl)aminomethane (Tris) hydrochloride, pH 8.5, and 100 mmol/L phenylmethylsulfonylfluoride (PMSF). Following a 10-minute centrifugation at 10,000 × g, the supernatant was centrifuged at 100,000 × g to prepare microsomes as described previously. Microsomes were harvested in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [120 mmol/L Tris-HCl, pH 6.5, 4% SDS, 5 mmol/L dithiothreitol (DTT), and 20% glycerol] followed by repetitive aspiration. This material was then heated in boiling water for three minutes, and the protein concentration was determined. Twenty milligrams of each protein extract were loaded onto a 10% SDS-PAGE mini-gel and run at 200 V. After transfer to nitrocellulose membrane (100 V, 1 h, 4°C), the membrane was washed three times with TBST (50 mmol/L Tris, pH 7.5, containing 150 mmol/L NaCl, 0.05% Tween 20) and then incubated in blocking buffer (TBST containing 5% Carnation nonfat dry milk) for one hour at room temperature. The nitrocellulose membrane was then incubated in primary antibody (COX-1, Santa Cruz, C-20; COX-2, Cayman, 160106) diluted 1:300 and 1:1000 in blocking buffer overnight at 4°C. Following washing, the membrane was incubated with horseradish-peroxidase-labeled secondary antibody (1:20,000; Vector Lab., Inc., Burlingame, CA, USA) for one hour and was washed in TBST, and labeling was visualized with chemiluminescence reagent (DuPont NEN, Boston, MA, USA) exposing the membrane to Kodak XAR-5 film.

### Micrography and quantitative image analysis

Photomicrographs were viewed with a Zeiss Axioskop microscope using either bright field or dark-field optics (Micro Video Instruments, Avon, MA, USA). Pictures were captured with a digital camera (Spot-Cam; Diag-



**Fig. 1. Reduced cortical volume in COX-2 inhibitor and COX-2  $-/-$  mice.** Mouse kidneys were harvested at P21 and stained with hematoxylin (A–D). Compared with control kidneys (A) and kidneys from mice treated with a COX-1 inhibitor (B), low-power magnifications of coronal kidney sections reveal a reduced cortical volume in COX-2 inhibitor-treated mice (C) and COX-2  $-/-$  mice (D). Rat kidney sections (E and F) were stained with an anti lipocortin antibody, which highlight glomeruli (brown). A similar reduction in cortical volume is seen in rats treated with the COX-2 inhibitor (F) compared with control kidneys (E).

nostic Instruments, Sterling Heights, MI, USA), and color composites were generated by using Adobe Photoshop v4.0 on a Power Macintosh. The diameter of glomeruli was measured perpendicularly to the renal capsule using BIOQUANT (R&M Biometrics, Nashville, TN, USA). Three coronal kidney sections from three individual animals from each group were analyzed.

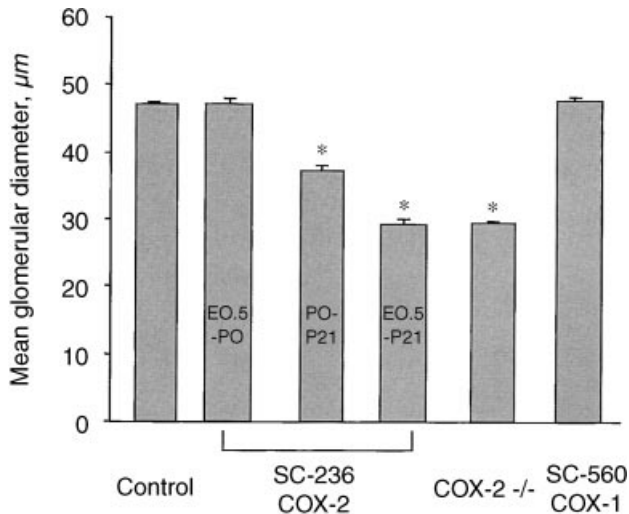
#### ***In situ* hybridization**

Mouse tissue was fixed in 4% paraformaldehyde overnight and embedded in paraffin.  $^{35}$ S-labeled antisense

and sense riboprobes from mouse COX-2 (597 bp) were hybridized to tissue sections and washed as previously described [6]. Slides were then dipped in emulsion (Ilford K5; Knutsford, Cheshire, England, UK) diluted 1:1 with 2% glycerol and exposed for four to five days at 4°C. After developing in Kodak D-19, slides were counterstained with hematoxylin.

#### **Immunohistochemistry**

Mouse tissue was fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections were cut at



**Fig. 2. Histologic analysis and histomorphometry of renal cortical development in control and experimental animals.** The mean glomerular diameter was determined in control, COX-1- or COX-2-selective inhibitors treated mice, and in COX-2  $-/-$  mice. In control animals and COX-1 inhibitor (SC58560)-treated mice, the mean glomerular diameter was similar, whereas it was significantly reduced by the COX-2 inhibitor (SC58236) either from conception to P21 or from P0 to P21. \* $P < 0.00001$  compared with control values.

4  $\mu\text{m}$  thickness, dewaxed in xylenes, and incubated for 30 minutes in methanol containing 0.3%  $\text{H}_2\text{O}_2$  to block endogenous peroxidase activity. Sections were then microwaved for three minutes in PBS containing 0.1 mol/L sodium citrate. Primary antibody was diluted 1:200 in TBST containing 1% bovine serum albumin (BSA) and 1% nonfat dry milk. The antibodies were visualized using Vector Stain Elite and counterstained with hematoxylin. For rat tissue, rats were exsanguinated under deep anesthesia with 50 mL/100 g heparinized saline (0.9% NaCl, 2 U/mL heparin, 0.02% sodium nitrite) through a transcardiac aortic cannula and fixed with glutaraldehyde-periodate acid saline (GPAS), as described previously [6]. Following dehydration with a graded series of ethanol and embedding in paraffin, immunostaining was performed as described previously in this article.

## RESULTS

### Effects of COX-1 and COX-2 inhibitors on renal development

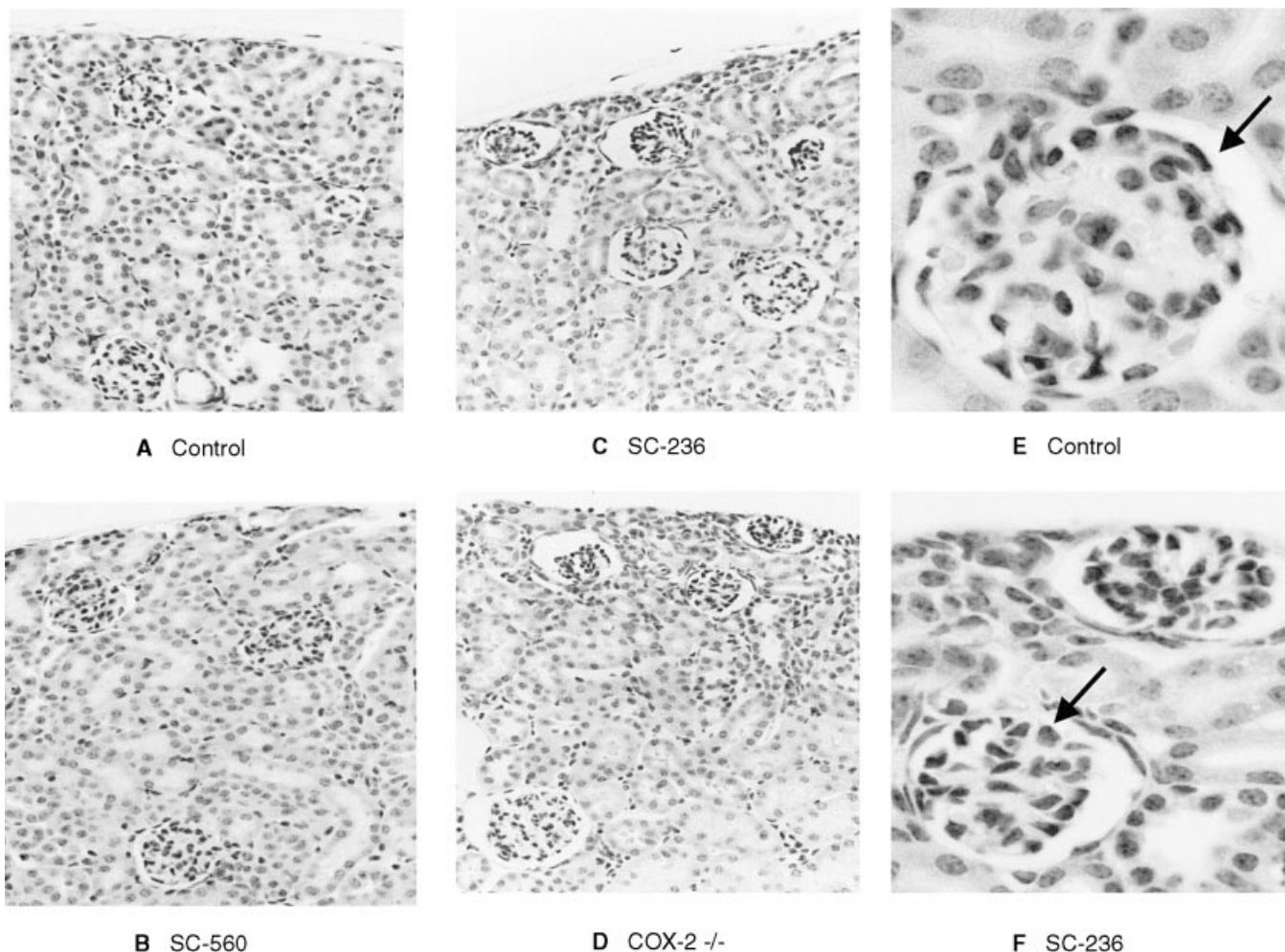
The effects of pharmacologic inhibition of COX-1 versus COX-2 on murine kidney development were investigated by treating pregnant mice from E0.5 (morning of the sperm positive day) with the COX-2 inhibitor SC58236 or the COX-1 inhibitor SC58560 until P0 or weaning at P21. At P0, no effects on renal morphology were observed in either COX-1 or -2 inhibitor-treated mice or in COX-2  $-/-$  mice. Treatment with the COX-2

inhibitor markedly reduced renal cortical volume, as seen on low-power views of kidneys at P21 compared with control kidneys (Fig. 1 A, C). No overt effect on kidney morphology is seen in animals treated with the COX-1 inhibitor (Fig. 1B). Similarly, P21 kidneys from COX-2  $-/-$  mice (Fig. 1D) showed reduced cortical volume despite maintaining normal papillary volume. Similar findings were observed in rats treated with SC58236 from E10.5 (inception of metanephric development) to P15 (Fig. 1 E, F).

Impaired renal cortical development in mice was accompanied by reduced mean glomerular cross-sectional diameter from  $47.01 \pm 0.41 \mu\text{m}$  in controls to  $29.35 \pm 0.42 \mu\text{m}$  in COX-2 inhibitor-treated mice ( $P < 0.0001$  vs. control,  $N = 411$ ; Fig. 2). This decrease was identical to that seen in COX-2  $-/-$  mice in which the glomerular diameter was  $29.35 \pm 0.68 \mu\text{m}$  ( $P < 0.0001$  vs. control,  $N = 320$ ). Glomerular cells were more densely packed, and podocytes within cortical glomeruli of COX-2 inhibitor-treated animals (Fig. 3E) and COX  $-/-$  mice were cuboidal, whereas podocytes within subcapsular glomeruli from control (Fig. 3F) mice were flattened. The administration of SC58236 also reduced glomerular diameter in developing rat kidney treated from E10.5 to P15. Even after perfusion fixation of rat kidneys and expansion of rat glomeruli, the diameter was significantly reduced from  $54.11 \pm 0.87 \mu\text{m}$  in control rats versus  $51.16 \pm 0.32 \mu\text{m}$  in SC58236-treated animals (control,  $N = 125$ ; experimental,  $N = 1441$ ,  $P < 0.01$ ). The small glomeruli did not represent a complete failure of glomerulogenesis because glomeruli from SC58236-treated rat pups were positive for factor VIII staining (endothelial cells) and Thy 1.1 staining (mesangial cells; data not shown). Furthermore, erythrocytes were present in the small glomeruli of nonperfused mouse kidneys. Tubules in the immediate subcapsular area were reduced in volume in COX-2 inhibitor-treated mice.

To determine the stage of renal development affected by the COX-2 inhibitor, we limited treatment of mice with SC58236 to P0 through P21 (0.6 mg/L SC58236 in the drinking water). Plasma levels for the COX-2 inhibitor were 18 and 8 mg/L for mother and pups, respectively. A similar but less pronounced reduction in glomerular diameter (22%) was observed in mice in which COX-2 inhibitor treatment was limited to the postnatal period ( $37.31 \pm 0.68 \mu\text{m}$  vs.  $47.01 \pm 0.67 \mu\text{m}$  in control,  $P < 0.0001$ ,  $N = 350$ ; and  $P < 0.0000001$  vs. E0.5 to P21; Fig. 3A). In contrast, when SC58236 was limited to the gestational period and stopped at P0, no effect on glomerular diameter was appreciated ( $47.1 \pm 0.65 \mu\text{m}$  in experimental animals). Thus, postnatal murine renal development is most sensitive to COX-2 inhibition.

Initial inspection suggested that COX-2 inhibition did not affect juxtamedullary glomerular size. This impression was morphometrically confirmed. The juxtamedul-



**Fig. 3. Histologic analysis and histomorphometry of renal cortical development in control and experimental animals at postnatal day 21.** Kidney sections from control and experimental animals at P21 were stained with hematoxylin. In control animals, cortical glomeruli exhibit a mature morphology, but they are still smaller in size compared with juxtamedullary glomeruli at P21 (A). A similar pattern is observed in animals treated with the COX-1 inhibitor (B). Up to three layers of tubules occupy the space between the subcapsular glomeruli and the renal capsule. (C) In COX-2 inhibitor-treated animals, subcapsular glomeruli exhibit greatly reduced size. The urinary space of Bowman's capsule was increased. In addition, the outermost layer of tubules appears thinned and immature. (D) A phenotype similar to mice treated with the COX-2 inhibitor including immature tubules and minuscule cortical glomeruli is seen in COX-2  $-/-$  mice. Podocytes within cortical glomeruli from controls are flattened (E; arrows), whereas podocytes within subcapsular glomeruli of COX-2 inhibitor-treated mice (F; arrows) are cuboidal.

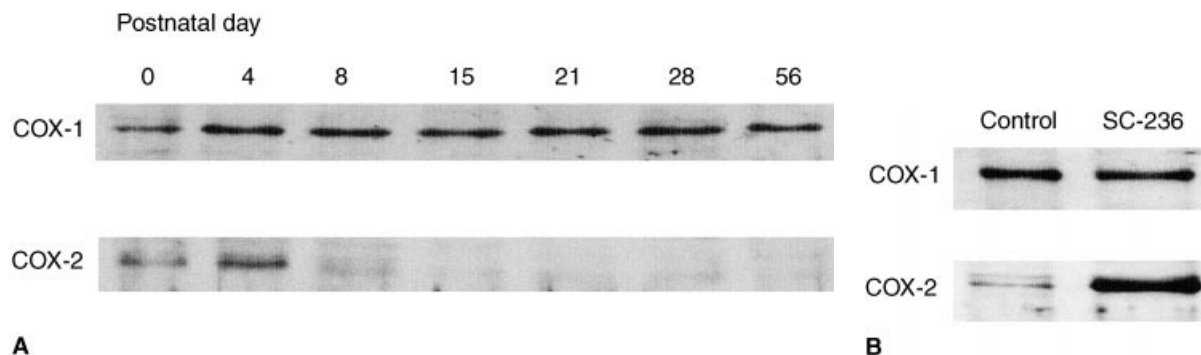
lary glomeruli diameter was  $47.11 \pm 0.56 \mu\text{m}$  and unchanged by the COX-2 inhibitor ( $46.59 \pm 0.38 \mu\text{m}$ , not significant vs. control) and slightly larger in COX-2  $-/-$  mice ( $49.71 \pm 0.59 \mu\text{m}$ ,  $P < 0.05$ ,  $N = 170$ ).

The effect of the COX-2 inhibitor was also tested in adult mice (12 weeks of age). The administration of SC58236 ( $0.6 \text{ mg/mL}$ )  $\times 12$  weeks did not affect mean glomerular cross-sectional diameter:  $48.9 \pm 0.63 \mu\text{m}$  in controls vs.  $50.02 \pm 0.59 \mu\text{m}$  ( $N = 230$ ) in experimental animals. No difference in glomerular diameter was observed in mice treated with the COX-1 inhibitor SC58560 throughout gestation and postnatal development ( $47.66 \pm 0.43 \mu\text{m}$ ,  $P < 0.0001$ ,  $N = 310$ ; Fig. 2). Likewise, the renal

cortex and medulla did not exhibit detectable changes following treatment with the COX-1 inhibitor (Fig. 3B).

#### Effects of COX-1-selective inhibitors on gastric mucosal prostaglandin $E_2$ synthesis

Because renal cortical development was not affected by administration of the COX-1-selective inhibitor, we assessed drug transfer from the mother to the pups by measuring COX-1 dependent  $PGE_2$  synthesis in the gastric mucosa of the pups [13]. Maternal SC58560 treatment reduced gastric  $PGE_2$  synthesis in the pups by 86%, from  $4.98 \text{ ng/mg} \pm 0.93$  protein of mucosal tissue in control pups, to  $0.58 \text{ ng/mg} \pm 0.64$  of mucosal tissue in SC58560-treated pups ( $P < 0.05$ ,  $N = 3$ ).



**Fig. 4. (A) Expression of COX-2 protein in total kidney microsomes during postnatal renal development (P0–P21) up to P56.** (Right upper panel) Expression of COX-1 protein increases from P0 to P4 and then is expressed at constant levels. Data represent pooled membranes from three mice at each time point. This experiment was repeated twice. (Lower panel) Expression of COX-2 protein increases from P0 to P4 and then declines rapidly to undetectable levels at subsequent time points. (B) Treatment with the COX-2 inhibitor induces expression of COX-2 in renal microsomes. Renal microsomes from neonatal mice exposed *in utero* to the COX-2 inhibitor were analyzed for expression of COX-1 and COX-2 immunoreactive protein. Expression of COX-1 was unchanged. Compared with expression of COX-2 protein in control microsomes, COX-2 is greatly increased in experimental animals.

### Expression and intrarenal localization of COX-2 in developing and adult mouse

The expression of COX-1 and COX-2 protein during murine renal development was examined by immunoblot (Fig. 4). COX-2 protein expression increased from P0 to P4 and then declined. Selectivity of the antibodies used in this study was confirmed by stripping the membranes and reprobing with COX-1-selective antibodies. COX-1 protein expression also increased from P0 to P4 but then remained constant during later stages of kidney development and adult life. Mice treated *in utero* with the COX-2 inhibitor exhibited a marked up-regulation of renal COX-2 protein at P0, supporting transplacental drug transfer (Fig. 4B). In contrast, the expression of COX-1 protein at P0 was not changed.

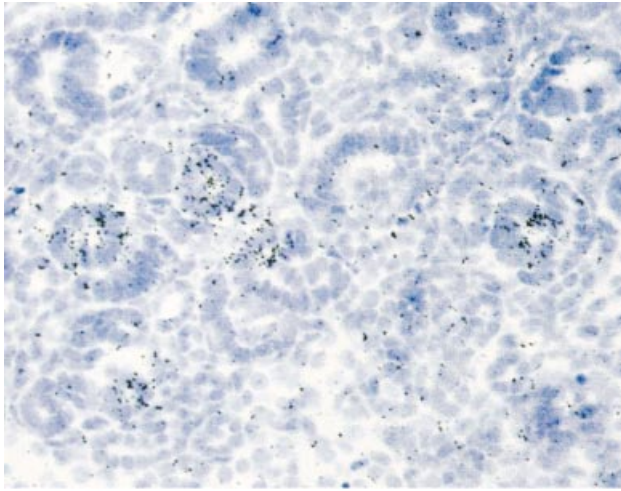
*In situ* hybridization in developing mouse kidneys localized COX-2 mRNA to tubular epithelial cells derived from the induced mesenchyme of the developing nephron and interstitial cells of the renal medulla. COX-2 mRNA expression was detected in renal vesicles, the earliest epithelial structure derived from the induced metanephric mesenchyme [15]. COX-2 mRNA expression predominated in developing tubule epithelial cells adjacent to nascent glomeruli (Fig. 5A), and COX-2 expression was detected in comma- and S-shaped bodies. In mature glomeruli, COX-2 mRNA is expressed in cells associated with the macula densa. No labeling of podocytes or the parietal layer of Bowman's capsule was seen. A prominent hybridization to juxtamedullary macula densa was observed as early as E14.5 and was also present in superficial cortical nephrons of adult kidneys (Fig. 5B). COX-2 immunostains of sections from control animals were unsuccessful; however, COX-2 immunoreactivity was detected (Fig. 5C) in kidneys from animals treated with a COX-2 inhibitor and localized to cells

within and adjacent to the macula densa, consistent with its up-regulation by immunoblot and supporting the *in situ* hybridization data.

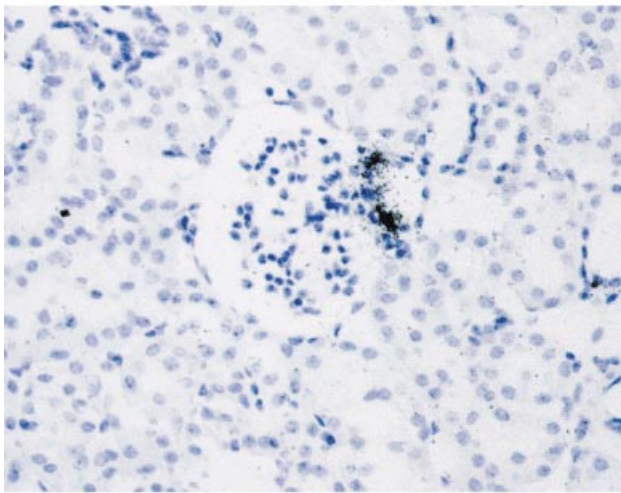
### DISCUSSION

The adult kidney is a major site of prostaglandin synthesis and action. Prostaglandins regulate a wide variety of renal functions, including glomerular and renal medullary blood flow, renin secretion, and salt and water transport in thick limb and collecting duct [16]. There is also evidence that prostaglandins are synthesized in the kidney during fetal and early postnatal life [17]. Targeted disruption of the gene encoding COX-2 suggests an important role for the COX-2 protein in nephrogenesis; however, the precise structural changes in this model remain uncharacterized [11, 12].

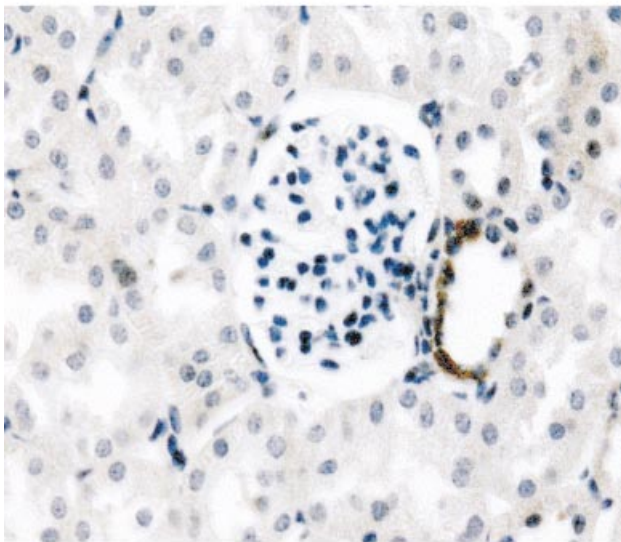
These present studies now extend these findings, demonstrating that COX-2- but not COX-1-selective NSAIDs also disrupt nephrogenesis, and for the first time to our knowledge define a discrete effect of COX-2 inhibition on glomerular size. The finding that COX-2 inhibitors impair renal development in both mice and rats argues against a species- or strain-specific effect of COX-2 inhibitors attributable to modifier loci or genetic background. This newly described effect of COX-2 inhibitors on glomerulogenesis was quantitatively identical to that we observed in transgenic COX-2  $-/-$  mice. The small subcapsular glomeruli also appear immature, as judged by the presence of cuboidal shaped podocytes in cortical glomeruli [18]. Furthermore, the subcapsular layer of tubules was thinned, and the tubular diameter was reduced. It remains to be determined whether or not this change in nephron development is secondary to the glomerular defect or an independent direct effect of COX-2 inhibition on nephron growth.



A P 4



B P 21



C P 21

The period during which COX-2 inhibitors interfere with glomerulogenesis was partially defined by studies examining their effects at different time periods during development. Limiting COX-2 inhibitor treatment to gestation (that is, stopping at birth) did not yield overt effects on kidney morphology examined at P21. In contrast, treatment with the COX-2 inhibitor throughout gestation to P21, or treatment from P0 to P21, resulted in severe impairment of the development of cortical glomeruli. Interestingly, animals treated throughout pregnancy had significantly smaller glomeruli than those in which SC58236 treatment started at P0. This supports the efficient transfer of this drug across the placenta. The less severe decrease in glomerular size observed in mice treated only during postnatal development may also be related to the time needed to reach steady-state levels of SC58236, which could require several days because of its long half-life [19]. The observation that stopping the drug at P0 results in normalization of glomerular size is consistent with the possibility of recovery of glomerular volume from these inhibitory effects in the postnatal period. The postnatal COX-2 inhibitor sensitivity corresponds to the peak in murine renal COX-2 expression at P4. We recently reported a similar pattern of COX-2 expression in rat kidney development, with maximal levels between the first and second postnatal week [8].

These studies confirm that as in the rat, rabbit, and dog [6, 20, 21], renal cortical COX-2 expression in the mouse is relatively restricted to the cells associated with the macula densa. Importantly, COX-2 inhibition is not associated with induction of novel sites of intrarenal COX-2 expression. Considering the similar glomerular developmental defect in mice and rats treated with the COX-2 inhibitor, as well as in mice with a targeted disruption of the COX-2 gene, and that COX-2 is expressed in the macula densa in both species, we speculate that prostanoids or other products resulting from COX-2 activity in the macula densa act in a paracrine manner to influence glomerular development. Although prostanoids modulate afferent arteriolar vasoreactivity [22] and hemodynamic effects could lead to reduced glomerular volume, the lack of effect of the COX-2 inhibitor administration on glomerular volume in adult mice argues against this mechanism. Effects of these prostanoids on glomerular proliferation and development seem more likely.



**Fig. 5. *In situ* hybridization showing COX-2 mRNA distribution in mouse renal cortex at P4 (A) and P21 (B).** (A and B) Brightfield illumination of mouse renal cortex at postnatal day 4 ( $\times 400$ ). Black grains depicting hybridization are seen over S-shaped bodies (A). At this stage of nephron formation, COX-2 mRNA was consistently seen over cells destined to become tubular epithelial cells. COX-2 mRNA localizes to the cells of the macula densa (B; P21). Expression in this structure is seen throughout renal development and in adult kidneys as well. (C) In kidneys from mice treated with the COX-2 inhibitor, COX-2 immunoreactive protein could be localized to cells within and adjacent to the macula densa (P21).

The isozyme-specific COX inhibitors used in this study have been characterized previously *in vitro* and *in vivo*. The selectivity of these compounds has been demonstrated *in vivo* in rats (SC58560 and SC58236) and mice (SC58560) [19, 23]. Significant plasma levels for the COX-2 inhibitor found in the plasma of the mouse pups treated postnatally in this study document efficient transfer of the drug, via milk to the neonatal mice. These levels have been shown to selectively inhibit COX-2 activity in rats [19]. Placental transfer of the drug and uptake into the fetus were not directly assessed in this study (because of insufficient plasma volumes available), but are suggested by the profound induction of COX-2 protein in renal microsomes harvested from mice at P0 exposed antenatally to SC58236 (Fig. 4B). The difference in glomerular size between E0.5- to P21-treated and P0- to P21-treated mice also supports placental transfer. Effective inhibition of COX-2 activity in this study is strongly suggested by the similar effect of this compound on glomerular size to that in COX-2  $-/-$  mice. In both COX-2 inhibitor-treated and COX-2  $-/-$  mice, changes in glomerular size do not appear until after P8. Interestingly, in neither COX-2-inhibitor treated mice nor COX-2  $-/-$  mice was an effect on juxtamedullary glomerular size observed. These similarities further support a similar mechanism of action for COX-2 inhibitors and COX-2 gene disruption with respect to renal development. These findings also suggest a special role for COX-2 in the development of subcapsular glomerular.

A COX-1 inhibitor had no effect on renal development, despite the fact that maternal treatment with the COX-1 inhibitor is accompanied by a significant reduction in gastric mucosal PGE<sub>2</sub> synthesis in the pups. Because gastric PGE<sub>2</sub> synthesis is COX-1 dependent [23], these findings also argue against a role for COX-1 in the observed effects on renal development and support the idea that they are specifically due to inhibition of COX-2. These data complement the recent study by Langenbach et al showing that COX-1 gene targeting does not affect nephrogenesis in mice [10].

In summary, these studies demonstrate, to our knowledge for the first time, that selective inhibitors of COX-2 and targeted disruption of the COX-2 gene have a similar effect on glomerular development and nephrogenesis. Use of a COX-1 selective inhibitor does not affect nephrogenesis. The effect of the COX-2 inhibitor is most pronounced in the postnatal period when superficial nephrons begin developing and COX-2 expression peaks (P4). This period of nephrogenesis in mice roughly corresponds to gestational weeks 24 to 32 in humans [24], which has been identified as critical with regard to NSAID-induced dysgenesis [1]. COX-2 is expressed near the glomerulus in the cells associated with the macula densa in the developing mouse, suggesting a role for local prostaglandin production in subcapsular glomeru-

logenesis. No effect of COX-2 inhibitors on glomerular diameter is observed in adult kidneys. The use of COX-2-selective inhibitors to prevent premature onset of labor in humans has been proposed [25]. This observation may have relevance to reports of renal dysgenesis in human neonates with a history of exposure to NSAIDs and should be considered when administering COX-2 inhibitors in the period of renal development in humans.

## ACKNOWLEDGMENTS

Dr. Breyer is the recipient of a Veterans Administration Career development award. This study was supported by National Institutes of Diabetes and Digestive and Kidney Diseases (NIDDK) grant 1-P50-DK-39261, George M. O'Brien Kidney Center (MDB, RCH, JAM), NIDDK Grant DK-37097 and funding from (Searle/Monsanto Pharmaceuticals, M.D.B.). Dr. Kömhoff is a recipient of a research grant (KO 1855/1-1) from the Deutsche Forschungsgemeinschaft. The authors thank L.S. Davis for expert advice on performing *in situ* hybridization, Drs. Karen Seibert and Peter Isakson (Searle/Monsanto Pharmaceuticals) for generously providing SC58236 and SC58560, and Dr. Jerry Muhammed measuring drug plasma levels.

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## APPENDIX

Abbreviations used in this article are: COX, cyclooxygenase; E, embryonic day; GPAS, glutaraldehyde-periodate acid saline; HPLC, high pressure liquid chromatography; NSAIDs, nonsteroidal antiinflammatory drugs; P, postnatal day; PGE, prostaglandin E; PMSF, phenylmethylsulfonylfluoride; RT-PCR, reverse transcription-polymerase chain reaction; TBS, Tris buffered saline.

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