Cyclooxygenase-2 Is Expressed in Vasculature of Normal and Ischemic Adult Human Kidney and Is Colocalized with Vascular prostaglandin E₂ EP4 Receptors

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Abstract. The study was performed to elucidate the distribution and cellular localization of cyclooxygenase (COX)-2 in human kidney and to address localization of downstream targets for COX-derived prostanoids. Cortex and outer and inner medulla tissue were obtained from control kidneys (cancer specimens), kidneys with arterial stenosis, and kidneys of patients who received angiotensin II inhibition or acetylsalicylic acid. Ribonuclease protection assay and Western blot test revealed that COX-1 and -2 mRNA and protein were expressed in all regions of human kidney (mRNA ratio, cortex:outer medulla:inner medulla COX-1 1:3:20 and COX-2 1:1:3). In adult kidney, immunohistochemical labeling for COX-2 was associated with smooth muscle cells in pre- and postglomerular vessels and with endothelium, particularly in vasa recta and medullary capillaries. Western blot test confirmed COX-2 expression in renal artery. COX-2 had a similar localization in fetal kidney and was additionally observed in Henle’s loop and macula densa. Human tissue arrays displayed COX-2 labeling of vascular smooth muscle in multiple extrarenal tissues. Vascular COX-2 expression was significantly increased in kidneys with arterial stenosis. COX-1 was colocalized with microsomal prostaglandin E₂ synthase (PGES) in collecting ducts, and PGES was also detected in macula densa cells. Vascular COX-2 was colocalized with prostaglandin E₂ EP4 receptors but not with EP2 receptors. Thus, renovascular COX-2 expression was a constitutive feature encountered in human kidneys at all ages, whereas COX-2 was seen in macula densa only in fetal kidney. Vascular COX-2 activity in human kidney and extrarenal tissues may support blood flow and affect vascular wall-blood interaction.

A rate-limiting step in prostaglandin formation is catalyzed by cyclooxygenase (COX). Two isoforms are recognized: constitutive COX-1 and inducible COX-2. Selective COX-2 antagonists have recently been developed and are in widespread clinical use. In contrast to expectations, COX-2 selective blockers exhibit adverse effects related to kidney and cardiovascular function; they have antinatriuretic properties; they lower GFR and RBF and can lead to acute renal failure; and they aggravate preexisting hypertension and may lead to adverse cardiovascular outcomes (1–6). Thus, the data indicate important physiologic roles for COX-2 in human cardiovascular and renal homeostasis, but the renal correlates of these clinical observations remain poorly understood. Both COX genes are expressed in human kidney (7,8), but the quantitative relation of COX-2 to COX-1 expression is not known. Whether there are differences in expression level over time or in kidney regions is also not known. Data on the cellular localization of COX-2 in human kidney are inconsistent.

COX-2 has been observed in thick ascending limb of Henle’s loop (cTAL), including the macula densa in fetal human kidney (8–10) and in the kidneys of children with Bartter’s syndrome (11), a location first described in rat kidney (12). Several studies did not detect COX-2 in the cTAL or macula densa of adult human kidneys (7,8,10), whereas other reports showed an age- or disease-dependent expression of COX-2 in macula densa of adult human kidneys (7,8,10), whereas other reports showed an age- or disease-dependent expression of COX-2 in macula densa of adult human kidneys (7,8,10), whereas other reports showed an age- or disease-dependent expression of COX-2 in macula densa of adult human kidneys (7,8,10). COX-2 has also been observed in renal vascular tissue (7–9), and observations indicate that intact prostaglandin synthesis is important for maintenance of renal function during renovascular hypertension with renal arterial stenosis (17–19). It is not known whether this dependence involves COX-2 activity.

Three major objectives of the study presented here were to determine the quantitative regional distribution and cellular localization of COX isozymes in normal adult human kidney and to compare to fetal kidney; to assess COX-2 in human kidneys with arterial stenosis and after pharmacologic interventions; and to elucidate colocalization of COX enzymes with...
downstream target molecules. We used quantitative mRNA and protein assays in combination with immunohistochemical analysis to address these issues in human nephrectomy samples.

Materials and Methods
Human Tissue Samples
All patients gave written informed consent to participate in the study. The study was approved by the local ethics committee. None of the cancer patients had received chemotherapy or radiation therapy before nephrectomy. Samples of normal kidney tissue were obtained from unaffected areas in nephrectomy specimens with tumors. Four kidneys with artery stenosis were obtained during the 3-yr sampling period. Kidneys were extirpated at the Department of Urology, Odense University Hospital, and immediately transported to the Institute of Pathology. Each kidney, with or without tumor, was divided into cortex, outer medulla, and papilla and frozen in liquid nitrogen. Tissue blocks were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Other sections were embedded and frozen in CO2-cooled isopentane and kept at −80°C until use. In three kidneys, it was possible to dissect a piece of the renal artery, which was immediately frozen. Paraffin-embedded human tissue arrays and fetal kidneys were obtained from the archive of the Department of Pathology, Odense University Hospital.

Human Umbilical Artery Endothelial Cell Culture
Primary cultures of human umbilical artery endothelial cells were established by collagenase treatment of umbilical cords. The cords were obtained from the Department of Obstetrics and Gynecology, Odense University Hospital, Denmark, after informed consent was obtained. Human umbilical artery endothelial cells were seeded on gelatin-coated tissue-culture plastic, and the cultures were maintained in EGM-2 medium (Clonetics, San Diego, CA) and kept at 37°C, 95% O2, 5% CO2. Cells were detached with trypsin-EDTA solution (Sigma, Rødovre, Denmark) and used until passage 7. Lipopolysaccharide (10 µg/ml, Sigma) was added to culture flasks for 5 h, and cell protein was isolated.

Molecular Protocols
Total RNA was isolated from tissue by columns (RNasey Midi Kit, Qiagen, Albertslund, Denmark). RNA was quantified by measuring optical density at 260 nM. RT-PCR was used to amplify sequences specific for human COX-1, COX-2, renin, and β-actin (20). All primers were synthesized with 5 restriction sites for BamHI (sense) and EcoRI (antisense) (Invitrogen, Scotland) to allow for directional cloning (vector pSP73, Promega-Ramcon, Birkerød, Denmark), sequencing, and in vitro transcription (20). Primers were as follows: for COX-1, sense: 5'-ATG TCA TCA GGG AGT CTC-3', antisense: 5'-AAG CAC AGG GTA GAA-3' (accession number xm011834, bases 1339 to 1514, 176 bp); and COX-2, sense: 5'-GTG AAA CCA TGG TAG AAG-3', antisense: 5'-AGT AGT ACT GTG GGA TTG-3' (accession number xm0173416, bases 1648 to 1924, 277 bp, with the 3' half of the amplified sequence not found in COX-1); renin, sense: 5'-ATG AAG AGG CTG ACA CTT-3', antisense: 5'-GAG AAA GCC ACT GAC TGT-3' (285 bp spanning two introns) (21); β-actin, sense: 5'-CCA AAG AGG GTA AGA TGC-3', antisense: 5'-CAC GAA AGC AAT GCT ATC-3' (accession number xm004814, bases 1392 to 1579, 188 bp).

Solution Hybridization and Ribonuclease Protection Assays
mRNA levels were estimated by solution hybridization followed by A/T1 ribonuclease protection assay as described (20). Protected probes were excised from dry gels and radioactivity was quantified in a β counter. COX-1 and -2 probes had the same specific radioactivity, which permitted direct comparison of isoform mRNA levels.

Immunohistochemical and Immunofluorescence Analysis of Kidney Sections
Processing of tissue for immunohistochemical analysis was essentially as previously described (22). Two COX-2 antibodies were used; rabbit anti-human directed against amino-terminal amino acids 50 to 111 (Santa Cruz, AH Diagnostics, Aarhus, Denmark), and rabbit anti-human against the carboxy-terminal amino acids 567 to 599, unique to COX-2 (Cayman Chemicals Co., AH Diagnostics, Aarhus, Denmark). Both antibodies have been used previously on human tissue (7,9,11,23). COX-1 antibody was from Santa Cruz (goat-anti-human). Antibodies directed against microsomal prostaglandin E synthase (PGES; rabbit anti-human), EP4 receptor (rabbit anti-human) and EP2 receptor (rabbit anti-rat) were from Cayman Chemicals. Antigen retrieval was carried out by microwave cooking in citrate buffer (Dako, Glostrup, Denmark) for 20 min or in a pressure cooker for 5 min. The sections were blocked with 5% dry milk in 0.05% Tween–Tris-buffered saline (TTBS), and then incubated with diluted primary antibodies overnight at 4°C. Next, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody directed against the relevant species (Dako). The Santa Cruz COX-2 antibody was visualized according to the Power Vision poly HRP amplification kit (Immuno-vision Technology Co., Brisbane, CA). Signals were visualized by incubation for 2 to 20 min with 0.01% diaminobenzidine (Dako) and 0.02% H2O2. For preabsorption controls, the antibody and 10 µg/ml peptide was incubated in 5% milk–TTBS for 2 h. Immunolabeling for EP4 receptor was carried out on unfixed cryosections that were incubated in 0.5% Triton-X–100 PBS, and next were blocked in 5% dry milk–PBS. The sections were incubated overnight at 4°C with EP4 primary antibody (1:50), and signals were visualized as described above. Double-immunofluorescence labeling for COX-1 and COX-2 was performed after blocking with 5% milk–TTBS. Diluted primary antibodies were incubated overnight at 4°C, and the sections were then incubated with Alexa-fluor 594 conjugated donkey–anti-goat antibody (1:250, Molecular Probes) followed by incubation with Alexa 488 conjugated goat–anti-rabbit antibody (1:200, Molecular Probes). Sections were inspected with an epifluorescence microscope (Olympus BX51).

Western Blot Test
Protein was isolated and quantitated as described (22). Twenty-microgram protein samples were used for COX-2 assays and 60-µg samples for COX-1 assays. Proteins were separated by 4–12% SDS-PAGE and electroblotted (Bio-Rad Laboratories, Copenhagen, Denmark) onto PVDF Immobilon membranes (Millipore, Glostrup, Denmark). Membranes were blocked and incubated with anti-COX-2 antibodies (1:1000) or COX-1 antibody (1:1000) in 5% dry milk–TTBS overnight. Membranes were incubated with HRP-coupled secondary antibody (1:2000) for 1 h. Bound secondary antibody was detected by enhanced chemiluminescence kit (ECL plus Western Blotting Detection System, Amersham Biosciences, Horsholm, Denmark) and exposed to x-ray film. Proteins for PGES detection were separated on a 10% SDS-PAGE gel and reacted with primary antibody (1:1000).
Statistical Analyses
All values are given as mean ± SEM. Unpaired t test was used to determine statistical difference when two groups of data were compared. P < 0.05 was considered statistically significant.

Results
Distribution of COX mRNA and Proteins in Adult Human Kidney Regions
By ribonuclease protection, both COX transcripts were detected in all regions of 11 adult human kidney samples that were selected from patients who did not receive any chronic medication before nephrectomy. COX-1 and -2 were expressed at equal levels in kidney cortex, but COX-1 was more abundant than COX-2 in outer and inner medulla (Figure 1, A and B). There was a significant cortical-medullary gradient for both isoforms. Compared with cortex, COX-1 was expressed at 3 and 20 times higher levels in outer and inner medulla, whereas COX-2 was modestly but significantly more highly expressed in inner medulla compared with cortex and outer medulla (Figure 1B). Renin mRNA was detected in cortex only, which confirmed correct separation of tissue (Figure 1A). The COX-2 antibodies, recognizing amino- and carboxy-terminal parts of human COX-2, reacted with a protein with the expected size of 72 kDa on Western blot tests of kidney cortex and inner medulla. Figure 1C shows the results with the COOH-terminal COX-2 antibody. There was no significant difference in COX-2 protein level between cortex and inner medulla. The COX-1 antibody detected a single protein with the anticipated molecular size of 70 kDa. COX-1 protein level was significantly higher in the inner medulla compared with cortex, similar to the data on mRNA distribution.

Immunohistochemical Localization of COX Isoforms in Adult and Fetal Human Kidney Sections
In adult kidney, COX-2 immunoreactivity was associated with preglomerular, glomerular, and postglomerular vessels (Figure 2, a, b, d, f, and j, k). Preglomerular vessels of all calibers from arteries to arterioles were labeled in the smooth muscle cell layer (Figure 2, b, d, and k; Figures 4A and 5B). In glomeruli, labeling was observed in parietal epithelial cells (Figure 2b) and in few intraglomerular cells, probably podocytes (Figure 4A) (8). In the postglomerular vasculature, labeling was associated with outer medullary vasa recta, as seen in the compound picture of a longitudinal section and in cross sections of outer medulla (Figure 2, a, f, and j).

The inner medullary capillary network was labeled for COX-2, which implies that COX-2 is expressed in endothelium (Figure 2, f and j). The immunopositive vessels were identified on the basis of the presence of erythrocytes in their lumen. In adult kidney, no COX-2 immunoreactivity was associated with collecting ducts, proximal tubules, loops of Henle, distal tubules, or medullary interstitium (Figure 2, a, f, and j). Notably, we did not observe immunolabeling in macula densa in any of the samples analyzed (Figure 2b). A second, COX-2–specific antibody directed against the NH2-terminal of human COX-2 was applied. The same pattern of labeling was obtained with this antibody (Figure 2, j and k). In two human fetal kidneys (gestational weeks 29 and 30), COX-2 immunoreactivity was associated with the parietal epithelial cells of Bowman’s capsule and with endothelium and vascular smooth muscle (Figure 1C).
Figure 2. Immunohistochemical labeling of human kidney sections for cyclooxygenase (COX)-2 and COX-1. (a) Compound picture showing COX-2 immunoreactive protein in vessels of an outer medullary vasa recta bundle. Scale bar = 200 μm. In renal cortex, COX-2 was observed in vascular smooth muscle (b, d, k) and in the parietal glomerular epithelium (b), but not in the loop of Henle or the macula densa (b). (c) COX-1 was located in a few mesangial cells in glomerulus. Scale bar = 50 μm. Cross section of inner medulla revealed (g) COX-1 immunoreactivity in collecting ducts and (f) COX-2 in vasa recta and capillaries. Incubation of the primary antibodies with peptides used to raise the antibodies prevented labeling of tissue sections (h, i). Application of a separate COX-2 antibody directed against the amino terminal of COX-2 produced a similar staining result as with the carboxy-terminal antibody used in a, b, d, and f (j, k). Human fetal kidney displayed COX-2 in loop of Henle and macula densa (l) and in vasculature (m).
COX-2 was not found in the nephrogenic zone, but was associated with loops of Henle, including macula densa cells of mature juxtamedullary glomeruli (Figures 2l and 4B), as previously reported (8–10).

In adult kidney, COX-1 immunoreactivity was associated with endothelial cells of arteries, with mesangial cells at the glomerular hilum and with cortical interstitial cells (Figure 2, c, e, and g). COX-1 immunoreactivity was detected in collecting duct principal cells in cortex and medulla (Figure 2, c and e). Omission of primary COX antibodies or preabsorption of antibodies with surplus of the respective peptides prevented labeling (Figure 2, h and i).

Next, it was addressed whether vascular expression of COX-2 was specific for the kidney. An array of adult human organs was labeled with the NH2-terminal COX-2 antibody (Figure 4C). Significant fluorescence signals were associated with submucosal arteries and arterioles in ventricle (m), gallbladder (n), jejunum (o), and urinary bladder (k). Arterial and arteriolar smooth muscle was labeled in skeletal muscle (j) and spleen (l) and in dermis, lung, and placenta (not shown).

Localization of Downstream Targets for COX Products in Human Kidney

Microsomal PGES is responsible for conversion of the COX product prostaglandin H2 to prostaglandin E2 (PGE2). PGES immunoreactivity in cortex was associated with the macula densa and not observed in adjacent epithelial cells (Figure 5A, a through c). PGES labeling was detected in a subset of cortical collecting duct cells and in all inner medullary collecting duct cells (Figure 5A, d and e). On Western blot test, the PGES antibody detected a single protein with the expected size of 16 kDa (Figure 5A, f).

Next, Gs-coupled vasodilator receptors for PGE2, EP2 and EP4, were localized in human kidney. EP4 labeling was detected in vascular smooth muscle cells of large (Figure 5B, h) and smaller afferent (Figure 5B, j) glomerular vessels (24).
Figure 4. Immunofluorescence analysis of cyclooxygenase (COX) in human kidney and nonrenal tissues. (A) Human kidney cortex was double-labeled for COX-2 (green, a) and COX-1 (red, b), and colocalization was detected in few endothelial cells in the afferent arteriole (yellow, c). Scale bar = 50 μm. Cross section (d) and longitudinal section (e) of human kidney outer medulla and inner medulla (f) labeled for COX-1 (red) and COX-2 (green), showing separate localization. Scale bar in d and e = 200 μm; f, 50 μm. (B) Labeling of human fetal kidney tissue at gestational week 30 for COX-2. COX-2 signals were associated with glomeruli (g), loops of Henle (g) arteries and arterioles (h), and vasa recta (i). The glomeruli are small compared with (A). Scale bar = 50 μm. (C) Labeling of a human tissue array for COX-2 (green fluorochrome) showed a vascular localization: skeletal muscle (j), bladder (k), spleen (l), ventricle (m), gallbladder (n), and small intestine (o). Scale bar = 50 μm.
adjacent serial sections, COX-2 was associated with the same smooth muscle cells as EP4 (Figure 5B, g and i). EP4 immunoreactivity was detected in vasa recta bundles (not shown). Immunopositivity for the EP2 receptor was associated with thin descending and ascending limbs of Henle’s loop (Figure 5C, l) (25). EP2 immunolabeling was detected in the post-macula densa distal convoluted tubule and in connecting tubule (Figure 5C, k). EP2 receptor labeling was associated predominantly with the basolateral aspect of the epithelial cells. EP2 immunoreactivity was not observed in any part of the renal vasculature or in the macula densa (Figure 5C, k, arrows).

**Effect of Renal Artery Stenosis, Angiotensin II (AngII) Inhibitors, and COX Inhibitors on COX Expression in Human Kidney**

COX expression was analyzed in kidneys with renal artery stenosis; in kidneys from patients who had preoperatively received angiotensin-converting enzyme (ACE) inhibitors, or AT1 receptor antagonists; and in patients who had received acetylsalicylic acid. COX-2 mRNA abundance was significantly higher in cortex and medulla from kidneys with arterial stenosis compared with control kidneys (Figure 6, A through C). Renin expression was strongly elevated in three of the four kidney cortices with arterial stenosis compared with six controls (Figure 6A). COX-1 and COX-2 expression was not changed in kidneys from patients who had received AT1 receptor blockers, ACE inhibitors, or acetylsalicylic acid (not shown). Kidneys with artery stenosis exhibited tubular atrophy and interstitial fibrosis. The vasculature displayed medial and intimal thickening and some arteriolar hyalinosis. COX-2 immunoreactivity was associated with vascular smooth muscle in the thickened media layer. In general, there was a poor morphologic resolution in the cortical labyrinth because of the end-stage nature of the kidneys. Therefore, it was not possible to identify the macula densa or any other specific tubular segment in these kidneys.

**Discussion**

In the present investigation, the major finding is that cyclooxygenase (COX-1 and COX-2) was expressed constitutively in all regions of adult human kidney with distinct cellular localization. COX-1 was associated with collecting ducts. COX-2 was associated with media smooth muscle and vascular pericytes of all segments of the pre- and postglomerular vasculature and was significantly elevated in kidneys from patients with renal artery stenosis. In fetal kidneys, COX-2 was expressed in vasculature and glomeruli, but also in loop of Henle and macula densa cells. Thus, COX-2 was detected consistently in tubular epithelium only in fetal kidneys, pregglomerular arterioles (i, j). Scale bar in (g) = 50 μm. (C) Localization of cAMP-coupled PGE2-EP2 receptor immunoreactive protein in human kidney. EP2 was associated with distal convoluted tubule (k) and loop of Henle (l). Arrows indicate the macula densa region where the EP2 signal waned (k).
whereas vascular expression was a constant feature of human kidney established during fetal life.

Because of the reported controversies on COX-2 localization in human kidney, the specificity of the COX-2 antibody was of major concern. Two separate anti-human COX-2 antibodies directed against different, specific epitopes yielded a similar pattern of histochemical labeling that was different from that of COX-1. Labeling was prevented by preabsorption with the peptides used for immunization. Both antibodies reacted with a protein with the expected molecular size of COX-2 on Western blot test of kidney tissue and renal artery, and the intrarenal distribution of COX-2 protein corresponded to COX-2 mRNA. Moreover, COX-2 immunopositive vessels were encountered in all kidney zones similar to COX-2 protein and mRNA. COX-2 has previously been observed in adult human kidney vasculature with a different COX-2 antibody (8) and also in nonrenal human arteries (23) and in vascular endothelium of several other species (26,27).

The vascular localization of COX-2 was not restricted to human kidney because an array of human organs displayed immunolabeling associated with media of arteries and arterioles. Together, the data indicate that renovascular expression of COX-2 is a general feature not causally related to the presence of adjacent malignant cells in the human kidney tissue analyzed. Moreover, despite different gender (three women, eight men), age (range, 45 to 83 yr, median 63 yr), tumor size, and histopathological tumor type (clear cell, transitional papillary tumor), there was very little variation between patients in intrarenal COX mRNA and protein levels. This suggests constitutive expression in adult human kidney. It is established that vascular COX-2 expression is stimulated by the shear stress-nitric oxide pathway, by AngII, and by inflammatory mediators and cytokines (28–33). COX-2 couples preferentially with prostacyclin (PGI) synthase and inducible PGE2 synthase (34), and COX-2 is the predominant source of PGI2 in vivo (2,23,35,36). Tonical vascular prostacyclin synthesis is crucial to antagonize thromboxane-mediated, injury-induced proliferation and thrombosis (37). The present observation of widespread vascular COX-2 offers an explanation for the inhibitory effect of COX-2 antagonists on prostacyclin synthesis in humans (2,23,35,36).

In kidneys with arterial stenosis, vascular COX-2 expression was augmented in cortex and medulla. Human kidneys with arterial stenoses release net prostaglandin to the systemic circulation and COX blockers cause a drop of GFR (19), plasma renin, and BP (17–19,38). Thus, vascular COX-2 activity, either from smooth muscle or from endothelium, may contribute to elevated renal prostaglandin synthesis under conditions with diminished blood flow to the kidney. PGE2 and/or PGI2 may serve to support vessel patency and blood supply to the ischemic kidney through interaction with vascular IP or EP4 receptors (present data) (24,39). AngII has been shown to induce COX-2 in smooth muscle (29), but COX-2 mRNA was not changed in nephrectomy samples from patients that received AngII receptor blockers or ACE inhibitors. However, because of the few samples analyzed, we cannot exclude a regulatory role of AngII on COX-2 in the human kidney.

In cortex, immunolabeling for PGES was associated with macula densa and collecting ducts and was not observed in the vasculature similar to rat (40). We did not observe any COX
isoform associated with the macula densa in the adult kidneys analyzed. This observation essentially confirms previous reports where COX-2 was absent in normal adult human kidney macula densa (7,8,11) but present in fetal kidney (9). COX-2 is thought to be induced in human macula densa in conditions with low physiologic, or compromised, transepithelial NaCl transport (9,10,11,13). Dependence of plasma renin on COX-2 has been shown in normal volunteers given a low-salt diet or furosemide (16) and in patients with Bartter syndrome (15). The data suggest that COX-2–mediated prostaglandin synthesis contributes to macula densa–mediated signal transfer to renin-producing granular cells in these situations. None of the patients in the study presented here had overt heart disease or received loop diuretics; all were elderly and presumably NaCl replete. Thus, we propose that COX-2 protein is below the detection limit in macula densa of sodium-replete humans but that it can be rapidly induced and generate substrate for PGES in states of low NaCl intake, diuretic treatment, or defect transport proteins.

In summary, the present data show constitutive expression of both COX isoforms in normal adult human kidney. COX-1 was colocalized with PGES in collecting ducts. COX-2 was localized in vascular smooth muscle and endothelium together with EP4 receptors both in kidney and extrarenal tissues. COX-2 was expressed in cTAL and macula densa in human fetal kidney during and after active nephrogenesis.

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