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Research Article

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Cyclooxygenase-2 Is Associated with the Macula Densa of Rat Kidney and Increases with Salt Restriction

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Abstract

The kidney is a rich source of prostaglandins. These eicosanoids, formed by cyclooxygenase-dependent metabolism of arachidonic acid, are important physiologic mediators of renal glomerular hemodynamics and tubular sodium and water reabsorption. Two separate isoforms of cyclooxygenase (COX) have now been identified: constitutive COX-1, encoded by a 2.8-kb mRNA, and mitogen-activated COX-2, encoded by a 4.0–4.5-kb mRNA. COX-2 expression increases during development and inflammation, but, except for brain, constitutive expression is low. It has been generally accepted that physiologic renal production of prostaglandins is mediated by COX-1. However, in the absence of inflammation, low levels of COX-2 mRNA are also detectable in the kidney. To examine the role of COX-2 in the kidney and determine its intrarenal localization, we used a 1.3-kb cDNA probe specific for the 3′ untranslated region of rat COX-2 and COX-2–specific antiserum. The COX-2–specific cDNA probe hybridized with a 4.4-kb transcript in total RNA from adult rat kidney. Immunoblots of microsomes isolated from kidney cortex and papilla indicated immunoreactive COX-2 in both locations. In situ hybridization and immunohistochemistry indicated that renal cortical COX-2 expression was localized to the macula densa of the juxtaglomerular apparatus and to adjacent epithelial cells of the cortical thick ascending limb of Henle. In addition, COX-2 immunoreactivity was detected in interstitial cells in the papilla. No COX-2 message or immunoreactive protein was detected in arterioles, glomeruli, or cortical or medullary collecting ducts.

When animals were chronically sodium restricted, the level of COX-2 in the region of the macula densa increased threefold (from 0.86±0.08 to 2.52±0.43/mm^2) and the total area of the COX-2 immunoreactive cells in cortex increased from 34 μm^2/mm^2 of cortex to 226 μm^2/mm^2 of cortex. The intrarenal distribution of COX-2 and its increased expression in response to sodium restriction suggest that in addition to its proposed role in inflammatory and growth responses, this enzyme may play an important role in the regulation of salt, volume, and blood pressure homeostasis. (*J. Clin. Invest.* 1994. 94:2504–2510.) Key words: prostaglandin synthase • prostaglandin • juxtaglomerular • macula densa • renin

Introduction

Prostaglandins serve as important physiologic modulators of vascular tone and salt and water homeostasis in the mammalian kidney. The conversion of arachidonic acid to prostaglandin G_2 and thence to prostaglandin H_2 by prostaglandin G_2/H_2 synthase (cyclooxygenase [COX][1]) is a key enzymatic step in the regulation of prostaglandin synthesis (1). The gene for constitutive prostaglandin G_2/H_2 synthase (COX-1) encodes a 2.7–2.9-kb transcript (2). In the kidney, immunoreactive COX-1 has been localized to arteries and arterioles, glomeruli, and collecting ducts. No immunoreactive COX-1 has been found in the proximal or distal convoluted tubules, Henle's loop, or macula densa (3).

Recent studies have demonstrated that in addition to COX-1, certain cells express a gene encoding a 4.0–4.4-kb transcript, COX-2, the expression of which is activated by mitogenic stimuli (4). Although the translation products of both COX-1 and COX-2 are of similar size (~ 73 kD) and possess similar cyclooxygenase activity, they share only ~ 66% homology in amino acid sequence (4–7). The message for COX-2 (also known as TIS-10) was first detected in phorbol ester–treated Swiss 3T3 cells (4). COX-2 mRNA expression also increased in these cells in response to serum, growth factors, and forskolin, and was superinduced in cycloheximide-treated cells (8).

Regulated COX-2 expression occurs during murine development (9); in adult animals COX-2 expression is seen predominantly during cell growth or in inflammatory states (7, 9–11). Recent studies have noted that kidney has low but measurable levels of COX-2 mRNA (12, 13). The present studies were therefore designed to localize the sites of COX-2 in the adult rat kidney.

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1. *Abbreviations used in this paper:* COX, cyclooxygenase; NO, nitric oxide.

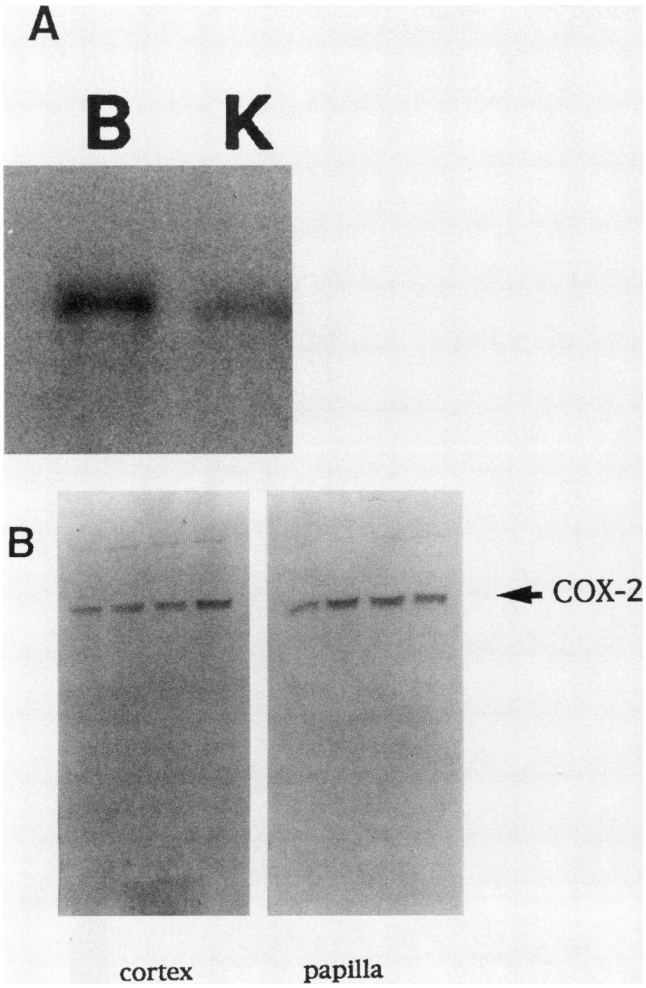


Figure 1. (*A*) Northern analysis of COX-2 mRNA expression. Total RNA (15 μg) from adult male Sprague-Dawley rat brain (*B*) and total kidney (*K*) were fractionated by electrophoresis, blotted onto nitrocellulose, and probed with a 1.3-kb ^{32}P-labeled cDNA Kpn1/Xho1 fragment of the 3′ untranslated region of rat COX-2. Ethidium bromide staining and densitometric scanning of the total RNA after electrophoresis were performed to demonstrate that an equivalent amount of ribosomal RNA was present in each lane. (*B*) Western blot analysis of COX-2 immunoreactivity in rat kidney. Microsomes (25 μg/lane) prepared from kidney cortex and papilla from four separate animals were electrophoresed in each lane, and the proteins were electrophoretically transferred to polyvinylidene difluoride membranes and probed with an antibody specific for COX-2. COX-2 (~ 73 kD) is indicated by the arrow.

Methods

Male Sprague-Dawley rats (200–250 g) were placed either on control rat chow or a rat chow deficient in sodium (< 25 mg/kg Na^+) (ICN Biomedicals, Irvine, CA) for >3 wk. Subsets of animals on regular rat chow were given 1% NaCl in their drinking water for 5–7 d.

Northern analysis. RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (14). RNA samples were electrophoresed in denaturing agarose gels and transferred to nitrocellulose. Nitrocellulose blots were hybridized as described previously with a 1.3-kb ^{32}P-labeled cDNA Kpn1/Xho1 fragment of the 3′ untranslated region of rat COX-2 (12).

In situ hybridization. Animals were anesthetized with Nembutol, and the kidneys were perfused in situ with 1% paraformaldehyde/phosphate-buffered saline and then removed and immersed in 4% paraformaldehyde, followed by graded alcohol dehydration. Tissues were embedded in parafin and 7-μm sections were cut.

Before hybridization, sections were deparaffinized, refixed in paraformaldehyde, and treated with 20 μg/ml proteinase K, washed with phosphate-buffered saline, refixed in 4% paraformaldehyde, and treated with triethanolamine plus acetic anhydride (0.25% vol/vol). Sections were then dehydrated in 100% ethanol.

For in situ hybridization, the 1.3-kb Kpn1/Xho1 cDNA fragment of the 3′ untranslated region of rat COX-2 was ligated into pBSK (−). The plasmid was linearized and sense and antisense RNA were transcribed from the flanking T7 or T3 promoters in the presence of [α-^{35}S]UTP, as described previously (15). In preliminary experiments, we determined that, as expected, there was no hybridization by sense RNA. Antisense RNA (5×10^5 cpm/μl) was used for subsequent in situ hybridization.

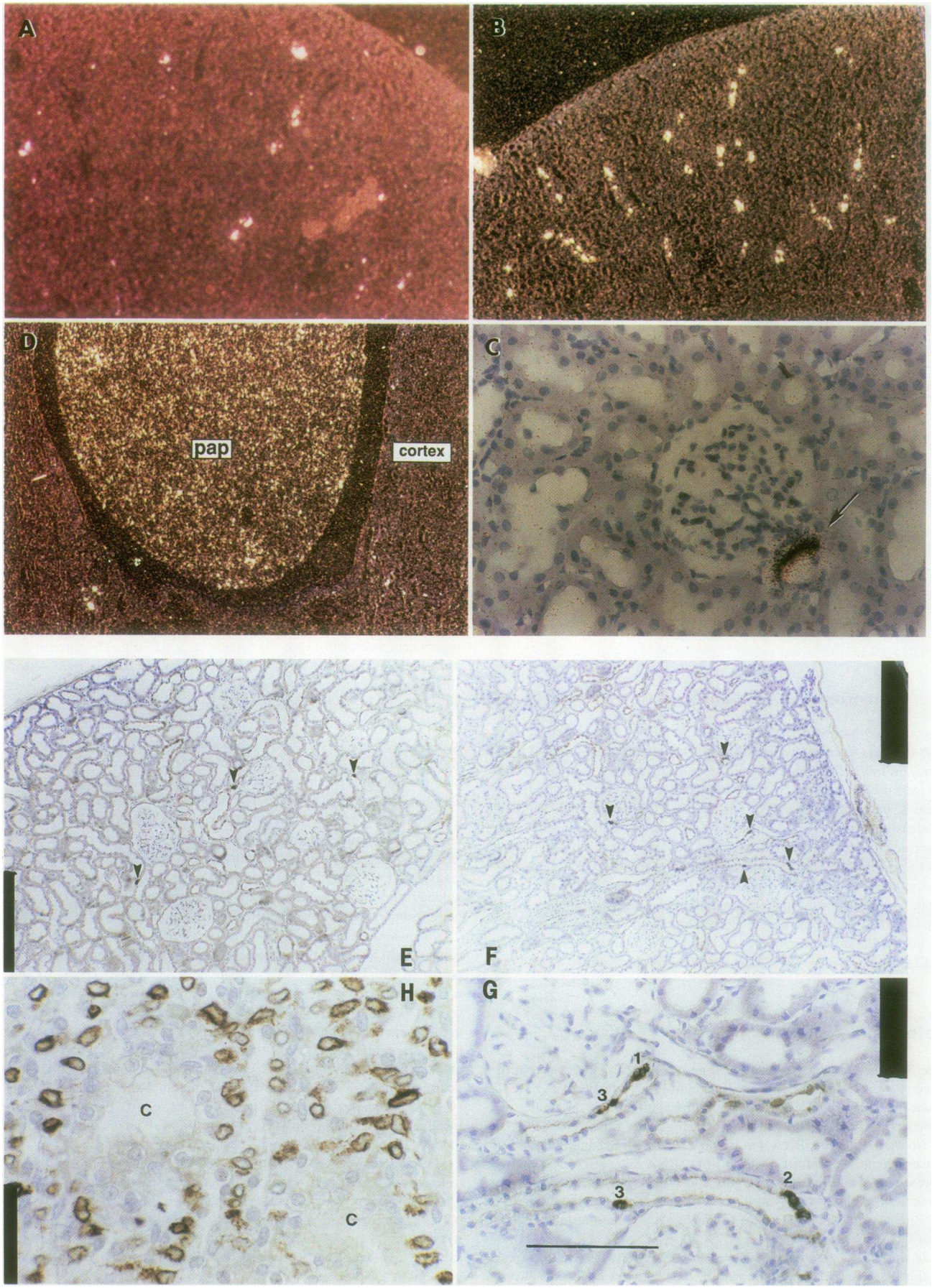
Anti-sense RNA was hybridized to the sections at 55°C for 18 h, as previously described (15). Sections were then washed at 50°C in 5× SSC + 10 mM β-mercaptoethanol for 30 min, followed by sequential washes in 50% formamide, 2× SSC, 100 mM β-mercaptoethanol for 60 min and 10 mm Tris, 5 mM EDTA, 500 mM NaCl (TEN). Sections were then treated with RNase (10 μg/ml) at 37°C for 30 min, followed by an additional wash with TEN. The sections were then washed twice with 2 × SSC and twice with 0.1× SSC (50°C) and dehydrated with graded ethanols containing 300 mM ammonium acetate.

For detection of the hybridized probe, slides were dipped in photoemulsion (K5; Ilford, Knutsford, UK) diluted 1:1 with 2% glycerol/water and exposed for up to 7 d at 4°C. After development in Kodak D19, slides were counterstained with hematoxylin and eosin. Photomicrographs were taken with a Zeiss Axioskop using both bright- and dark-field optics.

Western blotting. For immunoblots, kidneys were removed and the cortex and papilla were dissected, homogenized in 30 mM Tris/Cl, pH 8.0, 100 μm PMSF. After a 10-min centrifugation at 10,000 *g*, the supernatant was centrifuged for 60 min at 200,000 *g*. The microsomes were then resuspended in SDS-sample buffer, heated to 100°C for 5 min, and the proteins were separated on 7.5% SDS gels under reducing conditions and transferred to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). The blots were blocked overnight with 100 mM NaCl/50 mM Tris/Cl, pH 7.4, containing 5% nonfat dry milk, 3% albumin, and 0.5% Tween 20, followed by incubation for 16 h with rabbit antimurine polyclonal antiserum to COX-2 (Cayman Chemical Co., Inc., Ann Arbor, MI) at 1:100 dilution. The second antibody was a biotinylated goat anti–rabbit antibody, which was detected using avidin and biotinylated horseradish peroxidase (Pierce, Rockford, IL). We have previously determined that this antibody to COX-2 does not recognize COX-1 purified from ram seminal vesicles (16). For COX-1 immunolocalization in rat kidney, a polyclonal goat anti–sheep COX-1 antibody from Oxford Biomedical Research, Inc. (Oxford, MI) was used.

Immunohistochemistry. Kidneys were perfused in situ with saline containing 0.02% sodium nitrite and heparin (10 U/ml) and fixed by perfusion with FLPA (3.7% formaldehyde, 1.4% lysine, 0.01 M sodium meta-periodate, 0.04 M sodium phosphate, and 1% acetic acid) as described previously (17). The kidneys were then dehydrated with a graded series of ethanols and embedded in paraffin. Sections (4-μm-thick) were mounted on glass slides and immunostained with the polyclonal rabbit antimurine COX-2 antiserum (1:50 dilution). A polyclonal goat anti–sheep antibody was used for immunolocalization of COX-1 (Oxford Biomedical Research, Inc.). Vectastain ABC-Elite was used to localize the primary IgGs with a chromogen of oxidized diaminobenzidine, followed by a light toluidine blue counterstain.

For quantitation, COX-2–positive cells in cortex were counted. Multiple sections from each kidney were counted; an average of 489±15 glomeruli were present in control samples ($n = 3$) and 516±25 glomeruli were present in low salt samples ($n = 4$), and measured cortical areas were 72.1±1.9 vs 68.7±2.2 mm^2/sample. Only those cells unmistakably associated with a glomerulus were considered to be in the juxtaglomerular region. Quantitation of COX-2 immunoreactivity was performed using BIOQUANT real color image analysis system (R&M Biometrics,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Inc., Nashville, TN). Digitized color video images were adjusted to discriminate the brown diaminobenzidine reaction product and areas calculated as previously described (17). Quantitation of foci of antisense mRNA probe hybridization detected with light-field microscopy was similarly performed with this program.

Results

In initial studies, using a cDNA probe specific for rat COX-2, we determined constitutive expression of the 4.4-kb message in normal rat kidney, consistent with previous reports (12). For comparison, there were higher levels of constitutive COX-2 mRNA expression in rat brain, consistent with the studies of Yamagata et al. (18) (Fig. 1 *A*). Immunoblotting of microsomes from rat kidney cortex and papilla with an antiserum specific for COX-2 revealed immunoreactive protein with a molecular mass of ~ 73 kD in both locations, with greater relative abundance in papilla (Fig. 1 *B*). In some studies, immunoreactive COX-2 was clearly discriminated as an immunoreactive doublet in both cortex and papilla, similar to previous findings in rat preovulatory follicles (19) and human endothelial cells (20). Sirois and Richards (19) performed microsequencing after one-dimensional SDS-PAGE and identified identical amino-terminal sequences. These bands have been attributed to either posttranslational modification or alternative splicing (20).

To determine the intrarenal sites of COX-2 mRNA expression, in situ hybridization was performed. A restricted distribution of COX-2 mRNA was found in the cortex (Fig. 2 *A*). The cortical structures expressing COX-2 mRNA were identified as predominantly cells of the macula densa of the juxtaglomerular apparatus (Fig. 2 *C*). In situ hybridization also revealed COX-2 mRNA expression in papillary tip (Fig. 2 *D*). Immunohistochemical studies confirmed that a restricted subset of cells that were localized to outer- and midcortex expressed COX-2 (Fig. 2 *E* and 3 *A*). These cells were identified as cells of the macula densa and the adjacent cortical thick ascending limb of Henle. The immunoreactivity of stained cells was intense, but only one (and rarely two) COX-2–positive cell was observed per site. The majority of identified glomeruli sectioned through the juxtaglomerular apparatus did not have COX-2–positive cells in the macula densa. In the papilla, medullary interstitial cells were COX-2 positive, with the greatest incidence of immunoreactivity at the papillary tip (Fig. 2 *H*). No COX-2 immunoreactivity was detected in arterioles, glomeruli, or cortical or medullary collecting ducts. Consistent with previous reports (3), COX-1 immunoreactivity was greatest in medullary collecting ducts and medullary interstitial cells, with much less intense staining in cortical collecting tubules (Fig. 4). Importantly, no staining of macula densa or cortical thick ascending limbs was observed.

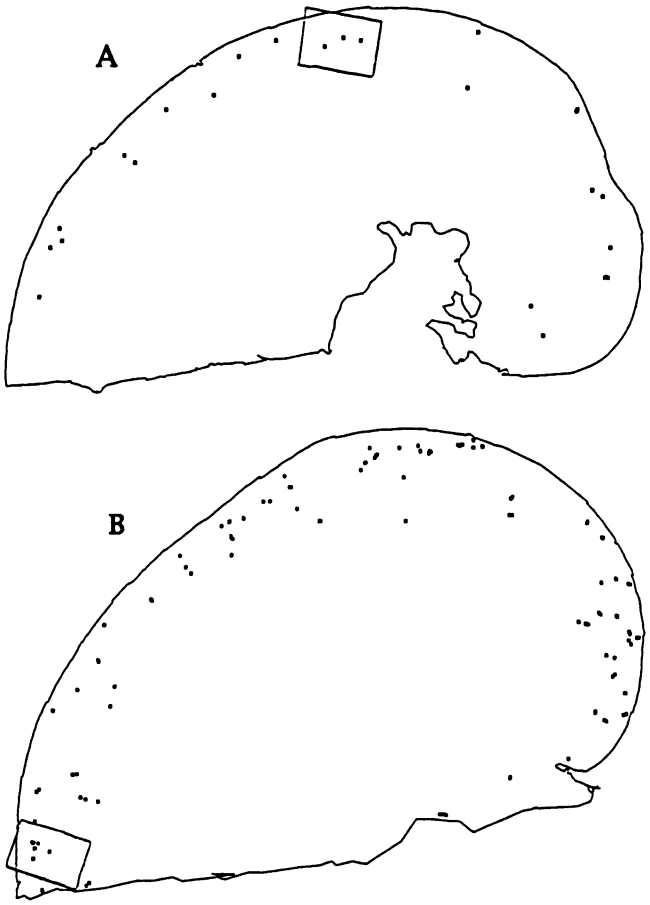

Figure 3. Topographic distribution of COX-2 immunoreactivity in kidney. Representative renal sections from control (*A*) and low salt (*B*) rats. The section profiles and locations of COX-2–positive cells (or cell clusters, which were not subdivided) were determined at a resolution of ±1 μm, as described in Methods. Rectangular outlines identify sites of Fig. 2, *E* and *F*. Papillae were not present in these sections.

Because the macula densa plays a central role in homeostatic regulation of intravascular volume by the kidney, additional animals were studied after either chronic volume expansion (1% NaCl in drinking water) or contraction (low sodium diet). Quantitative image analysis showed that the number of cells displaying immunoreactive COX-2 in the cortex was not detectably altered in the volume-expanded animals (data not shown); in contrast, following chronic (> 3 wk) dietary salt depletion, there was significantly more cortical COX-2 immunoreactivity (Figs. 2 *F* and 3 *B*), with an increase from

Figure 2. Localization of COX-2 in kidney sections from control and low salt rats. In situ hybridization of COX-2 mRNA (*A–D*). Dark-field photomicrographs demonstrating increased COX-2 message in specific foci within cortex of low salt kidney (*B*) compared with control (*A*). At a higher magnification (*C*), the COX-2 mRNA signal in renal cortex of low salt animals can be seen to be concentrated in the region of the macula densa (*arrow*). Substantial COX-2 mRNA signal is also detectable in the papillary tip (*D*). Cortex and papilla (*pap*) are noted. COX-2 immunoreactive protein (*E–H*). Typical sections of renal cortex (from rectangular regions outlined in Fig. 3, *A* and *B*) show paucity of COX-2 immunoreactivity in either control (*E*) or low salt (*F*) rats. Sparse populations of densely stained COX-2–positive cells (*arrows*) usually are singlets in control renal cortex (*E*) but are often grouped in low salt (*F*). At a higher magnification (*G*) (from the center of *F*), clusters of COX-2–positive cells are apparent within the macula densa (*1*), adjacent to the macula densa (*2*), and in the cortical thick ascending limb proximal to the macula densa (*3*). In the tip of the papilla (*H*), COX-2 is absent from collecting ducts (*c*) but is localized to cytoplasmic granules and the perinuclear cysternae of interstitial cells. Scale bar in *A, B,* and *D* = 750 μm; in *C* and *G* = 100 μm; in *E* and *F* = 375 μm; and in *H* = 60 μm.

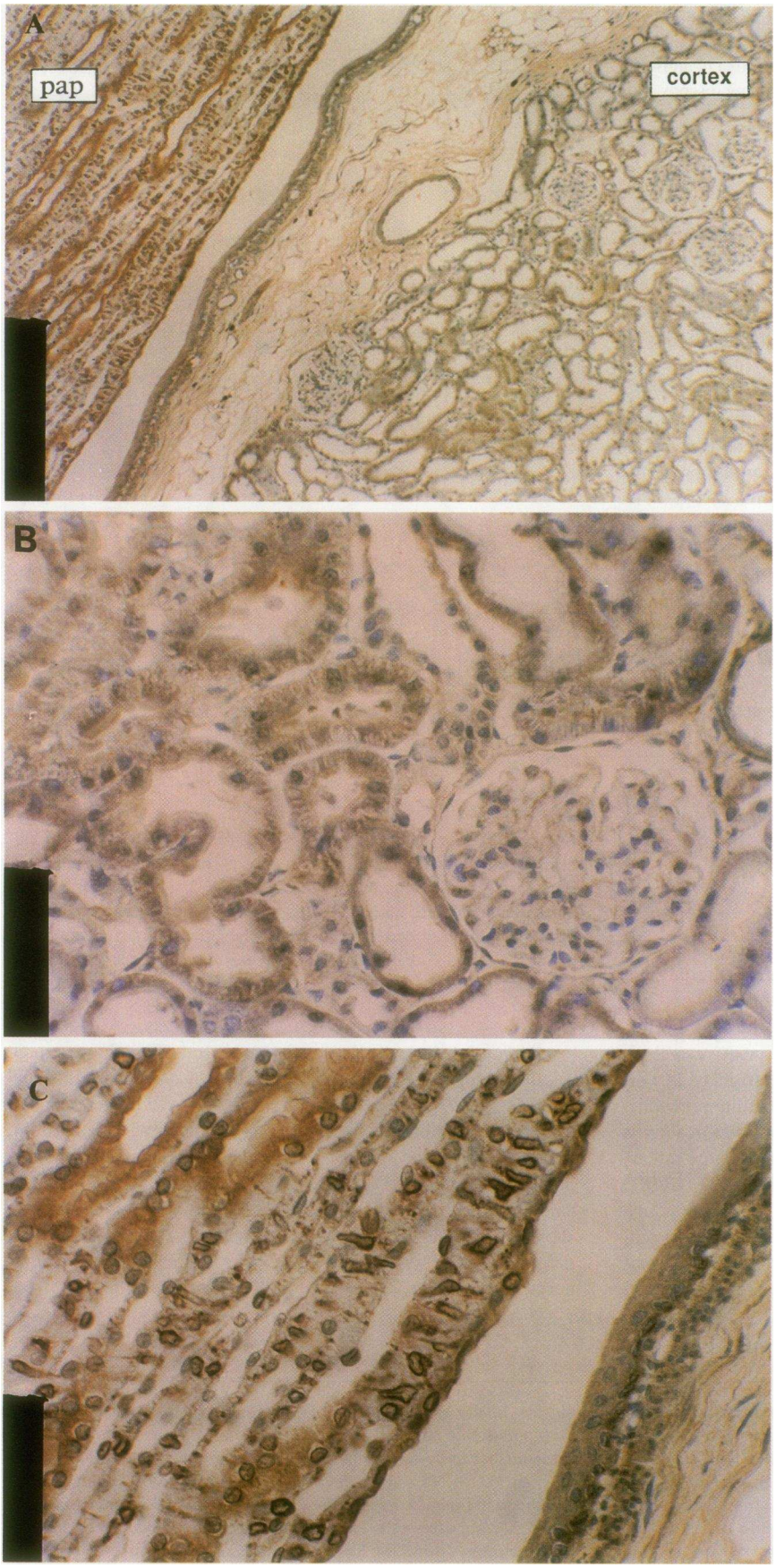

Figure 4. COX-1 immunoreactivity in control rat kidney. (*A*) Shown is a view of the midpapilla (*pap*) with surrounding cortex. Note the high levels of immunoreactivity in the inner medullary collecting ducts and interstitial cells. (*B*) Higher power view of the cortex shown in *A*. (*C*) Higher power view of the inner medulla shown in *A*. Scale bar (see Fig. 2) in *A* = 375 μm; and in *B* and *C* = 100 μm.

0.86±0.08 (n = 3) to 2.52±0.43 (n = 4) cell clusters of COX-2 immunoreactivity/mm^2 of cortex ($P < 0.025$) and an increase from 5±1.2 to 16.1±3.3% ($P < 0.05$) in the percentage of glomeruli noted to have an associated COX-2 immunoreactive macula densa. In addition to increased frequency of sites of COX-2–positive cells, more cells of each macula densa and associated thick limb were positive in the cortices of low salt animals (Fig. 2 *G*). The number of individual COX-2 immunoreactive cells increased from 0.95 cells/mm^2 of cortex to 4.99 cells/mm^2 of cortex, and the total area of the COX-2 immunoreactive cells in cortex increased from 34 μm^2/mm^2 of cortex to 226 μm^2/mm^2 of cortex. As indicated in Fig. 3 *B*, there were increases in outer- and midcortex immunoreactivity in the salt-depleted animals. In addition, in the low salt animals, cortical COX-2 immunoreactivity was also noted to be associated with juxtamedullary glomeruli. No obvious changes in the level or distribution of COX-2 immunoreactivity in the papilla could be discerned in the sodium-restricted animals. In situ hybridization also indicated increased expression of COX-2 mRNA in renal cortex from salt-depleted animals (Fig. 2 *B*), with the number of foci of hybridization in the cortex increasing from 4.7/mm^2 in control to 7.8/mm^2 in salt depletion.

Discussion

The present studies demonstrate localized expression of COX-2 mRNA and immunoreactivity in the cells of the macula densa and adjacent cortical thick ascending limb in the rat kidney cortex. In addition, COX-2 was found in medullary interstitial cells in the tip of the papilla. Although previous studies have shown alterations in COX-2 expression in macrophages and other cells during inflammatory states (10, 21), the present studies demonstrate constitutive expression of COX-2 mRNA and immunoreactive protein in the kidney and regulation by alterations in dietary sodium.

The papilla has long been recognized as a rich source of COX-1, and it has been proposed that papillary prostaglandins mediate vasa recta dilation to maintain medullary blood flow and antagonize vasopressin-mediated water and solute reabsorption (22). The present studies confirm previous work showing that COX-1 is in medullary collecting ducts and interstitial cells. We now show that COX-2 is also present in medullary interstitial cells although it was not present in collecting ducts; these results suggest a role for COX-2 in regulation of medullary prostaglandin production, since the medullary interstitial cells are known to be a rich source of prostaglandins (23).

The cortical distribution of COX-2 is distinct from that of COX-1. Whereas we show that COX-2 is present in macula densa and the adjacent cortical thick ascending limb, no COX-1 was present in these locations, and little COX-1 immunoreactivity was noted in the cortex. In the mammalian kidney, the macula densa has been shown to participate in tubuloglomerular feedback and renin release (24). Although early studies suggested that prostaglandins might mediate tubuloglomerular feedback, more recent studies have failed to implicate these compounds in the response (25–27). Of the four distinct types of signals that mediate renin release, sympathetic nervous system activation, humoral factors, alterations in intrarenal vascular tone, and alterations in NaCl concentration in the early distal tubule, only salt sensing involves the macula densa (28, 29). Decreased chloride reabsorption by the macula densa increases renin secretion (30). Recent studies have shown that an intact cyclooxygenase pathway is necessary for stimulation of renin release mediated by macula densa sensing of decreases in luminal NaCl (31–36). Because previous studies did not detect immunoreactive COX-1 in cortical thick limb or macula densa (3), the origin of prostaglandins mediating the macula densa response has been uncertain. The present studies suggest that the source of prostaglandins in this response is from COX-2 localized to the macula densa and adjacent thick limb cells.

PGE_2 is known to inhibit net chloride reabsorption in the (non–macula densa) cells of the thick ascending limb of Henle (37); therefore, locally produced prostaglandins may function as autocoids to inhibit macula densa transport directly. Alternatively, prostaglandins may affect signaling by the extracellular mesangial (Goormatigh) cells (38). Finally, although epithelial cells of more distal nephron structures produce predominantly PGE_2 (39), the profile of cyclooxygenase products produced by macula densa cells is unknown. Therefore, it is possible that these cells may produce PGI_2 (31) and/or other cyclooxygenase metabolites that directly stimulate renin secretion by juxtaglomerular cells.

In addition to COX-2, a constituitive calcium/calmodulin-dependent nitric oxide (NO) synthase is localized to the macula densa (40, 41), and NO appears to be a mediator of macula densa signaling (42). Recent studies indicating that NO augments COX-2 activity both in vitro and in vivo (43, 44) suggest that interactions among different macula densa signaling mechanisms may be involved in coordinated responses to alterations in luminal NaCl.

In summary, COX-2 mRNA and immunoreactive protein localize to the macula densa and adjacent cortical thick ascending limb in renal cortex, and chronic NaCl restriction increases expression of this enzyme. As selective inhibitors of COX-2 become available, it will be important to assess their effects upon the renin-angiotensin system and glomerular hemodynamics.

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