NS-398 Upregulates Constitutive Cyclooxygenase-2 Expression in the M-1 Cortical Collecting Duct Cell Line

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Abstract. The cortical collecting duct (CCD) is a major site of intrarenal prostaglandin E₂ (PGE₂) synthesis. This study examines the expression and regulation of the prostaglandin synthesizing enzymes cyclooxygenase-1 (COX-1) and -2 in the CCD. By indirect immunofluorescence using isoform-specific antibodies, COX-1 and -2 immunoreactivity was localized to all cell types of the murine M-1 CCD cell line. By immunohistochemistry, both COX-1 and COX-2 were localized to intercalated cells of the CCD on paraffin-embedded mouse kidney sections. When COX enzyme activity was measured in the M-1 cells, both indomethacin (COX-1 and -2 inhibitor) and the specific COX-2 inhibitor NS-398 effectively blocked PGE₂ synthesis. These results demonstrate that COX-2 is the major contributor to the pool of PGE₂ synthesized by the CCD. By Western blot analysis, COX-2 expression was significantly upregulated by incubation with either indomethacin or NS-398. These drugs did not affect COX-1 protein expression. Evaluation of COX-2 mRNA expression by Northern blot analysis after NS-398 treatment demonstrated that the COX-2 protein upregulation occurred independently of any change in COX-2 mRNA expression. These studies have for the first time localized COX-2 to the CCD and provided evidence that the intercalated cells of the CCD express both COX-1 and COX-2. The results also demonstrate that constitutively expressed COX-2 is the major COX isoform contributing to PGE₂ synthesis by the M-1 CCD cell line. Inhibition of COX-2 activity in the M-1 cell line results in an upregulation of COX-2 protein expression.

Nonsteroidal anti-inflammatory drugs (NSAID) are used extensively for their anti-inflammatory, antipyretic, and analgesic effects (1). These therapeutic effects stem from the ability of these drugs to inhibit the activity of the prostaglandin-forming enzymes termed cyclooxygenase-1 (COX-1) and -2, also known as prostaglandin endoperoxide H synthase-1 and -2. After the release of arachidonic acid (AA) from membrane phospholipids by cytosolic phospholipase A₂ (cPLA₂), COX isoforms convert AA to prostaglandin G₂ (PGG₂) (cyclooxygenase activity) and then in a separate active site, PGG₂ is reduced to PGH₂ (peroxidase activity). PGH₂ is then transformed to a biologically active prostanoid such as PGE₂, PGI₂, or thromboxane A₂ by specific synthases (2). Each prostaglandin-producing cell type synthesizes one major prostanoid. For example, PGE₂ is the major prostanoid produced in the kidney cortical collecting duct (CCD) (3). The collecting duct represents an important segment of the nephron for the fine regulation of salt and water reabsorption. The role of PGE₂ in modulating these and other renal processes is well documented (4–11).

The two COX isoforms are derived from two different genes and differ significantly in their regulation (12). The COX-1 enzyme is constitutively expressed in most cell types, where it plays a housekeeping role. COX-2 has been termed the inducible cyclooxygenase because of its rapid upregulation in response to a variety of stimuli, including growth factors, cytokines, and phorbol esters (13–16). Early observations of different expression and regulation of the COX isoforms coupled with the observation that the two COX isoforms were differentially inhibited by some NSAID (17) have led to the development of COX-2-specific inhibitors (18,19). The rationale for their development was the association of COX-1 with physiologically necessary prostanoids such as those involved in gastric cytoprotection and COX-2 with the production of prostanoids in inflammatory pathologic states such as rheumatoid arthritis. Surprisingly, studies on COX-1 null mice have revealed that the phenotypes of these mice are relatively normal, and the lack of COX-1 does not result in gastric ulcers or abnormal renal function (20). Although the COX-1 knockout mouse studies suggested a less important role for COX-1 than what previous evidence suggested, with respect to COX-2, the knockout study demonstrated that COX-2 produces prostanoids, which play a crucial role in kidney development (21). The kidneys of COX-2-disrupted mice were normal at birth; however, they did not proceed to develop properly after this point. This aberrant renal development was the major abnormal phenotype observed in these COX-2 knockout mice. In addi-
tion to inflammatory and developmental states, it is now known that some cell types constitutively express the COX-2 isoform under normal physiologic conditions. Within the kidney, constitutive COX-2 expression has been observed in the macula densa (22), cortical thick ascending limb (23), medullary interstitial cells, and inner medullary collecting ducts (24). Although this renal COX-2 expression is constitutive, it can also be upregulated. For example, in rats dietary salt restriction upregulates COX-2 in the cortex (22,24,25), while a high salt diet increases medullary COX-2 expression.

These recent findings concerning renal COX-2 expression have prompted us to investigate the effects of the new class of COX-2-specific NSAID on COX expression and activity in the collecting duct. The M-1 mouse CCD cell line represents a powerful tool for the study of the interaction between NSAID and the COX isoforms. The M-1 cell line was derived from collecting duct cells from the kidney of an SV40 transgenic mouse, and it has been characterized functionally, morphologically, and immunologically to reflect the intact CCD (26). This cell line has also been shown to constitutively express COX-2 (24). The availability of COX isoform-specific inhibitors has allowed us to evaluate the effect of specific isoform inhibition on M-1 CCD cell PGE2 synthesis. Indomethacin was chosen as a potent non-isoform-selective NSAID (27). NS-398 and resveratrol were chosen as COX-2- and COX-1-specific inhibitors, respectively (18,28,29). Unlike traditional NSAID, which target the cyclooxygenase activity of COX, resveratrol preferentially inhibits both the cyclooxygenase and peroxidase activities of COX-1. The ability of these cells to compensate for COX inhibition was assessed by measuring COX isoform protein and mRNA levels after NSAID treatment.

Materials and Methods

Cell Culture
M-1 mouse CCD cells (CRL-2038; American Type Culture Collection, Rockville, MD) passages 18 to 30 were grown at 37°C in 100-mm dishes in a medium consisting of Dulbecco’s minimal essential medium/F12 supplemented with 5% fetal bovine serum (FBS) and 1% penicillin, streptomycin, and Fungizone antibiotic-antimycotic solution (Life Technologies, Burlington, Ontario, Canada) in a humidified atmosphere containing 5% CO2. To evaluate the effect of various NSAID on COX isoform expression, the drugs indomethacin (Sigma Chemical Co., Mississauga, Ontario, Canada), NS-398 (Cayman Chemical, Ann Arbor, MI), and resveratrol (Sigma Chemical) were added to the media dissolved in DMSO. Drug concentration was in each case 10−5 M, and DMSO concentration was 0.02% (vol/vol).

Immunofluorescence Localization of COX-1 and COX-2 in M-1 Cells
M-1 cells were grown to confluence on glass coverslips, rinsed with phosphate-buffered saline (PBS), and fixed for 30 min at room temperature with 1:1 (vol/vol) PBS:10% phosphate-buffered formalin. The fixative was washed off, and the primary antibodies were applied diluted 1:50 in PBS + 10% FBS + 0.2% saponin (Sigma Chemical). The cells were incubated with the primary antibody solution at 37°C for 1 h. The COX-1 antibody was raised in a rabbit against a peptide corresponding to ovine COX-1 amino acids L274 to A288 and was provided by Drs. W. L. Smith and D. DeWitt (Michigan State University, East Lansing, MI). Two COX-2 antibodies raised against different peptides from the murine COX-2 protein were purchased from Cayman Chemical, and both were effective at detecting murine COX-2 by our methodology. The coverslips were washed with PBS + 0.2% saponin before application of the secondary antibody. The secondary antibody, CY3-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, Bio/Can Scientific, Mississauga, Ontario, Canada), was diluted 1:75 in PBS + 10% FBS + 0.2% saponin, applied to the coverslips, and incubated at 37°C for 30 min. The coverslips were washed with PBS + 0.2% saponin before being mounted on slides with PermaFluor fade-resistant mounting medium (Immunon Lipshaw, Pittsburgh, PA). The specificity of the COX-2 antibodies was tested by preincubation with the cognate peptide (Cayman Chemical). The COX-1 detection was controlled for by omission of the primary antibody. The results were captured under an Axiophot™ microscope (Carl Zeiss, Thornwood, NY), using Northern Eclipse 5.0 imaging software (Carl Zeiss).

Immunohistochemical Localization of COX-1 and COX-2 on Mouse Kidney Slices
Mice (CD-1, 25 to 30 g body wt, 10 to 12 wk old) from Charles River were sacrificed and the kidneys were immediately removed. The kidney was rinsed in PBS, decapsulated, sliced transversely, and placed in fixative. The fixative consisted of 2% paraformaldehyde and 0.2% picric acid in PBS, pH 7.4, and the fixation lasted 18 h at 4°C. The kidneys were paraffin-embedded, and 4-μm microtome sections were mounted onto SuperFrost Plus slides (VWR Canlab, Mississauga, Ontario, Canada). The sections were rehydrated and incubated in methanol + 0.3% H2O2 for 30 min at room temperature. The cells were permeabilized for 15 min in PBS + 0.3% Triton X-100 and blocked for 30 min in PBS + 1% skim milk powder. The COX-1 and COX-2 primary antibody (Cayman Chemical) was diluted in PBS + 0.1% Triton X-100 + 1% bovine serum albumin. For detection of the CCD via aquaporin-2 (AQP-2) labeling, a specific antibody obtained from Dr. Mark A. Knepper (National Institutes of Health, Bethesda, MD) was diluted to 40 ng/ml (30,31). The sections were incubated with these antibody solutions for 18 h at 4°C. The secondary antibody was biotinylated anti-rabbit IgG (Amersham Canada, Oakville, Ontario, Canada), which was diluted 1:100 in PBS + 1% bovine serum albumin + 0.1% Triton X-100; this incubation lasted 30 min at 37°C. After 10 min in 3% H2O2/PBS, the slides were incubated with streptavidin-linked horseradish peroxidase (HRP) diluted 1:50 in PBS. Diaminobenzidine (DAB) substrate (Sigma Chemical) was used to visualize the signals. The slides were counterstained with Mayer’s hematoxylin (VWR Canlab) before dehydration and mounting with Permount (Fisher Scientific, Nepean, Ontario, Canada). For double labeling of COX-1/AQP-2 and COX-2/AQP-2, the COX isoform labeling was performed first as described above, up to and including the visualization with DAB. After this step, HRP activity was quenched by incubating the slides in PBS + 3% H2O2 for 10 min at room temperature. The slides were rinsed with PBS and incubated for 30 min with an unconjugated donkey anti-rabbit IgG Fab fragment (Jackson Immunoresearch Laboratories) to block further detection of the COX isoform primary antibody. The AQP-2 antibody incubations were carried out as described above. For detection of the AQP-2 localization, metal-enhanced DAB (Sigma Chemical) was used. This reagent allowed for the rapid detection of AQP-2 via the development of an insoluble blue precipitate that is easily discernible from the DAB (brown color) used for the COX-1 and COX-2 localization. After the AQP-2 detection, the slides were rinsed in H2O, dehydrated, and mounted with Permount. The results were analyzed and captured.
using an Axiophot microscope (Carl Zeiss) and Northern Eclipse 5.0 imaging software.

**Enzyme Activity**

Confluent dishes of M-1 cells were harvested and resuspended in Dulbecco’s minimal essential medium/F12 media. The NSAID to be assessed were added dissolved in DMSO to give a final NSAID concentration of 10⁻⁵ M and a DMSO concentration of 0.02% (vol/vol). The following drugs were studied: indomethacin, NS-398, resveratrol, and the combination of NS-398 + resveratrol. The cells were incubated with the respective drugs for 30 min at 37°C before [1-¹⁴C]-arachidonic acid (AA) (50 mCi/mmol) (Amersham Canada) was added to give a final concentration of 10 μM, and the cells were further incubated at 37°C for 40 min. At the end of the incubation, the samples were centrifuged (5 min, 2000 × g), and the supernatant was retained for measurement of prostaglandin content. Protein samples from the cellular pellet were measured by the Bradford method to normalize each sample for cell number. The lipid fraction was extracted from the supernatant with diethyl ether/methanol/0.2 M citric acid solution (30:4:1 vol/vol/vol). This prostanoid and unreacted AA-containing fraction was separated on Silica Gel 60 thin-layer chromatography plates (VWR Canlab), using the organic phase of an ethyl acetate/2,2,4-trimethyl pentane/acetic acid/H₂O (11:5:2:10 vol/vol/vol/vol) solvent system. The presence of radiolabeled products was evaluated by autoradiography using Kodak BioMax MR film. Cold PGE₂ standard (Cayman Chemical) visualized with iodine vapor was used to confirm the identity of the major prostanoid (PGE₂) produced by the M-1 cells. Scanning densitometry was performed using the Image 1.47 program, and PGE₂ signal density was normalized to total protein content.

**Western Blotting**

NSAID were added to the media when the cells were plated and the cells were grown to confluence (approximately 72 h). Dishes of confluent cells were rinsed with cold PBS and harvested in PBS by scraping with a cell lifter. The harvested cells were centrifuged, the supernatant was aspirated, and the cell pellet was resuspended in 100 μM Tris, pH 7.4, containing 1 mM ethylenediaminetetra-acid and 1 mM ethylenglycol-bis-(β-aminoethyl ether)-N,N′-tetra-acid. The resulting solution was sonicated for 5 s with an Ultrasonics cell disrupter to lyse the cells. The cell lysates were spun at 10,000 × g for 10 min to pellet the nuclei and insoluble cytoskeleton. The supernatants were removed and assayed for protein content by the Bradford method (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Twenty-five micrograms of protein from each sample was denatured in boiling Laemmli buffer for 5 min and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a polyacrylamide gel consisting of a 4% stacking and a 10% resolving layer. Twenty-five micrograms of protein from each sample was denatured in boiling Laemmli buffer for 5 min and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a polyacrylamide gel consisting of a 4% stacking and a 10% resolving layer using a Mini-PROTEAN II apparatus (Bio-Rad Laboratories). After electrophoresis, the proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Canada) with the Mini-Trans blot system (Bio-Rad). The membranes were blocked overnight at 4°C in Tris-buffered saline-0.1% Tween 20 (TBS-T) supplemented with 5 or 10% fat-free dried milk for COX-1 and COX-2 detection, respectively. After rinsing away the blocking solution with TBS-T, the membranes were incubated with primary antibody diluted 1:2000 in TBS-T-2% milk for 90 min at room temperature. The COX-1 antibody was raised in a rabbit immunized with a peptide corresponding to amino acids L274 to A288 of mouse COX-1 (antibody and immunizing peptide were provided by Drs. W. L. Smith and D. DeWitt, Michigan State University). The COX-2 primary antibodies were purchased from Cayman Chemical. The specificity of the COX isoform-specific antibodies was tested by Western blotting using 50 ng of purified COX-1 and -2 electrophoresis standards per lane (Cayman Chemical). In addition, the antibodies were tested after preincubation with their respective immunizing peptides. After washes with TBS-T, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Promega, Madison, WI) diluted 1:2000 in TBS-T for 1 h at room temperature. Excess secondary antibody was washed away with TBS-T. The results were visualized after developing with the Amersham-enhanced chemiluminescence (ECL) reagents according to the manufacturer’s instructions (Amersham Canada). To ensure equal protein loading, the membranes were stripped as per the Amersham protocol, blocked for 3 h at room temperature in TBS-T-10% milk, rinsed with TBS-T, and incubated with anti-mouse β-actin antibody (Sigma Chemical) diluted 1:5000. After rinsing the membranes, the HRP-conjugated anti-mouse IgG secondary antibody (Amersham Canada) was diluted 1:2000, and incubation took place for 30 min at room temperature. After thorough washing, the results were visualized by the Amersham ECL protocol. Scanning densitometry was performed using the Image 1.47 program, and COX signal density was normalized to β-actin density.

**Northern Blotting**

Total RNA was isolated via the TriZol method (Life Technologies). Poly(A)⁺ RNA isolated from 100 μg of total RNA with Qiagen Oligoex™ columns was loaded onto 1% denaturing agarose gels and electrophoresed. The RNA was transferred to nylon membranes (Boehringer Mannheim, Laval, Quebec, Canada) and fixed by baking at 80°C under vacuum. A probe of 1.3 kb (25 ng) complementary to the portion of the mouse COX-2 cDNA between the XmnI restriction site at 492 nucleotides and the 3′ end was labeled with 32P-CTP (50 μCi) by random priming with the Prime-It kit (Stratagene Cloning Systems, La Jolla, CA). After a 2-h prehybridization at 42°C, the labeled probe was added, and the membrane was allowed to hybridize for 24 h at 42°C. After hybridization and high stringency washing, the radioactive signal was detected by autoradiography with Kodak X-OMAT AR film. The COX-2 probe was stripped from the membrane by boiling for 3 min in diethylpyrocarbonate-treated water. To control for the quantity of mRNA loaded per lane, α-tubulin mRNA levels were measured using a 32P-CTP (50 μCi)-labeled probe prepared and hybridized as for the COX-2 probe. Band intensity was quantified by scanning densitometry using the Image 1.47 program.

**Statistical Analyses**

Data are presented as mean ± SEM. Statistical analyses were performed using the Prism Graphpad 2.01 software and consisted of ANOVA followed by the Bonferroni post test. Differences were considered statistically significant at P < 0.05.

**Results**

**All M-1 Cell Types Express both COX Isoforms**

The M-1 cell line reflects the intact collecting duct in that it is a heterogeneous population with characteristics of principal and intercalated cells. Each of these cell types has been characterized with respect to their individual roles in tubular transport (26). Immunofluorescence experiments with COX isoform-specific antibodies were performed to investigate the distribution of the two isoforms in the M-1 cell line. Figure 1A is representative of the pattern of fluorescence associated with COX-1 immunoreactivity in a monolayer of confluent M-1
cells. The bright perinuclear/endoplasmic reticulum pattern of fluorescence is strikingly apparent in this micrograph. Figure 1B demonstrates the lack of fluorescence when the COX-1 primary antibody is omitted. Like COX-1, COX-2 is also expressed in all cell types (Figure 1C). Each cell is positive for COX-2, and the bright perinuclear ring reflects the increased content of COX-2 protein in the nuclear envelope and is consistent with previously published observations describing the intracellular location of this enzyme (32). Figure 1D establishes the specificity of the COX-2 immunofluorescence. The COX-2 signal is abolished when the primary antibody is preincubated with the immunizing peptide against which it was raised.

**In the Mouse Kidney Cortex, both COX-1 and COX-2 Isoforms Are Detectable in the Intercalated Cells of the CCD**

The distribution of COX-2 in the mouse kidney has not been characterized. Therefore, to demonstrate that the constitutive COX-2 expression by the M-1 cell line is not an artifact of the M-1 cell line immortalization or culture conditions, COX-1, AQP-2, and COX-2 immunoreactivity was localized on three serial 4-μm paraffin-embedded transverse sections of mouse kidney (Figure 2). Figure 2A illustrates COX-1 immunoreactivity in a subpopulation of cells in the cortex compatible with localization within CCD (indicated by arrows). The APQ-2 water channel has been well characterized as the mediator of vasopressin-induced principal cell apical membrane water permeability (30,31). We have used AQP-2 as a marker of collecting ducts to clearly establish the identity of the COX isoform-positive tubules. By labeling three serial sections with COX-1, AQP-2, and COX-2 antibodies, it was possible to confirm the localization of both COX-1and COX-2 to the CCD. The AQP-2 immunohistochemistry result displayed in Figure 2B was obtained on a section serial to that presented in Figure 2A for COX-1 (the arrows have been added to indicate AQP-2-positive tubules). The glomerulus on the right side of the picture serves as a reference point between these two

Figure 1. Indirect immunofluorescence demonstrating the pattern of cyclooxygenase (COX) isoform expression in cultured M-1 cells. (A) Using a COX-1 isoform-specific primary antibody, a perinuclear pattern of fluorescence was observed for COX-1 that is compatible with localization to the endoplasmic reticulum and nuclear envelope. (B) Omission of the COX-1 primary antibody results in complete loss of this labeling. (C) COX-2 was found to be most densely localized within the nuclear envelope, with less intense fluorescence associated with the endoplasmic reticulum. (D) Preincubation of the COX-2 primary antibody with the immunizing peptide abolishes all COX-2-related fluorescence. Magnification, ×1250.
sections as well as the following section where COX-2 has been localized. Figure 2C clearly demonstrates that in addition to COX-1, COX-2 is also expressed in the CCD. Since only a subpopulation of the CCD cells expressed the COX-1 and -2 isoforms, we sought to determine the identity of these cells through double labeling for AQP-2 with each of the COX isoforms.
isoforms. In Figure 2, D and E, we have localized both COX-1 and COX-2 respectively to the intercalated cells of the CCD. In each of these photos, the COX isoform was detected with DAB as the chromogen (indicated by arrows), whereas AQP-2 was visualized using metal-enhanced DAB as the chromogen (blue-colored cells). Two important observations can be made from Panels D and E. First, all CCD cells label for either AQP-2 or the respective COX isoform. Second, no cells label for both AQP-2 and a COX isoform. From these two observations, we conclude that because there are no unlabeled CCD cells, both COX isoforms are expressed by the \( \alpha \)- and \( \beta \)-intercalated cells. The lack of COX/AQP-2 colocalization demonstrates the absence of expression of both COX isoforms in the principal cells. Because there is no AQP-2/COX isoform colocalization, we can also conclude that there is no significant cross reactivity between the two detection systems that we have used. The evidence provided in Figure 2 presents for the first time the finding that the intercalated cells of the CCD constitutively express both COX-1 and COX-2.

COX-2 Is the Major Isoform Contributing to PGE\(_2\) Production by the M-1 Cell Line

The immunofluorescence micrographs (Figure 1) demonstrate that both COX isoforms are expressed by M-1 cells; however, it is also relevant to determine how much each of these isoforms contributes to the total prostanoid pool produced by these cells. This was addressed by measuring the activity of each isoform as evaluated by the conversion of \( [1-^{14}C] \)-AA to \( ^{14}C \)-PGE\(_2\) in the presence of the specific inhibitors. The lipid fraction isolated from M-1 cells was separated by thin-layer chromatography, and the resulting signal was detected by autoradiography. PGE\(_2\) was the only major prostanoid produced by this CCD cell line (Figure 3). Therefore, quantifying PGE\(_2\) synthesis gives a measurement of COX enzyme activity. The localization of the PGE\(_2\) band was performed by visualizing cold PGE\(_2\) on lane 1 using iodine vapor. The \( R_f \) value calculated for PGE\(_2\) was 0.14 \( \pm \) 0.004. Indomethacin was clearly effective at inhibiting both COX isoforms as it reduced PGE\(_2\) production to 13.7 \( \pm \) 3.2% of control \( (P < 0.01, n = 3) \). NS-398 effectively lowered PGE\(_2\) synthesis to 18.5 \( \pm \) 5.6% of control \( (P < 0.01, n = 3) \), indicating that COX-2 is the major prostaglandin-producing enzyme in these cells. Meanwhile, resveratrol treatment lowered M-1 cell COX activity to 60.0 \( \pm \) 7.3% of control \( (P > 0.05, n = 3) \). This reduction in PGE\(_2\) synthesis reflects the inhibition of COX-1 activity by resveratrol. The combination of inhibition with both NS-398 and resveratrol yielded a reduction in COX enzyme activity to 10.3 \( \pm \) 2.7% of control \( (P < 0.01, n = 3) \). The method of combining the two isoform-specific inhibitors to

![Figure 3](image-url)

**Figure 3.** Effect of nonsteroidal anti-inflammatory drugs (NSAID) on COX isoform activity in M-1 cells. (A) Representative autoradiograph of a thin-layer chromatography plate demonstrating radiolabeled prostaglandin E\(_2\) (PGE\(_2\)) production from \( ^{14}C \)-arachidonic acid (AA) substrate following preincubation with respective NSAID. Lane 1, cold PGE\(_2\) standard and \( ^{14}C \)-arachidonic acid (no M-1 cells); lane 2, control (no NSAID); lane 3, indomethacin \( (10^{-5} \text{ M}) \); lane 4, NS-398 \( (10^{-5} \text{ M}) \); lane 5, resveratrol \( (10^{-5} \text{ M}) \); lane 6, NS-398 \( (10^{-5} \text{ M}) \) + resveratrol \( (10^{-5} \text{ M}) \). (B) Bar graph depicting quantification of PGE\(_2\) production by densitometry. Bars 1 through 5 are representative of lanes 2 through 6 on the autoradiograph in Panel A. Results are means \( \pm \) SEM \( (n = 3) \). *\( P < 0.01 \) versus control.
inhibit PGE$_2$ production, as much as does indomethacin, indicates that each COX isoform is maximally inhibited by this concentration of the respective specific inhibitors.

**Inhibition of COX-2 Activity Leads to an Increase in COX-2 Enzyme Expression**

M-1 cells constitutively express COX-1 and COX-2 protein (Figures 1, 4, and 5). To assess the ability of these cells to compensate for reductions in prostaglandin concentrations, the M-1 cells were grown in the presence of nonspecific (indomethacin $10^{-5}$ M) and COX isoform-specific inhibitors (NS-398 $10^{-5}$ M and resveratrol $10^{-5}$ M). Confluent cells were harvested, and expression of COX isoforms was evaluated by Western blotting using COX isoform-specific antibodies as described in Materials and Methods. After culture in the presence of COX-2 inhibition, there was an upregulation in the expression of COX-2 protein (Figure 4). The extent of this COX-2 upregulation was 2.55 ± 0.16- and 2.14 ± 0.12-fold for indomethacin and NS-398, respectively ($P < 0.001$ versus control, $n = 4$). Although there was a trend for a greater effect in the presence of indomethacin (inhibits both isoforms), the difference between the effect of indomethacin and NS-398 (specific COX-2 inhibitor) was not statistically significant. The COX-1-specific inhibitor resveratrol did not have a statistically significant effect on COX-2 expression (0.53 ± 0.13-fold of control, $P > 0.05$). The increase in COX-2 protein expression associated with COX-2 inhibition was measured and found not to be significantly different at time points from 8 to 72 h of NSAID exposure (data not shown, $P > 0.05$). The quantification of COX-2 protein expression as presented in Figure 4C was performed by scanning densitometry. In each lane, the COX-2 level was normalized to the $\beta$-actin signal (COX-2

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**Figure 4.** Effect of NSAID administration on M-1 cell COX-2 expression. (A) Representative autoradiograph of a Western blot detecting COX-2. (B) The corresponding bands for $\beta$-actin protein expression were used to control for variations in loading the gels. Lane 1, control (no NSAID); lane 2, indomethacin ($10^{-5}$ M); lane 3, NS-398 ($10^{-5}$ M); lane 4, resveratrol ($10^{-5}$ M). (C) Bar graph depicting quantification of COX-2 Western blot results by densitometry. Results are means ± SEM ($n = 4$). *$P < 0.001$ versus control.

**Figure 5.** Effect of NSAID administration on M-1 cell COX-1 expression. (A) Representative autoradiograph of a Western blot detecting COX-1. (B) The corresponding bands for $\beta$-actin protein expression were used to control for variations in loading the gels. Lane 1, control (no NSAID); lane 2, indomethacin ($10^{-5}$ M); lane 3, NS-398 ($10^{-5}$ M); lane 4, resveratrol ($10^{-5}$ M). (C) Bar graph depicting quantification of COX-1 Western blot results by densitometry. Results are means ± SEM ($n = 4$). *$P < 0.05$ versus control.
Signal/β-actin signal). COX-2 upregulation was then expressed as a fold increase of the control values.

Identical results were obtained with both COX-2 antibodies obtained from Cayman Chemical. Each of these antibodies was raised against different peptides from the murine COX-2 protein. We have found that neither of these antibodies detects the Cayman Chemical COX-1 standard, whereas both detect the COX-2 electrophoresis standard sold by Cayman Chemical (data not shown). Additionally, the COX-2 signal detected by these antibodies can be abolished by preincubating the antibody with the immunizing peptide (Figure 6). These observations ensure that the COX-2 antibodies used in our experiments are specific for this COX isoform.

M-1 CCD Cell COX-1 Expression Is Not Affected by COX Isoform Inhibition

Following the same 72-h NSAID treatment described for COX-2 Western blot analysis, it was found that COX-1 isoform expression is not affected by the presence of the NSAID studied. Figure 5 demonstrates that although M-1 cells do express COX-1 protein constitutively, the level of expression of this COX isoform is not regulated by culture in the presence of the NSAID tested. The values for COX-1 expression expressed as a fold increase of the control that were obtained for M-1 culture in the presence of indomethacin, NS-398, and resveratrol were 0.85 ± 0.07, 1.07 ± 0.20, and 0.85 ± 0.23, respectively (P > 0.05 versus control). This COX-1 antibody raised against a peptide corresponding to mouse COX-1 amino acids L274 to A288 does not cross-react with the COX-2 standard (Cayman Chemical). The lack of any effect of indomethacin or NS-398 on the band intensity for COX-1 further illustrates an absence of cross reactivity toward COX-2.

The Increase in COX-2 Protein Expression Is Not Correlated with an Increase in COX-2 mRNA Levels

Given the preceding observations regarding the upregulation of COX-2 protein by the inhibition of COX-2 activity, it was of interest to study the effect of NS-398 treatment on levels of COX-2 mRNA expression. To assess whether the increase in COX-2 protein is dependant on an increase in COX-2 mRNA synthesis or stability, Northern blot analysis was performed. Using a 32P-labeled 1.3-kb fragment of the murine COX-2 cDNA as a probe, it was possible to detect a 4.2-kb signal matching the known size of the COX-2 mRNA (Figure 7A). In addition to the time points depicted in Figure 3, COX-2 mRNA levels were measured 2 h after addition of NS-398 to the culture media (data not shown). At no time point was there a significant increase in COX-2 mRNA expression compared with the control (P > 0.05 versus control). In the histogram (Figure 7C), COX-2 mRNA levels have been normalized to the corresponding α-tubulin band. α-Tubulin levels were mea-

![Figure 6. Preincubation of COX-2 antibody with the immunizing peptide completely blocks the signal for COX-2. This figure demonstrates the specificity of the COX-2 antibody used in our experiments. Both lanes were loaded with 25 μg of M-1 cell lysate prepared as described in Materials and Methods. As opposed to lane 1, before detection, the antibody in lane 2 was preincubated with an excess (by weight) of the immunizing peptide.](image)

![Figure 7. Time course for the regulation of M-1 cell COX-2 mRNA expression by the COX-2-specific inhibitor NS-398 (10^-5 M). (A) Lane 1, control (no NS-398); lane 2, 8 h; lane 3, 18 h; lane 4, 30 h; lane 5, 54 h. (B) Corresponding signals for α-tubulin mRNA detection. (C) Histogram depicting quantification of COX-2 mRNA signal by densitometry followed by normalizing with the α-tubulin signal. Results are means ± SEM (n = 3). *P < 0.05 versus control.](image)
sured to control for variability in the efficiency of isolating poly(A)⁺ RNA from one sample to the next as well as to control for variability in sample loading.

**Discussion**

In 1978, Smith and Bell localized COX immunoreactivity to the CCD in the rabbit, cow, guinea pig, rat, and sheep (33–35). This antibody did not detect COX in the macula densa, suggesting that it was more specific for COX-1 than COX-2. Additional studies that measured PGE₂ synthesis by the microdissected CCD demonstrated the prostaglandin-synthesizing ability of the CCD (3). That the COX-1 isoform is expressed in the rat and human CCD has been confirmed with isoform-specific antibodies (22,34). However, with respect to COX-2, the human and rat studies diverge with respect to the renal localization. The M-1 cell line has been well characterized by Stoos et al. (26). These cells exhibit an amiloride-sensitive Na⁺ transport and K⁺ secretion characteristic of principle cells as well as electrogenic H⁺ secretion characteristic of immunocytochemistry. The COX isoform signal in the M-1 CCD cell line matches the bright nuclear envelope/endo-somal reticulum pattern of fluorescence that is characteristic of the intracellular localization of this enzyme as reported by Morita et al. (32). Each of the COX isoforms is expressed in all CCD cell types as represented by the M-1 CCD cell line (Figure 1).

Our present study has demonstrated for the first time that in addition to COX-1, COX-2 is also expressed in the CCD. The demonstration of constitutively expressed COX-1 and COX-2 immunoreactivity in only a portion of the cells of the native CCD (Figure 2) is not unlike the situation in the macula densa, where only a small subpopulation of the cells are COX-2-positive (22). However, because the CCD is composed of principal, α-, and β-intercalated cell types, we sought to determine whether the subpopulation of COX isoform-positive cells corresponded to a specific cell type. The AQP-2/OX isoform double-labeling evidence we have presented (Figure 2, D and E) reveals that in the native CCD under normal conditions, COX-1 and COX-2 are expressed in the intercalated cells. Full comprehension of the role of COX-1 and COX-2 in the CCD will require an understanding of the localization and regulation of the PGE₂ receptors in this segment of the nephron. The effects of PGE₂ are mediated by a distinct class of receptors referred to as E-prostanoid (EP) receptors (36). The EP₁, EP₂, and EP₃ receptor subtypes have been localized in the CCD (37). Functional studies examining the effects of PGE₂ on salt and water transport have suggested that these receptors are present on the principal cells (4). Our results, which localize both COX isoforms to the intercalated cells, indicate that the effects exerted by PGE₂ on principal cell salt and water reabsorption represent a paracrine interaction between intercalated and principal cells. The interactions between the EP receptors, the regulation of COX isoform expression, and PGE₂ synthesis in this segment of the nephron represent an interesting field of study.

The comparison of studies of COX activity and inhibition in different cell types is complicated by several factors (38). These factors include: the use of exogenous versus endogenous AA as substrate, purified enzyme versus broken cells versus intact cells as a source of COX enzymes, instantaneous versus time-dependant inhibition, and the study of endogenously expressed COX versus vector-driven expression systems. Regardless of these limitations, the observations we make concerning COX isofrom activity within the system we have used are significant. The novel finding in our results is that both constitutively expressed COX isoforms in the CCD are functional and make significant contributions to PGE₂ synthesis by this nephron segment.

Our finding that COX-2 inhibition results in an upregulation in COX-2 expression is interesting in that it is the opposite of what was observed with respect to compensatory upregulation of the remaining COX isoform in cultured fibroblast cells from COX null mice (39). In addition to the compensatory COX isoform upregulation, there is also cPLA₂ protein overexpression. In our experiments, cPLA₂ overexpression was not detected by immunoblotting in the M-1 cells following COX-2 inhibition (S. Ferguson, R. Hébert, O. Laneuville, unpublished results). Also, our study found that COX-2 inhibition was necessary for stimulation of COX-2 upregulation. Although not statistically significant, the trend is for COX-2 expression to decrease in response to resveratrol treatment (COX-1 inhibition). The comparison between these two studies suggests that interactions between COX isoform expression and prostaglandin production is cell type-specific. This idea is supported by the observation in COX-1⁻/⁻ mice that there is no compensatory COX-2 upregulation in the stomach (20).

The NSAID-induced upregulation of COX-2 protein was not accompanied by any effect on COX-2 mRNA expression. This is in sharp contrast to the effect of inflammatory agents such as cytokines and phorbol esters, which stimulate increased COX-2 protein and mRNA expression (40). For example, interleukin-1α was shown to both stimulate COX-2 mRNA transcription and prolong the half-life of this message in human umbilical vein endothelial cells and the human ECV324 cell line (41). The mechanism responsible for the COX-2 regulation we have documented remains unknown but is most likely posttranscriptional. Evidence is available that allows for speculation on this subject. With the murine MC3T3-E1 osteoblast cell line, NS-398 was able to block cytokine-induced upregulation of COX-2 protein (42). This blockade was reversed by the addition of exogenous PGE₂. However, PGE₂ did not upregulate COX-2 protein in the absence of cytokine stimulation. The M-1 cell line expresses COX-2 constitutively, thus it is possible to study COX-2 regulation without any confounding effects induced by cytokine stimulation. Unlike the previously mentioned study, the NS-398-stimulated COX-2 upregulation that we observed is associated with an inhibition of PGE₂ synthesis.

NSAID are a powerful tool in controlling inflammation; however, their clinical usefulness is limited by their negative side effects: primarily gastric ulcers and secondarily kidney impairment. The kidney impairment that follows NSAID therapy may be divided into several categories. The hemodynamic effects of prostaglandins have been well studied. The vasodil-
latory effects of PGE₂ and PGI₂ are important in counteracting the effects of vasoconstricting peptides such as angiotensin II and endothelin I and renal sympathetic nerve activity (5–10). This is especially important in volume-depleted states in which the vasodilatory prostaglandins are critical for the maintenance of renal blood flow and GFR. Electrolyte imbalances can occur subsequent to vasoconstrictor-mediated reductions in GFR when NSAID inhibit PGE₂ and PGI₂ production in mesangial cells. COX-2 has also been implicated in the regulation of renal renin levels. Both low salt diet (25) and renovascular hypertension (35) studies have shown a link between macula densa COX-2 expression and renin content. In addition to the hemodynamic and endocrine effects of renal prostaglandins, they also inhibit sodium and water reabsorption in the collecting duct (4). Our study suggests that the effects of PGE₂ on transport in the collecting duct could be mediated through a paracrine interaction between principal and intercalated cells. We also demonstrate that in addition to inhibiting COX-2 activity, COX-2-specific NSAID also rapidly stimulate an increase in COX-2 protein expression. This finding suggests that after the metabolism and clearance of a dose of an NSAID, there could be a period in which COX-2 activity rises above baseline while the increased COX-2 protein expression persists in the absence of inhibitor.

In summary, in the cortex of intact kidney sections, COX-1 and COX-2 are localized to the intercalated cells of the CCD. We have found that although M-1 CCD cells express both COX isoforms, COX-2 is the major COX isof orm contributing to murine CCD prostaglandin production. Both the COX-1 and COX-2 isoforms were found in all cultured M-1 cell types. When COX activity was measured in the presence of commercially available isoform-specific inhibitors, it was observed that COX-2 inhibition significantly decreased PGE₂ synthesis compared with control. Meanwhile, COX-1 inhibition resulted in a smaller decrease in PGE₂ production. These results demonstrate the predominance of COX-2 activity in the production of prostaglandins by the CCD. Furthermore, inhibition of the cyclooxygenase activity of COX-2 with NS-398 or indomethacin resulted in increased COX-2 protein expression. This increase in COX-2 protein expression occurred independently of any change in the level of COX-2 mRNA expression. COX-1 protein expression was not affected by NSAID administration. Much of the previous research has focused on the role of COX-2 within the juxtaglomerular apparatus; however, within the kidney cortex, the CCD synthesizes the greatest quantity of PGE₂ per nanogram of dissected nephron segment protein (3). This PGE₂ is an important mediator controlling salt and water reabsorption by the CCD (43). Our new observations in conjunction with previously published work concerning the localization and regulation of COX-2 in the rat kidney demonstrate the importance of constitutively expressed, noninflammatory COX-2 in the kidney. When one takes into account the role that COX-2 plays in producing prostanoids required for normal renal development, hemodynamics, renin secretion, and now collecting duct function, questions arise as to how benign the new class of COX-2-specific inhibitors will be toward the kidney.

Accept the final content from the section below, which is the new version of the text:

**Pharmacological Effects of PGE₂ and PGI₂**

PGE₂ and PGI₂ have significant pharmacological effects on the kidney. They are known to counteract the effects of vasoconstricting peptides such as angiotensin II and endothelin I, and they play a crucial role in maintaining renal blood flow and GFR. Electrolyte imbalances can occur when renal prostaglandin synthesis is reduced by NSAIDs. Decreased COX-2 activity, especially in the medulla, can lead to a period of increased COX-2 expression in the absence of inhibitor.

In summary, in the cortex of intact kidney sections, COX-1 and COX-2 are localized to the intercalated cells of the CCD. We found that although M-1 CCD cells express both COX isoforms, COX-2 is the major COX isozyme contributing to murine CCD prostaglandin production. Both the COX-1 and COX-2 isoforms were found in all cultured M-1 cell types. When COX activity was measured in the presence of commercially available isoform-specific inhibitors, it was observed that COX-2 inhibition significantly decreased PGE₂ synthesis compared with control. Meanwhile, COX-1 inhibition resulted in a smaller decrease in PGE₂ production. These results demonstrate the predominance of COX-2 activity in the production of prostaglandins by the CCD. Furthermore, inhibition of the cyclooxygenase activity of COX-2 with NS-398 or indomethacin resulted in increased COX-2 protein expression. This increase in COX-2 protein expression occurred independently of any change in the level of COX-2 mRNA expression. COX-1 protein expression was not affected by NSAID administration. Much of the previous research has focused on the role of COX-2 within the juxtaglomerular apparatus; however, within the kidney cortex, the CCD synthesizes the greatest quantity of PGE₂ per nanogram of dissected nephron segment protein (3). This PGE₂ is an important mediator controlling salt and water reabsorption by the CCD (43). Our new observations in conjunction with previously published work concerning the localization and regulation of COX-2 in the rat kidney demonstrate the importance of constitutively expressed, noninflammatory COX-2 in the kidney. When one takes into account the role that COX-2 plays in producing prostanoids required for normal renal development, hemodynamics, renin secretion, and now collecting duct function, questions arise as to how benign the new class of COX-2-specific inhibitors will be toward the kidney.


