Cyclooxygenase expression in canine platelets and Madin-Darby canine kidney cells

Patricia A. Kay-Mugford, DVM, MSc; Sally J. Benn, MSc; Jonathan LaMarre, DVM, PhD; Peter D. Conlon, DVM, PhD

Objective—To examine cyclooxygenase (COX) expression in canine platelets and Madin-Darby canine kidney (MDCK) cells in culture.

Sample Population—Canine platelets and MDCK cells.

Procedure—Total RNA was recovered from isolated canine platelets and MDCK cells. Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR), using complementary DNA probes and primers designed from the human COX sequences, were used to determine COX-1 and -2 (cyclooxygenase isoforms 1 and 2) messenger RNA (mRNA) expression.

Results—Following northern blot analysis, canine platelets were found to express only the 2.8-kb COX-1 transcript; COX-2 was not detected. Canine MDCK cells expressed the 4.5-kb COX-2 transcript, in addition to the 2.8-kb COX-1 transcript. A single DNA band of 270 base pairs was identified following gel electrophoresis of the product obtained from RT-PCR of mRNA from canine platelets. Sequencing revealed that this PCR product was 90% homologous to a portion of the human COX-1 gene (Genbank M59979).

Conclusions and Clinical Relevance—Detection of COX-1 by RT-PCR of RNA obtained from canine platelets is a novel finding. The 90% homology of the PCR product with the human sequence suggests strong conservation between the canine and human COX-1 gene. Cloning and sequencing of the canine gene will be required to fully characterize homologous regions. Because of the importance of COX in the inflammatory process and as a potential target of currently available nonsteroidal anti-inflammatory drugs (NSAID), a better understanding of canine COX may improve our ability to use NSAID appropriately, achieve efficacy, and avoid potential adverse drug effects in dogs. (*Am J Vet Res* 2000;61:1512–1516)

Cyclooxygenase (COX) is a membrane-bound bifunctional enzyme that catalyzes the conversion of arachidonic acid to form prostaglandins.[1,2] This enzyme exists as 2 differentially regulated isoforms known as COX-1 and COX-2.[3,4] Isoform 1 is constitutively expressed and maintains homeostatic production of prostaglandins. Isoform 2 is primarily inducible by mitogens, cytokines, and growth factors and plays a role in the inflammatory process.[3,5,6]

The pattern of distribution of COX-1 and -2 **messenger RNA (mRNA)** and protein is known for tissues from various species. Nearly all cell types express COX-1 under basal conditions.[7-9] In certain tissues such as the brain, prostate, and renal cortex of various species, COX-2 is also constitutively expressed.[7,10,11]

Because COX is a therapeutic target of **nonsteroidal anti-inflammatory drugs (NSAID)**, recent scientific efforts have focused on the development of COX-2 selective inhibitors to suppress inflammation with lesser risk of adverse effects, often using in vitro cellular assays to evaluate anticipated efficacy and toxicity of these agents. It is hypothesized that gastric, renal, and coagulation-related toxicoses associated with the use of NSAID are primarily related to inhibition of homeostatic COX-1 in the gastrointestinal tract, kidney, and platelets, respectively. Interspecies differences in the distribution of COX isoenzymes is found in the gastrointestinal tract and renal tissues of humans, primates, dogs, and rodents, a finding that may help to explain differences in species susceptibility to NSAID-related toxicity.[10,12]

To our knowledge, there are limited data available on the **complementary DNA (cDNA)** sequence or cellular distribution of COX in dogs. We have examined COX expression, using northern blot analysis and **reverse transcription-polymerase chain reaction (RT-PCR)** in isolated canine platelets, which are commonly used to screen NSAID in vitro for their presumed activity against constitutive COX-1.[13-15] We have also determined the pattern of COX expression in **Madin-Darby canine kidney (MDCK)** cells, which may also provide a source of COX enzyme to use for canine NSAID screening assays. Although prostaglandin synthesis and COX expression by MDCK cells have been investigated, there are conflicting reports in the literature as to the pattern of expression.[16,17]

A better understanding of COX expression in dogs may improve our ability to use NSAID appropriately, achieve efficacy, and avoid potential adverse drug effects. Therefore, the purpose of the study reported here was to examine COX expression in isolated canine platelets and MDCK cells in culture.

Materials and Methods

Platelet isolation and RNA recovery—Single units of whole blood were collected from donor dogs by venipuncture into collection bags containing sodium citrate. Whole blood was centrifuged at 20 C for 4 minutes at 1,000 × g to prepare platelet-rich plasma. The platelet-rich plasma was expressed into a polyolefin bag,[a] using a plasma extractor,[b] and extraction was stopped when the RBC interface was 1 cm

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From the Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Ontario, Canada N1G 2W1. Dr. Kay-Mugford's present address is Schering-Plough Animal Health Corporation, Bldg U-23 Mailstop 4800, 1095 Morris Avenue, Union, NJ 07083-1982.
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from the top of the bag.[18] An aliquot was sent to the clinical pathology laboratory at the Ontario Veterinary College, University of Guelph, Ontario, for a full platelet count and differential WBC count. White blood cell and RBC contamination was consistently < 1%. The platelet-rich plasma was then transferred into dimethylpyrocarbonate-treated 10-ml volume siliconized glass tubes and centrifuged for 20 minutes at 2,000 × g to form a platelet pellet.[19] Total RNA was recovered and processed by use of a reagent[c] according to the manufacturer's protocol.

MDCK cell culture and RNA recovery—The MDCK cells[d] were maintained in monolayers at 37 C and 5% CO_2 in **Dulbecco Modified Eagle Medium (DMEM)**[e] containing 10% fetal bovine serum[f] and penicillin (50 U/ml)/streptomycin (50 U/ml).[e] Passage of cells was performed regularly with trypsin.[e]

Cells were seeded into 24 well plates[f] at 1×10^5 cells/well in DMEM. After 24 hours, the cells were incubated overnight in serum-free DMEM. The medium was removed and replaced with fresh serum-free DMEM. Total RNA was recovered and processed by use of a reagent[c] according to the manufacturer's protocol.

Northern blot analysis—The 1.8-kilobase fragments of the COX-1 and COX-2 cDNA were subcloned into the *Not I*, *Hind III*, *Eco R1*, and *Apa I* vector sites, respectively.[g,20] The fidelity of the cDNA fragments was verified by dye terminator cycle sequencing, using a DNA sequencer.[h] Total cellular RNA was isolated by use of a reagent,[c] as described by the manufacturer. Total RNA (20 µg for MDCK cells and 5 µg for canine platelets) was subjected to electrophoresis in 0.8% agarose gels, transferred to nylon membranes,[i] and prehybridized for 1 hour in 0.5% **sodium dodecyl sulfate solution (SDS)**, 5× Denhardt solution, 6× sodium chloride/sodium phosphate buffer solution, 50% formamide,[j] and denatured salmon sperm DNA[k] (10 mg/ml). The human COX-1 and COX-2 probes were radiolabeled by use of [α-^{32}P] dCTP[l] and a random primer labeling kit,[m] as per the manufacturer's instructions. The ^{32}P-labelled cDNA were incubated for 16 hours with the membranes in a solution identical to the prehybridization solution. Membranes were then washed twice (15 minutes each) at 42 C in 2× **sodium chloride/sodium citrate buffer solution (SSC)** and 1% SDS and once in 0.1× SSC and 0.1% SDS at 55 C for 30 minutes for COX-1. Each membrane was probed for COX-1, stripped with 0.1% SDS, and rehybridized with the probe for COX-2 on separate occasions. Membranes were also rehybridized with a probe for murine 7S RNA[21] that served as a load control. Phosphoimaging with commercially available software[n] was used to image the radioactive blots.

Reverse transcription and polymerase chain reaction—Ribonucleic acid (1 µg) obtained from canine platelets, as previously described, was reverse-transcribed with reverse transcriptase[e] in the presence of 10 m*M* deoxynucleotides,[o] random primers, Oligo (dT) primer,[p] deoxyribonuclease,[e] 10× deoxyribonuclease buffer,[e] 2 µl dithiothreitol (0.1*M*),[e] and 3' (antisense) primer (50 pmol/µl) in dimethylpyrocarbonate-treated water. Resultant cDNA was diluted 5 times. In a thin-walled PCR tube, 10 µl of the cDNA was amplified in a final volume of 100 µl containing 2 µl of deoxynucleotide (10 m*M*), 5.0 ml of $MgCl_2$ (50 m*M*),[e] 0.2 ml of *Taq* DNA polymerase,[e] 10 µl of 10× PCR buffer,[e] 2 µl of (50 pmol/µl) sense and antisense primers, and sterile double distilled water. The primers used to amplify the COX cDNA were as follows: COX-1 sense, 5' to 3', AGATGGCAGCAGAGTTGGGAG and antisense, 3' to 5' ACAGGTCTTGGTGTTGAGG; and COX-2 sense, 5' to 3' CAACTACCTGCGCACTGT and antisense, 3' to 5' GCCACTGAAGTGTAGCCT. The amplification conditions were 94 C for 3 minutes for 1 cycle; 94 C for 30 seconds (denaturation), 55.5 C for 30 seconds, and 72 C for 1 minute (primer annealing and extension) for 40 cycles; and 72 C for 5 minutes. The PCR products were identified following electrophoresis on a 2% agarose gel containing ethidium bromide (10 mg/ml)[k] in tris-acetate buffer. The remaining 90 µl of sample was purified, using a PCR purification kit,[q] and the identities of the COX-1 and COX-2 products were confirmed by dye terminator cycle sequencing, using a DNA sequencer.[h]

Results

Northern blot analysis—Canine platelet mRNA expressed the 2.8-kb COX-1 transcript, but COX-2

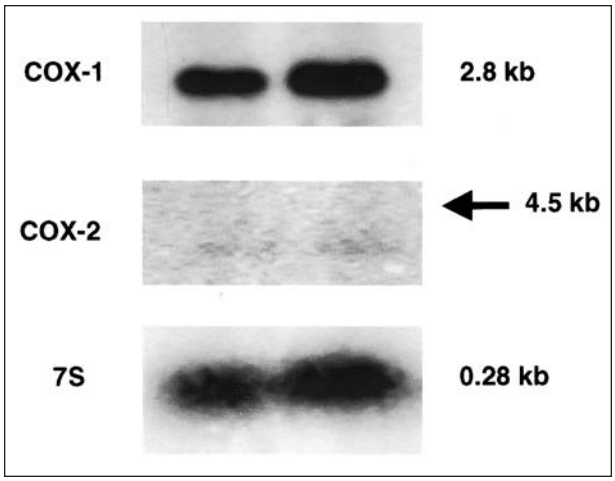

Figure 1—Northern blot analysis of total RNA (5 µg/lane) from canine platelets, using human complementary DNA (cDNA) probes. The cDNA probes of cyclooxygenase (COX) isoform 1 (top panel; COX-1) and 2 (center panel; COX-2) were used. Notice that prior to use of the cDNA probe for COX-2, the residual platelet COX-1 transcript was visible even after repeated stripping of the membrane. The expected band size of COX-2 was 4.5 kb (arrow). A 7S cDNA probe (lower panel) was used as a load control. Results are representative of 3 northern blot analyses.

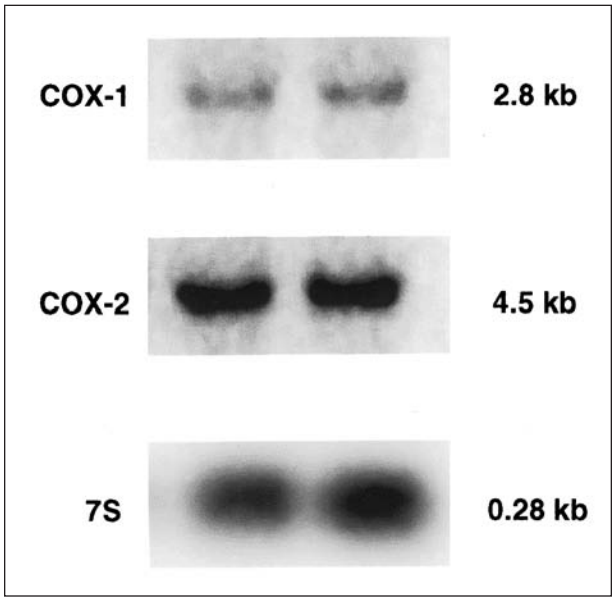

Figure 2—Northern blot analysis of total RNA (20 µg/lane) from Madin-Darby canine kidney cells, using human cDNA probes. The cDNA probes of COX-1 (top panel) and -2 (center panel) were used. A 7S cDNA probe (lower panel) was used as a load control. Results are representative of 3 northern blot analyses.

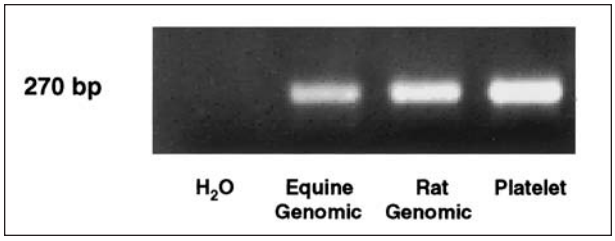

Figure 3—Reverse transcription-polymerase chain reaction (RT-PCR) of canine COX-1. The RNA obtained from canine platelets was reverse-transcribed into DNA and amplified by PCR, using sense and antisense primers for human COX-1. Sterile water (H_2O) served as a negative control, and equine and rat genomic DNA served as positive controls.

CTGCTGGAGACTTGATGCCTTGGAATTCTACCCGGGGCTTCTTCTGGAGAAGTGCC
TCCAAACTCCATCTTTGGAGAGAGTATGATAGAAATTGGGGCTCCCTTCTCCCTTA
GGGCCTCCTAGGGAATCCCATCTGTTCTCCAGAGTACTGGAAGCCAAGCACATTCG
TGGTGAGATGGGCTTCAATATGGTCAAGACAGCCACACTGAAGAAGCTGGTCTGC
TCAACACCAAGACCGGTCA

Figure 4—A portion of the canine COX-1 DNA sequence obtained by dye terminator cycle sequencing of the product obtained from RT-PCR of canine platelet messenger RNA (mRNA). This sequence is 90% homologous to the equivalent portion of the human COX-1 gene.

mRNA was not detected (**Fig 1**). The MDCK cells strongly expressed the 4.5-kb COX-2 transcript, in addition to the 2.8-kb COX-1 transcript (**Fig 2**).

RT-PCR—The DNA bands of 270 base pairs were identified following gel electrophoresis of the products obtained from RT-PCR of mRNA from canine platelets (**Fig 3**). Restriction mapping and dye terminator cycle sequencing revealed that the PCR product from the canine platelet product was 90% homologous to a portion of the human COX-1 gene (Genbank M59979). The sequence obtained from the canine platelets was determined (**Fig 4**). Amplification of COX-2 by RT-PCR, using mRNA recovered from MDCK cells or canine platelets, was unsuccessful.

Discussion

Platelets are the only cells that exclusively express COX-1 in all species studied.[8,13,14] In our study, we also report this expression pattern in canine platelet mRNA following northern blot analysis.

Canine platelets are generally considered functionally similar to human platelets in that they may aggregate in response to adenosine diphosphate, thrombin, and collagen.[22] However, unlike human platelets, the thromboxane A_2-responsiveness of canine platelets appears to be inherited as an autosomal recessive trait.[22-24] Although most dogs have platelets that form thromboxane A_2 from exogenous arachidonic acid as a product of the COX pathway, they fail to aggregate or secrete in response to it.[22,25] Thus, theoretically, the exposure of platelets from different breeds of dogs to NSAID may not inhibit platelet aggregation in a predictable manner. Quantitative measurement of thromboxane A_2 synthesis by canine platelets is considered to be a biochemical index of COX-1 activity.[15] However, COX gene expression has not been evaluated in canine platelets, as its similarity to that of other species is assumed.[15,24,25] Thus, our ability to verify COX-1 mRNA expression in canine platelets and lack of detectable COX-2 mRNA by northern blot analysis is notable.

Our detection of COX-1 by RT-PCR of mRNA obtained from canine platelets, using primers designed from the human COX-1 sequence, is a novel finding. The 90% sequence similarity between the 270 base pair PCR product and the human portion of the gene suggests strong conservation between the canine and human COX-1 gene. Previously, comparison of the human COX amino acid sequences among species has demonstrated 60 to 70% homology.[2,5] Cloning and sequencing of the canine gene is required to fully characterize homologous regions and the functional implication of these findings.

Prostaglandin synthesis and COX expression by MDCK cells has been investigated.[26] However, pertinent studies have produced variable results for expression of COX in MDCK cells. Our detection of COX-2 message in unstimulated MDCK cells by northern blot analysis concurs with a previous study that examined COX mRNA in MDCK cells. In that study, COX-2 mRNA was detected in low amounts in resting MDCK cells by semiquantitative PCR, using primers taken from a consensus sequence of mouse, rat, and human cDNA; however, COX-1 mRNA was undetectable.[16] Also, in that study, the authors reported that they were unable to detect COX-1 and -2 mRNA by northern blot analysis by use of murine cDNA probes. These results may reflect the lack of species homology between the murine and canine cDNA probe sequences used for northern blot analysis or difficulties in detection of low amounts of COX expression. Because we were able to use northern blot analysis with human cDNA probes to demonstrate expression of COX-1 in MDCK cells, it is possible that there is greater homology between the canine and human COX-1 sequences than the canine and murine COX-1 sequences. This possibility is supported by the results achieved by alignment of the nucleotide sequence of the PCR product, which we amplified from canine COX-1 with known Genbank sequences (National Center for Biotechnology). When the known human and murine COX-1 sequences were compared with the sequence of the canine PCR product, the homology was greater between the canine and human sequences than between the canine and murine sequences (**Fig 5**).

Our findings of apparent constitutive COX-2 expression, in addition to COX-1 expression by MDCK cells, conflicts with a previous publication that reported constitutive expression of COX-1 and induction of COX-2 in MDCK cells following incubation with 12-0-tetradecanoyl-phorbol-13-acetate. Polyclonal COX-1 and COX-2 antibodies were used in that study to perform immunodetection of COX protein.[17] It is possible that these results were the result of cross-reactivity between COX-1 and COX-2 antibodies rather than individual detection of each COX isoform.

In our study, we were unable to amplify COX-2 by RT-PCR of canine platelet mRNA. This was not unexpected, as platelets express only COX-1 in other

Figure 5—Comparison of the alignment of the DNA sequence obtained from RT-PCR of canine platelet mRNA with known Genbank sequences (National Center for Biotechnology) for human and murine COX-1. Homology was greater between canine and human sequences than between canine and murine sequences (only exact base pair matches are shown).

species.[8,13,14] However, we were also unable to amplify COX-2 from reverse-transcribed mRNA obtained from MDCK cells. This was surprising, because COX-2 was detected in MDCK cells by northern blot analysis. Therefore, it seems likely that the experimental conditions under which PCR was conducted were inappropriate, and further refinement is required to detect COX-2 in canine cells.

Our laboratory has previously examined COX expression in canine DH82 monocyte and macrophage cells in culture, demonstrating constitutive COX-1 expression in untreated cells, and dramatic increases in COX-2 mRNA expression following exposure to lipopolysaccharide.[27] These findings are consistent with other studies of human and rodent monocyte and macrophage cells, in culture and recovered from live subjects, which also have similar patterns of COX expression.[20,28,29] This provides supportive evidence that expression and regulation of inducible (inflammatory) canine COX-2 is similar to other species. However, constitutive canine COX-2 expression may differ, as reflected by our detection of COX-1 and COX-2 mRNA in unstimulated MDCK cells in culture and previously documented interspecies differences in renal localization of COX isoforms.[9,10,12]

Adverse renal effects of nonselective NSAID are thought to be caused by reduced prostaglandin synthesis through COX-1 inhibition.[1,2,12] However, COX-2 is upregulated in the macula densa following salt or volume depletion in healthy dogs and rodents, a phenomenon that does not seem to occur in humans or primates.[11,30] The consequences of selective COX-2 inhibition under these circumstances are as yet unknown.[11,12,30]

Several in vitro assay models have been developed to assess the potential efficacy and toxicity of NSAID that are commonly used in humans by evaluating their ability to inhibit either COX-1 or COX-2 in human cells.[31-33] However, only 2 studies have examined NSAID inhibition of canine COX isoenzymes.[15,27] The affinity of NSAID for the inducible canine COX-2 enzyme was evaluated through effects on inflammatory prostaglandin production in the canine DH82 monocyte and macrophage cell line following lipopolysaccharide stimulation.[15,27] It may also be feasible to anticipate the potential physiologic and clinical effects of COX-2 selective NSAID on constitutive COX-2 expression in dogs by studying the in vitro effects of NSAID on prostaglandin synthesis in MDCK cells. Although it is possible that the MDCK cell line may have aberrant expression of COX and, thus, may not be representative of in vivo COX expression in dogs, further study is required to explore the clinical relevance of our findings.

[a]PL 732 Plastic transfer pack container, Baxter Corp, Mississauga, ON, Canada.
[b]Fenwal 4R4414, Baxter Corp, Mississauga, ON, Canada.
[c]TRIzol, Gibco BRL, Life Technologies, Burlington, ON, Canada.
[d]Madin-Darby Canine Kidney cells, American Type Culture Collection, Rockville, Md.
[e]Gibco BRL, Life Technologies, Burlington, ON, Canada.
[f]ICN Biomedical Inc, Aurora, Ohio.
[g]pBluescript SK, Stratagene GmbH Heidelberg, Germany.
[h]ABI Prism 377, Bio-Rad Laboratories, Richmond, Calif.
[i]Hybond N, Amersham Life Sciences, Oakville, ON, Canada.
[j]Fisher Scientific, Nepean, ON, Canada.
[k]Boehringer-Mannheim Canada, Laval, QC, Canada.
[l]Amersham Life Sciences, Oakville, ON, Canada.
[m]Rediprime random primer labeling kit, Amersham Life Sciences, Oakville, ON, Canada.
[n]GS-250 Molecular Imager with Molecular Analyst Version 2.1 software, Bio-Rad Laboratories, Richmond, Calif.
[o]Pharmacia Biotech, Baie d'Urfe, QC, Canada.
[p]Promega, Madison, Wis.
[q]QIA quick PCR purification kit, Qiagen Inc, Mississauga, ON, Canada.

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