



2006-09-19

Studies on Cyclooxygenase-1, its Structure and Splice Variants, and Modulation of Cyclooxygenase-2 by Inducible Nitric Oxide Synthase and Novel Phytochemicals.

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**STUDIES ON CYCLOOXYGENASE-1, ITS SPLICE VARIANTS, AND
MODULATION OF CYCLOOXYGENASE-2 BY INDUCIBLE NITRIC OXIDE
SYNTHASE AND NOVEL PHYTOCHEMICALS**

by
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A dissertation submitted to the faculty of
Brigham Young University
In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Chemistry and Biochemistry
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December 2006

BRIGHAM YOUNG UNIVERSITY

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ABSTRACT

STUDIES ON CYCLOOXYGENASE-1, ITS SPLICE VARIANTS, AND MODULATION OF CYCLOOXYGENASE-2 BY INDUCIBLE NITRIC OXIDE SYNTHASE AND NOVEL PHYTOCHEMICALS

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Department of Chemistry and Biochemistry

Doctor of Philosophy

Cyclooxygenases (COXs) are of important therapeutic value as they are the target site of aspirin-like drugs. Here I report nine new COX-1 splice variants in **chapter 1**, which I characterized with regard to heme-binding and other properties.

Inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) are co-inducible in many tissues following mitogenic and proinflammatory stimulation. In **chapter 2**, I investigate the physical and enzymatic properties of human COX-2 and iNOS and demonstrate that, despite reports to the contrary by another laboratory, they do not interact.

The only reported COX-1 splice variant to exhibit cyclooxygenase activity has been isolated from dog brain and is termed COX-3. It contains an in-frame insertion of intron 1. However the existence of human COX-3 remains questionable since intron 1 is out of frame. Two putative in-frame human COX-3 isozymes, COX-1b₂ and COX-1b₃, (herein designated as COX-3-72 and COX-3-50) have been reported in the literature, but

only one of them, COX-3-72, has been characterized. In **chapter 3**, COX-3-50 and COX-3-72 are reported to be over-expressed and determined to be active cyclooxygenases. COX-3-72 and, to a greater extent, COX-3-50, were stimulated by rofecoxib at physiological concentrations. A similar rofecoxib-stimulated COX activity is observed in quiescent A549 cells. Immunoblot and immunoprecipitation analysis suggest that human platelet and potentially A549 cells, contain a COX-3-50 like protein.

Lonicera japonica is used as an anti-inflammatory treatment in traditional Chinese medicine. Its working mechanism is not well known. In **chapter 4**, I report that extracts from this herb inhibit COX-2 by three mechanisms: direct inhibition, transcriptional and post-transcriptional down regulation.

COX-1 and COX-2 are similar to each other in their crystallographic structures. One of the most striking differences is that there are eight amino acids immediately following the signal peptide in COX-1 which are not found in COX-2. The function of this sequence is unknown. In **chapter 5**, I found that deletion of these amino acids decreased COX-1 V_{\max} by approximately 4-fold, but had little effect on other properties of the enzyme.

Selecting bacteria transformed with recombinant plasmids is a laborious step in gene cloning experiments. This selection process is even more tedious when large numbers of clones need to be screened. In **appendix I**, I describe an ultra fast plasmid screening method. This new method was frequently used in the experiments performed in chapters 2-6.

ACKNOWLEDGMENTS

I am deeply grateful to Dr. Daniel L. Simmons for his mentorship during my graduate studies. This work wouldn't have been possible without his help.

I appreciate the valuable assistance from my graduate committee. Their insightful questions and suggestions have assisted my approach and analysis of my research.

It would have been inconceivable without the love, comfort and support of my wife, Kairong and our family members.

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ABBREVIATIONS

AA	arachidonic acid
COX	cyclooxygenase
HPLC	high performance liquid chromatography
iNOS	inducible nitric oxide synthase
IP	immunoprecipitation
K_M	Michaelis-Menton constant
NSAIDs	non-steroidal anti-inflammatory drugs
PBL	peripheral blood leukocytes
PG	prostaglandin
RP	reversed phase
RT	reverse transcriptase
TLC	thin layer chromatography
V_{max}	maximum velocity

General Introduction

Cyclooxygenases

Cyclooxygenases are encoded by two genes: COX-1 and COX-2. COX-1 is a largely constitutive expressed gene, whereas COX-2 is highly inducible (1, 2).

Prostaglandins are a group of biologically active compounds that are oxidatively metabolized from arachidonic acid (AA). They play important roles in human physiology in both health and disease (1). COX isozymes catalyze the first step in producing prostaglandins by synthesizing endoperoxide containing prostaglandin G₂ (PGG₂). PGG₂ is then converted to PGH₂ by the peroxidase site of cyclooxygenases.

PGH₂ is not stable and is further metabolized to different bioactive prostaglandins (Fig G-1). NSAIDs are inhibitors of cyclooxygenases. Except for aspirin, which acetylates serine 530 of COX-1 and an analogous serine in COX-2, NSAIDs compete with AA for binding in the COX active site to inhibit COX-1 and COX-2 (1, 3).

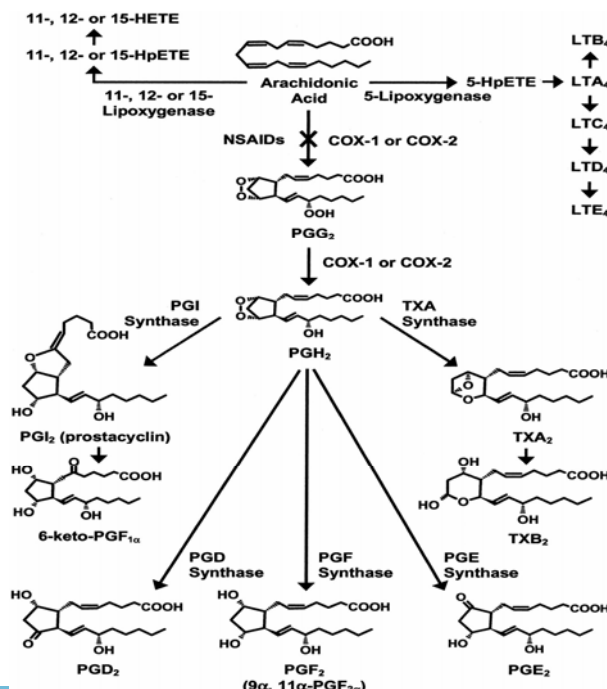


Fig. G-1. The arachidonic acid cascade. The fate of arachidonic acid in cells is taken from Simmons *et al.* (1). Arachidonic acid is metabolized by lipoxygenases to HETEs and HPETEs or by cyclooxygenases to PGG₂. NSAIDs block the synthesis of prostaglandin G₂. Prostaglandin H₂ spontaneously rearranges or is enzymatically isomerized, oxidized, or reduced to yield bioactive prostaglandin isomers.

COX-1 and COX-2 in all species are each about 600 amino acids in size (1). COX-1 and COX-2 share about 60% amino acid identity in all species and have very similar crystallographic structures. A gene duplication event gave rise to these paralogous COX isoforms early in or prior to vertebrate speciation (4). In humans, the COX-1 gene is located on chromosome 9q32–q33.3 and COX-2 is on chromosome 1q25.2–q25.3 (1). There are 11 exons and 10 introns in the human COX-1 gene, while COX-2 has 10 exons and 9 introns. Both genes share conserved intron/exon boundaries except for the presence of an extra intron inserted at the 5' end of the COX-1 gene (intron 1). Intron 1 is located in the N-terminal signal peptide of the protein. COX-1 and COX-2 also contain the same four functional regions: signal peptide, dimerization domain, membrane binding domain and catalytic domain (Fig. G-2). The catalytic domain contains both a cyclooxygenase active site and a peroxidase active site. In COX-1, the signal peptide is encoded by exon 1 and 2, while the dimerization domain is encoded by exon 3. Exon 4 encodes the membrane binding domain and exons 6-11 encode the catalytic domain. The effect of alternative splicing on COX structure and activity is predictable based on the relationship between exons and protein domains (1, 3, 4).

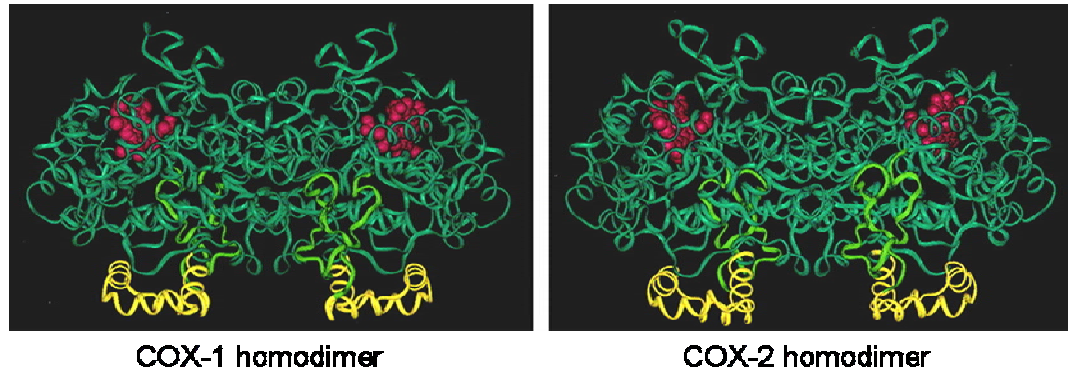


Fig. G-2. Crystallographic structures of ovine COX-1 (left) and murine COX-2 (right) homodimers. The crystallographic structure of ovine COX-1 and the murine COX-2 structure were from Protein Data Bank file 1PRH and file COX5 (5, 6). Functional domains are shown in different colors: 1) membrane binding domain (yellow); 2) dimerization domain (green); catalytic domain (blue) heme (red). The open cleft of the peroxidase active site is observable at the top of each monomer.

COX-2 and iNOS

In addition to the potential of affecting COX activity by altering domains of COX by splicing, COX activity can also be altered by protein-protein interactions. Inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) are co-inducible in many tissues following mitogenic and proinflammatory stimulation (7). Although nitric oxide (NO) does not bind COX heme (7), it can potentially modulate COX activity through nitrosylation of cysteines (8, 9). The question of whether or not cross-talk occurs between iNOS and COX-2 has been the subject of intense debate (10). COX-2 activity and expression is regulated by NO, and iNOS protein expression is also influenced by prostanoids, the products of COX-1 and COX-2. NO and its effector cyclic GMP regulate the function of several down stream target proteins, including transcription factors such as nuclear factor NF- κ B and activator protein-1. This is the key pathway by which NO may influence COX-2 expression (11).

Alternative splicing

Approximately 1×10^5 different proteins are expressed in human cells, yet our genome contains approximately 1/3 that number of genes. Proteome diversity is achieved in part by alternative splicing of over sixty percent of transcribed genes in humans (12). Alternative splicing not only leads to the expression of protein isoforms but can also regulate mRNA translation and stability. It can control localization as about twenty percent of alternative splicing happens in untranslated regions (13, 14).

Alternative splicing is required for apoptosis, sex determination, axon guidance, cell excitation and contraction, and many other cellular and developmental processes (15).

Many diseases, such as growth hormone deficiency, Frasier syndrome, Parkinson's disease, cystic fibrosis, retinitis pigmentosa, spinal muscular atrophy, and myotonic dystrophy are thought to involve alternative splicing in their pathogenesis (16). In recent years, some cancer specific alternative splicing events have been reported without genomic mutations, involving transcription factors, cell signal transducers, and components of the extracellular matrix (17).

There are seven types of alternative splicing in mRNA (4): alternative exons may be included with constitutive exons (Fig. G-3-1); exon switching may happen between two alternative exons in transcripts (Fig. G-3-2); part of an exon, either 5' end or 3' end, may be removed (Fig. G-3-3 and Fig. G-3-4); and alternative promoters or alternative polyadenylation may also occur (Fig. G-3-5 and Fig. G-3-6). Finally, intron sequences (Fig. G-3-7) may be retained in some transcripts. Intron retention is very important in the cyclooxygenases' gene regulation and expression.

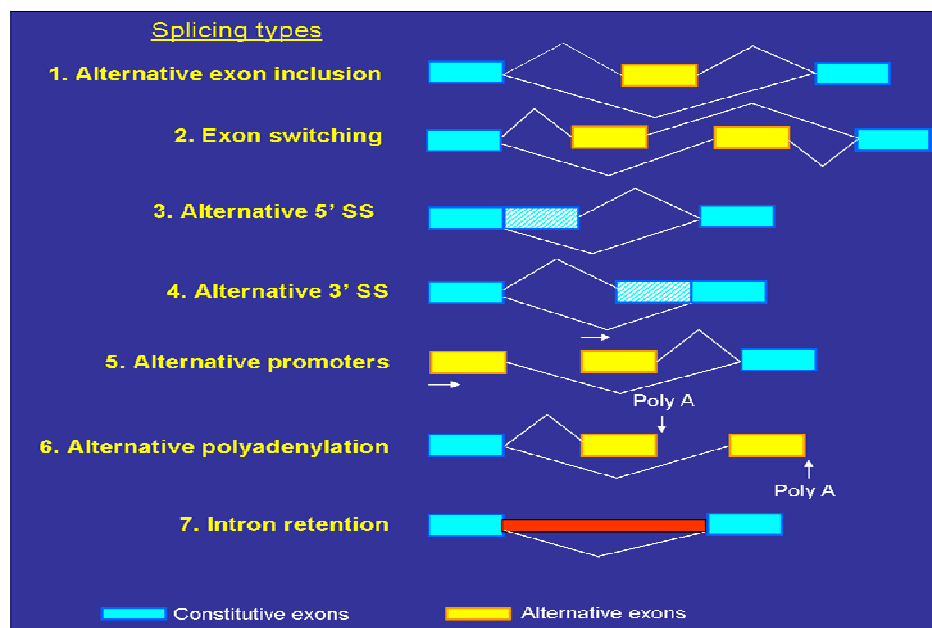


Fig. G-3. Alternative splicing in eukaryotes. The figure is taken from Roos *et al.* and revised (4). Constitutive exons are shown as blue boxes, alternative exon cassettes are shown as yellow boxes. A retained intron is indicated by a red box. Splicing is indicated by white lines, the upper set indicating default splicing and the lower set indicating alternative splicing.

Prior to this work, a limited number of COX splice variants were known. This dissertation addresses the biochemical and structural properties of many new COX-1 splice variants isolated from human tissue. The role of the amino terminal end of the COX-1 protein is specifically explored in studies of potential human COX-3 splice variants that retain an additional 31 amino acids on their N-termini, relative to COX-1. Another study addresses the role of 8 amino acids found at the N-termini of COX-1 that are not present in COX-2. In addition to these studies on COX-1, this dissertation evaluates COX-2's potential interaction with iNOS in human cells, and reaches a conclusion that challenges an existing paradigm that these proteins physically interact in cells. Finally, COX-2 selective inhibitions are important pharmacological treatments for inflammatory disease. Here, honeysuckle extracts, are reported to contain compounds

that, in addition to acting as COX-2 inhibitors, down-regulate COX-2 expression at the transcriptional and post-transcriptional levels.

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CHAPTER 1

The effects of exon skipping on heme-binding, glycosylation, and catalytic activities of human cyclooxygenase-1 splice variants

Abstract

Cyclooxygenases (COX) are encoded by two genes, COX-1 and COX-2. Four COX-1 splice variants and two COX-2 splice variants are known. Here nine new COX-1 splice variants are identified by screening human cDNA libraries, and by doing RT-PCR analysis of human cells and tissues. Each new variant involved some form of exon skipping or intron retention. A dichotomy exists between COX-1 and COX-2 splice variants in that the former, but not latter, produce potential heme-binding proteins that could be enzymatically active. These studies address this question. When expressed in insect and mammalian cell systems, glycosylation was observed with all new found COX-1 splice variants except one which is not expected to enter the endoplasmic reticulum, while no prostaglandins were formed by any new variants as determined by radioimmunoassay, HPLC and TLC assays. Heme-binding and peroxidase activity were easily detected in microsome preparations from insect cells infected with viruses encoding COX-1, COX-2 and selected COX-1 splice variants. Co-expression of one variant (Ex5) with COX-1 stimulated COX-1 cyclooxygenase activity. We conclude that some of the new reported human COX-1 alternative splice variants are transcribed at significant levels in specific cells or tissues, bind heme, contain residual peroxidase activity, and may activate or otherwise influence COX activity.

Introduction

Cyclooxygenases (COXs) are important therapeutic targets of aspirin-like drugs and are encoded by two genes: COX-1 and COX-2. COX-1 is a largely constitutive “housekeeping” gene, whereas COX-2 is highly inducible (1, 2).

Recent work lead to the identification of a canine COX-1 splice variant, termed COX-3 (also known as COX-1b) that retains intron 1. COX-3 produces a novel enzymatically active COX enzyme when ectopically expressed in insect cells (2). This enzyme is more potently inhibited by some NSAIDs and analgesics, such as acetaminophen and dipyron, than mouse COX-1 and COX-2.

Another alternatively spliced COX-1 mRNA was isolated from a human lung tumor and contains a deletion of part of exon 9 (3). This deletion eliminates an *N*-glycosylation site at residue 409, which is essential for proper folding of the enzyme and enzyme activity. Consistent with this fact, this variant possesses no prostaglandin synthesis activity. However, the sequence that provides the proximal histidine ligand to heme is not deleted in this variant, suggesting the possibility of heme binding and catalytic activity. A second COX-1 alternative splice variant was found in the EGV-6 rat tracheal cell line (4). This variant has been proposed to arise from a putative alternative promoter in intron 2 and encodes a predicted nonsense protein.

Two other COX-1 splice variants, Partial COX or PCOX proteins, exhibiting exon skipping were found in canine brain and lack exons 5 through 8. This deletion removes part (219 amino acids) of the globular catalytic region that contains both the cyclooxygenase and peroxidase active sites (2). However, like the partial exon 9 deletion

above, these proteins retain the proximal ligand to heme. The PCOX-1 forms are PCOX-1a and PCOX-1b, where PCOX-1a also retains intron-1 which is absent in PCOX-1b. Thus, five COX-1 splice variants have been reported to date from three different mammalian species and four of these have the potential to bind heme, but all, except for COX-3, lack cyclooxygenase activity.

Alternative splicing is also observed for COX-2 and the first example of COX alternative splicing was the report of retention of intron 1 in the COX-2 mRNA (5). More recently, a COX-2 variant, designated COX-2a, has been found that differs from the human COX-2 sequence by a deletion of part of exon 5 (from position +458 to +567) (6). This introduces a frame shift in the COX-2a sequence resulting in a TAA stop codon at position +490. COX-2a is elevated in platelets isolated from patients after coronary artery bypass grafting. A second splice variant, COX-2b, was identified in myometrium from women in labor and in rat macrophage mRNA (7). This splice variant retains intron 7 sequence, which also introduces a frame-shift that deletes most of the COX-2 catalytic domain. COX-2a and COX-2b lack catalytic domains and cannot possess activity as cyclooxygenases or peroxidases, but may have regulatory roles in the cell through heterodimerization with COX-2 via their dimerization domains.

A dichotomy therefore exists between COX-1 and COX-2 in that most splice variants of the former have the potential to produce heme-binding proteins that could be enzymatically active, whereas variants of the latter lack cyclooxygenase catalytic domains. By analyzing RNA from a variety of human tissues and cells, we have investigated exon-skipping in human COX-1 transcripts. Here we report new human COX-1 splice variants, many of which fit stringent criteria for biological and

pharmacological relevance: 1) they are expressed at significant levels in specific human cells or tissues and, in some cases, are inducible; 2) they encode COX-like proteins that are readily expressed in ectopic systems; 3) ectopically expressed proteins bind membrane, target either to the lumen of endoplasmic reticulum where they are N-glycosylated or to the cytosolic surface of that organelle; and 4) these proteins bind heme and have residual peroxidase activity.

Materials and Methods

Unless otherwise stated all basic protocols used were from the Manual on Molecular Cloning by Sambrook and Russell (8).

cDNA library construction and screening, RT-PCR amplification, and DNA sequencing. Isolation of RNA, library construction, and screening methods have been described elsewhere (9). Briefly, human cDNA was synthesized from mRNA isolated from ovarian tumor samples and freshly drawn peripheral blood mononuclear cell (PBMC). For reverse transcription-coupled polymerase chain reaction (RT-PCR) analysis, primers were designed for amplification of the COX-1 coding region. The sense primer (5'-CTGCGTCCCGCACCCCAGCA-3') corresponded to nucleotides -20 to -8 of the human COX-1 sequence. The antisense primer (5'-TCAGAGCTCTGTGGATGGTCGCTCCACAGC -3') corresponded to nucleotides 1768-1799, ending at the stop codon. Amplicons were cloned into pCR2.1-TOPO and transformed into competent *E. coli* (Invitrogen), and plasmids were isolated and amplicons were verified by DNA sequencing.

For analysis of variants from human brain, poly (A) RNA (2.5ug) (Ambion) was reversed transcribed with oligo dT and the cDNA was PCR amplified using the same

primers described above. A library of these amplicons was then constructed in the vector Lambda Zap II (Invitrogen). To isolate variants with skipped exons, duplicate filters of the amplified library were differentially screened with ^{32}P labeled human COX-1 cDNA or with ^{32}P labeled oligonucleotides corresponding to specific COX-1 exons. Plaques that hybridized to COX-1 but not to the exon specific oligonucleotides were isolated, plaque purified, in vivo excised and sequenced. Multiple screenings of the library were carried out with oligonucleotides specific for exons five, six and eight.

Expression of COX-1 variants in Sf9 cells and COS-7 cells. For ectopic expression and assessment of enzyme activity of COX-1 variants in Sf9 cells, complete coding regions, verified by DNA sequencing, were cloned into the baculovirus expression vector pBlueBac 4.5/V5-His according to the manufacturer's instructions (Invitrogen). For analysis of protein expression, Sf9 cells ($\sim 1 \times 10^6$) were infected with viral stocks at a multiplicity of infection (moi) of 3 (2).

In some cases, to assess N-glycosylation of individual splice variants, tunicamycin was added to a final concentration of 10 $\mu\text{g}/\text{ml}$ to insect cells 1 h after infection. Cells were then cultured and harvested after 48 h. Levels of recombinant protein expression were determined by immunoblot analysis, and COX activity in intact cells was determined by radioimmunoassay (RIA) (2), reverse phase high performance liquid chromatography (HPLC) and thin layer chromatography (TLC).

For immunoblot analysis of protein expression, total protein (20 μg) from cells was resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with ovine COX-1 mAb (Cayman Chemical). Primary antibodies (1:2,000) were incubated with membranes overnight. Blots were processed with

appropriate secondary antibody (1:2,000) from Sigma. Densitometry of the blot images was performed using the AlphaImage 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA).

COX-1 and its splice variants cloned into the pcDNA3.1 vector were transiently transfected into COS-7 cells using lipofectamine with Plus Reagent (Invitrogen). Cells were harvested 48 h post-transfection by scraping, and were washed once with PBS. To measure N-glycosylation of COX variants in mammalian cells, tunicamycin was added to COS-7 cells to a final concentration of 10 µg/ml 4h after transfection. Cells were then cultured for an additional 48 h. Assessments of COX-1 and splice variant expression and activities were done by TLC and RIA using intact cells as described for insect cells (above).

Cyclooxygenase activity assays.

a. Radioimmunoassay (RIA). Infected Sf9 cells (0.2×10^6 cells; 100µl) were incubated with arachidonic acid (100 µl, 60 µM) for 15 minutes at 37°C. Supernatants were assayed for COX activity by RIA for PGE₂ as previously described (2). At least three separate assays were performed for every protein analyzed and each was done in triplicate.

b. Reverse Phase-HPLC. As described previously (10), infected Sf9 cells were harvested and resuspended in buffer (100 mM Tris/HCl, 0.5 mM phenol, pH 8.0 and 1 µM hematin). Equal numbers of cells expressing approximately equal levels of protein, as determined by immunoblotting, were used in each assay. Cells expressing COX-1 or COX-1 variants were incubated with 0.25 µCi [uniformly labelled-¹⁴C] arachidonic acid

in a total volume of 1ml for 20 min at 37°C. The reaction was terminated by adding 10µl glacial acetic acid to a final pH of 4 and protein was precipitated by the addition of 2 ml of acidified methanol and 125 µl of methylene chloride. Arachidonic acid and its metabolites were extracted into ether (2x4 ml) and dried.

Products were analysed by reverse phase-HPLC using a Waters Symmetry C18 5 µm column (0.46 cm×25 cm) eluted at a flow rate of 1 ml/minute with the following solvents: methanol/water/acetic acid (60:40:0.01, v/v) for 10 minutes. This was followed by increasing methanol up to 100% (100:0:0.01) for 20 minutes followed by acidified methanol for 10 min. Fractions were collected every 12 sec and quantified using a liquid scintillation counter (Tri-CARB 2100 TR).

c. Thin-layer chromatography (TLC). As described before (11, 12), infected Sf9 cells or transfected COS-7 cells were incubated with 0.75 µCi [uniformly labeled-¹⁴C] arachidonic acid for 30 min at 37 °C and reactions were terminated by addition of acetic acid to pH 4. Arachidonic acid products released into the incubation medium were isolated through chromatography on reverse phase Sep-Pak columns (Waters) and the eluted products were dried and resuspended in methanol. For each assay, equal amounts of radioactive products were applied on loading strips of Whatman linear-K preadsorbent TLC plates. Chromatograms were developed using the upper organic phase of an ethyl acetate/ 2, 2, 4-trimethylpentane/ acetic acid/ water mixture (165:75:30:150, v/v/v/v) which was shaken and then resolved in a separation flask. Plates were air dried (5 min) under vacuum (75°C, 10 min) to remove residual solvent and were exposed overnight to X-ray film (XB-1, Kodak).

Heme binding assays. Sf9 cells were infected with high titer viral stocks (moi = 3) and cultured for 48-72 hrs. Cells were scraped, sedimented to remove tissue culture media, and rinsed in phosphate buffered saline (PBS) with protease inhibitors (PI) (Roche, Germany). Equal aliquots (2×10^7 cells) of COX-1, COX-2 or of each variant were resuspended in 5 ml of PBS. Hematin (50 μ l, 2 mM) was added and cells were incubated at 37°C for 1 hr. Unbound hematin was removed by washing the cells twice in 5 ml of PBS. Washed cells (5 ml) were then suspended in glass test tubes and photographed. Photographs were analyzed for color density using AphaDigiDoc software (Alpha Innotech). Intracellular hematin was then extracted and measured (13, 14). Washed cell pellets were suspended in acidified acetone (1 ml) by vigorous vortexing and allowed to incubate on ice for two hours. Water and ether (1 ml of each) were then added and the heme extracted into the ether layer. The ether fraction was then washed with 200 μ l of 5% NaCl in water. Phosphate buffer, 1 ml at pH 8.0, was then added, and the organic fraction removed and evaporated to dryness at 56°C. Extracted heme was resuspended in 100 μ l of pyridine to which was added 1 μ l of 0.1 M $K_2Fe(CN)_6$, 100 μ l of 0.2 M NaOH, 100 μ l of 40% pyridine and 6mg of solid sodium thionite. The concentration of heme was measured spectrophotometrically at an absorbance of 584 nm. The same experiment was performed on subcellular fractions of cells derived through sonication to evoke lysis followed by differential centrifugation to isolate nuclei (1000 x g for 10 minutes), mitochondria (10,000 x g for 20 minutes), and cytosol containing microsome (the supernatant of the mitochondria preparation).

Heme bound to protein was also measured by low temperature electron paramagnetic resonance spectrometry (EPR). Cells were first treated with hematin and

washed as described above. The cells were then gently sedimented in EPR tubes, media was removed, and the cells flash frozen. EPR spectra were obtained with a Bruker E500 spectrometer at 4° K.

Peroxidase assays. For peroxidase assays, Sf9 cells were infected with viral stocks (moi = 3) and cultured for 48 to 72 hrs. Five different assays, employing different co-reductants, were used to test for the peroxidase activity of COX-1 variants. Microsomal protein (200 µg) from infected Sf9 cells was added in each of the 5 assays to a microcuvette (1 cm path length) containing 500 µl of 100 mM Tris/HCl, 0.5 mM phenol (pH 8.0), hematin (1 µM final) and 200 µM ABTS. After 1 min of equilibration, the reaction was initiated by the addition of 1 µl of 20 mM H₂O₂. The reaction was recorded spectrophotometrically (417 nm) at room temperature for 10 min.

The tetramethylphenyldiamine (TMPD) peroxidase assay was performed as described by Kulmacz et al., 1987 (15). Briefly, microsomes were prepared, resuspended in 150 µl of PBS containing 1 µM hematin followed by incubation at 37°C for 30 min. The reaction cocktail contained 0.1 M Tris (pH 8.0), 0.2 mM TMPD, 9 µM H₂O₂ and 200 µg total microsomal protein. The reaction was initiated by the addition of H₂O₂. Oxidation of TMPD was then spectrophotometrically measured at an absorbance of 610 nm for 10 min.

The guaiacol peroxidase assay was performed on microsomes prepared as described above (16). The reaction cocktail contained 1 ml of 0.1 M sodium phosphate (pH 7.0), 5 mM guaiacol, 60 µM H₂O₂, and 200 µg total microsomal protein. Absorbance was read at 480 nm for 10 min at room temperature. In one experiment, stopped flow analysis was used to analyze peroxidase activity by this method.

The 10-acetyl-3, 7-dihydroxyphenoxazine assay (17) reaction was done in a final volume of 1ml containing 200 µg microsomal protein, 0.1 M Tris, pH 8.0, 9 µM H₂O₂, and 50 µg 10-acetyl-3, 7-dihydroxyphenoxazine. The reaction was incubated at room temperature for 30 minutes and fluorescence was measured at an excitation of 560 nm with emission detection at 590 nm.

The Amplex Red Hydrogen Peroxide Assay (Molecular Probes; Eugene, Oregon) was performed exactly as described by the manufacturer. This assay differs from those above in that, rather than directly quantifying the co-reduction of substrates by COX-1 variants as a measurement of peroxidase activity, it measures the amount of hydrogen peroxide that remains in a reaction cocktail following incubation with 200 µg of microsomal protein. The assay is also described in detail by Votyakova *et al.* (18).

Ex5 and COX-1 co-expression assays. Sf9 cells ($\sim 5 \times 10^6$) were simultaneously infected with Ex5 and COX-1 viral stocks of an MOI of 3 to co-express those two proteins. Cells were collected after 48 h infection for Western blot and RIA.

Results

Characterization of COX-1 alternative splicing variants. By constructing and screening human cDNA libraries, and by doing RT-PCR analysis of human cells and tissues, we identified nine COX-1 variants which are expressed at significant levels in a cell specific fashion (Fig.G-1). Each new variant involved some form of exon skipping including: skipping of exons 2-3 (Ex2-3); skipping of exon 3 (Ex3), skipping of exon 3 and part of exon 9 (Ex3+ExP9); skipping of exon 5 (Ex5); skipping of exons 3-5 and part of exon 9 (Ex3-5+ExP9); skipping of exons 3-8 (Ex3-8); skipping of exons 3-9 (Ex3-9);

skipping of exon 10 (Ex10); skipping of exon 3 and retention of intron 1 (Intron1+ Ex3). These new COX-1 splice variants are summarized in Figure 1-1.

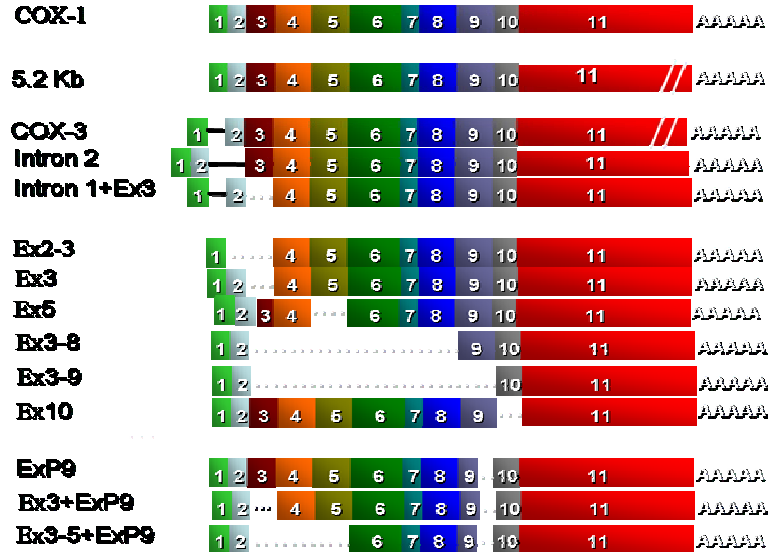


Fig. 1-1. Diagrammatic representation of human COX-1 and COX-1 splice variants. Exons are denoted in different shading, and skipped exon deletions are shown by dots. Retained introns are indicated by a solid black line. The 5.2kb variant is a fully processed COX-1 mRNA resulting from alternative polyadenylation. COX-3, Intron 2 and Intron 1+Ex3 have retained introns. Exon skipping is observed in Ex2-3, Ex3, Ex5, Ex3-8, Ex3-9, and Ex10. ExP9, Ex3+ExP9 and Ex3-5+ExP9 have part of exon 9 being removed.

Some of the variants described here were transcribed at higher levels than others in human cells. As a representative example, COX-1 was amplified from human PBL cDNA. The resulting fragments were cloned in pcDNA3.1 (Invitrogen) and then screened for COX-1 as described (2). Thirty clones that hybridized to a COX-1 probe were isolated and subsequently characterized by automated DNA sequencing. Five of the thirty clones were found to be ExP9 which has been previously isolated by others (3). Five clones were found to be Ex3, and one clone was Ex3+ExP9. Thus, assuming equal amplification, the mRNA levels of individual COX-1 splice variants individually constitute 3-17% of COX-1 transcripts, with all the variants together constituting roughly

38% of total COX-1 transcripts (data not shown). In some cases, splice variants were inducible. In explanted PBL cells, Ex3+ExP9 was induced at levels that exceeded COX-1 by the treatment of interleukin-1 β (IL-1 β) or with a mixture of IL-1 β , TNF α and IFN γ (cytomix) other pro-inflammatory mediators for 36 hours (Fig. 1-2a).

Some of the variant mRNAs are expressed in a marked tissue specific fashion. Of the tissue and cells being examined, Ex5 was only present in human ovarian tissue and ovarian cancer specimens. Analysis by RT-PCR indicated that the mRNA level of the Ex5 splice variant in human ovarian tissue and ovarian cancer specimens is about 10-20% of the level of COX-1 mRNA as determined by densitometry using the AlphaImage 2000 Documentation and Analysis System, and no difference in expression level was observed between ovarian tumors, ovarian benign tumor and ovarian normal tissue (Fig. 1-2b). In contrast, Ex3-5+ExP9, Ex3-8, Ex3-9 and Ex10 have so far only been found in a human brain cDNA library. However, ExP9 was found in all human tissue we screened. Along with COX-3 mRNA and a splice variant that retains part of intron 2 (to be reported elsewhere), the extra splice variant is commonly co-expressed with COX-1 in human tissue and cell lines at about 10-20% of the level of COX-1 as determined by densitometry.

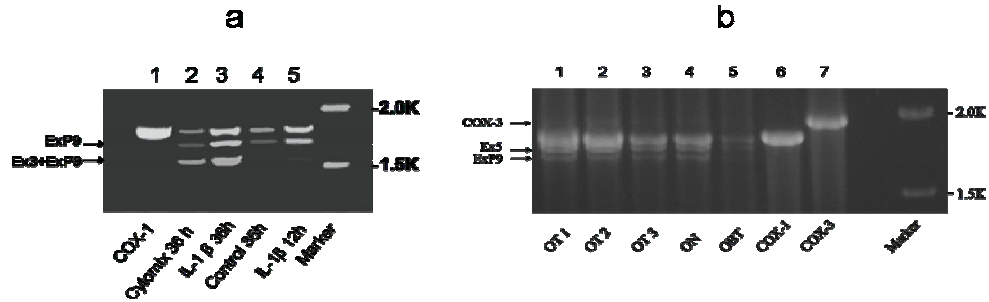


Fig. 1-2. PCR amplification of COX-1 and COX-1 variants. **2a:** Variants in human PBL. PBL cells were treated by cytomix (a mixture of IL-1 β , TNF α and IFN γ) and IL-1 β for 12 h or 36 h. Arrows indicate COX-1 variants: ExP9 and Ex3 (upper); ExP9+Ex3 (lower). **2b:** COX-1 splice variants in human ovarian tissues. Lane 1-3, three ovarian tumors; lane 4, ovarian normal tissue; lane 5, ovarian benign tumor; lane 6, COX-1 control; lane 7, COX-3 control. Arrows indicate COX-1 variants: COX-3 (upper); Ex5 (middle); ExP9 (lower).

Ectopic expression of COX-1 splice variants in insect and mammalian cells.

COX enzymes are intraluminal residents of the endoplasmic reticulum and depend on N-linked glycosylation for proper folding and activity. COX-1 and COX-2 have both been successfully expressed in insect and mammalian cells systems. However, the activity of COX-1 is frequently lower than that of COX-2 when expressed in insect cells (14). The cause of this is unknown but may be due to differences between the two enzymes in achieving proper folding in this system. Glycosylation and activity was, therefore, measured for human COX-1 splice variants in both insect and mammalian cell systems.

Insect cells (Sf9) infected with recombinant baculovirus expressing COX-1, ExP9, PCOX-1a and PCOX-1b and COS-7 cells transfected with COX-1, Ex3+ExP9, ExP9 and Ex3 recombinant plasmids in pcDNA3.1 were assayed for protein expression by Western blotting with a COX-1 monoclonal antibody and a COX-2 anti-peptide antibody. Based on their observed levels of expression, most splice variants were expressed as efficiently in these cell systems as COX-1 and COX-2 (Fig. 1-3). Posttranslational N-linked

glycosylation of COX-1 variants was compared with that of COX-1 by using tunicamycin to inhibit core glycosylation. All splice variants were predicted to be glycosylated except Ex2-3, which lacks a functional N-terminal signal peptide. Immunoblot analysis confirmed that COX-1 and its splice variants were all glycosylated, whether expressed in insect or mammalian cells (Fig. 1-3a and 1-3b). The Ex2-3 variant was an exception to this rule. Its expression was insensitive to tunicamycin. However, the Ex2-3 protein did appear to experience posttranslational modification as evidenced by the fact that it migrated as a doublet upon SDS-PAGE. In the course of our studies, we consistently observed that the majority of COX-1 protein, and frequently that of its splice variants, is expressed in a non-glycosylated form in insect cells (Fig. 1-3a). This is likely due to the massive amounts of COX-1 protein produced in this cell system resulting either in improper folding or a lack of translocation into the endoplasmic reticulum. We analyzed canine COX-3 protein by mass spectrometry for post-translational modifications and found that it was phosphorylated on Thr 165 (data not shown). Thus phosphorylation or other events may be responsible for post-translational modification of Ex2-3.

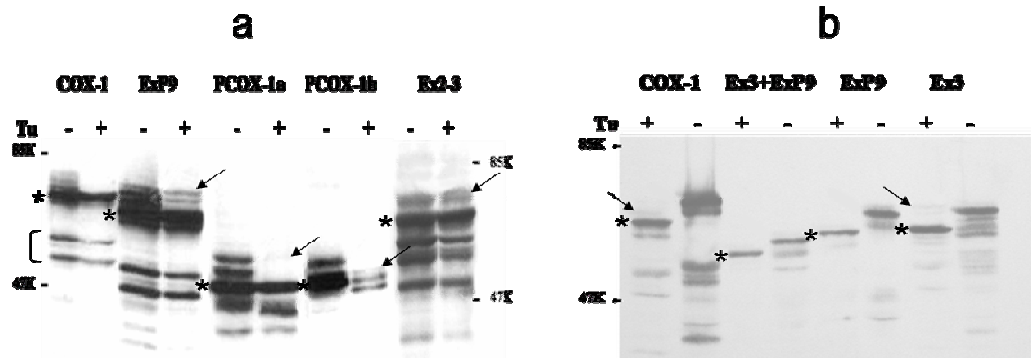


Fig. 1-3. Expression in insect and COS-7 cells. 3a: Immunoblots demonstrate the expression of COX-1, Ex 9P, PCOX-1a, and PCOX-1b in insect cells treated with (+) and without (-) tunicamycin. Asterisks indicate non-glycosylated COX-1 and other variants. The protein bands that migrate slower than the non-glycosylated proteins are modified forms of COX-1 and its splice variants. Arrows indicate modified COX-1 and other variants which are not affected by the treatment of tunicamycin. Overexpression of COX-1 and its variants consistently resulted in the detection of immunoreactive proteins that are smaller than non-glycosylated COX forms (example indicated by brackets). These likely represent proteolytic breakdown products or proteins resulting from incorrect initiation or termination of translation. **3b:** Immunoblots demonstrate the expression of COX-1, Ex3+ExP9, ExP9 and Ex3 in COS-7 cells treated with (+) and without (-) tunicamycin.

Cyclooxygenase activity. Sf9 cells infected by COX-1 and COX-1 variants were assayed for COX activity using RIA for PGE₂. No cyclooxygenase activity was detected for any tested COX-1 variant containing a skipped exon (Fig. 1-4a).

Sf9 insect cells infected with COX-1, COX-2, Ex2-3, Ex3, ExP9, Ex3+ExP9 Ex5, Ex10, PCOX-1a and PCOX-1b were also measured by RP-HPLC for cyclooxygenase activity by incubating whole Sf9 cells with [uniformly labeled -¹⁴C] arachidonic acid and analyzing released prostaglandins by HPLC coupled to scintillation counting. COX-1 and COX-2 completely oxidatively metabolized the radio labeled arachidonic acid while variants containing a skipped exon had no effect. Representative chromatograms comparing COX-1 with PCOX-1b are shown (Fig. 1-4b).

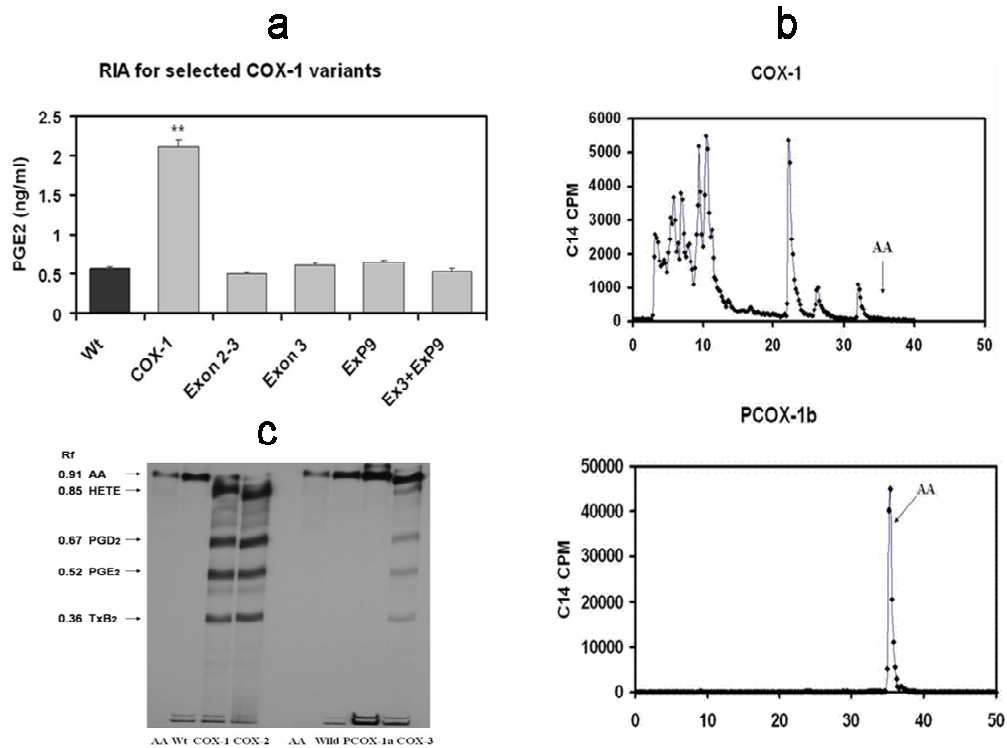
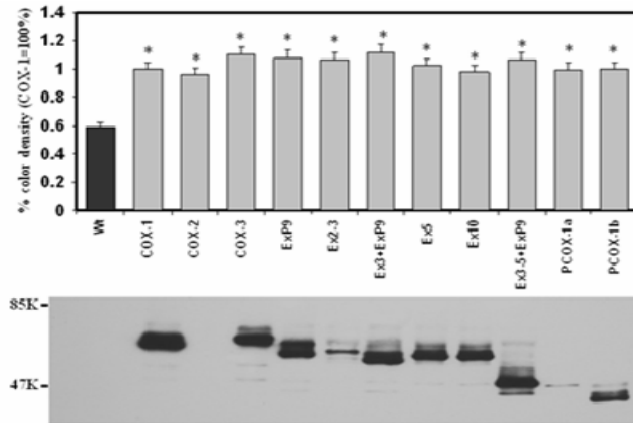
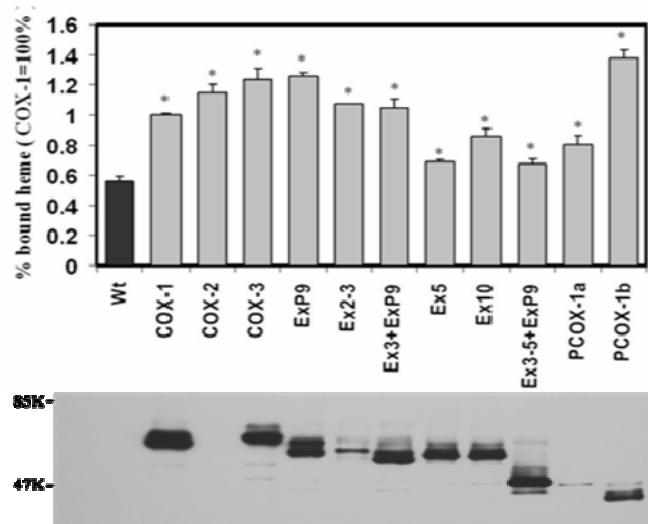
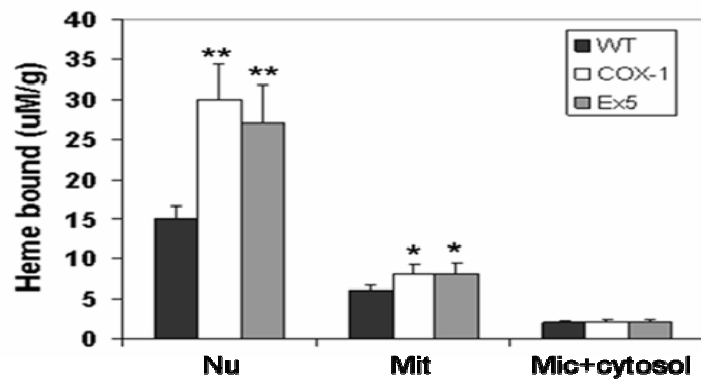


Fig. 1-4. RIA, Reverse phase-HPLC and TLC analysis of products formed by COX-1 and COX-1 variants. **4a:** RIA assay of cyclooxygenase activity of COX-1 (positive control), Wild type baculovirus infected cells (negative control), and selected COX-1 variants. No cyclooxygenase activity was shown on COX-1 variants. **4b:** Products from COX-1 and PCOX-1a were analyzed by reverse phase-HPLC. Fractions were measured with a scintillation counter. Data (counts per minute) (y axis) are shown as a function of elution time(x axis). **4c:** An autoradiogram of thin-layer chromatography separation of the products obtained after incubation of [uniformly- ^{14}C] arachidonic acid with Sf9 cells infected with COX-1, COX-2, COX-3 and PCOX-1a. COX-1 and COX-2 show very high activities. COX-3 cyclooxygenase activity is about 20% of COX-1. No cyclooxygenase activity was observed for PCOX-1a.

Thin layer chromatography (TLC) coupled with autoradiography was also done to further confirm these results (Fig. 1-4c). In this analysis, canine COX-3 activity was ~20% of that of COX-1, which is consistent with RIA data previously published for this COX-1 variant (8), whereas PCOX-1a lacked detectable COX activity. Thus, other than COX-3, no COX-1 splice variants showed PG production as measured by these different methods.

Heme binding assays. Because of a negative finding for COX activity, Sf9 cells expressing COX-1 splice variants were assessed for heme binding. When incubated in the presence of exogenous heme, Sf9 cells expressing COX-1, COX-2 or COX-1 splice variants became visibly brown compared to cells infected with wild-type virus or with a virus encoding a non-heme protein, nucleobindin (unpublished data). The color density of cell pellets was quantified by photography and densitometry (Fig. 1-5a). Intracellular heme was then measured by extraction with organic solvent and found to be significantly higher for COX-1, COX-2, and all tested COX-1 variants than for cells infected with wild type virus (Fig. 1-5b). To determine whether this represented an increase in heme bound to intracellular protein, rather than simply increased levels of free intracellular pools of heme, cells expressing COX-1, Ex5 or wild type virus were treated with 20 μ M hematin for 30 minutes, vigorously washed to remove extracellular hematin, lysed by sonication, and fractionated into nuclei, mitochondria, and cytosol containing microsomes. The majority of heme was associated with the nuclear and mitochondrial membranous fractions and was significantly elevated in cells expressing COX-1 and Ex5 versus wild type virus (Fig. 1-5c).

a**b****c**

d

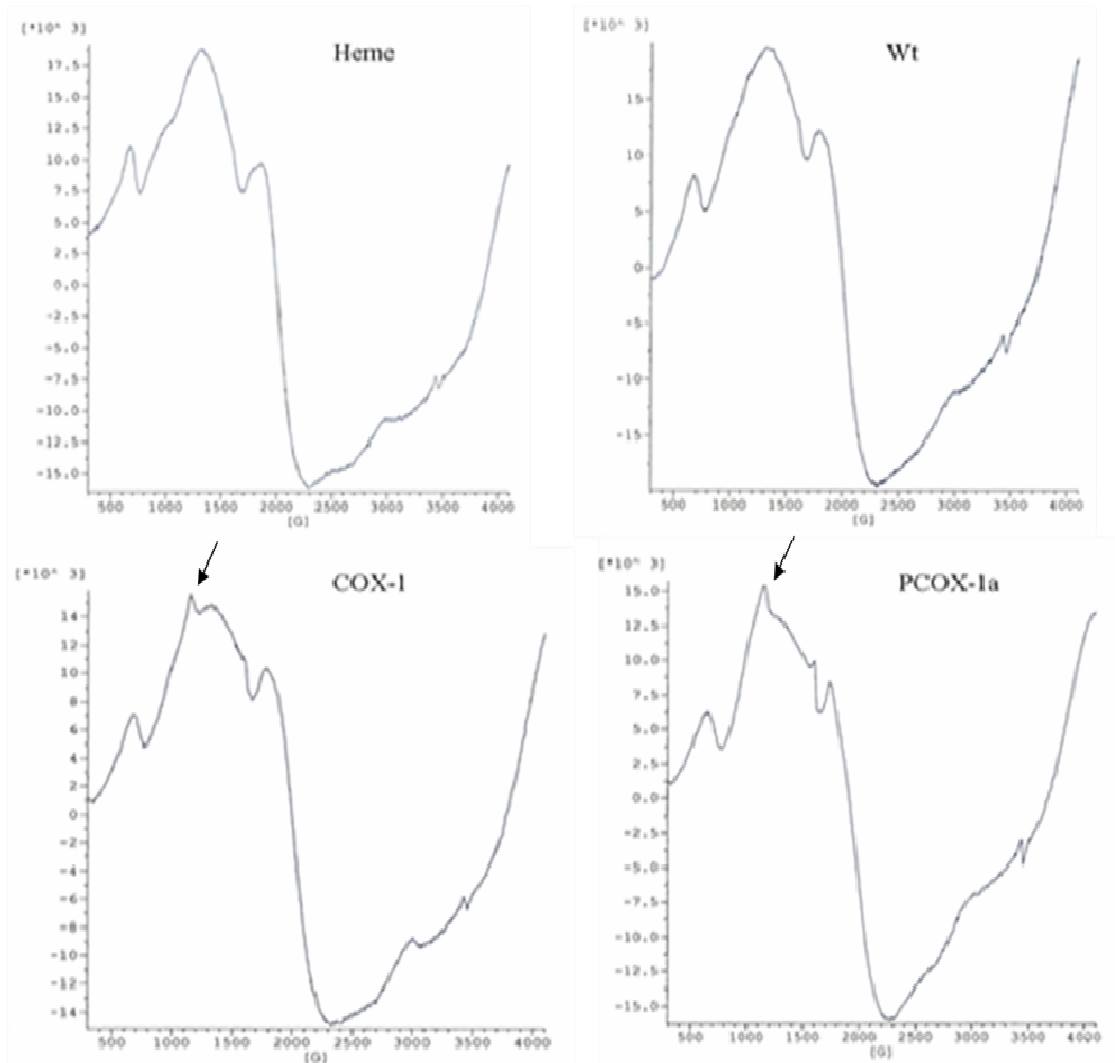


Fig. 1-5. COX-1 variants and heme binding assays. **5a:** The color density of exogenous heme bound to Sf9 cells infected by wild type, COX-1, COX-2 and COX-1 splice variants virus were measured. COX-1 variants cell color density was significantly higher (T-test, *, $p < 0.05$) than wild type cells. **5b:** Intracellular heme in wild-type-infected and recombinant expressed in Sf9 cells was extracted and measured. COX-1 variants contained significantly higher heme (T-test, *, $p < 0.05$) than wild type cells. **5c:** Heme bound to nuclei, mitochondria, and cytosol containing microsomes was measured. The majority of heme was associated with the nuclear and mitochondrial membranous fractions and was significantly elevated in cells expressing COX-1 and Ex5 versus wild type virus (T-test, **, $p < 0.001$). **5d:** Low-temperature EPR data on whole cells overexpressing COX-1 or PCOX-1. A specific resonance peak at roughly 1200 G as indicated by arrows was observed for both COX-1 and PCOX-1b that is diagnostic of protein bound high spin ferric heme. This peak was not observed in wild type cells or in a hematin solution.

To further confirm that heme was protein bound, low-temperature EPR was performed on whole cells overexpressing COX-1 or PCOX-1a. The latter possesses a deletion of 219 amino acids in its catalytic domain from skipping of exons 5-8. This is the largest deletion of any of the COX-1 variants evaluated and is predicted to have a profound impact on the structure of the COX catalytic domain that could potentially affect heme binding. A specific resonance peak at roughly 1200 G was observed for both COX-1 and PCOX-1b that is diagnostic of protein bound ferric heme. This peak was not observed in wild type cells or in a hematin solution (Fig. 1-5d).

Peroxidase assays. COX enzymes are evolutionarily derived from peroxidases and possess distinct cyclooxygenase and peroxidase active sites. The dual finding that COX-1 variants lack COX activity yet bind heme raised the question of whether they might still possess peroxidase activity. Five different methods that employed different co-reductants or strategies for measuring peroxidase activity were used to test the peroxidase activity of COX-1 variants. TMPD, guaiacol, 10-acetyl-3, 7-dihydroxyphenoxazine and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTs) were employed as co-reductants (Fig. 1-6). To reduce confounding levels of endogenous cellular peroxidase pools, assays were performed on isolated microsomes from infected sf9 cells. Prior to assay, the level of expression of each variant in the microsomal preparations were measured by immunoblotting in order to equalize the amount of immunoreactive protein used in each assay. With H₂O₂ used as substrate and TMPD, guaiacol, 10-acetyl-3, 7-dihydroxyphenoxazine, or ABTs used as co-reductant, we readily detected peroxidase activity of COX-1, COX-2, and COX-3, as would be expected from their ability to synthesize prostaglandin H₂ and its derivatives.

Importantly, however, COX-1 variants also exhibited peroxidase activity. In many cases these levels equaled or exceeded the activity of COX-1 and COX-2, but were co-reductant dependent. For example, Ex3+ExP9, demonstrated peroxidase levels that were comparable to COX controls when guaiacol, 10-acetyl-3, 7-dihydroxyphenoxazine, or ABTs were used as coreductants, but lower levels when TMPD was used. PCOX-1b, however, consistently exhibited peroxidase activity that was greater than that of COX-1 in all assays that were employed.

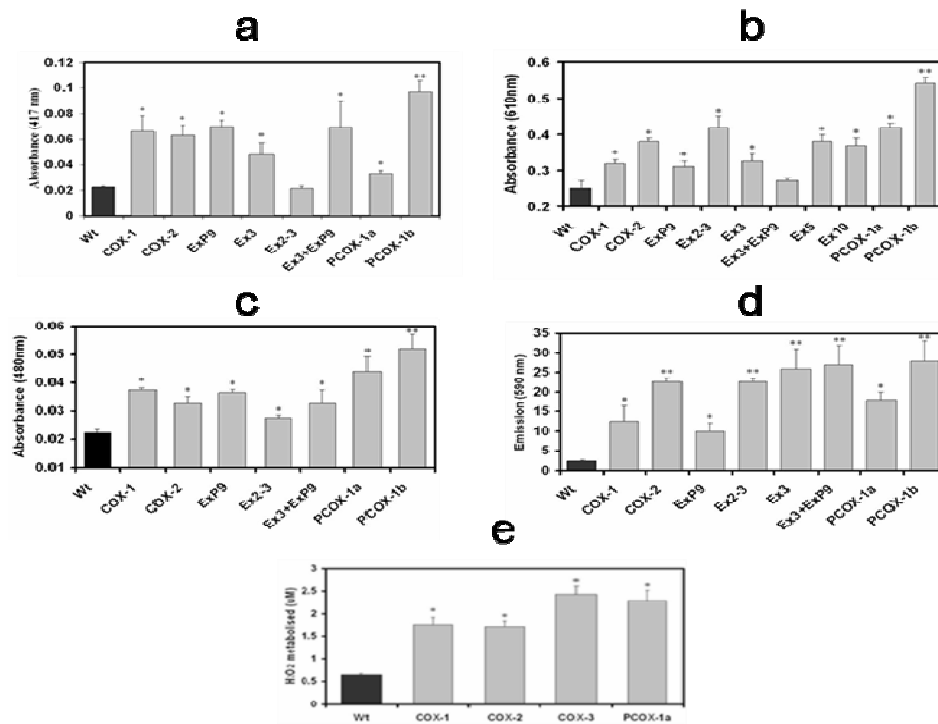


Fig. 1-6. Peroxidase assays. Peroxidase activity of COX-1 variants was tested by three assays based on absorbance (Fig. 5a, Fig. 5b and Fig. 5c), two assays based on fluorescence (Fig. 6d, Fig. 6e) and one on stopped flow coupling with absorbance (Fig. 6f). **6a:** ABTS peroxidase assay. **6b:** TMPD peroxidase assay. **6c:** Guaiacol peroxidase assay. **6d:** 10-acetyl-3, 7-dihydroxyphenoxazine peroxidase assay. **6e:** Amplex Red peroxidase assay. ABTS assay was measured at 470nm; TMPD peroxidase assay was measured at 610 nm; guaiacol peroxidase assay was measured at 480 nm; and the 10-acetyl-3, 7-dihydroxyphenoxazine assay and the Amplex Red hydrogen peroxide assay were measured by excitation at 550nm and detection at 590nm, respectively. All of the COX-1 variants measured contain statistically significant higher peroxidase activity than cells infected with wild type virus (T-test, *, p<0.05).

Ex5 and COX-1 co-expression assays. The finding that COX-1 splice variants lack COX activity but bind heme and possess a type of peroxidase activity suggested that they could modulate COX activity either through heterodimerization or modulation of redox tone. To test this, Ex5, which possesses the EGF module of the dimerization domain, was co-infected with COX-1 into Sf9 cells and cyclooxygenase activity was measured by RIA as a function of increasing Ex5 expression. Because COX-1 expression decreases with increasing Ex5 expression due to promoter competition, data were normalized for the level of COX-1 protein. Co-expression of increasing amounts of Ex5 with COX-1 produced a concomitant stimulation of COX-1 activity under saturating levels of arachidonate substrate that peaked at more than two-fold that of COX-1 control (Fig. 1-7).

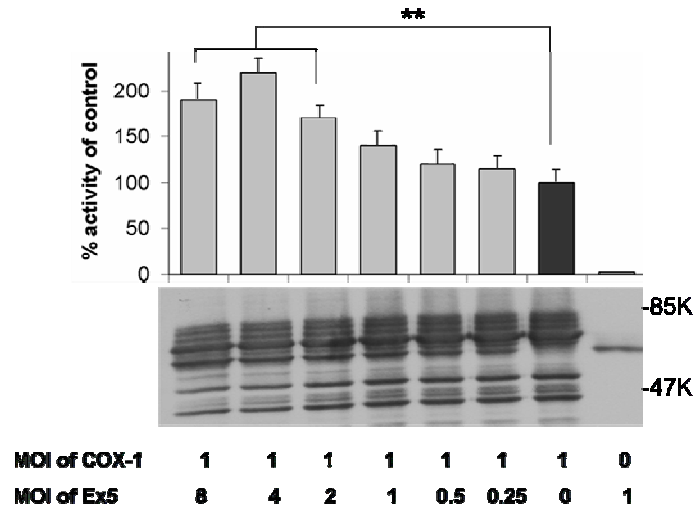


Fig. 1-7. Co-expression of Ex5 with COX-1 and increased COX-1 activity. Ex5 and COX-1 were co-expressed in Sf9 cells. Cyclooxygenase activity was measured and normalized for the level of COX-1 protein. Co-expression of increasing amounts of Ex5 with COX-1 produced a concomitant stimulation of COX-1 activity under saturating levels of arachidonic acid that peaked at more than two-fold that of COX-1 control (T-test, **, $p < 0.001$). The data shown here represent three experiments.

Discussion

In these studies we show that human tissues and cells produce many more COX-1 splice variants than were previously known. All the splice variants that resulted from exon skipping, except for Ex10 resulted in in-frame deletions producing proteins with possible enzymatic function. Indeed, studies in other systems show that splice variants can encode new proteins, new therapeutic targets, and new regulators of physiological and developmental processes. However, the biological role and importance of splice variants is unequivocally demonstrated by identifying, characterizing, or purifying the protein product of the splice variant from tissues and cells. Thus far this has been accomplished for relatively few splice variants of any gene. For shortlived, or non-abundant enzymes involved in signaling, such as COX enzymes, this is particularly

difficult and is complicated by the fact that COX-1 and its variants are difficult to dissociate and purify from membrane. Moreover, COX-1 splice variants are difficult to detect by immunoblotting because many of them migrate in SDS-PAGE with proteolytic breakdown products of COX. However, by immunoblotting techniques we have previously identified expression of one of the most abundant of mammalian COX-1 splice variants, PCOX-1a, in dog brain (2). Its function, though, is unknown.

Biochemical data reported here suggest that human COX-1 splice variants are biologically relevant. They are expressed at significant levels in cell- and tissue-specific fashions, are in some cases inducible, bind heme, and, in one instance, they may modulate COX-1 catalytic activity upon co-expression. As examples of biologically relevant levels of expression, the human COX-1 ExP9 variant is found at about 10% of the level of COX-1 in most of the human tissues and cell lines we have examined. Others have shown that treatment of cultured human lung fibroblasts with transforming growth factor β 1, interleukin 1β , and tumor necrosis factor α cause a two fold up-regulation of the ExP9 mRNA as compared to the intact COX-1 transcript (3). Additional human variants reported here show more striking patterns of cell-specific expression or inducibility (Fig. 1-2). For example, Ex5, was found only in normal ovarian tissue and human epithelial ovarian tumor specimens (Fig. 1-2b). For unknown reasons, vertebrate brain tissue is known to make many splice variants (2) and the same is true with regard to splice variants of COX-1. PCOX-1a and PCOX-1b mRNAs were previously found in high amounts in canine brain. In these studies we identified Ex3-5+ExP9, Ex3-8, Ex3-9 in human brain. Although expressed at lower levels than canine PCOX-1a and PCOX-1b, these human variants were cloned from whole brain and it is possible that they are

expressed at higher levels in specific regions of the brain, or in certain cells or developmental stages. An example of marked induction was observed for the human splice variant Ex3+ExP9, which was found to be highly elevated within 36 hours following treatment with cytokines (a mixture of IL-1 β , TNF α and IFN γ) in PBMC. In untreated cells, this splice variant was present at about 3% of the level of COX-1 mRNA but increased following stimulation to levels that exceeded that of COX-1 (Fig. 1-2a). Together these data suggest that splice variant expression is not haphazard or due to faulty RNA processing, but instead reflects a biological role for these transcripts and their translation products.

Our studies have previously noted that intron placement in COX genes demarks boundaries of the major domains of these proteins (19). In COX-1, exons 1 and 2 encode the signal peptide, exon 3 encodes the EGF module that constitutes the majority of the dimerization domain, exon 4 encodes the membrane binding domain, and exons 5-11 encode the catalytic domain with its two active sites (1). Skipping of exons 2-5 or exon 7 produces in-frame deletions of these structures as does the simultaneous skipping of exons 6 and 8 (19). Skipping of a specific exon can evoke removal or alteration of proper subcellular localization, membrane binding, dimerization etc. This is demonstrated by Ex2-3 which lacks an amino terminal signal peptide and dimerization domain and is predicted to be on the cytosolic surface of the endoplasmic reticulum or other membrane structures. This was confirmed by subcellular fractionation and the demonstration that Ex2-3 microsomal protein was insensitive to the action of tunicamycin (Fig. 1-3a). However, higher molecular weight electrophoretic moieties of this protein were observed

for this protein that likely reflect modifications other than N-linked glycosylation. We have identified one such candidate in the phosphorylation of Thr 165.

One unusual finding of our studies was that nine out of the thirteen COX-1 splice variants we have characterized (Fig. G-1) contain a deletion of either exon 3 or exon 5 which encode the dimerization domain. Exon 3 encodes the majority of this structure (domain 1), which contains three disulfide bonds reminiscent of epidermal growth factor. A fourth disulfide bond, in dimerization domain 2, encoded by exon 5, links the dimerization domain with the globular catalytic domain (1). Theoretically, skipping these exons, particularly exon 3, may produce monomers. This could prevent direct binding of these variants to COX-1 where they could potentially act as direct modulators of COX. However, our finding that the Ex-5 variant increases COX-1 catalytic activity (Fig. 1-7) suggests that this is either not the case for splice variants containing deletion of exon 5 or that these variants can alter COX activity in *trans*, potentially through altering redox tone.

The finding that COX-1 splice variants bind heme was surprising for some of the variants that contain deletions in their catalytic domains. Five separate assays detected varying levels of the ability of these proteins to catalyze peroxide reduction (Fig. 1-6, a-e). The peroxidase activities of splice variants were co-reductant dependent. Ironically, PCOX-1a and PCOX-1b exhibited the highest activities in some of these assays even though they contain the largest deletions in their active sites (Fig. 1-6, a-e). The degree of peroxidase activity for each of these variants may differ from COX-1 or from each other if lipid peroxides instead of hydrogen peroxide were used as substrate.

A previous study done by Schneider *et al.* (20) did not detect peroxidase activity of ExP9, which they attributed to the inability of the enzyme to bind heme cofactor. However, they did not test this possibility. Our experiments, in contrast, show that ExP9 protein binds heme and possesses peroxidase activity, which varies with the co-substrate used (Fig. 1-6, a-c). This discrepancy may be due to the fact that previous studies used 30µg of microsomal protein from COS-7 cells while we used 200µg of microsomal protein from insect cells. Moreover, insect cells produce much more recombinant protein than COS-7 cells and this property may further increase our ability to detect peroxidase activity.

At present, it is unclear whether the elevated peroxidase activity associated with expression of COX-1 splice variants represents the actual roles of these proteins in nature. Apart from having detectable peroxidase activity, COX-1 variants described here could possibly have other heme-mediated catalytic actions. Further studies, including purification of individual COX-1 splice variants, are needed in order to fully define their catalytic roles.

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CHAPTER 2

Inducible nitric oxide synthase-cyclooxygenase-2 interaction in human cells: An unlikely pairing

Abstract

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are co-induced in A549 human lung cells following treatment with endotoxin or cytokines. Similar co-induction of iNOS and COX-2 in murine RAW cells has been reported to result in physical interaction of the two proteins, causing activation of COX-2 via S-nitrosylation. However, co-immunoprecipitation and enzyme activity studies do not support this proposed interaction in human lung A549 cells as well as in human cell lines from other tissue sources. The physical and enzymatic properties of COX-2 and iNOS suggest that a lack of interaction is the rule in humans to which the murine findings may be an exception.

Introduction

Inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) are co-inducible in many tissues following mitogenic and proinflammatory stimulation. Although NO does not bind COX heme (1), it can potentially modulate COX activity through nitrosylation of cysteines (2, 3). The question of whether or not cross-talk occurs between iNOS and COX-2 has been the subject of intense debate (4). Recently, Kim *et al.* found that iNOS and COX-2 physically interact in LPS/ IFN γ - induced murine macrophage RAW264.7 cells as well as when mouse iNOS and human COX-2 were transfected into human embryonic kidney cells (HEK293T) (5). However, iNOS is a cytosolic protein whereas COX-2 is an intraluminal resident of the endoplasmic

reticulum (ER). They, therefore, are separated by an ER membrane making direct contact unlikely (6). As previous results were in a murine system or used mouse iNOS, we have analyzed human COX-2 and iNOS in HEK293T, human lung carcinoma cells (A549 cells), HeLa cells and Scept 1 cells where they are co-transfected or coinduced by IL-1 β /LPS/ IFN γ . When analyzed by a variety of methods, human iNOS and COX-2 do not interact, suggesting that any cross-talk between the two enzyme systems would need to be mediated by diffusible products (i.e. NO and prostanoids) between the two enzyme systems and not by enzyme contact.

Materials and methods

Cells. A549 and HEK293T cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in F-12 nutrient mixture with 10% fetal bovine serum (Hyclone, Logan, UT).

Reagents. IL-1 β and IFN- γ were from Sigma; 1400W was from Alexis Biochemicals (San Diego, CA); iNOS antibody was from BD Biosciences (Franklin Lakes, NJ); COX-2 antibodies were from Cayman Chemical (Ann Arbor, MI) and BD Biosciences.

Plasmids constructions. For assessment of human iNOS and human COX-2 binding, complete coding regions, verified by DNA sequencing, were cloned into the pcDNA3.1 vector according to manufacturer's instructions (Invitrogen).

Immunoprecipitation assays. Coimmunoprecipitation experiments were done according to Kim *et al*, (5). Briefly, 5 x 10⁶ A549 cells were plated in 75 cm² culture dishes. Cells were induced by IL-1 β and IFN- γ at 10 ng/ml of each for 18 h or co-transfected with 15 μ g pcDNA3.1-COX-2 and 15 μ g pcDNA3.1-iNOS according to the

manufacturer's protocol and cultured for 48h. Cells were collected and lysed in lysis buffer as described (5). Supernatants were precleared with sepharose A/G resin, then incubated with COX-2 or iNOS antibodies at 4°C overnight. Protein A-sepharose was then added and mixed by rotation for 1h at 4°C. Resin was washed three times with lysis buffer at 10-fold bed volume following which the resin was boiled in protein sample buffer and immunoprecipitates were analyzed by Western blot.

Western blot. For immunoblot analysis of COX-2 and iNOS protein, total protein (15 µg) from cells was resolved on 8% or 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane, and probed with COX-2 or iNOS primary antibodies (1:2,000) for a minimum of 1 h at 4°C. Blots were then processed with appropriate secondary antibodies (1:2,000) followed by development in chemiluminescent reagent (PerkinElmer Life Sciences, Boston, MA). Densitometry of images captured on X-Ray film (PerkinElmer Life Sciences) was performed using the AlphaImage 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA).

Radioimmunoassay (RIA) of COX-2 activity. A549 cells (1×10^6) seeded in 10 cm² culture dishes were induced or co-transfected as described above. In some cases, iNOS specific inhibitor, 1400w, was added to media concomitant with Il-1β and IFN-γ treatment or transfection of COX-2 and iNOS plasmid. For stimulation with NO, 100µM GSNO was added for 3h prior to cell harvesting. Cells were washed twice in serum free F-12 media and incubated with arachidonic acid (1 ml, 30 µM) for 15 minutes at 37°C. Supernatants were assayed for COX activity by RIA for PGE₂ as previously described (7). Four independent assays were done in quadruplicate.

Results

Analysis of ectopically expressed COX-2 and iNOS in HEK293T cells, HeLa and A549 cells. To measure whether human COX-2 and iNOS interact as detected by co-immunoprecipitation, each was expressed individually or together in HEK293T, HeLa, and A549 human cell lines. Ectopically expressed proteins were measured 48h after transfection by Western blot analysis (Fig. 2-1).

Immunoprecipitation of COX-2 in cells over-expressing both COX-2 and iNOS pulled down COX-2 but not iNOS regardless of the human cell system used to express these proteins (Fig. 2-1). However, the amount of COX-2 pulled down by the COX-2 antibody (from BD Biosciences) used by Kim *et al.* to demonstrate COX-2-iNOS interaction was only about 2% of the level of COX-2 pulled down by a COX-2 antibody from Cayman Chemical (Fig. 2-2, lane 2 and lane 3). Antibodies from both sources were used in subsequent immunoprecipitations of COX-2 to measure iNOS interaction. Immunoprecipitation of iNOS was attempted with the antibody used by Kim *et al.* to assess COX-2-iNOS interaction, and a protein of the appropriate molecular weight for iNOS was very faintly detected (Fig. 2-2, lane 3). However, this protein was also detected when probed with an anti-COX-2 antibody, suggesting that it is due to a non-specific antibody interaction.

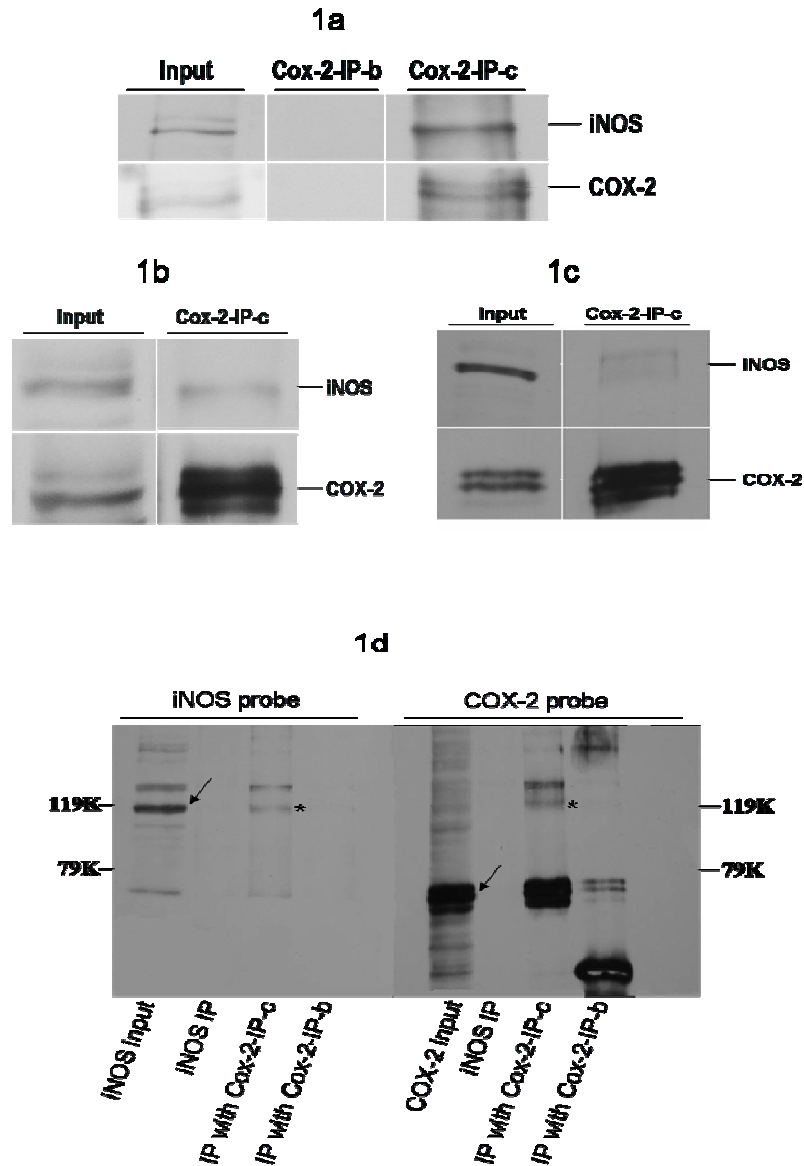


Fig. 2-1. Ectopic overexpression and coimmunoprecipitation assays of COX-2 and iNOS in HEK293T (1a), HeLa (1b) and A549 cells (1c, 1d). COX-2 was immunoprecipitated by COX-2 polyclonal antibody from Cayman Chemical (Cox-2-IP-c) and BD Biosciences (Cox-2-IP-b). Western blots were probed by iNOS antibody (upper panels of Figs. 1a, 1b, and 1c) and COX-2 monoclonal antibody (lower panels of Fig 1a, 1b, and 1c). The anti-COX-2 antibody reported by Kim failed to immunoprecipitate COX-2 (Cox-2-IP-b in Fig 1a). The Cayman Chemical antibody, in contrast, immunoprecipitated COX-2 and an immunoreactive protein was observed in the region of ~118 KDa when the blot was probed with anti-iNOS sera (Figs 1a, 1b, and 1c). This band, however, appears to be a non-specific protein that is detected by either the iNOS or COX-2 antibodies used to probe the immunoblot and which utilized a common secondary antibody (Lanes 3 and 7 from the right in Fig. 1d). Arrows indicate iNOS and COX-2 and an asterisk indicates the non-specific band.

Analysis of interaction between cytokine-induced COX-2 and iNOS in

ScupT1 and A549 cells. To control for artifacts that could occur in protein processing or subcellular localization due to ectopic overexpression, COX-2 and iNOS interaction was also evaluated in two human cell systems where these two proteins are coinducible. COX-2 and iNOS expression is strongly induced in A549 lung carcinoma cells after treatment with IL-1 β /LPS/ IFN γ at 10 ng/ml for 18 h (Fig. 2-2, lane 1) and occurs to a lesser extent in ScupT1 lymphoma cells following the same treatment. When immunoprecipitation was done on induced cells from these two systems, as described above for transfected cells, COX-2 but not iNOS was pulled down by COX-2 polyclonal antibody (Fig. 2-2), and no iNOS immunoprecipitation was observed using anti-iNOS antisera (Fig. 2-2a, lane 4).

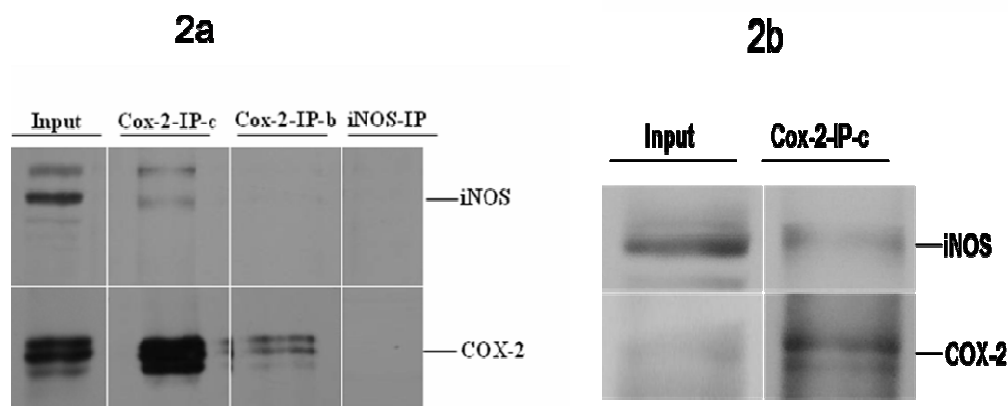


Fig. 2-2. Expression and coimmunoprecipitation assays of COX-2 and iNOS in cytokine-induced A549 and ScupT1 cells. COX-2 was immunoprecipitated by COX-2 polyclonal antibody from Cayman Chemical (Cox-2-IP-c) and COX-2 antibody from BD Biosciences (Cox-2-IP-b) in A549 cells (2a) and Scup T1 cells (2b). iNOS was immunoprecipitated by iNOS antibody from BD Biosciences. Western blots were probed by iNOS polyclonal antibody (upper panel in 2a and 2b) and COX-2 monoclonal antibody (lower panel in 2a and 2b).

The effects of GSNO and 1400w on cytokine-induced COX-2 activity in A549

cells. The NO donor, GSNO, indiscriminately stimulates COX-1 and COX-2 activity via nitrosylation (7) whereas the iNOS inhibitor, 1400w, was reported by Kim *et al.* to specifically attenuate COX-2 activity. The latter effect was postulated to be due to prevention of nitrosylation of cysteine 526 through direct interaction between iNOS and COX-2 (5). Cytokine-induced A549 cells contain high levels of induced COX-2 and iNOS (Fig. 2-3) but lack detectable COX-1 and, therefore, were used to detect the effects of these agents on COX-2 activity. Assays were done in the presence of exogenous arachidonic acid to saturate COX-2 enzyme in order to measure the total catalytic activity of the enzyme. Treatment with GSNO (100 μ M) for three hours had no effect on COX-2 expression and caused a modest increase in COX-2 activity in the presence of exogenous arachidonic acid (30 μ M) as expected (Fig. 2-3, lane 2, 3, 5). However, rather than diminishing COX-2 activity in the presence of exogenous arachidonic acid as anticipated, treatment with 1400w (100 μ M) also caused a slight increase in COX-2 activity similar to GSNO. Also, when 1400w was coadministered with GSNO, a slight increase in COX-2 activity was observed similar to that caused by treatment with GSNO alone. Thus, in the presence of exogenous arachidonic acid, 1400w had no inhibitory effect on cytokine-induced COX-2 activity in A549 cells (Fig. 2-3, lanes 4-5).

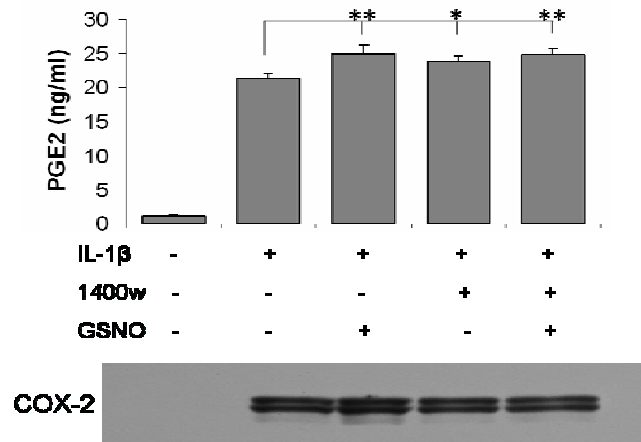


Fig. 2-3. The effect of exogenously generated NO and iNOS inhibition by 1400w on cytokine-induced COX-2 activity. A549 cells were activated by IL-1 β and IFN- γ at 10 ng/ml for 18 h with the presence of 1400w (100 μ M). GSNO, exogenous NO donor, was included in media for three hours before 30 μ M arachidonic acid in fresh media was added and PGE₂ was measured. Data were pooled from four independent determinations, each done five times (T-test, *P<0.05; **P<0.001).

The effects of GSNO and 1400w were also measured on PGE₂ synthesized from endogenous arachidonic acid in cytokine-induced A549 cells to determine whether the mode or level of arachidonic acid delivery to the enzyme affected the action of these agents. A modest non-statistical increase in activity was seen for GSNO-treatment; however, 1400w produced a significant decline in enzyme activity that was not prevented by co-treatment with GSNO (Fig. 2-4).

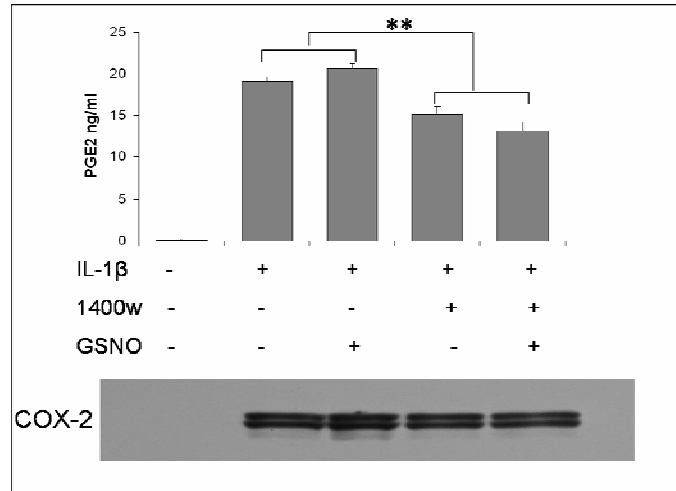


Fig. 2-4. Effect of GSNO and 1400w on COX-2 activity utilizing endogenous arachidonic acid in cytokine stimulated A549 cells. A549 cells were activated by IL-1 β and IFN- γ at 10 ng/ml for 18 h in the presence of 1400w (100 μ M). GSNO, exogenous NO donor, was added to media for three hours before the media was collected for PGE₂ RIA analysis. Data were pooled from four independent determinations, each done five times (T-test, *P<0.05; **P<0.001)

Inhibition of recombinant human COX-2 activity by 1400w in sf9 insect cells.

The fact that 1400w had no effect on COX-2 activity in the presence of high levels of exogenous arachidonic acid, but inhibited COX-2 activity utilizing endogenous arachidonic acid, suggested that 1400w may be an inhibitor of COX-2 at the high concentration (100 μ M) used in these experiments. COX-2 inhibitors may act either at the COX active site or by affecting the redox state of the enzyme and can be out-competed by high levels of substrate, which also alter the enzyme's redox state by the generation of prostaglandin peroxides. Tested against human COX-2 expressed recombinantly in insect cells, 1400w was found to be an inhibitor of COX-2 at concentration as low as 10 μ M if 1 μ M arachidonic acid is applied (Fig. 2-5). The fact that 1400w did not entirely inhibit COX-2 activity may represent different

subpopulations of glycosylated forms of COX-2 overexpressed in Sf9 cells, only one of which is inhibited by 1400w.

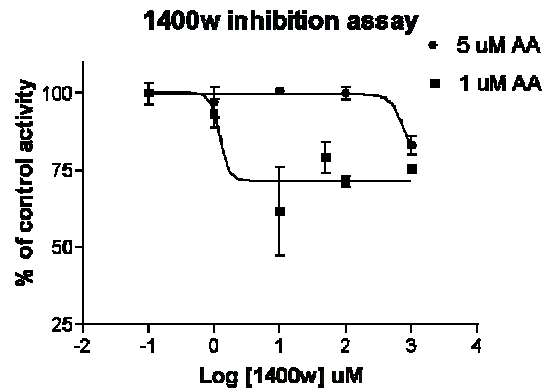


Fig. 2-5. Inhibition of COX-2 activity by 1400w. The effects of 1400w on COX-2 activity were tested in Sf9 cells overexpressing human COX-2. COX-2 activity was measured by the formation of PGE₂ after exposure to exogenous 5 μ M or 1 μ M arachidonic acid (AA) for 10 min. Data are expressed as mean \pm SEM ($n = 3$).

The effect of GSNO and 1400w on ectopically expressed COX-1 and iNOS activity. The studies of Kim *et al.* suggested that iNOS interaction was specific for the COX-2 isozyme and not for COX-1. We tested this by overexpression of human iNOS and human COX-1 in A549 cells in the presence of exogenous arachidonic acid. As with COX-2 measured in the same system (Fig. 2-2), COX-1 activity was stimulated by 1400w and was not inhibited (Fig. 2-6).

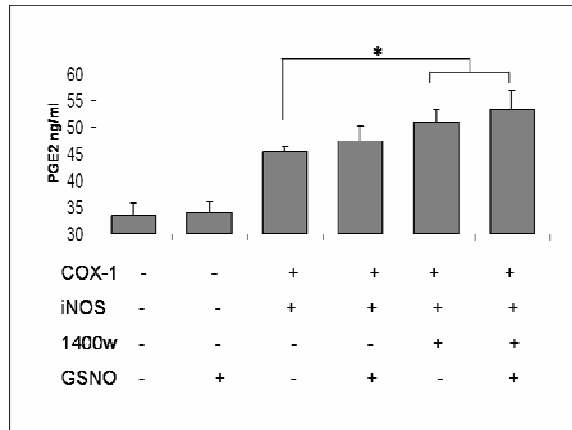


Fig.2-6. The effect of exogenously added NO and 1400w treatment on ectopically expressed COX-1 activity. A549 cells were co-transfected by COX-1 and iNOS and cultured for 48 h in the presence of 1400w (100 μ M). GSNO, exogenous NO donor, was added to media for three hours before 30 μ M arachidonic acid in fresh media was exposed to cells for ten minutes and PGE₂ measured. Data were pooled from four independent determinations, each done five times (T-test, *P<0.05; **P<0.001)

Discussion

Nitric oxide from NOS isozymes and prostaglandins from cyclooxygenases can function to regulate similar or opposing physiological processes. In the cardiovascular system, in particular, expression of prostaglandins and nitric oxide requires coordination to properly influence vascular tone. Moreover, both enzyme systems contain highly inducible members (COX-1 and iNOS) that are co-induced in some contexts. Together these facts raised the question of whether there is “cross-talk” between iNOS and COX-2 (9). Some investigators found that NO could down-regulate COX-1 and COX-2 catalytic ability (10), whereas others found that it stimulated it (8). In both cases, the effect of NO was not specific for either COX isozyme and was presumed to occur through the action of cell-diffusible NO and not through specific transfer of NO to COX through NOS-COX interaction.

Recently, Kim *et al.* reported that iNOS binds to COX-2 in induced mouse RAW264.7 cells and transfected human HEK293T cells. In their transfection studies they ectopically co-expressed human COX-2 and murine iNOS, or used recombinantly altered forms of these enzymes. In the present studies we tested ectopically expressed or cytokine induced human COX-1 and human iNOS in a variety of human cells lines, but found no evidence for direct interaction of human iNOS and human COX-2. The discrepancy between the results of the two studies may lie in species differences of the cell systems used (murine versus human), or the fact that we paired human COX-2 and iNOS as opposed to murine iNOS and human COX-2 in their cell system. It is possible that, for unknown reasons, murine iNOS somehow can translocate into the ER to bind to COX-2 in murine and human cells, while human iNOS cannot. Alternatively, non-specific interaction may occur between the two proteins following solubilization of the ER membrane. These possibilities, however, remain to be demonstrated.

In the course of these studies we identified a number of variables that could result in an artefactual interpretation of iNOS-COX-2 interaction. One regards antibodies used for co-immunoprecipitation experiments. Although we used the immunoprecipitation procedure of Kim *et al.* exactly as described, we found that both the COX-2 and iNOS antibodies employed only weakly precipitated their cognate proteins. Moreover, in our coimmunoprecipitation experiments where iNOS antibodies were used to probe immunoblots containing proteins pulled down by anti-COX-2 sera, we always observed a detected protein that migrated in SDS-PAGE in the region of iNOS (Fig. 2-1, lane 3 and Fig. 2-2 lane 3). The protein, however, does not exactly migrate with iNOS and also is present when the blot was probed with COX-2 antibody (Fig. 2-1d), which makes us

believe it is not iNOS but a nonspecific protein present in the COX-2 antibodies used for immunoprecipitation.

Another phenomenon we observed was that 1400w weakly stimulated COX-2 catalytic activity in over-expression assays, rather than inhibiting activity as found by Kim *et al.* These ectopic co-expression assays were done using exogenous arachidonic acid substrate and a relatively short exposure time to 1400w (3 hours). In contrast, PGE₂ produced in cell media using endogenous arachidonic acid as substrate was found to be detectably inhibited in the presence of 1400w (Fig. 2-5) as shown by Kim *et al.* One possibility for this result, as proposed by Kim *et al.*, is that 1400w inhibits COX-2 activity by preventing COX-2 nitrosylation, since 1400w inhibits NO synthesis dose dependently. However, the fact that the high levels of 1400w used in these studies (100 uM), weakly inhibit the activity of COX-2 expressed in Sf9 cells *in vitro* (Fig. 2-5) and that GSNO, a strong NO donor, failed to stimulate the production of PGE₂ in the presence of 1400w in induced A549 cells provides an alternative explanation that the inhibition of 1400w on the PGE₂ produced from endogenous substrates is due to weak direct weak inhibition of COX-2 by 1400w. This is further indicated by the fact that PGE₂ production in cytokine induced A549 cells was not lower than vehicle-treated cells when 1400w was removed before exogenous arachidonic acid incubations, even though the cells were treated with 1400w for 18 hours (Fig. 2-3).

As previously noted, iNOS is made as a cytosolic protein which, under some conditions, can translocate to a subdomain in perinuclear membrane containing misfolded or aggregated proteins called the aggresome, where it is likely faces the cytosol (11). The COX-2 isozyme, on the other hand, is in the lumen of the endoplasmic reticulum (ER).

At best, this places COX-2 and iNOS on opposite sides of membranes where they could not interact. COX-2 is directed into the lumen of the ER by its N-terminal signal peptide encoded by exon 1 of the gene. Exons 2 and 3 encode the EGF dimerization module and membrane binding domains respectively (12). Almost all of the recombinantly altered forms of COX-2 employed by Kim *et al.* in their pull-down assays had their exon-1 region deleted, thus producing COX-2 variants that will no longer translocate into ER and will be cytosolic. Interpretation of iNOS interaction based on those constructs should be interpreted with caution. Other constructs employed by Kim *et al.* involved removal of all or part of exons 1-3, which will not only make COX-2 cytosolic but also is predicted to produce proteins that are monomers and non-membrane binding. These forms are very different in structure than COX-2 *in vivo* and pull-down assays with anti-iNOS antibody may not reflect *in vivo* interactions.

Regardless of these findings that failed to find a direct interaction between human iNOS and COX-2, we did observe that NO weakly stimulated COX-2 activity when exogenous arachidonic acid was applied to cytokine-induced A549 cells. As shown by others previously, this implies that NO can modulate COX-2 catalytic activity at least to some extent. However, in human cells this appears to be through cell-diffusable NO, not through direct iNOS and COX-2 binding. This effect of NO is also not specific for COX-2 as shown by the fact that ectopically expressed COX-1 activity in A549 cells was equally stimulated (Fig. 2-6). Kim *et al.* noted that COX-1 and iNOS do not bind to each other, further underscoring the fact that this action of NO occurs through diffusion. The activation of both COX-1 and COX-2 by NO makes sense because

cysteine 526, shown by Kim *et al.* to be the important nitrosylation site in COX-2 necessary for activation, is also highly conserved in COX-1.

Our studies find NO to be a weak stimulator of COX activities, with COX-1 and COX-2 activity only increasing about 15% following treatment of 100 μ M GSNO when the enzyme is measured at near V_{max} . Thus drugs designed for the purpose of blocking nitrosylation of COX-1 or COX-2 activity may not be effective at reducing inflammation or other unwanted processes where these enzymes are involved.

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CHAPTER 3

Putative in-frame human COX-3 variants produce structurally and catalytically distinct COX isozymes

Abstract

Cyclooxygenases (COXs) are of important therapeutic value as they are the target site of aspirin-like drugs. A catalytically active COX-1 splice variant in dog, COX-3, has been reported that retains intron 1. The same transcript occurs in humans; however, intron 1 is out of frame producing a frameshift and nonsense protein. Two putative in-frame human COX-3 transcripts, COX-1b₂ and COX-1b₃, (herein designated as COX-3-72 and COX-3-50) have been reported in the literature, but the encoded protein of only one of them, COX-3-72, has been characterized. This study analyzes COX-3-50 protein and compares it with COX-3-72. When COX-3-72 and COX-3-50 were over expressed in insect and mammalian cell systems, these proteins differed in their pattern of N-glycosylation, protein stability and enzyme activity. COX-3-50 was unstable and was more poorly expressed, yet was more than ten times more active than COX-3-72. Moreover, COX-3-72, and to a greater extent, COX-3-50, were stimulated by treatment with rofecoxib at physiological concentrations. A similar rofecoxib-stimulated COX activity is observed in quiescent A549 cells. Immunoblot and immunoprecipitation analysis suggest that human platelets and potentially A549 cells contain a COX-3-50 like protein. Polymerase chain reaction analysis of A549 transcripts shows that this protein does not occur by RNA editing but by some other process, such as ribosomal frameshifting. Our data, together with previous published results, suggest the existence

of one or more COX-3 proteins in humans which could be activated by rofecoxib at physiological concentrations.

Introduction

Cyclooxygenases (COXs) are encoded by two genes: COX-1 and COX-2. COX-1 is constitutively expressed, whereas COX-2 is largely inducible (1,2). Recent work has led to the identification of a canine COX-1 splice variant, termed COX-3 that retains intron 1. COX-3 produced a novel active enzyme when ectopically expressed in insect cells. This enzyme was found to be inhibited by NSAIDs and analgesics such as acetaminophen and dipyron better than mouse COX-1 and COX-2 (2). Acetaminophen is often used for analgesic and anti-pyretic purposes because it causes fewer gastrointestinal problems than commonly used NSAIDs (1).

Human COX-3 mRNA is transcribed at readily detectable levels in heart, brain, kidney, liver, skeletal muscle, stomach, and small intestine as well as in various sub-regions of human brain (2, 3). However the existence of human COX-3 protein remains questionable since intron 1 of human COX-1 is 94 nt in length and its complete retention would shift the frame of the encoded protein (2, 3, 4, 5). Although human COX-3 is out of frame, some immunoprecipitation assessments suggest the existence of COX-3 protein in human tissues (2).

Two types of in-frame COX-3 mRNAs have been recently reported in the literature (3, 6). They retain the intron 1 but are missing either a guanine at position 72 (COX-3-72) or a cytosine at position 50 (COX-3-50). Thus, these two COX-3 transcripts contain a self-rectifying shift in the reading frame, which allows the translation of a full

length COX protein in Sf9 cells. Single nucleotide polymorphisms (SNP) and RNA editing have been proposed as mechanisms to explain the correction of frame in these COX-3 transcripts (2, 3). Only ectopically expressed human COX-3-72 has been characterized at the protein level, and was demonstrated to possess most of the structural and catalytic properties of canine COX-3 (3). Notable differences were that human COX-3-72 was more catalytically active than canine COX-3 and less sensitive to analgesic/antipyretics and some NSAIDs (3). Here we compare human COX-3-50, COX-3-72 and COX-1 proteins and demonstrate that the position of the self-rectifying frameshift significantly influences the structural and catalytic properties of the resulting COX-3 variant produced. Furthermore, our research, together with our previous results (2), indicates the existence of one or more endogenous COX-3 proteins in human platelets and possibly A549 cells. Additionally, over-expressed COX-3-50 and COX-3-72 are activated by the COX-2 selective drugs rofecoxib (Vioxx) and (to a lesser extent) celecoxib (Celebrex). Rofecoxib was withdrawn from the market because of an increased risk of cardiovascular problems, the reasons for which are still not well known. We postulate that rofecoxib's ability to stimulate a COX-3 activity in the vascular system, particularly in platelets, could be responsible for increased risk of cardiovascular adverse events in humans.

Materials and Methods

Ectopic expression of COX-1, COX-3-50 and COX-3-72 variants in Sf9 cells.

For ectopic expression and assessment of enzyme activity of COX-3 in Sf9 cells, complete coding regions, verified by DNA sequencing, were cloned into the baculovirus expression vector pBlueBac 4.5/V5-His according to manufacturer's instructions

(Invitrogen). For analysis of protein expression, Sf9 cells ($\sim 1 \times 10^6$) were infected with viral stocks at a multiplicity of infection (moi) of 3 (2).

In some cases, to assess N-glycosylation of individual splice variants, tunicamycin was added at a final concentration of 10 $\mu\text{g/ml}$ to insect cells 1 h after infection. Cells were then cultured and harvested after 48 h. The level of recombinant protein expression was determined by immunoblot analysis, and COX activity in intact cells was determined by radioimmunoassay (RIA) (2).

For immunoblot analysis of protein expression, total protein (20 μg) from cells was resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with COX-1 mAb as described above.

Drug inhibition assays and V_{max} and K_{m} determinations. Sf9 cells were infected with high titer viral stocks (moi of 3) and cultured for 48 h to express COX-1, COX-3-50, or COX-3-72. Cells were pelleted by centrifugation and washed in serum free-media, after which the level of ectopically expressed protein was evaluated by immunoblot. Cell numbers were then adjusted so that equal amounts of COX isozymes and enzyme activity were used in subsequent drug inhibition assays. For these assays, cells were preincubated with test compounds for 30 min at 25°C and arachidonic acid (final concentration, 30 μM) was then added for an additional 10-min incubation at 37°C. Cells were then pelleted and the supernatant was removed and assayed for COX activity by RIA of PGE₂. For each test compound, assays were performed multiple times in triplicate, inhibition curves were constructed, and IC₅₀ values were determined using PRISM 3.0 (GraphPad, San Diego).

For V_{\max} and K_M determinations, initial velocities were determined by stopping cyclooxygenase enzyme reactions by transferring reaction tubes into boiling water 15 seconds after the addition of arachidonic acid. This length of time for substrate incubation was determined by doing time-course assays for each COX isozyme preparation.

Production of polyclonal anti-COX-3 antibodies. Polyclonal anti-COX-3 antibody corresponding to the N-terminal 13 amino acids of COX-3-50 and COX-3-72 (MSRECDPGARWG) was previously made in our laboratory (2). Another antibody directed against the last 12 amino acids (LSPSSLSSAGSL) of the intron-1 encoded sequence contained in COX-3-50 and COX-3-72 was generated. The cognate peptide was coupled to keyhole limpet hemocyanin as carrier and injected into rabbits. The antibody against N-terminus COX-3 isozymes is designated H1253, and the antibody against C-terminal end of the intron-1 encoded sequence in these enzymes is designated K0040.

In some cases antibody was purified by coupling the cognate peptide to Sulfolink coupling gel (Pierce) according to the manufacturer's instructions. Serum (15 ml) was applied to the resin and incubated overnight after which the resin was washed and bound antibodies were eluted in glycine buffer (pH 2) and equilibrated to pH 7.4.

Detection of COX-3-related proteins cells by Western blotting. Total protein (20 μ g) from A549 cells or platelet preparations was analyzed by Western blotting using murine COX-1 mAb (Cayman Chemical, Ann Arbor, MI) and rabbit COX-3 antipeptide polyclonal antibodies (pAb). Blots were processed with appropriate anti-mouse secondary antibody (1:2,000) or anti-rabbit secondary antibody (1:10,000) from Sigma.

Densitometry of X-ray film-captured luminescent images was performed using the AlphaImage 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA).

Detection of 71- and 72-kDa COX-3-related proteins in human platelets by immunoprecipitation. For immunoprecipitation experiments of COX-3, human platelets were collected and homogenized. Microsome fractions were prepared and solubilized in ice-cold lysis buffer (100 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and protease inhibitor cocktail). The supernatants (800 μ l) were precleaned for nonspecific binding with 50 μ l protein A–Sepharose, then mixed overnight at 4°C with COX-3 antibody (H1253). After the addition of 80 μ l of protein A–Sepharose, the immunoprecipitates were mixed for another 1 hour at 4°C. The mixture was washed three times with the buffer described above. The pellet was then boiled in 10 μ l SDS-loading dye containing β -mercaptoethanol. The released protein was loaded on to a 10% SDS PAGE gel, electrophoresed, transferred to nitrocellulose membrane, and probed with murine COX-1 mAb (Cayman Chemical) at 1:2,000 dilution overnight at 4°C. Blots were then processed with rabbit-anti-mouse secondary antibody (1:2,000) from Sigma. Densitometry of the autoradiographic images were with AlphaImage 2000 Documentation and Analysis System.

RT-PCR amplification, and DNA sequencing. Isolation of RNA has been described (7). Human cDNA was synthesized from mRNA isolated from A549 cells by reverse transcription-coupled polymerase chain reaction (RT-PCR). To analyze for COX-3-50 and COX-3-72 expression, primers were specifically designed for amplification of the COX-1 intron 1 region. The sense primer (5'-

CTGCGTCCCGCACCCCAGCA-3') corresponded to nucleotides -20 to -8 of the human COX-1 sequence. The antisense primer (5'- CCGGAGCAAGAGACTCCCTGCAGA - 3') included five nucleotides of the C-terminal intron 1 and 19 nucleotides of the N-terminal exon 2. Amplicons were cloned into pCR3.1-TOPO and transformed in competent *E. coli* (Invitrogen) and verified by DNA sequencing.

Results

Over-expression of Human COX-3-50 and COX-3-72 in Sf9 cells and measurement of enzyme activity. COX-3 mRNA in mammalian species is defined as the COX-1 mRNA that additionally retains intron-1 and, in some species, also possesses a significantly longer 3' untranslated region (2, 3). Retention of intron-1, which is 94 nucleotides in length in human COX-3 mRNA, would introduce a frame-shift and produce a non-sense protein. However, human COX-3-50 and COX-3-72 forms of this sequence have been reported that exhibit postulated RNA editing events which remove single nucleotides in the intron-1 portion of COX-3 mRNA at positions 50 or 72, respectively (3). These deletions produce a self-rectifying shift in the reading frame that will potentially produce functional COX-1 related isozymes that retain their signal peptides plus 31 amino acids encoded by intron 1. The predicted amino acid sequences of the 31 amino acids encoded by intron-1 of COX-3-50 and COX-3-72, which differ by only 6 internal residues, are shown in Figure 3.1.

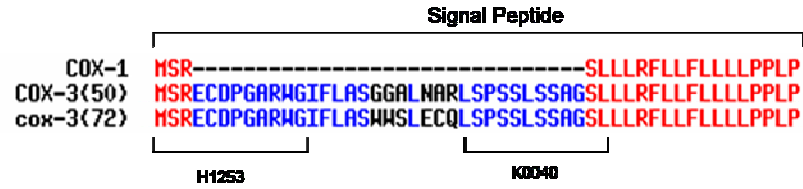


Fig. 3-1. N-terminal protein sequence of COX-1, COX-3-50 and COX-3-72. Exons 1 and 2 of the COX-1 gene encode the N-terminal hydrophobic signal peptide. Thus retention of intron1 results in sequence inserted into the signal peptide of COX-1. The blue and black sequences represent amino acids encoded by intron 1 and sequence highlighted in red is of the N-terminal signal peptide. Amino acids highlighted in black represent residues that differ between COX-3-50 and COX-3-72. Antibody used in these studies against the N-terminal amino acids of intron 1 is designated H1253 and antibody against C-terminal amino acids of intron 1 is designated K0040.

COX enzymes are intraluminal residents of the endoplasmic reticulum and depend on N-linked glycosylation for proper folding and activity. Retention of intron 1 theoretically could prevent COX-3-50 and COX-3-72 expression by preventing export of these mRNAs from the nucleus or by targeting these proteins to another subcellular compartment, preventing glycosylation. To test if these COX-3 proteins are properly expressed, Sf9 cells were infected with recombinant baculovirus expressing COX-1, COX-3-50 and COX-3-72 and cell homogenates were assayed for protein expression by Western blotting. COX-1 mAb and H1253 were used to probe for COX-3-50 and COX-3-72.

This analysis demonstrated that both COX-3-50 and COX-3-72 are expressed in insect cells, although COX-3-72 is expressed at much higher levels than COX-3-50 (Fig. 3-2a). COX-3-50 and COX-3-72 are glycosylated on asparagine similar to COX-1, as this glycosylation can be prevented by tunicamycin treatment (Fig. 3-2a, lower). Like canine COX-3, the signal peptide was also retained in COX-3-50 and COX-3-72, since these proteins were detected by H1253 (Fig. 3-2a, lower, lane 2-4 and Fig. 3-2b, lane 4-

5). Retention of the first 15 nucleotides of intron 1, which are highly conserved in mammalian species, are sufficient to prevent cleavage of the signal peptide (Fig. 3-2b, middle, lane 3).

Purified and unpurified preparations of H1253 were used in our analyses and produced the unexpected finding that unpurified COX-3 antibody detected COX-3-50 significantly better than COX-3-72 and purified antibody produced the opposite result (Fig. 3-2b, upper, lane 3-4). None of these preparations detected COX-1 (Fig. 3-2b, lane 2), suggesting that unpurified and purified H1253 preparations recognize different conformational epitopes specific for COX-3-50 and COX-3-72, respectively.

Conformational/structural differences between COX-3-50 and COX-3-72 were further evidenced by the fact that COX-3-50 was expressed much more poorly than COX-3-72, COX-1 or its variant that retained 15 nucleotides. It also consistently exhibited an abundance of distinct apparent proteolytic break-down products not shared with the other COX proteins. Treatment with tunicamycin additionally showed that although all the COX proteins are N-linked glycosylated, the COX-3-50 protein showed an altered pattern of N-linked glycosylation relative to the other COX forms. COX-3-50 also differed with the other COX proteins in its level of cyclooxygenase activity. Despite the fact that COX-3-50 is expressed much more poorly than COX-3-72, it was found to be at least 10-fold more active than COX-1 and COX-3-72 (Fig. 3-2a, upper, lane 3) when account is taken of the differing levels of protein expression. Determinations of K_M and V_{max} for COX-3-50 and COX-3-72 isozymes were performed that further demonstrated this difference (Fig. 3-2c). Although COX-3-50 enzyme was present in this assay at a level that was estimated by immunoblot to be only one fifth that of COX-1 and

COX-3-72, it had a V_{\max} value double that of COX-1 and significantly greater than COX-3-72. In contrast, the K_M values for COX-3-50 and COX-3-72 were determined to be half that of COX-1 (Fig. 3-2c), suggesting greater affinity for substrate.

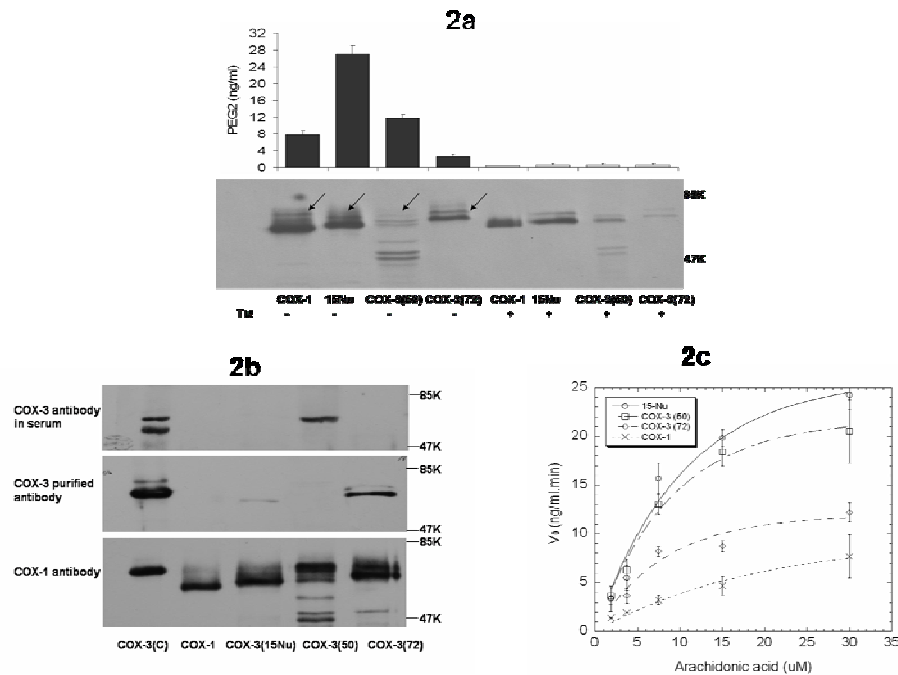


Fig. 3-2. COX-3 over expression in insect cells. **2a.** Western blots showing the expression of COX-1, COX-3-15, COX-3-50 and COX-3-72 in insect cells treated with (+) (*lower, right*) and without (-) tunicamycin (*lower, left*). Arrows indicate glycosylated forms of COX-1 that are not present in cells treated with tunicamycin. Murine monoclonal antibody to ovine COX-1 (Cayman Chemical) was used to probe the blot. Because of the low level of expression of COX-3-50, five times more cellular protein was loaded in the gel to enable viewing. The upper panel shows COX activity in insect cells expressing COX-1, COX-3-15, COX-3-50 and COX-3-72. The same amount of cellular protein was used in each assay. Cells in the upper and lower panel were treated with (+) and without (-) tunicamycin as described in Materials and Methods. **2b.** Western blot showing the retention of signal peptide in COX-3. COX-3 antibodies in unpurified serum, affinity purified anti-COX-3 preparations (see Materials and Methods), and COX-1 monoclonal antibody were used to probe COX-1, COX-3-15, COX-3-50 and COX-3-72 on the blot. **2c.** K_M and V_{\max} value of COX-1, COX-3-15, COX-3-50 and COX-3-72 were determined by RIA. Cyclooxygenase K_M and V_{\max} values were determined by measuring the activity in the presence of 0.93-30 μM arachidonic acid and fitting the values to the Michaelis-Menten equation by using Kaleidagraph software (Synergy Software). Data were expressed as mean \pm SEM ($n = 3$)

Inhibition of COX-3-50 and COX-3-72 by NSAIDs, acetaminophen and

coxibs. Canine COX-3 differs from COX-1 in sensitivity toward some NSAIDs and analgesics (2). Therefore, common analgesic/antipyretic drugs and NSAIDs were tested for their ability to inhibit activity of human COX-1, COX-3-50 and COX-3-72. Analyses were done in the presence of exogenously added AA at 30 μ M concentrations. Neither COX-3-50 nor COX-3-72 was appreciably inhibited by acetaminophen. The COX-3-72 protein, in fact, exhibited a modest dose-dependent trend toward stimulation (Fig. 3-3). Human COX-1 and COX-2 are similarly affected by this drug (data not shown). However, COX-3-50 and COX-3-72 were inhibited by NSAIDs such as ibuprofen where they demonstrated only slight differences in comparison to COX-1 (Fig. 3-3). An exception was aspirin, which preferentially inhibited COX-3-72 over COX-1 and COX-3-50 by about 7-fold. The increased sensitivity toward aspirin was previously reported for canine COX-3 (2) and COX-3-72 by Qin et al (1). Analysis of coxibs produced the unexpected finding that COX-3-50, but not COX-3-72 or COX-1, were stimulated with very low concentrations of rofecoxib ($<1\mu$ M [Fig. 3-3]). At higher concentrations, ($>1\mu$ M) a much more modest stimulation of COX-1 and COX-3-72 was also observed. The overall results indicate that COX-3-50 and COX-3-72 possess COX activity that differs pharmacologically from COX-1 and COX-2.

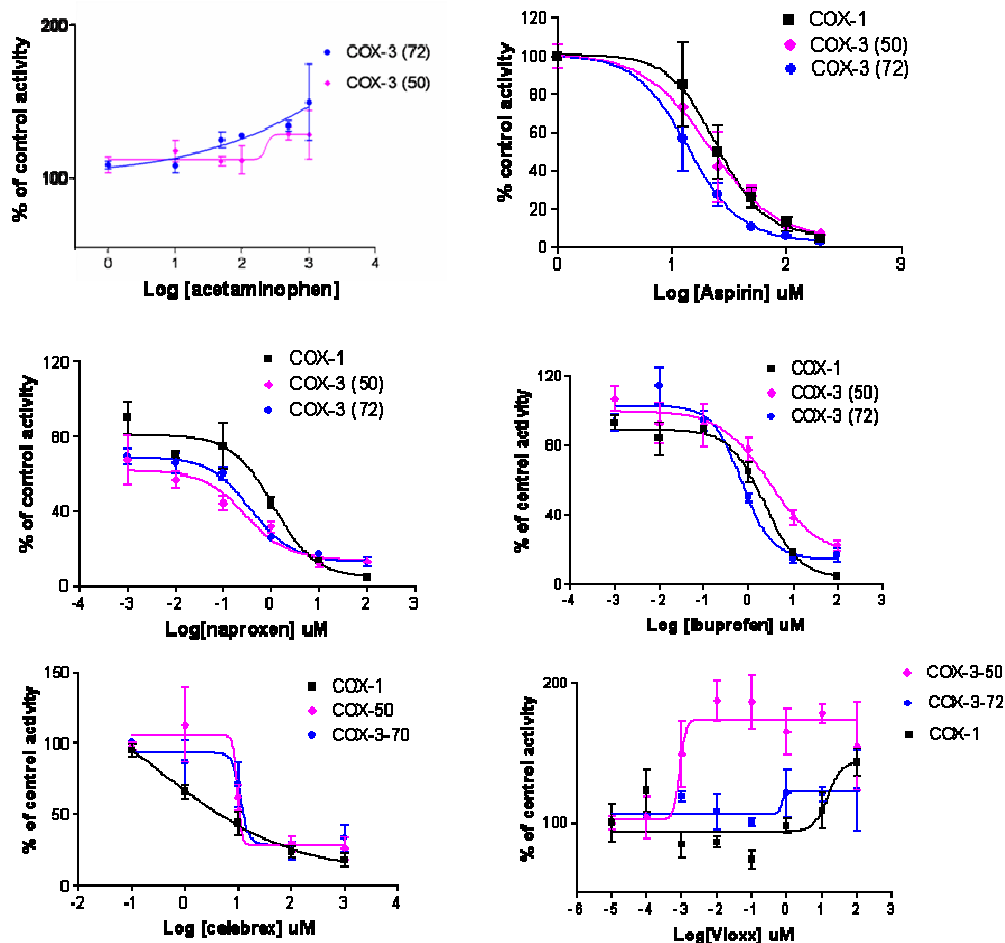


Fig. 3-3. Drug inhibition studies. The effects of acetaminophen, aspirin, celecoxib and rofecoxib on COX-1 (black), COX-3-50 (pink), and COX-3-72 (blue) activity in Sf9 cells. COX activity was measured by the formation of PGE₂ after exposure to exogenous 30μM arachidonic acid for 10 min. Data are expressed as mean ± SEM (*n* = 3).

Analysis of COX-3 expression in A549 cells. The human lung carcinoma line A549 has been used extensively in studies of COX because it is highly inducible for COX-2 expression by a variety of cytokines and mitogens. Quiescent cells also contain a low-basal level of an uncharacterized COX activity that is presumed to be related to COX-1. Western blotting analysis of human A549 cells by two COX-3 antipeptide polyclonal antibodies detected the presence of distinct 65- and 72-kDa putative COX-3

proteins (Fig. 3-4). H1253, developed against N-terminal sequence of intron 1, detected both 65- and 72-kDa bands, whereas K0040, developed against C-terminal sequence of intron 1, detected only the 65-kDa protein. The 65-kDa protein detected by H1253 was more prominent than K0042 which may suggest that the binding efficiency of H1253 is better than K0042, and could account for why the 72-kDa protein was not detected by K0040.

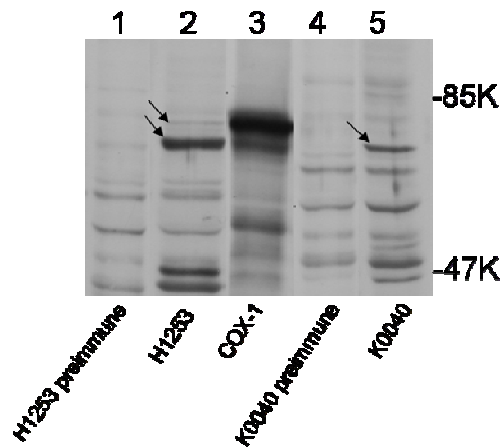


Fig. 3-4. Western blot on A549 cells. COX-3 polyclonal antibodies raised against the N-terminal (H1253) and C-terminal (K0040) protein sequence of intron 1 in immunized rabbit serum were used to probe the blot. Two immunoreactive proteins were detected by H1253 and one was detected by K0040 as indicated by arrows.

Analysis of COX-3 expression in human platelets. Human platelet is a rich source of COX-1 protein and a potential source for COX-1 splice variants. Western blotting analysis of human platelets (Fig. 3-5) detected the presence of 65-, 71-, and 72-kDa COX-3 bands by using two COX-3 antibodies as done for A549 cells (above). When platelet protein was subjected to an immunoprecipitation pull-down assay (done by Lamar Roos) using H1253 or its pre-immune antisera as the antibody, 71- and 72-kDa

proteins detected by anti-COX-1 antisera were pulled down by the former but not the latter (Fig. 3-5b).

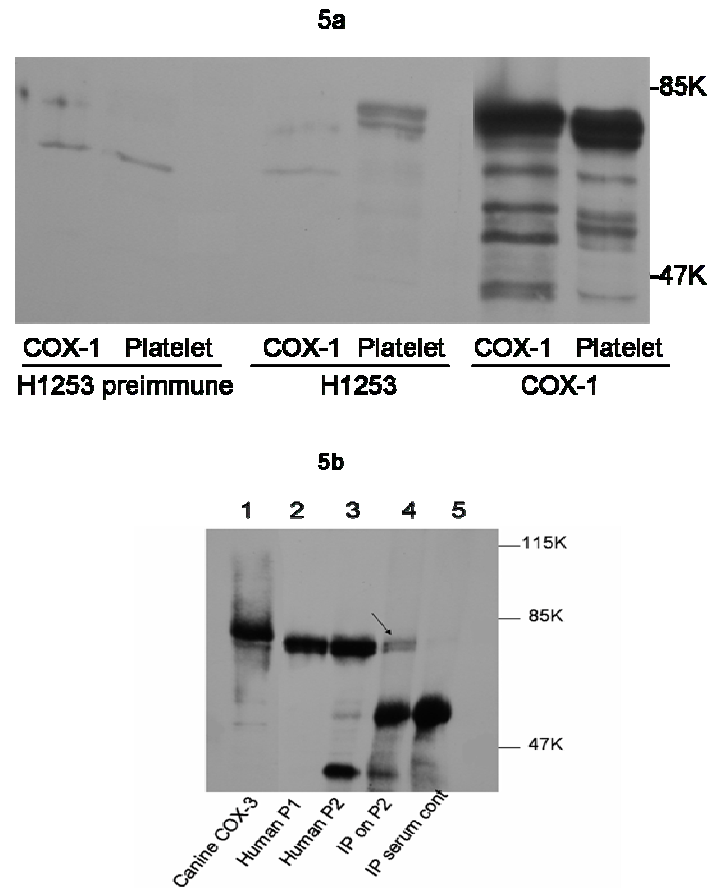
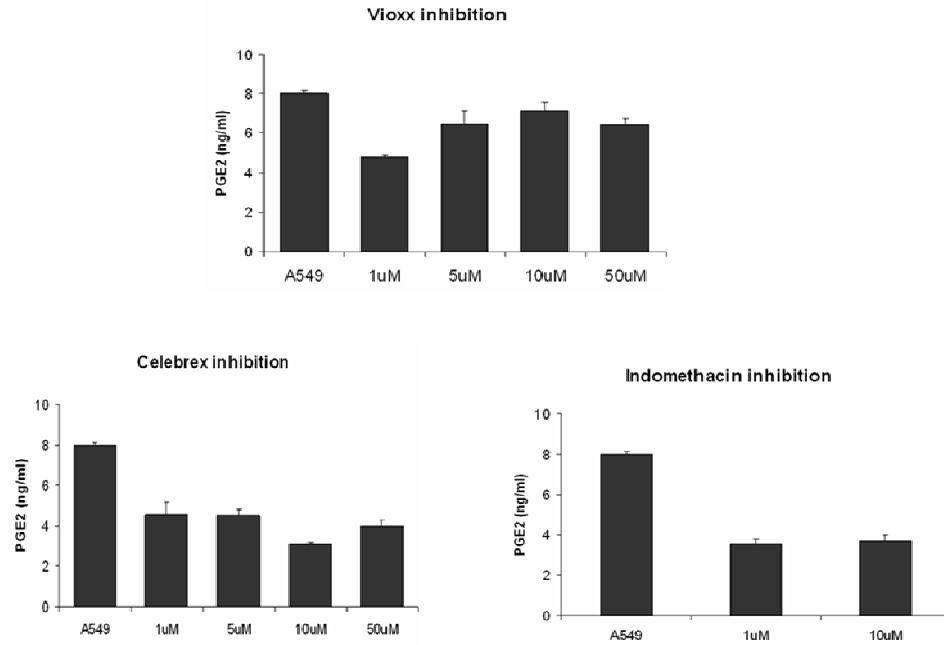


Fig. 3-5. Western blotting and immunoprecipitation assays of putative COX-3 proteins in human platelets. **5a:** H1253 (lane 1-2), K0040 (lane 3-4) and COX-1 (lane 5-6) monoclonal antibodies were used as primary antibodies for development of Western blots which detected 65-, 71-, and 72-kDa putative COX-3 related proteins denoted by arrows. Human COX-1 transfected in COS-7 cells was used as positive control (lane 1,3 and 5). **5b:** H1253 was used to pull down potential COX-3 proteins from human platelet lysate. COX-1 monoclonal antibody (mAb) was used as primary antibody to develop the Western blot. Lane 1 is canine COX-3 used for molecular weight comparison. Lane 2 and 3 are human platelet COX-1 as detected in two different patients. Lane 4 shows two COX-3 related proteins (designated by an arrow) in the immunoprecipitate brought down by H1253 COX-3 antibody. Lane 5 is the immunoprecipitate brought down by a 2-fold excess of pre-immune serum. Densitometry of the image was performed using an AlphaImage 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA). Immunoprecipitation experiment was by Lamar Roos.

Modulation of cyclooxygenase activity in A549 cells by rofecoxib. The finding in A549 cells of proteins ~72,000 Da in size by the H1253 antibody raised the possibility that these cells expressed a low level of a COX-3-like protein. The antibody used in this experiment was unpurified, which reacts primarily with the COX-3-50 form of COX-3 (Fig 3-2b). This form also is selectively stimulated by rofecoxib (Fig. 3-3). Therefore, COX activity in non-stimulated, quiescent A549 cells was measured in the presence and absence of rofecoxib, celecoxib, and indomethacin as control. The IC₅₀ value for COX-2 inhibition in these cells by rofecoxib was determined to be 0.17 μ M (data not shown). In quiescent cells, this highly selective COX-2 inhibitor inhibited 40% of COX activity in A549 cells at 1 μ M (Fig. 3-6a, lane 1-2), likely due to residual expression of COX-2 under these cell-culture conditions. With increasing levels of rofecoxib (5, 10 and 50 μ M) stimulation of PGE₂ production was observed. Celecoxib and indomethacin, in contrast, did not demonstrate this stimulatory effect and both agents inhibited COX activity to a greater extent than rofecoxib. Previous experiments by Lamar Roos also generated stimulation by rofecoxib of COX activity, but to a greater extent than seen in our studies (Fig. 3-6b). In the Roos' studies, cells may have been more quiescent and thus contained lower levels of interfering COX-2 expression.

6a



6b

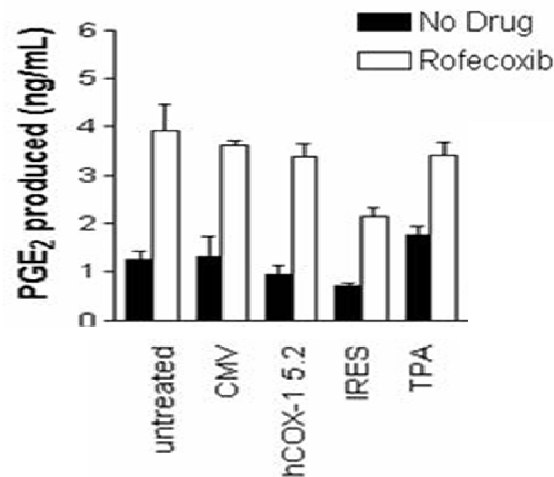


Fig. 3-6. A549 cells Rofecoxib stimulation assays. **6a:** The effects of rofecoxib, celecoxib and indomethacin on A549 cell COX activity. COX activity was measured by the formation of PGE₂ after treatment with the drugs for 30 minutes followed by exposure to exogenous 30μM arachidonic acid for 10 minutes. Data are expressed as mean ± SEM (*n* = 3). **6b:** The effects of rofecoxib on quiescent A549 cells as done by Lamar Roos. COX activity was measured after rofecoxib treatment as described in 6a.

Assessment of RNA editing of intron 1 in COX-3 transcripts from A549 cells.

The COX-3-50 and COX-3-72 isoforms have been reported to result by either RNA editing or because of the presence of single-nucleotide polymorphisms (SNPs) in intron 1. The finding in A549 cells of a putative COX-3 immunoreactive protein and a rofecoxib stimulated COX activity, suggested the presence of a COX-3 isoform in these cells. To evaluate this possibility, RNA was extracted from quiescent A549 cells and measured for the presence of COX-3 mRNA, which was readily detected (data not shown). This mRNA was then evaluated for the presence of single-nucleotide deletions that would occur if either RNA editing or SNPs were present. To do this, 5 independent PCR amplifications were performed to generate amplicons spanning the COX-3 intron 1 region. These amplicons were individually cloned into pCR3.1-TOPO, transformed into competent *E. coli*, and 10 clones from each of the five amplicons were purified and sequenced. No single nucleotide deletions were detected.

Discussion

The data above suggest that COX-3-72 and COX-3-50, two in-frame COX-3 reported in the literature, differ structurally and enzymatically, despite the fact that their only difference is six amino acids located in the intron 1 region. By Western blot analysis, non-purified H1253 COX-3 antibody preferred binding to COX-3-50 while affinity purified H1253 preferred COX-3-72 (Fig. 3-4), suggesting that the intron-1 region can form at least two alternative stable conformations. Because the protein was electrophoresed through a denaturing gel this conformation would likely result from refolding of the small domain containing the sequence of the cognate peptide used to develop the antibody. Other tell-tale signs of structural difference included the low level

of expression of COX-3-50 in Sf9 cells relative to the other COX-1 related proteins examined. Furthermore, COX-3-50 was different from the other forms in its distinctly different pattern of its breakdown products and pattern of glycosylation (Fig. 3-2a). Retention of the signal peptide with an embedded intron-1-encoded sequence represents an addition of over 50 amino acids to the amino terminus of the protein and could understandably have significant structural effects as a result. These effects could be evoked either by altering subcellular localization or kinetic or thermodynamic properties of folding. However, it is intriguing that COX-3-72 differs little in its expression level or glycosylation pattern with either COX-1 or another variant that contains only 15 nucleotides of intron 1. Yet COX-3-50, which differs from COX-3-72 by only 6 internal residues encoded by intron 1 is so dramatically affected by this small change.

The COX-3-50 and COX-3-72 are not only different structurally, but are also different in their cyclooxygenase catalytic activities. Despite being poorly expressed, COX-3-50 is significantly more active than COX-1 or any of its variant forms we have thus far examined. It is also significantly more active than COX-2 (data not shown). This high activity may answer a question regarding COX-3 mRNA expression in humans, which is frequently found at lower levels (~10%) than that of COX-1. Expression of a low level enzyme with high catalytic activity could result in a physiologically significant pool of COX enzyme activity. Moreover, this pool of enzyme activity would be difficult to detect in comparison to COX-1.

The retention of the signal peptide and intron-1 encoded sequence to the amino terminus of COX-3-50 and COX-3-72 appears to also result in steric changes that affect either or both cyclooxygenase and peroxidase active sites in the enzyme. Most noticeable

was the finding of increased sensitivity toward aspirin in COX-3-72 (Fig. 3-3) and stimulation of COX-3-50 and COX-3-72 by the coxibs celecoxib and rofecoxib. The 2-fold stimulation by rofecoxib of COX-3-50 when measured at Vmax may be significant from a medical viewpoint. Stimulation of this COX isoform occurred at concentrations well below at 1 μ M, which is peak plasma level of this drug under normal dosing regimens *in vivo*.

COX-2 enzyme inhibitors were developed based on the hypothesis that selective inhibition of COX-2 would decrease pain and inflammation without associated gastrointestinal and bleeding risks (1). However, rofecoxib (Vioxx) and valdecoxib (Bextra), two COX-2 inhibitors were withdrawn from the market due to excess cardiovascular risk in 2004 and 2005. The withdrawal of rofecoxib and valdecoxib raises great concern about cardiovascular side effects of COX-2 inhibitors and other NSAIDs which inhibit both COX-1 and COX-2. Very few data have been published regarding the reason of this increased risk. Suppression of prostacyclin (PGI₂) but not thromboxane A₂ biosynthesis by coxibs is a popular theory to explain this increase (8). Our data provide an alternative to this hypothesis and suggests that not all COX-2 inhibitors may have a negative cardiovascular impact.

Because the human COX-3 mRNA has been shown to be highly expressed in the vascular system, including in the heart and microvasculature of the brain (2, 3, 9), persistent stimulation of this activity could create a proinflammatory state in the vasculature resulting in atherosclerosis, a condition considered by many investigators to be a form of inflammation (10). Alternatively stimulation of COX-3 activity in platelet

could theoretically increase thromboxane A₂ synthesis, thus increasing thrombotic events leading to myocardial infarction or stroke.

Thus, our finding may explain the cardiovascular risks associated with rofecoxib use and may also allow the testing of other coxibs, non-selective NSAIDs, or other drugs with regard to their ability to stimulate COX-3 and thus potentially negatively impact the cardiovascular system.

Our finding of potential COX-3 proteins in human platelets and A549 cells is problematic given that we found no evidence for either SNPs or RNA editing that could result in correction of the reading frame shift generated by intron-1 insertion in COX-3. However, analysis of the intron-1 sequence demonstrates a canonical slippery site for -1 ribosomal frame-shifting five codons upstream from the COX-3-50 deletion site. This sequence, GGGGAATTTTCTT, conforms exactly to the viral sequences for -1 ribosomal framsehsifting and may provide a mechanism by which a COX-3-50 like protein is made in humans (11). Further studies, including characterization of COX-3 sequence in human platelets and A549 cells, are needed in order to fully prove the existence of COX-3 in humans, to define its catalytic roles, and to determine how the protein is made.

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CHAPTER 4

Trifunctional inhibition of COX-2 by extracts of *Lonicera japonica*: direct inhibition, transcriptional and post-transcriptional down regulation.

Abstract

The anti-inflammatory properties of aqueous extracts from *Lonicera japonica* (LJ) flower, an anti-inflammatory treatment in traditional Chinese medicine, were tested by radioimmunoassay of cyclooxygenase isoenzyme-generated prostaglandin E₂ (PGE₂) synthesis as well as by Western and Northern blot analysis of COX-2 protein and mRNA expression, respectfully. Boiled LJ aqueous extracts directly inhibited both COX-1 and COX-2 cyclooxygenase activity, while non-boiled extracts stimulated both enzymes. Boiled LJ extracts also inhibited expression of IL-1 β -induced COX-2 protein expression and suppressed its mRNA induction by IL-1 β in A549 cells. Suppression of COX-2 mRNA induction required a significantly higher dose of aqueous extract than did suppression of protein expression, indicating that compounds in the extract act translationally or post-translationally at lower doses and transcriptionally or post-transcriptionally at higher doses. Direct inhibition of COX isoenzymes as well as down-regulation of COX-2 mRNA and protein may represent the mechanism by which this ancient herbal treatment decreases inflammation.

Introduction

The aqueous extract from *Lonicera japonica* (LJ) flower has been used in Chinese traditional medicine for treating fever, arthritis and infectious diseases for thousands of years. Previous studies have shown that LJ could act as an anti-inflammatory agent through regulation of NF- κ B activation, since it inhibits the appearance of nuclear NF- κ B

p65 and the action of I- κ B in the liver of lipopolysaccharide (LPS) challenged rats (1). LJ has also been found to inhibit LPS induced inflammatory responses such as induction of tumor necrosis factor (TNF- α) and inducible nitric oxide synthase as well as by inhibiting PAR2 agonist induced myeloperoxidase activity in paw tissue (2).

Cyclooxygenase (COX) isoenzymes, COX-1 and COX-2, promote inflammation through the production of prostaglandins (3). Whereas COX-1 is constitutive, COX-2 is highly inducible and is readily elevated in tissues in response to proinflammatory stimuli, including cytokines and bacterial LPS (4). COX-2 converts arachidonic acid to prostaglandin H₂, which is then further metabolized to various prostaglandins, prostacyclin, and thromboxane A₂ (3). Thus, COX-2 is thought to be the isoform responsible for the production of proinflammatory prostanoids in many models of inflammation (4). COX-2 over-expression is also seen in human malignancies including colorectal cancer, lung cancer, and breast cancer (5). COX-2 selective NSAIDs have been used to inhibit intestinal neoplasia in mice and human (6).

Whether the analgesic and anti-inflammatory effects of LJ can be attributed to inhibition of COX-2 activity or expression has not been previously examined. Here we show that LJ can either directly stimulate or inhibit COX-1 and COX-2 activity, depending on how the extract is made. Moreover, boiled LJ extract down-regulates COX-2 by two mechanistically distinct pathways.

Material and Methods

Reagents. Dried *Lonicera japonica* flower bought from a local health food store was boiled in phosphate buffered saline (PBS) for 5 minutes to yield boiled aqueous extracts or stirred at 4 °C overnight to get non-boiled aqueous LJ extracts. IL-1 β was

purchased from Sigma. Penicillin, streptomycin, F-12 nutrient mixture and Grace's insect medium were obtained from Invitrogen. Fetal bovine serum (FBS) was from Hyclone and polyclonal and monoclonal COX-2 antibodies were purchased from Cayman Chemical.

Cell lines and culture. A549 lung carcinoma cells were maintained in F-12 nutrient mixture with 10% FBS. Sf9 insect cells were grown in Grace's insect medium supplemented with 10% FBS. Culture media contained 100U/ml penicillin and 100ug/ml streptomycin.

Expression of human COX-1 and human COX-2 in baculovirus-infected insect cells. For radioimmunoassay (RIA), human COX-1 and human COX-2 coding regions, verified by DNA sequencing, were cloned into the baculovirus expression vector pBlueBac 4.5/V5-His (Invitrogen). Recombinant plaques were identified, purified and high titer viral stocks were prepared following the protocol of the manufacturer. Sf9 cells (5×10^6) were infected with viral stocks at a multiplicity of infection (moi) of 3. COX-1 and COX-2 expression was determined by RIA of prostaglandin E₂ using intact cells and by Western blot of cell lysates.

LJ Cyclooxygenase Inhibition Assays. Sf9 cells were infected with recombinant baculovirus encoding human COX-1 or COX-2 and cultured for 48 h. Cells (2×10^5 , 100μl) were suspended in serum free medium and preincubated with LJ extracts (50μl) for 30 min at 25°C. Arachidonic acid (final concentration of 30 μM in a final volume of 200μl) was then added for an additional 10-min incubation at 37°C. Cells were pelleted by centrifugation and assayed for COX activity by RIA of PGE₂. A minimum of three independent assays for each isoenzyme and each drug preparation

were performed and each was done in triplicate. Inhibition curves were constructed and IC₅₀ values were determined using PRISM 3.0 (GraphPad, San Diego, USA).

Western blot analysis. Total protein (15 µg) from cells was resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and probed with COX-2 or actin primary antibodies (1:2,000) for a minimum of 1 h at 4°C. Blots were processed with appropriate secondary antibodies (1:2,000). Densitometry of the autoradiographic image was performed using an AlphaImage 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA).

Northern blot. Total RNA was isolated from cell cultures using the RNeasy kit (Qiagen, Valencia, CA, USA). Five micrograms of total RNA was separated on a 1.0% agarose gel containing formaldehyde and RNA was transferred to membrane and cross-linked by UV light. Prehybridization was performed in QuickHyb solution (Stratagene, La Jolla, CA, USA) for 1h at 65°C. Human COX-2 and GAPDH cDNA probes were labeled with [³²P]-CTP using random priming and hybridized to the blots overnight at 65°C. Blots were washed twice in 2 x SSC with 0.01% of SDS and exposed to X-OMAT Blue XB-1 film (Kodak, Rochester, NY, USA) for autoradiography. Film images were recorded and analyzed using AlphaDigiDoc (Alpha Innotech, CA, USA).

Results

Cytotoxicity of LJ extract to A549 cells. Cytotoxicity of LJ boiled and non-boiled extracts to A549 cells was measured by trypan blue assay. Cell viability was not significantly affected by LJ extracts up to concentrations of 100 mg/ml (data not shown). Concentrations of LJ extracts below this threshold were used in subsequent experiments.

LJ extracts inhibit COX-1 and COX-2 Prostaglandin E₂ (PGE₂) synthesis.

Boiled LJ and non-boiled extracts were tested for their ability to inhibit prostaglandin synthesis activity of COX-1 and COX-2. In Sf9 cells, PGH₂ produced by COX-1 and COX-2 non-enzymatically rearranges to PGE₂. Thus analysis of PGE₂ directly assesses COX activity. Analyses of human COX-1 and COX-2 over-expressed in Sf9 cells were done at Vmax for the enzymes by adding exogenously arachidonic acid at 30μM concentrations.

Prior to analyses, boiled and non-boiled extracts were evaporated to dryness and solutes were collected and weighed. Solute were then redissolved in water at 0.2g : 1ml and diluted to specific concentrations. Both COX-1 and COX-2 were inhibited by boiled LJ extracts (Fig. 4-1 top), while non-boiled LJ extracts greatly stimulated COX-1 and did not inhibit COX-2 (Fig. 4-1 bottom). Moreover, COX-2 was more sensitive (by approximately four fold) to boiled LJ extracts than COX-1 (Fig. 4-1). Boiled LJ extracts inhibited COX-2 with an IC₅₀ value of 15 mg/ml, whereas IC₅₀ values for COX-1 were 56 mg/ml.

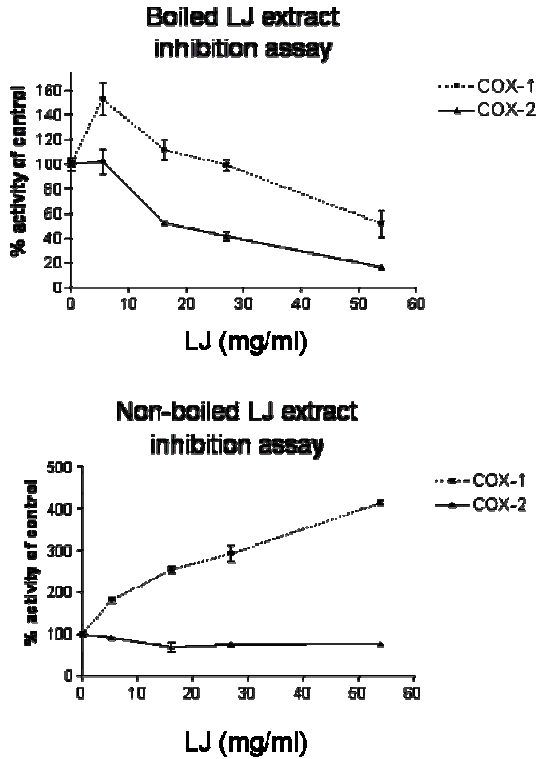


Fig. 4-1. Inhibition of PGE₂ synthesis by LJ aqueous extracts. The effects of boiled LJ extracts and non-boiled extracts on COX-1 (■), and COX-2(▲) activity in insect cells. COX activity was measured by the formation of PGE₂ after exposure to exogenous 30 μM arachidonic acid for 10 min. Data are expressed as mean ± SEM (*n* = 3-5).

Boiled LJ extracts inhibit IL-1β-induced COX-2 protein expression.

Activation of COX-2 is one of the critical events mediating inflammation (3). Therefore, LJ extract's effect on COX-2 mRNA and protein expression following IL-1β treatment was investigated. A549 cells were treated with various concentrations of boiled LJ extracts during induction of COX-2 by treatment with IL-1β (5 ng/ml for 3 h). Treated cells were divided into two aliquots and analyzed for COX-2 protein by Western blot and mRNA expression by Northern blot, respectively. Boiled LJ extracts alone did not induce or repress baseline COX-2 protein expression (Fig. 4-2). However, IL-1β-induced COX-

2 protein expression was inhibited by boiled LJ extracts in a dose dependent manner (Fig. 4-2). The IC₅₀ value for COX-2 protein down-regulation was 5.0 mg/ml.

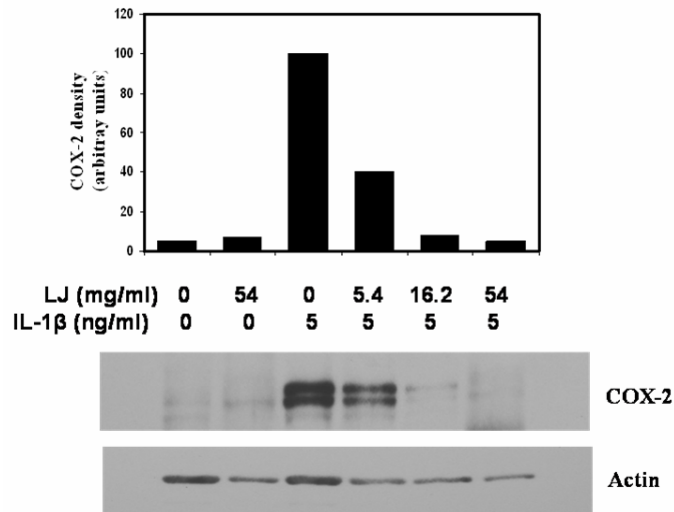


Fig.4-2. Effect of boiled LJ extracts on IL-1β induced COX-2 protein over-expression. A549 cells were treated with boiled LJ extracts and IL-1β for 3 h. Whole cell extracts were resolved on SDS-PAGE and transferred to nitrocellulose membrane. Western blot was done using COX-2 and actin antibodies as described in Materials and Methods.

Boiled LJ extracts down regulate IL-1β-induced COX-2 mRNA

transcription. Northern blot analysis was carried out to evaluate COX-2 mRNA expression in A549 cells treated with various concentrations of LJ extracts in the presence or absence of IL-1β as described above. Induction of COX-2 mRNA was observed after exposure to IL-1β for 3 h (Fig. 4-3, lane 3). No significant inhibition of COX-2 transcription occurred at 5.4 or 16.2 mg/ml of extract, a concentration at which COX-2 protein was strongly down-regulated (Fig. 4-3, lane 4 and lane 5). However, at 54 mg/ml, COX-2 mRNA was completely down regulated. The degree of down regulation observed was comparable to that evoked by 20 μM dexamethasone (Fig. 4-2, lane 6 and lane 7), a known down regulator of COX-2 mRNA transcription and message stability.

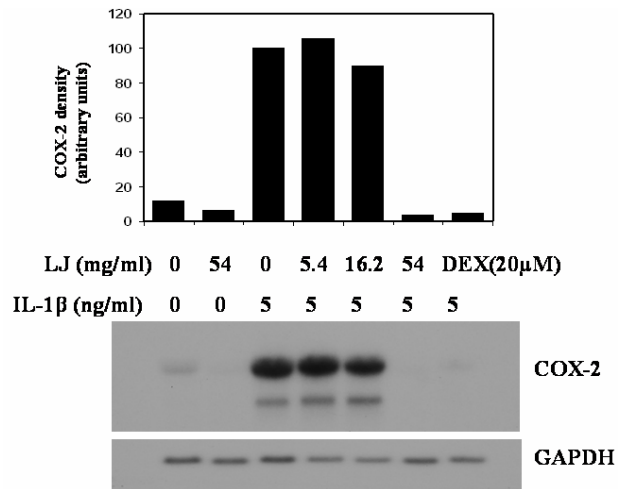


Fig. 4-3. Effect of boiled LJ extracts on IL-1 β induced COX-2 mRNA transcription. A549 cells were treated with LJ boiled extracts and IL-1 β for 3h. A549 cells treated with 20 μ M dexamethasone (DEX) and IL-1 β were used as a positive control for transcriptional down-regulation and COX-2 mRNA destabilization. Total cellular RNA was isolated; 5 μ g of RNA was added to each lane. The Northern blot was probed sequentially for COX-2 mRNA and GAPDH.

Discussion:

The current study investigated the effect of LJ aqueous extracts on inhibition of cyclooxygenase activity and COX-2 expression. Boiled LJ extract inhibits both COX-1 and COX-2 activity, favoring COX-2 inhibition by four fold. This boiled extract, the method by which LJ tea is made in China, contains endogenous NSAID-like compounds. The identity of these is unknown, but salicin and other salicylate derivatives have long been known to exist in willow bark and other plants. Boiled LJ extract could be an effective NSAID because it shows preferential inhibition toward COX-2. It would not likely have the gastric sparing properties of highly selective COX-2 inhibitors, such as rofecoxib, but it may not possess the reported cardiovascular risks that have been reported for this drug (11).

In contrast to boiled extracts, non-boiled extract actually stimulates COX-1 activity. A number of organic compounds, such as acetaminophen at some doses, do the same (7). Stimulation by non-boiled LJ is likely due to the presence of phenolic or other compounds that can act as co-reductants in the COX enzymatic redox cycle (3). We postulate that boiling in air may oxidize these compounds.

In addition to containing NSAID-like compounds, boiled extract down-regulates COX-2 mRNA expression. Previously, the aqueous extract from *Lonicera japonica* flower was shown to inhibit NF- κ B activation through reducing I- κ B degradation (1). Ochnaflavone, a natural biflavonoid isolated from *Lonicera japonica* Thunb., was found to inhibit LPS induced iNOS expression through NF- κ B in RAW264.7 cells (8). COX-2 is frequently co-induced with iNOS in RAW264.7 cells (9). Thus although unexamined in this study on ochnaflavone, COX-2 may also have been down-regulated since Il-1 β induced COX-2 expression is also known to involve NF- κ B. The COX-2 transcriptional inhibition capacity of boiled LJ extracts seen in our studies may be due to biflavonoids or structurally similar compounds acting through NF- κ B.

Boiled LJ extract also inhibited COX-2 protein expression at lower doses than those that produce inhibition of COX-2 mRNA expression. COX-2 protein induction was inhibited by 60% and 90% at 5.4mg/ml and 16.2mg/ml while COX-2 mRNA was not detectably inhibited at those two concentrations. These findings suggest that boiled LJ extract also contains compounds which inhibit COX-2 post-transcriptionally. Only one other known natural product, radicicol, is known to have this effect (10). Purified from the culture broth of fungus strain KF9, radicicol has a variety of clinically important medical uses, including as an antibiotic, anti-inflammatory and anti-tumor agent. It

inhibits c-src tyrosine kinases in lipopolysaccharide-stimulated macrophages. The active gradients in LJ could function the same as radicicol or be structurally and functionally different. However, the compounds in LJ may have similar therapeutic potential in inflammation and neoplasia.

In summary, the data show the existence of three different compounds in LJ which directly, transcriptionally and post-transcriptionally inhibit COX-2 to potentially decrease inflammation. Since COX-2 is one of the most critical proteins related to inflammation, the inhibition of COX-2 by LJ extracts represents a potential therapeutic approach to the treatment of inflammatory diseases.

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CHAPTER 5

Studies on the N-terminus of COX-1 and its effect on cyclooxygenase-1 catalytic activity

Abstract

Cyclooxygenases (COX) are encoded by two genes: COX-1 and COX-2. Even though COX-1 and COX-2 in all species share only sixty percent sequence identity they are very similar to each other in their crystallographic structures. One difference in the primary structure of these two isozymes is the presence of eight amino acids in the amino-terminal region of COX-1 that are not present in COX-2. This amino acid sequence, in fact, forms the amino terminus of the processed COX-1 protein following cleavage of the signal peptide and its function is unknown. In this study, a human COX-1 deletion variant with this sequenced removed was constructed, transfected into mammalian cells, and studied in parallel with human COX-1. No differences were observed in N-linked glycosylation or in the level of expression of the mutant relative to COX-1. The mutant was enzymatically active as determined by radioimmunoassay and showed the same sensitivity toward aspirin as COX-1. However, whereas the K_M for the enzyme remained the same as COX-1, its apparent V_{max} decreased four fold. We conclude that the COX-1 specific amino-terminal sequence has a subtle but detectable effect on COX-1 catalysis.

Introduction

Cyclooxygenases (COX) are rate-limiting enzymes in the production of prostaglandins from arachidonic acid (1). Two isoforms of this enzyme exist in many

species: COX-1 and COX-2. COX-1 is constitutively expressed while COX-2 is highly inducible in response to a variety of agents including pro-inflammatory cytokines and mitogens. Both COX-1 and COX-2 have a molecular weight of approximately 71K and are 63% identical in sequence (2). COX-1 and COX-2 share the same four functional regions: an amino-terminal signal peptide, dimerization domain, membrane binding domain, and catalytic domain (3). Crystallographic structures show a striking tertiary and quaternary structural similarity between COX-1 and COX-2 (4, 5).

In spite of this structural similarity, COX-1 and COX-2 exhibit differences in sensitivity towards different NSAIDs. The difference is the result of a change in one of the 24 amino acid residues which line the hydrophobic cyclooxygenase active site - Ile at position 523 in COX-1 and Val at position 523 in COX-2. A mutation of Ile 523 to Val in the COX-1 enzyme makes it sensitive to COX-2-selective inhibitors and site-directed mutation of Val 523 to Ile in COX-2 shows inhibitor binding comparable to that seen in COX-1 (6, 7, 8). The Ile/Val substitutions result in pharmacological and biochemical differences between COX-1 and COX-2 that have permitted the development of COX-2 selective inhibitors (coxibs).

Based on primary sequences alone, the major differences between COX isoforms occur at the amino and carboxy terminal ends. COX-1 has 8 amino acids inserted at its amino terminal end not found in COX-2 and COX-2 has 18 amino acids inserted in its carboxyterminal region not found in COX-1 (9, 10). The effect of deleting the 18 amino acids in the COX-2 carboxyl terminus has been investigated by others (11). The role of the additional 8 amino acids in the amino-terminal end of COX-1 is unknown. This sequence begins at the +1 position relative to the N-terminal hydrophobic signal peptide

cleavage site. Co-translational cleavage of the signal peptide results in this sequence being located at the amino terminus of the processed protein, immediately upstream of the dimerization domain of the COX-1 protein (3).

In this study, a human COX-1 deletion mutation that removes almost all of this amino-terminal sequence was constructed and studied in parallel with COX-1. Specifically, the COX-1 deletion mutant differed from full length COX-1 only in deletion of seven of the eight amino acids immediately following the signal peptide cleavage site. The eighth residue, a valine (Val 32) was retained in order to keep a hydrophobic residue at the N-terminus of the process protein. The results suggest that the deletion of the seven amino acids coding sequence does not affect COX-1 expression in mammalian cells nor the enzyme's sensitivity towards aspirin. However, the seven amino acid deletion decreases the apparent V_{max} of the enzyme.

Materials and Methods

Materials. COX-1 monoclonal antibody was obtained from Cayman Chemical (Ann Arbor, MI). Arachidonic acid, Tween-20 and all other reagents were obtained from Sigma-Aldrich if not designated otherwise.

Construction of the COX-1 deletion mutation ($\Delta 7aa$). Human COX-1 in pcDNA 3.1 vector was used as a template. The following primers were designed for PCR amplification to make the 21 nucleotide deletion mutation: the sense primer (5'-ATGAGCCGGAGTCTCTTGCTCTGGTTCTTGCTGTTCTGCTCCTGCTCCC GCCGCTCCCCGTCCTGCTCGCGGACAATCCCTGTTGTTACTATCCATGCCAGCAC-3') corresponded to nucleotides 0 to 126 from human COX-1 sequence with a 21 nucleotide

deletion; and the antisense primer (5'-TCAGAGCTCTGTGGATGGTCGCTCCACAGCA-3') corresponded to nucleotides 1768-1799, which are begin 31 nucleotides upstream of the COX-1 stop codon and continue to the stop codon. For ectopic expression and assessment of enzyme activity of $\Delta 7aa$ and COX-1, coding regions were cloned into linearized pCR3.1-TOPO vector (Invitrogen). Plasmids were isolated and sequenced to assure that the proper mutation was made without the introduction of other unwanted changes.

Transfection of COX-1 and $\Delta 7aa$ into COS-7, CHO, HEK239T and A549 cells. COX-1 and $\Delta 7aa$ cloned into the pcDNA3.1 vector were transiently transfected into COS-7 cells using lipofectamine with Plus Reagent (Invitrogen). Cells, harvested 48 h post-transfection by scraping, were washed once with PBS prior to being counted in a hemocytometer. In some experiments equal numbers of cells from transfection experiments were solubilized in protein sample buffer for immunoblot analysis. In other instances, as designated in figure legends, cell protein was quantified by Bio-Rad Protein Assay (Bio-Rad) and equal amounts of protein were compared on immunoblots. To measure transfection efficiency, COX-1 and $\Delta 7aa$ constructs were cotransfected with a transfer vector containing a cDNA encoding firefly luciferase. Cell aliquots of these co-transfected cells were lysed and measured for luciferase activity by luminometry.

To measure N-glycosylation of COX variants in mammalian cells, tunicamycin was added to cells to a final concentration of 10 $\mu\text{g/ml}$ 4h after transfection. Cells were then cultured for an additional 48 h.

Immunoblot analysis of $\Delta 7aa$ and COX-1 expression. For immunoblot analysis of protein expression, total protein (20 μg) from cells was resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and probed with COX-1 murine mAb (Cayman Chemical). Primary antibodies (1:2,000) were incubated with membranes overnight at 4°C. Blots were processed with appropriate rabbit-anti-mouse secondary antibody (1:2,000) from Sigma. Densitometry of images was performed using the AlphaImage 2000 Documentation and Analysis System (Alpha Innotech, San Leandro, CA).

Radioimmunoassay (RIA) measurement of cyclooxygenase activity of COX-1 and $\Delta 7aa$. Transfected cells (0.1×10^6 cells; 100 μl) were incubated with arachidonic acid (final concentration 30 μM) for 15 minutes at 37°C. Supernatants were assayed for COX activity by RIA for PGE₂ as previously described (12). Three separate assays were performed for every protein analyzed and each was done in triplicate.

Aspirin inhibition assay. For measurement of irreversible inhibition of the COX active site by aspirin, transfected cells were mixed and incubated with aspirin for 30 min at 25°C. The mixture was then incubated with arachidonic acid (final concentration, 30 μM) for an additional 15 minutes at 37°C. Supernatants were assayed for COX activity by RIA for PGE₂. At least three separate assays were performed for every protein analyzed and each was done in triplicate.

Determining K_M and V_{max} value of COX-1 and $\Delta 7aa$. K_M and V_{max} were determined by both oxygen uptake and RIA. Oxygen uptake was assayed polarographically at 37°C as described previously (13). One unit of cyclooxygenase

activity has an optimal velocity of 1 nmol of O₂/min. For determination of K_M and V_{max} values by RIA, arachidonic acid (30 μM) was added to cells and the cyclooxygenase enzyme reaction was stopped after 15 seconds by transferring reaction tubes into boiling water. Cyclooxygenase K_M and V_{max} values were determined by measuring prostaglandin synthesis activity in the presence of 0.93-30 μM arachidonic acid and fitting the values to the Michaelis-Menten equation by using Kaleidagraph software (Synergy Software).

Results

Construction of the COX-1 deletion mutation (Δ7aa). The PCR products amplified from human COX-1 containing the desired mutations were inserted into the pcDNA3.1 vector. The integrity of the resulting transfer vector constructs was verified by restriction enzyme digestion and DNA sequencing. Shown in figure 5-1, are the N-terminal eight amino acids (bold style) of COX-1 not found in COX-2. The eight amino acids are located between the signal peptide (italic style) and the dimerization domains (regular style) of COX-1.

HCOX-2	<i>mlarallcavlalshia</i> npccshpcq
HCOX-1	<i>msrslllrfliflllpplpvlladpgaptpvnpccyyycq</i>
Δ7aa	<i>msrslllrfliflllpplpvlla</i> vnpccyyycq

Fig. 5-1. N-terminal sequence of human COX-1, COX-2 and Δ7aa. N-terminal sequence of COX-1, COX-2 and Δ7aa are shown. The signal peptide is in italics. Residues in regular font are the beginning of the COX dimerization domain and residues in bold are the eight amino acids of HCOX-1 not present in HCOX-2.

Detection of COX-1 and $\Delta 7aa$ over expressed in mammalian cells.

Polyacrylamide gel electrophoresis and subsequent visualization by immunoblot of the transfected cells revealed a major band at ~ 72 kDa for native COX-1 and the $\Delta 7aa$ mutant. Comparison of the degree of expression of each protein showed that COX-1 and the $\Delta 7aa$ mutants were expressed to similar levels in mammalian cells systems (Fig. 5-2). Transfected CHO and HEK293 cells produced more COX-1 and $\Delta 7aa$ mutant protein than COS-7 and A549 cells (Fig. 5-2). This result was due to different transfection efficiencies as shown by co-transfection with a luciferase reporter gene (data not shown). In all cell lines examined, ectopically expressed $\Delta 7aa$ protein migrated slightly faster than COX-1 in SDS PAGE, reflecting the deletion of amino-terminal sequence or alteration of posttranslational modification. The smaller size of $\Delta 7aa$ protein also indicated that removal of sequence adjoining the signal peptide cleavage site did not prevent co-translational signal peptide removal.

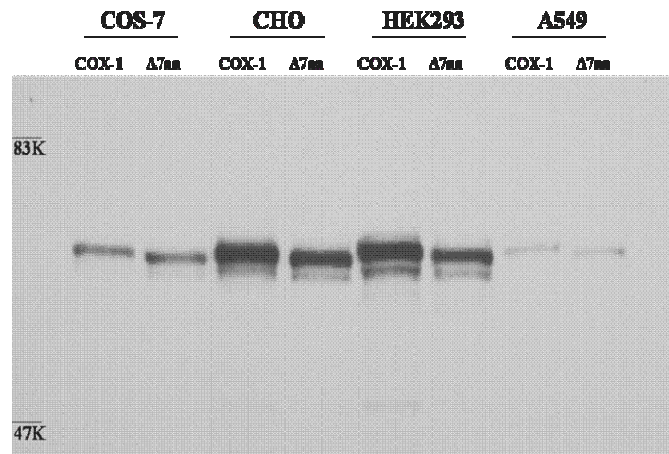


Fig. 5-2. COX-1 and $\Delta 7aa$ protein expression in mammalian cells. Western blots showing the expression of HCOX-1 and $\Delta 7aa$ in COS-7, CHO, HEK293 and A549 cells. Equal amount of protein (15 μ g) from lysate were electrophoresed on each lane. A monoclonal antibody to ovine COX-1 (Cayman Chemical) was used to probe the COX-1 and $\Delta 7aa$ blot.

Other than cleavage of the signal peptide, the only other known posttranslational modification of COX-1 is N-linked glycosylation. Because of the size difference noted in Fig 5-3, as well as the importance of N-linked glycosylation in governing proper COX-1 folding and activity, the glycosylation state of COX-1 and $\Delta 7aa$ mutant was measured in the presence and absence of tunicamycin. As in the previous experiment, $\Delta 7aa$ was smaller than COX-1 in the presence and absence of tunicamycin, indicating that this size difference is due to the deletion of the 7 amino acids and not an effect on posttranslational modification (Fig. 5-3). However, the size difference observed is about 1.3 KDa, which is slightly larger than the calculated size of the deleted seven amino acids (0.6 KDa) (Fig. 5-3).

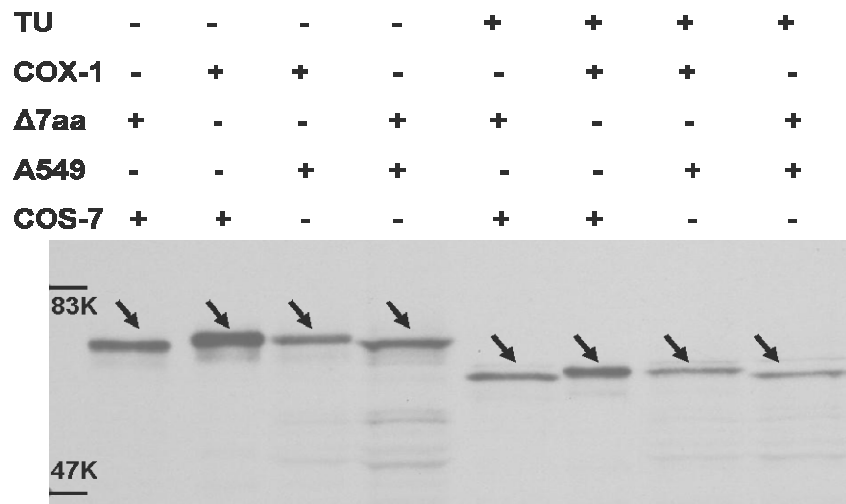


Fig. 5-3. COX-1 and $\Delta 7aa$ over-expression in A549 and COS-7 cells in the presence and absence of tunicamycin. Ectopically expressed COX-1 and $\Delta 7aa$ in A549 and COS-7 cells treated with (+) and without (-) tunicamycin were measured by immunoblotting with anti-COX-1 mAb used a probe. Immunoreactive proteins are indicated by arrows. Because of unequal transfection efficiencies between cell types, the levels of proteins have been adjusted so that they are similar in each lane.

Radioimmunoassay (RIA) on Cyclooxygenase activity of COX-1 and $\Delta 7aa$.

To assess the effects of the $\Delta 7aa$ mutation on cyclooxygenase catalysis, we measured

cyclooxygenase activity for both proteins in the presence of 30 μ M exogenous arachidonic acid. In each assay, the COX activity in one hundred thousand transfected cells was measured and each assay was repeated 5 times. By this method the Δ 7aa mutant was found to produce equal amounts of PGE₂ as COX-1. Furthermore, the PGE₂ made from the Δ 7aa mutant and native COX-1 was significantly higher than that produced by cells transfected with a control plasmid (**P< 0.001) (Fig. 5-4).

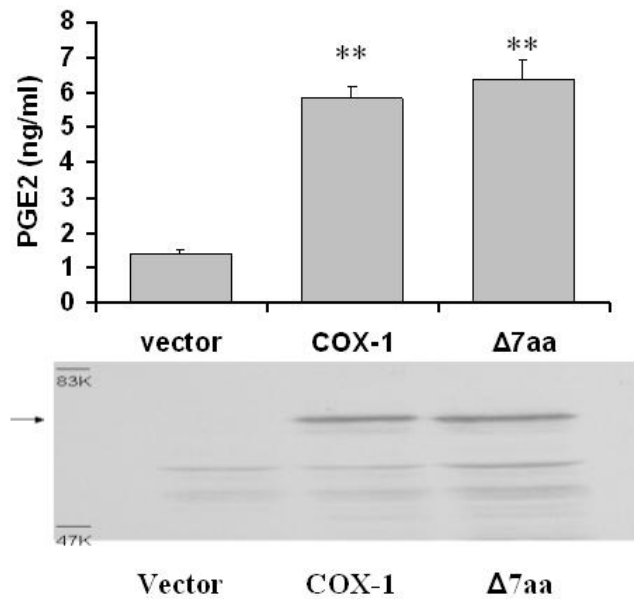


Fig. 5-4. RIA analysis of PGE₂ formed by COX-1 and Δ 7aa. Radioimmunoassay of PGE₂ produced by ectopically expressed COX-1 and Δ 7aa in COS-7 cells was performed with COS-7 cells transfected with pcDNA3.1 empty vector used as negative control. Immunoblot analysis was done to assure that equal amounts of COX-1 and Δ 7aa were used in the RIA (lower panel). Data are expressed as mean \pm SEM ($n = 5$). (**P< 0.001)

Aspirin inhibition assay. Aspirin covalently acetylates the COX active site and COX-1 and COX-2, whose COX active sites differ in dimension and contour, exhibit differences in the ability of aspirin to acetylate them. Therefore, COX-1 and $\Delta 7aa$ were compared in their susceptibility to aspirin acetylation. Enzymes were incubated with various concentrations of aspirin for 30 minutes and were then exposed to exogenously added arachidonic acid (30 μ M) and PGE₂ measured. COX-1 showed the same sensitivity to aspirin as $\Delta 7aa$ (Fig. 5-5).

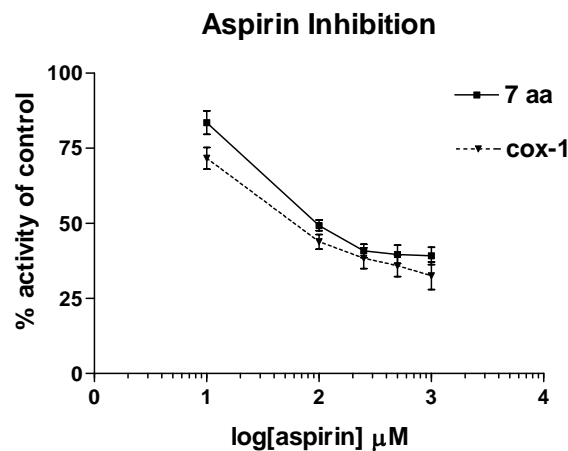


Fig. 5-5. Aspirin inhibition studies. The effect of aspirin on COX-1 (◆) and $\Delta 7aa$ (■) activity in COS-7 cells. Transfected cells were preincubated with aspirin. Activity was measured by the formation of PGE₂ after exposure to exogenous 30 μ M arachidonic acid. Data are expressed as mean \pm SEM ($n = 3$)

Determining K_M and V_{max} value of COX-1 and $\Delta 7aa$. To assess the effects of the mutation on cyclooxygenase catalysis, we also measured the K_M and V_{max} for arachidonic acid (Fig. 5-6). To enable comparison of the V_{max} values obtained in this assay, cell number was carefully quantified, and equal numbers of transfected cells were used in each assay. The determination of V_{max} of COX-1 was 52.3 μ M \pm 3.92 and V_{max}

of $\Delta 7aa$ was $15.9 \mu M \pm 0.86$. The determination of K_M of COX-1 was $1.90 \mu M \pm 0.56$ and K_M of $\Delta 7aa$ was $1.55 \mu M \pm 0.35$.

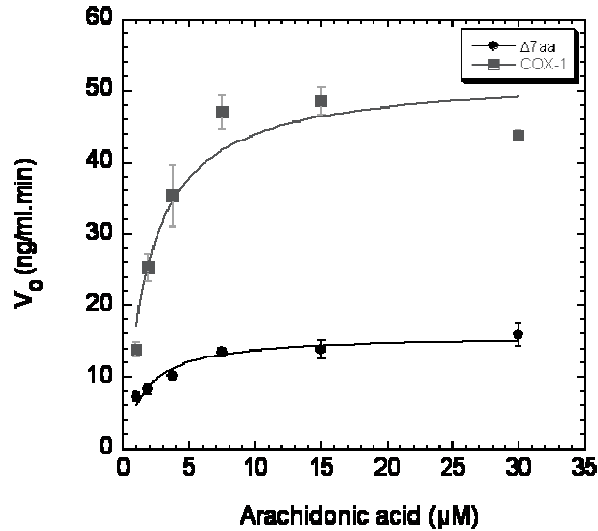


Fig. 5-6. Km-Vmax assay of HCOX-1 and $\Delta 7aa$. K_M and V_{max} value of COX-1 and $\Delta 7aa$ were determined by oxygen uptake and RIA and gave similar results. Only the RIA determined value is shown here. Cyclooxygenase activity was measured with 1.9-30 μM AA and values were fit to the Michaelis-Menten equation. Data are expressed as mean \pm SEM ($n = 3$)

Discussion:

The aim of the current study was to investigate the function of eight amino acids unique to COX-1 which compose the amino-terminal end of the fully processed protein. Removal of seven of these eight amino acids produced a catalytically active COX enzyme that expressed with equal efficiency to COX-1 in four mammalian cell lines. Removal of the signal peptide was not affected in the deletion mutation as evidenced by its smaller molecular weight relative to COX-1. However, the size difference between COX-1 and $\Delta 7aa$ (1.3K) was larger than expected (0.6K) (Fig. 5-3). Slight aberrancy in migration is commonly seen in SDS PAGE which could account for this result. Alternatively, it could result from the removal of an unknown posttranslational

modification that is located in the removed 7 amino acids. The size difference observed is not caused by N- glycosylation, since tunicamycin treated cells show the same size difference as untreated cells. Other possibilities that could theoretically account for this size difference include phosphorylation or O-glycosylation. Therefore the COX-1 sequence was analyzed in NetOGlyc 3.1 Server which analyzes for potential O-glycosylation sites. Prediction results showed that there could be an O-glycosylation modification on Thr-30 that was deleted in $\Delta 7aa$. However, COX-1 has not yet been demonstrated to traffic to the Golgi region where this type of modification would likely occur.

In most respects the polypeptide produced from the deletion mutation was functionally the same as COX-1. Equal amounts of PGE₂ were made from cyclooxygenase activity of COX-1 and $\Delta 7aa$ in COS-7 cells and both enzymes were equally sensitive toward aspirin. Kinetically, there is no significant difference between the K_M value for COX-1 and the deletion mutation, but the native COX-1 enzyme had a V_{max} value that was 4-fold higher than that of the mutant, indicating an affect of this sequence on catalysis. Paradoxically the enzyme activity of COX-1 and $\Delta 7aa$ in the presence of near-saturating concentrations of arachidonic acid (30 μ M) is essentially identical when measured for 10 minutes (Fig. 5-4). Cyclooxygenases undergo fairly rapid autoinactivation during catalysis (1). Thus, although $\Delta 7aa$ exhibits a lower V_{max} than COX-1 when instantaneous velocities are measured, it may be more long lived as a catalyst.

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Appendix I

Screening large numbers of recombinant plasmids: modifications and additions to alkaline lysis for greater efficiency

Selecting bacteria transformed with recombinant plasmid is a laborious step in gene cloning experiments. This selection process is even more tedious when large numbers of clones need to be screened. We describe here modifications to the ultra fast plasmid preparation method described previously by Law and Crickmore (1). The modified method is coupled to an efficient PCR step to rapidly determine orientation of the inserts. Compared to traditional methods of analysis requiring growth of overnight cultures, plasmid isolation and restriction enzyme digestion to determine orientation (2) this procedure allows for the analysis and storage of a large number of recombinants within a few hours.

Law and Crickmore described a simplified alkaline lysis procedure. Briefly, recombinant bacterial colonies (~1 mm in size) growing on agar plates are picked into 100µl of pre-warmed lysis buffer (5 mM EDTA, 10% w/v sucrose, 0.25% SDS 100 mM NaOH, 60 mM KCl and 0.05% bromophenol blue) and incubated at 37°C for 5 min. The tube is then placed on ice for 5 min followed by centrifugation in a microfuge for 1min. Supernatant (20 µl) is then electrophoresed on an agarose gel, where the electrophoretic mobility of potential recombinants is compared with a plasmid control (vector without an insert). Recombinant plasmids typically exhibit slower migration in the gel compared to vector.

This method requires centrifugation and has the drawback of RNA contamination resulting from incomplete alkaline hydrolysis. When gels are used with

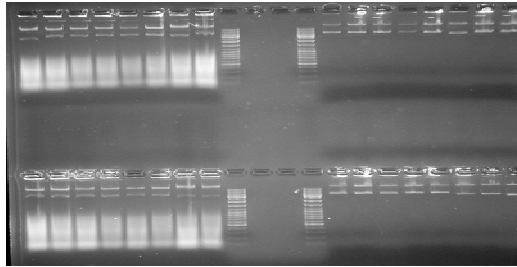
multiple rows of wells, this low molecular weight undegraded RNA tends to migrate through the wells in front of them to mask plasmid DNA (Fig. A-1a). We have found that pancreatic RNase is still catalytically active in the alkaline lysis buffer and can be used to remove this unwanted RNA. This modification also avoids the cumbersome steps of chilling and centrifugation, greatly shortening the procedure and making it more useable in microtiter plate format (Fig. A-1b).

To further greatly streamline plasmid isolation, orientation of inserts, and long term storage of the clones, we use the following process.

Rather than inoculate bacterial colonies directly into lysis buffer (which requires one to go back to the original plates for further operation), bacterial colonies are picked into 15 μ l of phosphate buffered saline (PBS) in Eppendorf tubes containing an appropriate antibiotic. The bacterial suspension in PBS is mixed well and 10 μ l is added to 10 μ l of our modified lysis buffer (MLB), 10mM EDTA, 20% w/v sucrose, 0.5% SDS, 200mM NaOH, 120mM KCl, 0.1% bromophenol blue and 0.2mg/ml RNase. This is simply incubated for 5 min at 37 °C and electrophoresis is performed by the method of Law & Crickmore. Either during electrophoresis or after identification of recombinant clones, the orientation of clones is determined by PCR amplifying 1.0 μ l of the original bacterial suspension in PBS using two primers, one in the vector and the other in the insert (Fig. A-2). PCR is done with short extension time (12s) and only clones in the proper orientation synthesize a product. Because of the short extension time, PCR amplification (40 cycles) is completed in 1 hour. Desirable clones identified by this method can then be amplified in large scale cultures by inoculating 1 μ l bacteria from the original bacterial suspension in PBS. For long term storage, 200 μ l of sterile 50%

glycerol (in LB) is added to the remaining 2-3 μ l of bacterial suspension in PBS, mixed well by briefly vortexing, and stored at -80 °C. When a very large number of clones need to be analyzed the procedure can be carried out in 96 well micro titer plates (Fig. A-1b). From bacterial colonies to long term storage of analyzed plasmids takes three hours and the process can be robotically mechanized.

1a



1b

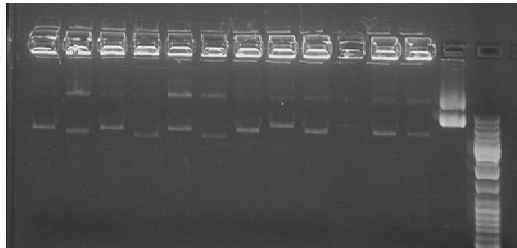


Fig. A-1. Comparison of clones analyzed by Law and Crickmore and modified methods. 1a.

Comparison of bacterial colonies analyzed by the original method (left side lanes 1-16,) and by the modified method (right side lanes 1-16). Electrophoreses was deliberately done for a short period so that long smears of low molecular weight of undegraded RNA are observed (on left side of the gel) containing plasmid DNA isolated by Law and Crickmore. Upon longer electrophoreses to detect mobility difference, these smears tend to migrate through the wells in front of them to mask the migrating plasmids. Plasmids on the right are by the modified method. **1b.** Clones analyzed by the modified method using 96 well micro titer plates. Putative recombinant clones were analyzed by the modified method using 96 well plates. For this procedure the incubation at 4 °C and the subsequent centrifugation step was omitted.

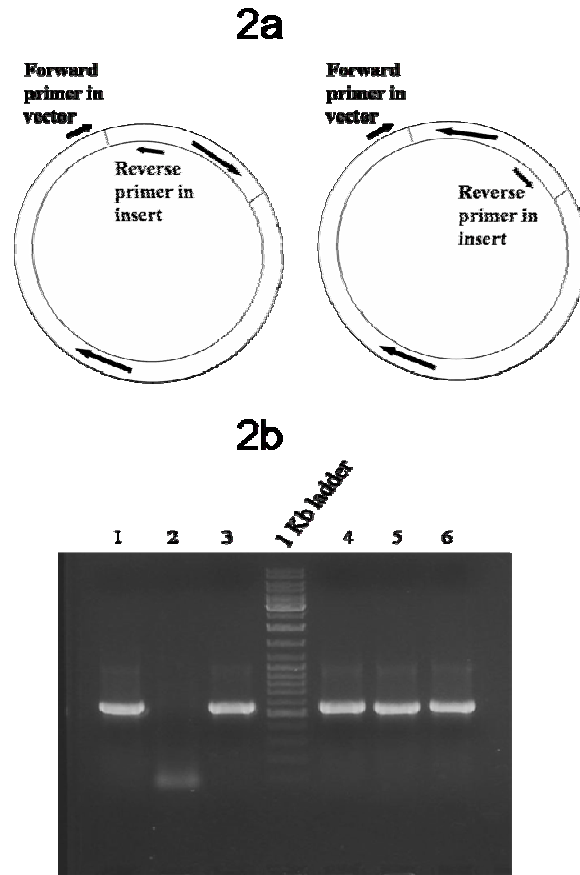


Fig. A-2. PCR based analysis of recombinant clones. **2a:** Recombinant clones that carried an insert were analyzed by PCR using 1.0 μ l of the remaining bacterial suspension as the template. Orientation of the insert was determined by PCR amplifying 1.0 μ l of the original bacterial suspension in PBS using two primers, one in the vector and the other in the insert. **2b:** An amplicon of 550 bp indicates that the clone is in the right orientation (lane 1, 3, 4, 5 and 6 of Fig. A-2a). An insert in the reverse orientation will not give an amplification product (lane 2, Fig. A-2a).

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