PHARM REV

Cyclooxygenase Isozymes: The Biology of Prostaglandin Synthesis and Inhibition

DANIEL L. SIMMONS, REGINA M. BOTTING, AND TIMOTHY HLA

The Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah (D.L.S.); The William Harvey Research Institute, St. Bartholomew's and William and Mary Medical College, London, England (R.M.B.); and Center for Vascular Biology, Department of Physiology, University of Connecticut School of Medicine, Farmington, Connecticut (T.H.)

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Address correspondence to: Daniel L. Simmons, Department of Chemistry and Biochemistry, E280 BNSN, Brigham Young University, Provo, UT 84604. E-mail: dan_simmons@byu.edu

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Abstract——Nonsteroidal anti-inflammatory drugs (NSAIDs) represent one of the most highly utilized classes of pharmaceutical agents in medicine. All NSAIDs act through inhibiting prostaglandin synthesis, a catalytic activity possessed by two distinct cyclooxygenase (COX) isozymes encoded by separate genes. The discovery of COX-2 launched a new era in NSAID pharmacology, resulting in the synthesis, marketing, and widespread use of COX-2 selective drugs. These pharmaceutical agents have quickly become established as important therapeutic medications with potentially fewer side effects than traditional NSAIDs. Additionally, characterization of the two COX isozymes is allowing the discrimination of the roles each play in physiological processes such as homeostatic maintenance of the gastrointestinal tract, renal function, blood clotting, embryonic implantation, parturition, pain, and fever. Of particular importance has been the investigation of COX-1 and -2 isozymic functions in cancer, dysregulation of inflammation, and Alzheimer's disease. More recently, additional heterogeneity in COX-related proteins has been described, with the finding of variants of COX-1 and COX-2 enzymes. These variants may function in tissue-specific physiological and pathophysiological processes and may represent important new targets for drug therapy.

I. Cyclooxygenase Isozymes

A. Prostaglandins and Cyclooxygenase

Prostaglandins, potent bioactive lipid messengers derived from arachidonic acid (AA[1]), were first extracted from semen, prostate, and seminal vesicles by Goldblatt

[1]Abbreviations: AA, arachidonic acid; AD, Alzheimer's disease; CLASS, Celecoxib Long-Term Arthritis Safety Study; COX, cyclooxygenase; EP, prostaglandin E receptor; ER, endoplasmic reticulum; FDA, U.S. Food and Drug Administration; GFR, glomerular filtration rate; GI, gastrointestinal; HETE, hydroxyeicosatetraenoic acid; IFN, interferon; IL, interleukin; kb, kilobase(s); LDS, linoleate diol synthase; LPS, lipopolysaccharide; MELISSA, Meloxicam Large-Scale International Study Safety Assessment; MI, myocardial infarction; NFκB, nuclear factor-κB; NO, nitric oxide; NSAID, nonsteroidal anti-inflammatory drug; OA, osteoarthritis; PG, prostaglandin; PGHS, prostaglandin H synthase; PCOX, partial COX; PIOX, pathogen-inducible oxygenase; POB, perforation, obstruction, or bleed; POX, peroxidase; PPAR, peroxisome proliferator-activated receptor; PUB, perforation, ulcer, or bleed; RA, rheumatoid arthritis; SELECT, Safety and Efficacy Large-Scale Evaluation of COX-Inhibiting Therapies; SNP, single nucleotide polymorphism; TALH, thick ascending limb of Henle; TNF, tumor necrosis factor; TX, thromboxane; UTR, untranslated region; VEGF, vascular endothelial growth factor; VIGOR, Vioxx Gastrointestinal Outcomes Research Study.

and von Euler in the 1930s and shown to lower blood pressure and cause smooth muscle contraction. Bergström and colleagues purified the first prostaglandin isomers during the 1950s and 60s, and in 1964, van Dorp et al. and Bergstrom et al. independently identified AA, a 20-carbon tetraenoic fatty acid ($C20:4\omega6$) as the precursor to prostaglandins. The cyclooxygenase reaction through which AA is enzymatically cyclized and is oxygenated to yield endoperoxide-containing prostaglandin G_2 (PGG_2) was later identified by Samuelsson and colleagues (Hamberg and Samuelsson, 1973; Hamberg et al., 1974) (Fig. 1). The enzyme, cyclooxygenase (COX) that catalyzes this cyclooxygenation reaction also reduces a hydroperoxyl in PGG_2 to a hydroxyl to form PGH_2 via a separate peroxidase active site on the enzyme. Isomerases and oxidoreductases produce various bioactive prostaglandin isomers using PGH_2 as substrate as shown in Fig. 1.

Until 1976 (Hemler and Lands, 1976; Miyamoto et al., 1976), when purified COX preparations were first described, tissue homogenates were used as a source of COX enzyme activity, which was frequently referred to
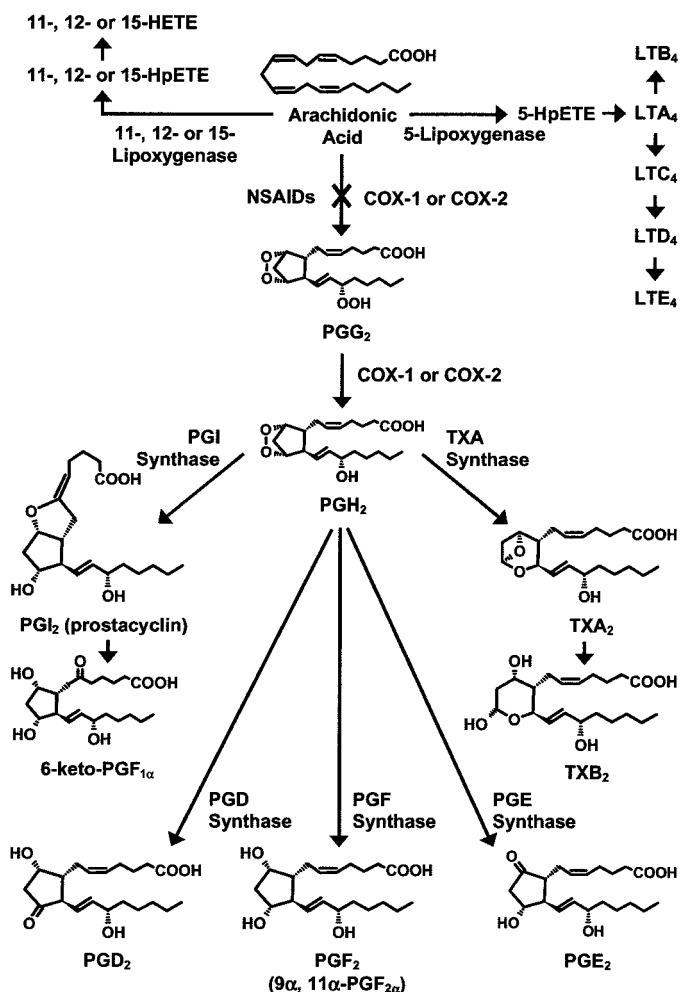
FIG. 1. The arachidonic acid cascade. The fate of arachidonic acid in cells as it is metabolized by lipoxygenases to HETEs and hydroperoxyeicosatetraenoic acids (HPETEs) or by cyclooxygenases to prostaglandin H_2 via the short-lived hydroperoxyl-containing intermediate prostaglandin G_2. NSAIDs block the synthesis of prostaglandin G_2. Prostaglandin H_2 spontaneously rearranges or is enzymatically isomerized, oxidized, or reduced to yield bioactive prostaglandin isomers, some of which are shown. [Reprinted with permission from *Clinician's Manual on COX-2 Inhibition* second edition (Vane JR, Botting R, Emery P, and Hawkey CJ, eds) Science Press Ltd., London, 2002].

at that time as a prostaglandin synthetase. Because the COX enzyme reaction does not require ATP, the nomenclature was later changed to synthase. The COX enzyme, also known as prostaglandin H synthase (PGHS) or prostaglandin endoperoxide synthase (E.C. 1.14.99.1), was identified as the major enzyme in the oxidative conversion of AA to PGG_2 and PGH_2 (Smith and Lands, 1972; Hamberg et al., 1974), with seminal vesicles of sheep, bovines being a rich enzyme source (Smith and Lands, 1972). Thus, purification of PGHS enzyme to homogeneity was first achieved from the sheep (Hemler and Lands, 1976; van der Ouderaa et al., 1977) and bovine (Miyamoto et al., 1976; Ogino et al., 1978) seminal vesicles. This purified enzyme migrated in the region of approximately 67 kDa in SDS-polyacrylamide gel electrophoresis and contained cyclooxygenase and peroxidase activities, which were later found to be

at separate sites (Marshall and Kulmacz, 1988). Since detergents such as Tween 20 were needed to solubilize the enzyme, it was classified as an integral microsomal membrane protein.

In 1971, John Vane used a cell-free homogenate of guinea pig lung to demonstrate that aspirin, indomethacin, and salicylate, popular nonsteroidal anti-inflammatory drugs (NSAIDs), were inhibitors of this enzyme—thus defining the mechanism of action of this important class of drugs.

B. Early Evidence for Multiple Cyclooxygenases

Researchers, beginning in the early 1970s, speculated on whether there was more than one COX enzyme. Flower and Vane (1972) postulated the existence of an acetaminophen-inhibitable COX activity that was in dog brain but not in rabbit spleen. The same year, two catalytically distinct prostaglandin synthase activities were reported to be present in acetone powder extracts of sheep vesicular glands (Smith and Lands, 1972). Studies of autoinactivation rates of COX, inhibition by NSAIDs, and time course profiles of PGE_2 and $PGF_{2\alpha}$ synthesis led Lysz and colleagues (1982, 1988) to propose that rabbit and mouse, but not rat brain, contained two forms of COX.

It was, however, through the study of prostaglandin induction by mitogens and proinflammatory agents, as well as prostaglandin down-regulation by glucocorticoids, that the most provocative data regarding the potential of more than one COX were obtained. The phenomenon that was observed by many laboratories was that prostaglandin synthesis and release in some situations, such as in activated platelets, occurs within a few minutes after stimulation. In other cases, such as in mitogen-stimulated fibroblasts, prostaglandin synthesis may take hours to occur. In 1985, Habenicht and colleagues (1985) reported that platelet-derived growth factor treatment of Swiss 3T3 cells resulted in an early (10 min) and a late (2–4 h) peak in induction in prostaglandin synthesis. Only the late peak was blocked by cycloheximide, leading to the conclusion that platelet-derived growth factor-stimulated PG synthesis occurred by "direct effects on the PG-synthesizing enzyme system, one involving a protein synthesis-independent mechanism and another that requires rapid translation of cyclooxygenase". The activities revealed in Habenicht's early study were indicative of an endogenous COX enzyme (COX-1) and an inducible enzyme (COX-2). Many other laboratories at this time did similar studies on induction of prostaglandin synthesis, but with only nucleic acid and antibody probes to the seminal vesicle form of COX to work with, many investigators observed the paradoxical phenomenon that, in many instances, prostaglandin induction occurred without an increase in the seminal vesicle COX—an enzyme which was found to be present in most cells and tissues investigated (DeWitt and Smith, 1988). Frequently only marginal increase in sem-

inal vesicle COX was observed despite robust increase in PG synthesis. Similar anomalies in which PG synthesis and seminal vesicle COX did not coincide were observed with regard to the action of glucocorticoids, which strongly decreased PG synthesis but typically had little to no effect on seminal vesicle COX levels. Various postulates were proposed that were consistent with these observed phenomena, the most common of which was that changes in substrate delivery were responsible for these fluctuations in PG synthesis.

In 1989, Rosen et al. used low-stringency Northern blot hybridization with an ovine seminal vesicle COX cDNA as probe to detect a 4.0-kb RNA, in addition to the known 2.8-kb mRNA encoding seminal vesicle COX. This 4.0-kb mRNA was inducible and paralleled induction of enzyme activity. These investigators concluded that "the larger mRNA may encode for a cyclooxygenase" encoded by a distinct gene. In 1990, Needleman and colleagues (Fu et al., 1990) studying lipopolysaccharide (LPS) stimulation of monocytes concluded that these "cells may contain two pools of COX, each with a differential sensitivity to LPS or DEX (dexamethasone)." During this time, Young, Macara, and colleagues (Han et al., 1990) identified, by using giant two-dimensional protein gel electrophoresis, proteins immunoreactive with COX-1 antibodies that were induced in v-*src*-transformed cells. The evidence in these and other early studies was consistent with distinct inducible and constitutive COX isozymes encoded by separate genes but was also compatible with other explanations.

C. Studies of Cell Division and the Discovery of Cyclooxygenase-2

The answer to the mechanism of how COX enzyme activity rapidly increases PG induction in inflammation and in other physiological contexts came from studies of cell division. In the late 1980s, Simmons et al. (1989) and Herschman (Varnum et al., 1989) and colleagues independently identified immediate-early genes in fibroblast-like cells activated by mitogens. Genes found by Simmons in chicken (Simmons et al., 1989; Xie et al., 1991) and mouse (Simmons et al., 1991) were activated by the v-*src* oncogene, phorbol esters and serum. Herschman used Swiss 3T3 cells to identify tetradecanoyl-13-phorbol acetate-inducible sequences (or TIS genes), which were also induced by other mitogens (Varnum et al., 1989; Kujubu et al., 1991). In 1991, each laboratory independently reported that one of their sequences encoded a new inducible COX enzyme. Also contributing to the identification of COX-2 in 1991, Young and colleagues (O'Banion et al., 1991) reported a partial predicted sequence of COX-2 from a murine cDNA. The inducible enzyme cloned in these studies is now most frequently referred to as COX-2 and the seminal vesicle form of the enzyme as COX-1.

Herschman and colleagues expressed the mouse TIS10 cDNA in heterologous cells and showed that in-

creased prostaglandin E_2 synthesis was induced by this cDNA (Fletcher et al., 1992). Ectopic overexpression was also done by Young's laboratory (O'Banion et al., 1992) and Meade et al. (1993), who also demonstrated the importance of the 3'-untranslated region of the COX-2 mRNA in governing overexpression of the enzyme.

Using mouse COX-2 sequences as probe, Hla and Neilson (1992) identified and published the sequence of the human homolog of TIS10/CEF147, which they named COX-2. Overexpression of human COX-2 cDNA in Cos cells also induced COX enzymatic activity, similar to that of TIS10, and this activity was inhibited by nonsteroidal anti-inflammatory drugs (Hla and Neilson, 1992). The human COX-2 cDNA was widely expressed as an inducible gene in nonimmortalized vascular and inflammatory cells.

D. Structure of Cyclooxygenase-1 and Cyclooxygenase-2

Pure preparations of the COX-1 enzyme obtained from seminal vesicles were instrumental in the elucidation of the primary structure of this enzyme by molecular cloning. Both the N terminus and the internal sequences following limited trypsin digestion of the sheep seminal enzyme were reported (Chen et al., 1987). Roth and colleagues (1975), using sheep and bovine seminal vesicle enzyme preparations, showed that aspirin acetylated the COX enzyme. The region of the active site residues and the determination of the serine acetylated by aspirin were elucidated by sequencing ^{3}H-aspirin-labeled peptides of the sheep seminal vesicle enzyme (Roth et al., 1980; van der Ouderaa et al., 1983; DeWitt et al., 1990); however, molecular cloning by three different laboratories ultimately elucidated the complete primary structure of the COX-1 enzyme (DeWitt and Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988).

Sequence analysis of COX-1 cDNAs indicated that they contained an open reading frame of ~1.8 kb, which contained all the polypeptide sequences from protein microsequencing efforts (Roth et al., 1980, 1983; DeWitt and Smith, 1988), including the aspirin acetylation site (DeWitt et al., 1990). These data strongly suggested that the isolated cDNA clone encoded the sheep seminal vesicle COX enzyme. In addition to ovine COX-1, the gene for the human homolog of this enzyme was also cloned (Yokoyama and Tanabe, 1989). These cloning efforts were followed by the demonstration that the cDNA for sheep seminal vesicle COX exhibited both cyclooxygenase and peroxidase activities upon ectopic overexpression in mammalian and insect cells (DeWitt et al., 1990; Funk et al., 1991; Shimokawa and Smith, 1992a,b; Meade et al., 1993). Mutagenesis experiments were conducted to identify critical residues for catalysis, such as the heme coordination sites, aspirin acetylation sites, etc. (DeWitt, 1990; Shimokawa et al., 1990; Shimokawa and Smith, 1992b)

Northern blot analysis with cDNA probes of COX-1 identified a major mRNA species of 2.8 kb and a minor

species of 5.2 kb in human endothelial cells (Hla, 1996). Further sequence analysis of a human endothelial cell-derived cDNA, which encoded the 3′-end of the 5.2-kb mRNA, indicated that the 5.2-kb cDNA and the 2.8-kb cDNAs represent alternatively polyadenylated mRNA species with differing lengths of the 3′-UTR (Hla, 1996). These alternative polyadenylation states were also confirmed in cDNAs encoding the 3′ of the 5.2-kb mRNA from a human megakaryoblastic cell line (Plant and Laneuville, 1999).

The predicted amino acid sequences of COX-2 cloned in chicken and mammals showed it to possess approximately 60% amino acid identity with COX-1 (Simmons et al., 1991). COX-1 and COX-2 were found to be approximately 600 amino acids in size in all species.

The COX-1 and/or -2 cDNA sequences from many organisms, including bony and cartilaginous fish, birds, and mammals have been characterized. Furthermore COX genes appear to be expressed in invertebrates, such as coral and sea squirts, where two COXs have been identified in two different species of each phylum (Valmsen et al., 2001, Jarving et al., 2004). These data suggest that the cyclooxygenase pathway was present in early invertebrate speciation in the animal kingdom. Known COX sequences are aligned in Fig. 2, and domains and residues essential to their function are designated. From an evolutionary standpoint COX-1 and COX-2 appear to have resulted from a gene duplication event that occurred early in or before vertebrate speciation (Fig. 3, courtesy of G. A. Fitzgerald and T. Grosser) as reviewed recently by Jarving et al. (2004).

Cyclooxygenases in unicellular organisms, insects, or the plant kingdom have not been identified; however, COX enzymes have recently been determined to be members of a larger fatty acid oxygenase family that includes pathogen-inducible oxygenases (PIOXs). These latter enzymes have been identified in monocotyledon and dicotyledon plants, *Caenorhabditis elegans*, and bacteria (*Pseudomonas*) (Fig. 4). Like COXs, PIOXs oxygenate polyunsaturated fatty acids using molecular oxygen (Sanz et al., 1998; Hamberg et al., 1999; Koeduka et al., 2000). They also introduce a hydroperoxyl moiety into the fatty acid, which is introduced at the α carbon by PIOXs to form $2R$-hydroperoxy fatty acids. Generation of α-peroxyl-fatty acids by PIOXs has been proposed to be a signaling response in these organisms to activate genes needed for antipathogen defense (Sanz et al., 1998).

COXs and PIOXs share approximately 30% sequence identity, and PIOXs contain conserved critical residues needed for fatty acid oxygenation seen in COX. Sequence similarity to COXs in the region of the Tyr385 has also been found in the enzyme linoleate diol synthase (LDS) from fungus (Oliw et al., 1998; Su et al., 1998; Hörnsten et al., 1999). This enzyme is a homotetrameric ferric hemeprotein that catalyzes the dioxygenation of linoleic acid to $(8R)$-hydroperoxylinoleate and isomerization of

this latter compound to $(7S,8S)$-dihydroxylinoleate. Like COXs, the enzyme is known to form ferryl intermediates and a tyrosyl radical. PIOXs and LDSs are clearly related to peroxidases; however, there is no evidence that PIOXs or LDS possess peroxidase activity. These findings lead to the conclusion that PIOXs, LDS, and COXs each represent distinct subfamilies of fatty acid oxygenases that are descended from ancient peroxidases. If they descend from a common peroxidase progenitor, LDS, PIOXs and COXs have additionally gained hydrophobic pockets for binding and oxygenation of fatty acid substrates; however, the PIOX and LDS branches of this fatty acid oxygenase family have a degenerate peroxidase active site, that likely functions solely to activate the enzyme. PIOXs and LDSs, therefore, are predicted to perform primarily the dioxygenation reaction typical of the COX active site (see below). Since PIOXs and LDS are found in plants, bacteria, fungus, and lower animals, the fatty acid oxygenase progenitor of COXs and PIOXs is predicted to exist very early in evolution, underscoring the concept that generation of oxygenated fatty acids by these enzymes represents an evolutionarily ancient mechanism of cell signaling.

Recently a cyclooxygenase enzyme from the protozoan *Entamoeba histolytica* has been identified that lacks structural similarity with other COXs, PIOXs, or LDS enzymes, but makes PGE_2 from arachidonic acid (Dey et al., 2003). Therefore, prostaglandin-synthesizing enzymes distinct from the COX lineage characterized in vertebrates, coral, and sea squirts appear to have arisen during speciation of some organisms.

Landmark studies by Garavito and colleagues (Picot et al., 1994) elucidated the tertiary and quaternary structure of COX-1. Early studies in the 1970s showed that COX-1 was likely a dimer and was tightly bound to microsomal membranes; however, the topology of the enzyme in microsomal membranes was unknown. At crystallographic resolution, Garavito's studies described COX-1's distinct domains for dimerization, membrane binding, and catalysis. A fourth domain, the N-terminal signal peptide, which is clearly evident in the primary structure of COX-1, was not observed because this sequence is cotranslationally cleaved from the nascent polypeptide by microsomal signal peptidase.

Crystallographic structures of COX-2 have been obtained by Luong et al. (1996), Bayly et al. (1999), and Kurumbail et al. (1996) and show striking similarity with COX-1. In fact, all known COX enzymes share the same functional domains. Outstanding reviews of COX structure and enzyme kinetics have recently been written (Garavito and DeWitt, 1999; Marnett, 2000; Smith et al., 2000) and thus only the essential aspects of these topics needed to understand the pharmacology of NSAIDs are discussed here.

The structures of COX-1 and COX-2 predict that both enzymes are located in the lumen of the nuclear enve-

Sequence Alignment of COX isoforms

```
                  1                                                    50
    HumanCOX1     .......... .......... ..........  MSR.SLLLRF LLFLLLL...
    SheepCOX1     .......... .......... ..........  MSRQSISLRF PLLLLLL...
   CanineCOX3     MSREFDPEAP RNPLRLPGEP RMPGPALTSR  SAGGSRLHRW PLLLLLLLLL
   CanineCOX1     .......... .......... ..........  MSRGSRLHRW PLLLLLLLLL
   RabbitCOX1     .......... .......... .......MSR  SSPSLRLPVL LLLLLLLLLP
    MouseCOX1     .......... .......... ..........  MSRRSLSLWF PLLLLLLL.P
      RatCOX1     .......... .......... ..........  MSRRSLSLQF PLLLLLLL.L
   RainbowCOX1    .....MSAAY IIFALLYWED APAEGAYAVN  LTMREC.VVW VWACILLQRL
    BrookCOX1     .......... .......... ..........  ..MRGL.VVC VWACILLQRL
    ZfishCOX1     .......... .......... ..........  ..MRECNFLL KWTVILLLSV
     SharkCOX     .......... .......... ..........  .........M EAARIILLLL
    HumanCOX2     .......... .......... ..........  .......... ........ML
   CanineCOX2     .......... .......... ..........  .......... ........ML
    HorseCOX2     .......... .......... ..........  .......... ........ML
   RabbitCOX2     .......... .......... ..........  .......... ........ML
 GuineaPigCOX2    .......... .......... ..........  .......... ........ML
    SheepCOX2     .......... .......... ..........  .......... ........ML
      CowCOX2     .......... .......... ..........  .......... ........ML
    MouseCOX2     .......... .......... ..........  .......... ........ML
      RatCOX2     .......... .......... ..........  .......... ........ML
     MinkCOX2     .......... .......... ..........  .......... ........ML
  ChickenCOX2     .......... .......... ..........  .......... ........ML
   RainbowCOX2    .......... .......... ..........  .......... .....MNRVI
    BrookCOX2     .......... .......... ..........  .......... .....MNKVV
 PhomomallaCOXA   .......... .......... ..........  .......... ..MKAFLHIV
 GfruticosaCOXA   .......... .......... ..........  .......... ...MVAKFVV
 GfruticosaCOXB   .......... .......... ..........  .......... ...MVGG GNGMSVKLVV
  CaninePCOX1a    MSREFDPEAP RNPLRLPGEP RMPGPALTSR  SAGGSRLHRW PLLLLLLLLL


                  51                                                   100
    HumanCOX1     PPLPVLLADP GAPTPVNPCC YYPCQHQGIC  VRFGLDRYQC DCTRTGYSGP
    SheepCOX1     SPSPVFSADP GAPAPVNPCC YYPCQHQGIC  VRFGLDRYQC DCTRTGYSGP
   CanineCOX3     PPPPVLPAEA RTPAPVNPCC YYPCQHQGIC  VRFGLDRYQC DCTRTGYSGP
   CanineCOX1     PPPPVLPAEA RTPAPVNPCC YYPCQHQGIC  VRFGLDRYQC DCTRTGYSGP
   RabbitCOX1     PPPPVLPADP GAPAPVNPCC YFPCQHQGVC  VRVALDRYQC DCTRTGYSGP
    MouseCOX1     PTPSVLLADP GVPSPVNPCC YYPCQNQGVC  VRFGLDNYQC DCTRTGYSGP
      RatCOX1     PPPPVLLTDA GVPSPVNPCC YYPCQNQGVC  VRFGLDHYQC DCTRTGYSGP
   RainbowCOX1    PTCRGEEVED AST.VVNPCC YYPCQNWGVC  VRFGIDRYEC DCTRTGFYGQ
    BrookCOX1     PTCRGEEVKD VSTNVVNPCC YYPCQNWALC  VRFGIDRYEC DCTRTGFYGQ
    ZfishCOX1     SFCAGEE.SP TSSNTANPCC YYPCQNQGIC  VRYGLERYEC DCTRTGYYGE
     SharkCOX     PLCFLKMADT TA...INPCC YYPCQNKGIC  VNVGKEGYEC DCTRTGYYGV
    HumanCOX2     ARALLLCAVL ALSHTANPCC SHPCQNRGVC  MSVGFDQYKC DCTRTGFYGE
   CanineCOX2     ARALVLCAAL AVVRAANPCC SHPCQNQGIC  MSTGFDQYKC DCTRTGFYGE
    HorseCOX2     ARALLLCVAL ALGHAANPCC SNPCQNRGVC  MSVGFDQYQC DCTRTGFYGE
   RabbitCOX2     ARALLLCAAV ALSHAANPCC SNPCQNRGVC  MTMGFDQYKC DCTRTGFYGE
 GuineaPigCOX2    ARALLLCAAL ALGQAANPCC SNPCQNRGEC  LSVGFDRYKC DCTRTGYYGE
    SheepCOX2     ARALLLCAAV .VCGAANPCC SHPCQNRGVC  MSVGFDQYKC DCTRTGFYGE
      CowCOX2     ARALLLCAAV ALSGAANPCC SHPCQNRGVC  MSVGFDQYKC DCTRTGFYGE
    MouseCOX2     FRAVLLCAAL GLSQAANPCC SNPCQNRGEC  MSTGFDQYKC DCTRTGFYGE
      RatCOX2     FRAVLLCACP GLSHAANPCC SNPCQNRGEC  MSIGFDQYKC DCTRTGFYGE
     MinkCOX2     ARAGLLCASL SPPHAANPCC SNPCQNQGVC  MSIGFDQYMC DCSRTGFYGE
  ChickenCOX2     LPCALLAALL AAGHAANPCC SLPCQNRGVC  MTTGFDRYEC DCTRTGYYGE
   RainbowCOX2    CIILLLAVGL YFCEGVDPCC AQPCENRGLC  NSKGFDNYEC DCTRTGYYGK
    BrookCOX2     CIILLLTVGL YFCEGVDPCC AQPCENRGLC  NSKGFDNYEC DCTRTGYYGK
 PhomomallaCOXA   VLIIFSGLFW HEVESVNPCC SFPCENGAVC  VDDG.DTYTC DCTRTGYYGV
 GfruticosaCOXA   FLGLQLILCS VVCEAVNPCC SFPCESGAVC  VEDG.DKYTC DCTRTGHYGV
 GfruticosaCOXB   FLGFQMALCS VLCGAVNPCC SFPCENGAVC  VDDG.DEYTC DCTRTGHHGV
  CaninePCOX1a    PPPPVLPAEA RTPAPVNPCC YYPCQHQGIC  VRFGLDRYQC DCTRTGYSGP
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EGF-like domain/dimerization domain (1)

FIG. 2. Alignment of COX isoenzymes. Known COX sequences that are complete are aligned. Signal peptide sequences, potential glycosylation sites, and some important residues that function in catalysis are in bold. Sequence encoded by intron-1 in COX-3 and PCOX-1a is underlined. Dimerization and membrane binding domains are denoted with a heavy underline. All sequence carboxyterminal to dimerization domain 2 constitutes the catalytic domain.

lope and endoplasmic reticulum. Structural aspects of each of the four domains (shown in Fig. 2 and the crystallographic structures of Fig. 5) of COX-1 and COX-2 lead to this conclusion.

1. Amino-Terminal Signal Peptide. Nascent COX-1 and COX-2 polypeptides are directed into the lumen of the endoplasmic reticulum by amino-terminal signal peptides. Although cleaved from the nascent polypep-

Glycosylation site   101   Potential Glycosylation site (COX1)   150

```
   HumanCOX1  NCTIPGLWTW LRNSLRPSPS FTHFLLTHGR WFWEFVNA.T FIREMLMRLV
   SheepCOX1  NCTIPEIWTW LRTTLRPSPS FIHFMLTHGR WLWDFVNA.T FIRDTLMRLV
  CanineCOX3  NCTIPELWTW LRNSLRPSPS FLHFLLTHGR WFWEFINA.T FIRDMLMRLV
  CanineCOX1  NCTIPELWTW LRNSLRPSPS FLHFLLTHGR WFWEFINA.T FIRDMLMRLV
  RabbitCOX1  NCTVPDLWTW LRSSLRPSPT FVHYLLTHVR WFWEFVNA.T FIRDTLMRLV
   MouseCOX1  NCTIPEIWTW LRNSLRPSPS FTHFLLTHGY WLWEFVNA.T FIREVLMRLV
     RatCOX1  NCTIPEIWTW LRSSLRPSPS FTHFLLTHGY WIWEFVNA.T FIREVLMRLV
 RainbowCOX1  NCTIPEFWTR VHQQLKPSPD VVHYILTHFH WLWNLINR.T FMRDWLMRVV
   BrookCOX1  NCTIPEFWTR IHQQLKPSPD VVHYILTHFH WLWNLINR.T FMRDWLMRMV
   ZfishCOX1  NCTIPELWTR VYRLLKPSPN VVHYILTHFD WLWDLINR.S FLRDWLMRKV
    SharkCOX  NCTFPFSWSR VHKFLKPSPS SMHHVLTHYK WLWYIINNIS FFSDTLMRLV
   HumanCOX2  NCSTPEFLTR IKLFLKPTPN TVHYILTHFK GFWNVVNNIP FLRNAIMSYV
  CanineCOX2  NCSTPEFLTR IKLYLKPTPN TVHYILTHFK GVWNIVNNIP FLRNTIMKYV
   HorseCOX2  NCSTPEFLTR IKLFLKPTPN TVHYILTHFK GVWNIVNSFP FLRNAVMKYV
  RabbitCOX2  NCSTPEFLTR IKLLLKPTPD TVHYILTHFK GVWNIVNSIP FLRNSIMKYV
GuineaPigCOX2 NCTTPEFLTR IKLLLKPTPN TVHYILTHFK GVWNIVNNIP FLRNAIMIYV
   SheepCOX2  NCTTPEFLTR IKLLLKPTPD TVHYILTHFK GVWNIVNKIS FLRNMIMRYV
     CowCOX2  NCTTPEFLTR IKLLLKPTPN TVHYILTHFK GVWNIVNKIS FLRNMIMRYV
   MouseCOX2  NCTTPEFLTR IKLLLKPTPN TVHYILTHFK GVWNIVNNIP FLRSLIMKYV
     RatCOX2  NCTTPRFLTR IKLPLKPTPN TVHYILTHFK GVWNIVNNIP FLRIQSMRYV
    MinkCOX2  NCSTPEFLTR VKLLLKPTPN TVHYILTHFK GVWNIVNKIP FLADVIMKYV
 ChickenCOX2  NCTTPEFFTW LKLILKPTPN TVHYILTHFK GVWNIINNIS FLRDTIMRYV
 RainbowCOX2  NCTTPEFLTW IKISLKPAPN TIHYILTHYK GLWNVINKIT FVRNAIMSYV
   BrookCOX2  NCTTPEFLTW IKISLKPAPN TVHYILTHYK GLWNVINKIT FVRNAIMSYV
PhomomallaCOXA NCEKPSWSTW LKSFIKPSEQ TKHFMLTHFG WFWWIVNNVQ FIRDPIMRAA
GfruticosaCOXA NCEKPNWSTW FKALIAPSEE TKHFILTHFK WFWWIVNNVP FIRNTVMKAA
GfruticosaCOXB DCEKPNWSTW FKGLIAPSEE TKHFILTHFN WFWWIVNNVP FIRNTVMKAA
 CaninePCOX1a  NCTIPELWTW LRNSLRPSPS FLHFLLTHGR WFWEFINA.T FIRDMLM...
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Membrane-binding domain

   151   200

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   HumanCOX1  LTVRSNLIPS PPTYNSAHDY ISWESFSNVS YYTRILPSVP KDCPTPMGTK
   SheepCOX1  LTVRSNLIPS PPTYNIAHDY ISWESFSNVS YYTRILPSVP RDCPTPMGTK
  CanineCOX3  LTARSNLIPS PPTYNIAHDY ISWESFSNVS YYTRVLPSVP QDCPTPMGTK
  CanineCOX1  LTARSNLIPS PPTYNIAHDY ISWESFSNVS YYTRVLPSVP QDCPTPMGTK
  RabbitCOX1  LTVRSNLIPS PPTYNLDYDY ISWEAFSNVS YYTRVLPSVP KDCPTPMGTK
   MouseCOX1  LTVRSNLIPS PPTYNSAHDY ISWESFSNVS YYTRILPSVP KDCPTPMGTK
     RatCOX1  ITVRSNLIPS PPTYNTAHDY ISWESFSNVS YYTRILPSVP KDCPTPMGTK
 RainbowCOX1  LTVRSNLIPS PPTFNSKYGY LSWESYSNVS YYTRILPPVP EDCPTPMGTK
   BrookCOX1  LTVRSNLIPS PPTFNSKYGY LSWESYSNVS YYTRILPPVP EDCPTPMGTK
   ZfishCOX1  LTVRANLIPS PPTYNSRYDY LNWEAYSNIT YYTRILPPVP NDCPTPMGTK
    SharkCOX  LTVRANIIPS PPTYNSDYTY VSWEGYSNIS YLTRLLPPVP KDCPTPTGTQ
   HumanCOX2  LTSRSHLIDS PPTYNADYGY KSWEAFSNLS YYTRALPPVP DDCPTPLGVK
  CanineCOX2  LTSRSHLIES PPTYNVNYGY KSWEAFSNLS YYTRALPPVP DDCPTPMGVK
   HorseCOX2  LVSRSHLIES PPTYNAQYGY KSWESFSNLS YYTRALPPVA DGCPTPMGVK
  RabbitCOX2  LTSRSHMIDS PPTYNVHYNY KSWEAFSNLS YYTRALPPVA DDCPTPMGVK
GuineaPigCOX2 LTSRSHLIDS PPTYNAHYGY KSWEAFSNLS YYTRALPPVA DDCPTPMGVK
   SheepCOX2  LTSRSHLIES PPTYNVHYSY KSWEAFSNLS YYTRALPPVP DDCPTPMGVK
     CowCOX2  LTSRSHLIES PPTYNVHYSY KSWEAFSNLS YYTRALPPVP DDCPTPMGVK
   MouseCOX2  LTSRSYLIDS PPTYNVHYGY KSWEAFSNLS YYTRALPPVR DDCPTPMGVK
     RatCOX2  LTSRSHLIDS PPTYNVHYGY KSWEAFSNLS YYTRALPPVA DDCPTPMGVK
    MinkCOX2  RTSRSHCIEP PPTYNVHYAY KSWEAFSNLS YYTRALPPVA DDCPTPMGVK
 ChickenCOX2  LTSRSHLIDS PPTYNSDYSY KSWEAYSNLS YYTRSLPPVG HDCPTPMGVK
 RainbowCOX2  LTPRSHLVDS PPTYNADYGY KSWEAYSNLF YYTRTLPPLP KDCPTPMGTA
   BrookCOX2  LTSRSHLVDS PPTYNADYGY KSWEAYSNLS YYTRTLPPLP KDCPTPMGTA
PhomomallaCOXA YFSRTDFIPV PHVYTSYHEY ATMEAHYNRT HFARTLPPVP KNCPTPFGVS
GfruticosaCOXA YFSRTDFVPV PHAYTSYHDY ATMEAHYNRS YFARTLPPVP KNCPTPFGVA
GfruticosaCOXB YFSRTDFIPV PHAYTSYHEY ATMEAHYNRS YFARTLPPVP KNCPTPFGVA
 CaninePCOX1a  .......... .......... .......... .......... ..........
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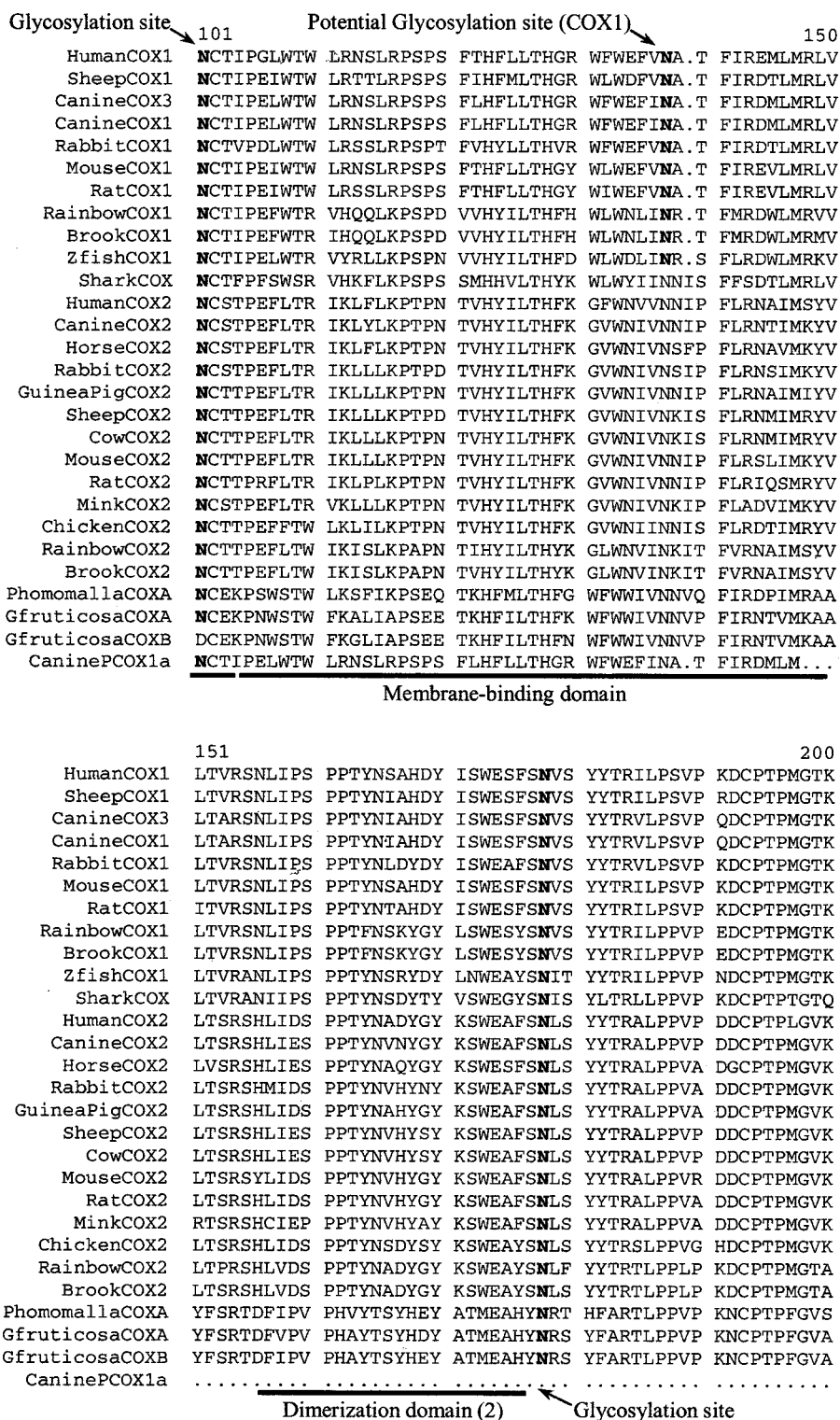Dimerization domain (2)   Glycosylation site

FIG. 2B

tide, these hydrophobic peptides show a size difference between COX-1 and COX-2 that, until recently, has been of unknown biological significance. The signal peptide for COX-1 is always 22 to 26 amino acids in length with a large hydrophobic core comprised of four or more leucines or isoleucines (Fig. 2). COX-2's signal peptide is 17 amino acids long in all species and appears to be less hydrophobic. In vitro translation experiments demonstrate that COX-1 is rapidly translocated into the lumen of canine pancreatic microsomes, whereas COX-2 is inefficiently translocated (Xie et al., 1991). Immediately following the signal peptide in COX-1 are eight amino acids that are not found in COX-2 (Fig. 2). The function of this sequence is unknown. Recently, as described be-

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                                              Distal histidine
                 201                                                   250
    HumanCOX1    GKKQLPDAQL LARRFLLRRK FIPDPQGTNL MFAFFAQHFT HQFFKTSGKM
    SheepCOX1    GKKQLPDAEF LSRRFLLRRK FIPDPQGTNL MFAFFAQHFT HQFFKTSGKM
   CanineCOX3    GKKQLPDAQL LGRRFLLRRK FIPDPQGTNL MFAFFAQHFT HQFFKTSGKM
   CanineCOX1    GKKQLPDAQL LGRRFLLRRK FIPDPQGTNL MFAFFAQHFT HQFFKTSGKM
   RabbitCOX1    GKKQLPDAQV LAHRFLLRRT FIPDPQGTNL MFAFFAQHFT HQFFKTSGKM
    MouseCOX1    GKKQLPDVQL LAQQLLLRRE FIPAPQGTNI LFAFFAQHFT HQFFKTSGKM
      RatCOX1    GKKQLPDIHL LAQRLLLRRE FIPGPQGTNV LFAFFAQHFT HQFFKTSGKM
  RainbowCOX1    GKSVLPDPKL VVEKFLLRRQ FRRDPRGTNL MFAFFAQHFT HQFFKTRNSM
    BrookCOX1    GKSVLPDPKL VVEKFLLRRQ FRPDPKGTNL MFAFFAQHFT HQFFKTRNSM
    ZfishCOX1    GKIKLPDPKL LVEKFMLRRN FRLDPQGTNL MFAFFAQHFT HQFFKTHNRV
     SharkCOX    GYKKLPDSEQ LAEEFLLRRK FIPDPQGSNL MFAFFAQHFT HQFFRTDLDR
    HumanCOX2    GKKQLPDSNE IVEKLLLRRK FIPDPQGSNM MFAFFAQHFT HQFFKTDHKR
   CanineCOX2    GKKELPDSKE IVEKFLLRRK FIPDPQGTNM MFAFFAQHFT HQFFKTDHKR
    HorseCOX2    GKKELPDSKE IVEKFLLRRK FIPDPQGTNM MFAFFAQHFT HQFFKTDPKR
   RabbitCOX2    GKKELPDSKD VVEKLLLRRK FIPDPQGTNM MFAFFAQHFT HQFFKTDLKR
GuineaPigCOX2    GKKELPDSNE VLEKVLLRRK FIPDPQGTNM MFAFFAQHFT HQFFKSDQKR
    SheepCOX2    GRKELPDSKE VVKKVLLRRK FIPDPQGTNL MFAFFAQHFT HQFFKTDIER
      CowCOX2    GRKELPDSKE VVKKVLLRRK FIPDPQGTNL MFAFFAQHFT HQFFKTDFER
    MouseCOX2    GNKELPDSKE VLEKVLLRRE FIPDPQGSNM MFAFFAQHFT HQFFKTDHKR
      RatCOX2    GNKELPDSKE VLEKVLLRRE FIPDPQGTNM MFAFFAQHFT HQFFKTDQKR
     MinkCOX2    GKKELPDSKE IVEKFLLRRK FIPDPQGTNM MFAFFAQHFT HQFFKTDHKR
  ChickenCOX2    GKKELPDSKL IVEKFLLRRK FIPDPQGTNV MFTFFAQHFT HQFFKTDHKK
  RainbowCOX2    GRAVLPDVKL VVEKVLLRKR FIPDPQGSNL MFAFFAQHFT HQFFKSDFMK
    BrookCOX2    GRAVLPDVKL VVEKVLLRKR FIPDPQGSNL MFAFFAQHFT HQFFKSDLKK
PhomomallaCOXA   GKKILPPAEE VANKFLKRRE FIADHRNTSW LFMFFAQHFT HQFFKTVHH.
GfruticosaCOXA   GKKELPPAEE VANKFLKRGK FKTDHTSTSW LFMFFAQHFT HEFFKTIYH.
GfruticosaCOXB   GKKELPPAEE VANRFLKREK FKTDHTNTSW LFMFFAQHFT HEFFKTIYH.
  CaninePCOX1a   .......... .......... .......... .......... ..........


                 251                                                   300
    HumanCOX1    GPGFTKALGH GVDLGHIYGD NLERQYQLRL FKDGKLKYQV LDGEMYPPSV
    SheepCOX1    GPGFTKALGH GVDLGHIYGD NLERQYQLRL FKDGKLKYQM LNGEVYPPSV
   CanineCOX3    GPGFTKALGH GVDLGHIYGD NLDRQYQLRL FKDGKLKYQV LDGEMYPPSV
   CanineCOX1    GPGFTKALGH GVDLGHIYGD NLDRQYQLRL FKDGKLKYQV LDGEMYPPSV
   RabbitCOX1    GPGFTKALGH GVDLGHIYGD SLERQYHLRL FKDGKLKYQV LDGEVYPPSV
    MouseCOX1    GPGFTKALGH GVDLGHIYGD NLERQYHLRL FKDGKLKYQV LDGEVYPPSV
      RatCOX1    GPGFTKALGH GVDLGHIYGD SLERQYHLRL FKDGKLKYQV LDGEVYPPSV
  RainbowCOX1    GLGFTRALGH GVDAGNVYGD NLVRQLNLRL LKDGKMKYQV VKGEVYPPTV
    BrookCOX1    GLGFTSALGH GVDAGNVYGD NLVRQLNLRL LKDGKMKYQV VKGEVYPPTV
    ZfishCOX1    GLGFTKGLGH GVDAGHIYGD SLDRQLELRL HKDGKLKYQV LNGDIYPPTV
     SharkCOX    GPGFTKALGH GVDLTHIYGD SLERQHHLRL FKDGKLKYQV VNGEVFPPSV
    HumanCOX2    GPAFTNGLGH GVDLNHIYGE TLARQRKLRL FKDGKMKYQI IDGEMYPPTV
   CanineCOX2    GPAFTKGLGH GVDLNHVYGE TLDRQHKLRL FKDGKMKYQV IDGEVYPPTV
    HorseCOX2    GPAFTKGLGH GVDLSHIYGE TLDRQHKLRL FKDGKMKYQI INGEVYPPTV
   RabbitCOX2    GPAFTKGLGH GVDLNHIYGE TLDRQHKLRL FKDGKMKYQV IDGEVYPPTV
GuineaPigCOX2    GPAFTTGLAH GVDLSHIYGE TLDRQHKLRL FKDGKMKYQI IDGEMYPPTV
    SheepCOX2    GPAFTKGKNH GVDLSHVYGE SLERQHNRRL FKDGKMKYQM INGEMYPPTV
      CowCOX2    GPAFTKGKNH GVDLSHIYGE SLERQHKLRL FKDGKMKYQM INGEMYPPTV
    MouseCOX2    GPGFTRGLGH GVDLNHIYGE TLDRQHKLRL FKDGKLKYQV IGGEVYPPTV
      RatCOX2    GPGFTRGLGH GVDLNHVYGE TLDRQHKLRL FQDGKLKYQV IGGEVYPPTV
     MinkCOX2    GPGFTKGLGH GVDLSHVYGE TLDRQHKLRL FKDGKMKYQV IDGEVYPPTV
  ChickenCOX2    GPGFTKAYGH GVDLNHIYGE TLERQLKLRL RKDGKLKYQM IDGEMYPPTV
  RainbowCOX2    GPAFTKALGH GVDLNHVYGD TLERQHKLRL FKDGKLKYRV LNGEVYPPLV
    BrookCOX2    GPAFTKALGH GVDLNHVYGD SLERQHKLRL FKDGKLKYQV LNGEVYPPLV
PhomomallaCOXA   SPAFSWG.NH GVDVSHIYGQ GVERENKLRA FKDGKLKSQM INGEEYPPYL
GfruticosaCOXA   SPAFTWG.NH GVDVSHIYGQ DMERQNKLRS FEDGKLKSQT INGEEWPPYL
GfruticosaCOXB   SPAFTWG.NH GVDVSHIYGQ DIERQNKLRS FQDGKLKSQM INGEEWPPYL
  CaninePCOX1a   .......... .......... .......... .......... ..........
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FIG. 2C

low, variants of COX-1 have been identified in which retention of all or part of intron-1 results in a retained signal peptide in COX-1, altering the biological properties of the enzyme (Chandrasekharan et al., 2002). Also, one coral isozyme has seven amino acids inserted in the same location; however, in this case, the insertion appears to change the location of the cleavage site in COS-7 cells rather than to affect retention of the signal peptide (Jarving et al., 2004).

2. Dimerization Domain. COX-1 and COX-2 dimers are held together via hydrophobic interactions, hydrogen bonding, and salt bridges between the dimerization domains of each monomer. Heterodimerization of COX-1 and COX-2 subunits does not occur. The dimerization domain is encoded by approximately 50 amino acids near the amino terminus of the proteolytically processed protein (Figs. 2 and 5). Three disulfide bonds hold this domain together in a structure reminiscent of epidermal

```
                    301                                              350
    HumanCOX1       EEAP.VLMHY PRGIPPQSQM AVGQEVFGLL PGLMLYATLW LREHNRVCDL
    SheepCOX1       EEAP.VLMHY PRGIPPQSQM AVGQEVFGLL PGLMLYATIW LREHNRVCDL
    CanineCOX3      EEAP.VLMHY PRGILPQSQM AVGQEVFGLL PGLMLYATLW LREHNRVCDL
    CanineCOX1      EEAP.VLMHY PRGILPQSQM AVGQEVFGLL PGLMLYATLW LREHNRVCDL
    RabbitCOX1      EEAP.VLMHY PRGVPPRSQM AVGQEVFGLL PGLMLYATLW LREHNRVCDL
    MouseCOX1       EQAS.VLMRY PPGVPPERQM AVGQEVFGLL PGLMLFSTIW LREHNRVCDL
    RatCOX1         EQAS.VLMRY PPGVPPEKQM AVGQEVFGLL PGLMLFSTIW LREHNRVCDL
    RainbowCOX1     AEAA.VNMRY PQETPVGQRM AIGQEVFGLL PGLTMYATLW LREHNRVCDI
    BrookCOX1       AEAP.VNMRY PQGTPVGQRM AIGQEVFGLL PGLTMYATLW LREHNRVCDI
    ZfishCOX1       LHAQ.VKMSY PPSVPPEQQL AIGQEVFGLL PGLGMYATLW LREHNRVCEI
    SharkCOX        KEAP.IQMKY PSTLPEEKRL AIGHDTFGLI PGLMMYATIW LREHNRVCDI
    HumanCOX2       KDTQ.AEMIY PPQVPEHLRF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    CanineCOX2      KDTQ.VEMIY PPHVPEHLQF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    HorseCOX2       KDTQ.VEMIY PPHIPEHLRF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    RabbitCOX2      KDTQ.VEMIY PPHIPAHLQF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    GuineaPigCOX2   KETQ.VEMMY PPYIPEHARF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    SheepCOX2       KDTQ.VEMIY PPHIPEHLKF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    CowCOX2         KDTQ.VEMIY PPHVPEHLKF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    MouseCOX2       KDTQ.VEMIY PPHIPENLQF AVGQEVFGLV PGLMMYATIW LREHNRVCDI
    RatCOX2         KDTQ.VDMIY PPHVPEHLRF AVGQEVFGLV PGLMMYATIW LREHNRVCDI
    MinkCOX2        KDTQ.VEMIY PPHVPEHLRF AVGQEVFGLV PGLMMYATIW LREHNRVCDV
    ChickenCOX2     KDTQ.AEMIY PPHVPEHLQF SVGQEVFGLV PGLMMYATIW LREHNRVCDV
    RainbowCOX2     REVG.AEMHY PPQVPEEHRF AVGHEHFGLV PGLMMYATIW LREHNRVCDV
    BrookCOX2       REVG.AEMHY PPQVPEEHRF AVGHEHFGLV PGLMMYATIW LREHNRVCDV
    PhomomallaCOXA  KDVDGLKMQY LENTAEEQKF ALGHPFFSML PGLFMFATLW LREHNRVCMI
    GfruticosaCOXA  KDVDNVTMQY PPNTPEDQKF ALGHPFYSML PGLFMYASIW LREHNRVCTI
    GfruticosaCOXB  KDVDNVTMQY PPNTSEDQKF ALGHPFYGML PGLFMYASIW LREHNRVCTI
    CaninePCOX1a    .......... .......... .......... .......... ..........


                    351                                              400
    HumanCOX1       LKAEHPTWGD EQLFQTTRLI LIGETIKIVI EEYVQQLSGY FLQLKFDPEL
    SheepCOX1       LKAEHPTWGD EQLFQTARLI LIGETIKIVI EEYVQQLSGY FLQLKFDPEL
    CanineCOX3      LKAEHPTWGD EQLFQTARLI LIGETIKIVI EEYVQQLSGY FLQLKFDPEL
    CanineCOX1      LKAEHPTWGD EQLFQTARLI LIGETIKIVI EEYVQQLSGY FLQLKFDPEL
    RabbitCOX1      LKAEHPTWDD EQLFQTTRLI LIGETIKIVI EEYVQQLSGY FLQLKFDPEM
    MouseCOX1       LKEEHPTWDD EQLFQTTRLI LIGETIKIVI EEYVQHLSGY FLQLKFDPEL
    RatCOX1         LKEEHPTWDD EQLFQTTRLI LIGETIKIII EEYVQHLSGY FLQLKFDPEL
    RainbowCOX1     LKAEHPTWGD EQLFQTARLI VIGETIRIVI EEYVQHLSGY LLDLKFDPVL
    BrookCOX1       LKAEHPTWGD EQLFQTARLI VIGETIRIVI EEYVQHLSGY LLDLKFDPVL
    ZfishCOX1       LKQEHPTWGD EQLFQTARLI IIGETIRIVI EEYVQHLSGY RLKLHFDPTL
    SharkCOX        LKEEHPVWSD EQLFQTTRLI LIGETIKIVI EDYVQHLSGY NFRMMFNPEL
    HumanCOX2       LKQEHPEWGD EQLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    CanineCOX2      LKQEHPEWDD ERLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    HorseCOX2       LKQEHPEWDD ERLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    RabbitCOX2      LKQEHPEWDD EQLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    GuineaPigCOX2   LKQEHPEWDD ERLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    SheepCOX2       LKQEHPEWGD EQLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    CowCOX2         LKQEHPEWGD EQLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    MouseCOX2       LKQEHPEWGD EQLFQTSRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    RatCOX2         LKQEHPEWDD ERLFQTSRLI LIGETIKIVI EDYVQHLRGY HFQLKFDPDL
    MinkCOX2        LKQEQGEWDD ERLFRRSRLI LIGETIKIVI EDYVRHLSGY HFSLKFDPEL
    ChickenCOX2     LKQEHPEWDD EQLFQTTRLI LIGETIKIVI EDYVQHLSGY HFKLKFDPEL
    RainbowCOX2     LRQEHPEWDD ERIFQTTRLI LIGETIKIVI EDYVQHLSGY HFQLKFDPEL
    BrookCOX2       LRQEHPEWDD ERIFQTTRLI LIGETIKIVI EDYVQHLSGY HFQLKFDPEL
    PhomomallaCOXA  LRKEHPHWED ERIYQTAKLI ITGETIKIVI EDYINHLANY NMKLRYDPQL
    GfruticosaCOXA  LRKEHPHWVD ERLYQTGKLI ITGELIKIVI EDYVNHLANY NLKLTYNPEL
    GfruticosaCOXB  LRKEHPHWED ERLYHTGKLI ITGELIKIVI EDYVNHLANY NLKITYDPEL
    CaninePCOX1a    .......... .......RLV LTGETIKIVI EEYVQQLSGY FLQLKFDPEL
```

FIG. 2D

growth factor. A fourth disulfide bond links the dimerization domain with the globular catalytic domain. The presence of disulfide bonds, which require an oxidizing environment, is consistent with the location of COXs inside the lumen of the nuclear envelope, ER, or Golgi, which have redox states that are significantly more oxidized than cytosol.

3. Membrane Binding Domain. COX isozymes associate with the intraluminal surface of microsomal membranes in an unusual fashion. Rather than employing transmembrane spanning sequences or covalently bound lipids for attachment, COX isozymes contain a tandem series of four amphipathic helices, which creates a hydrophobic surface that penetrates into the upper portion of the luminal side of the hydrophobic core of the lipid bilayer (Figs. 2 and 5). These helices are encoded by approximately 50 amino acids found immediately carboxy-terminal to the dimerization domain (Fig. 2). The helices allow COX dimers

```
                      Active site tyrosine        Glycosylation site
                   401                                                450
    HumanCOX1      LFGVQFQYRN RIAMEFNHLY HWHPLMPDSF KVGSQEYSYE QFLFNTSMLV
    SheepCOX1      LFGAQFQYRN RIAMEFNQLY HWHPLMPDSF RVGPQDYSYE QFLFNTSMLV
    CanineCOX3     LFSAQFQYRN RIAMEFNQLY HWHPLMPDSF WVGSQEYSYE QFLFNTSMLT
    CanineCOX1     LFSAQFQYRN RIAMEFNQLY HWHPLMPDSF WVGSQEYSYE QFLFNTSMLT
    RabbitCOX1     LFSVQFQYRN RIAMEFNHLY HWHPLMPDSF QVGSQEYSYE QFLFNTSMLV
    MouseCOX1      LFRAQFQYRN RIAMEFNHLY HWHPLMPNSF QVGSQEYSYE QFLFNTSMLV
    RatCOX1        LFRAQFQYRN RIALEFNHLY HWHPLMPDSF QVGSQEYSYE QFLFNTSMLV
    RainbowCOX1    LFKSTFQYRN RIAVEFKQLY HWHPLMPDSF HIDGDEVPYS QFIFNTSIVT
    BrookCOX1      LFKSTFQYRN RIAVEFNQLY HWHPLMPDSF HIDGDVVSYS QFIFNTSIVT
    ZfishCOX1      LFNSQFQYQN RISVEFNQLY HWHPLMPDSF YIDGDHIQYS KFIFNTSILT
    SharkCOX       LFTEHFQYSN RIAVEFDHLY HWHPLMPDSF IVKGQDFSYK DFLFNTDILL
    HumanCOX2      LFNKQFQYQN RIAAEFNTLY HWHPLLPDTF QIHDQKYNYQ QFIYNNSILL
    CanineCOX2     LFNQQFQYQN RIAAEFNTLY HWHPLLPDTL QIDDQEYNFQ QFIYNNSILL
    HorseCOX2      LFNQQFQYQN RIAAEFNTLY HWHPLLPDTF QIDDQEYNFQ QFLYNNSILL
    RabbitCOX2     LFNQQFQYQN RIAAEFNTLY HWHPLLPDTF QIDDQQYNYQ QFLYNNSILL
    GuineaPigCOX2  LFNQQFQYQN RIASEFNTLY HWHPLLPDTF QIDDQVYNFQ QFLYNNSILV
    SheepCOX2      LFNQQFQYQN RIAAEFNTLY HWHPLLPDVF QIDGQEYNYQ QFIYNNSVLL
    CowCOX2        LFNQQFQYQN RIAAEFNTLY HWHPLLPDVF QIDGQEYNYQ QFIYNNSVLL
    MouseCOX2      LFNQQFQYQN RIASEFNTLY HWHPLLPDTF NIEDQEYSFK QFLYNNSILL
    RatCOX2        LFNQQFQYQN RIASEFKTLY HWHPLLPDTF NIEDQEYTFK QFLYNNSILL
    MinkCOX2       LFNQQFQYQN RIAAEFNTLY HWHPLLPDTL QIDDQEYNFQ QFVYNNSILL
    ChickenCOX2    LFNQRFQYQN RIAAEFNTLY HWHPLLPDTF QIHNQEYTFQ QFLYNNSIML
    RainbowCOX2    LFNQRFQYQN RIAAEFNTLY HWHPLMPETF SIEDRAYTYP QFVFNNSLVT
    BrookCOX2      LFNQRFQYQN RIAAEFNTLY HWHPLMPDTF SIEDRAYTYP QFVFNNSLVT
    PhomomallaCOXA VFSRNYDYDN RIHLEFNHLY HWHPFSPDQF NISGTTYTIN DFMYHPEIVV
    GfruticosaCOXA VFDHGYDYDN RIHVEFNHMY HWHPFSPDEY NISGSTYSIQ DFMYHPEIVV
    GfruticosaCOXB VFDHGYDYDN RIHVEFNHMY HWHPFSPDEY NISGSTYSIH EFMYHPEIVV
    CaninePCOX1a   LFSAQFQYRN RIAMEFNQLY HWHPLMPDSF WVGSQEYSYE QFLFNTSMLT
                                       Proximal heme ligand

                   451                                                500
    HumanCOX1      DYGVEALVDA FSRQIAGRIG GGRNMDHHIL HVAVDVIRES REMRLQPFNE
    SheepCOX1      DYGVEALVDA FSRQPAGRIG GGRNIDHHIL HVAVDVIKES RVLRLQPFNE
    CanineCOX3     HYGIEALVDA FSRQSAGRIG GGRNIDHHVL HVAVETIKES RELRLQPFNE
    CanineCOX1     HYGIEALVDA FSRQSAGRIG GGRNIDHHVL HVAVETIKES RELRLQPFNE
    RabbitCOX1     DYGVEALVDA FSRQSAGRIG GGRNIDHHVL HVAVEVIKES REMRLQPFNE
    MouseCOX1      DYGVEALVDA FSRQRAGRIG GGRNFDYHVL HVAVDVIKES REMRLQPFNE
    RatCOX1        DYGVEALVDA FSRQRAGRIG GGRNFDYHVL HVAEDVIKES REMRLQSFNE
    RainbowCOX1    HYGVEKLVDA FSRQCAGQIG GGRNIHPVVT NVAEGVIEES RTLRLQPFNE
    BrookCOX1      HYGVEKLVDA FSRQYAGQIG GGRNIHPVVT KVAEGVIEES RTLRLQPFNE
    ZfishCOX1      HYGLEKLVEA FSIQPAGQIG GGHNIHPVVS GVAERVIVES RELRLQPFNE
    SharkCOX       NLGVDALVES FSKQIAGRIG GGRNIHQSLL HIAIATIEHG RLLRFQPYNE
    HumanCOX2      EHGITQFVES FTRQIAGRVA GGRNVPPAVQ KVSQASIDQS RQMKYQSFNE
    CanineCOX2     EHGLTQFVES FSRQIAGRVA GGRNVPAAVQ QVAKASIDQS RQMKYQSLNE
    HorseCOX2      EHGLTQFVES FSRQIAGRVA GGRNVPAAAQ KIAKASIDQS REMKYQSLNE
    RabbitCOX2     EHGLTQFVES FTRQIAGRVA GGRNVPPAVQ KVAKASIDQS RQMKYQSLNE
    GuineaPigCOX2  EHGLTQFVES FTKQIAGRVA GGRNVPLAVQ RVAKASIEHS RKMKYQSLNE
    SheepCOX2      EHGVTQFVES FTRQIAGRVA GRRNLPAAVE KVSKASLDQS REMKYQSFNE
    CowCOX2        EHGLTQFVES FTRQRAGRVA GGRNLPVAVE KVSKASIDQS REMKYQSFNE
    MouseCOX2      EHGLTQFVES FTRQIAGRVA GGRNVPIAVQ AVAKASIDQS REMKYQSLNE
    RatCOX2        EHGLAHFVES FTRQIAGRVA GGRNVPIAVQ AVAKASIDQS REMKYQSLNE
    MinkCOX2       EHGLTQFGES FSRQIAGRVA GGRNVPAAVQ QEQRASIDQS RQMKYQSLNE
    ChickenCOX2    EHGLSHMVKS FSKQSAGRVA GGKNVPAAVQ KVAKASIDQS RQMRYQSLNE
    RainbowCOX2    EHGINNLVES FTKQIAGRVA GGRNLPPALV GVAAKALEHS RDMRYQSLNA
    BrookCOX2      EHGITNLVES FTKQIAGRVA GGRNLPPALV AVAAKALEHS RDMRYQSLNA
    PhomomallaCOXA KHGMSSFVNA MSSGLCGKMS .HHNHGQYTL DVAVEVIKYQ RKLRMQSFNN
    GfruticosaCOXA KHGMSSFVDS MSKGLCGQMS .HHNHGAYTL DVAVEVIKHQ RELRMQSFNN
    GfruticosaCOXB KHGMSSFVDS MSKGLCGQMS .HHNHGAYTL DVAVEVIKHQ RELRMQSFNN
    CaninePCOX1a   HYGIEALVDA FSRQSAGRIG GGRNIDHHVL HVAVETIKES RELRLQPFNE
```

FIG. 2E

to float on the surface of the lumen of the ER/nuclear envelope, with the majority of the protein protruding into the luminal space of these compartments. The membrane binding domain also forms the mouth of a narrow hydrophobic channel that is the cyclooxygenase active site.

4. Catalytic Domain. Carboxy-terminal to the membrane binding domain in COX primary structures is the catalytic domain, which comprises 80% (approximately 480 amino acids) of the protein and contains two distinct enzymatic active sites.

a. Peroxidase Active Site. The catalytic domain is globular with two distinct intertwining lobes. The interface of these lobes creates a shallow cleft on the upper surface of the enzyme (i.e., the surface furthest from the

```
                 501                                                550
   HumanCOX1     YRKRFGMKPY TSFQELVGEK EMAAELEELY GDIDALEFYP GLLLEKCHPN
   SheepCOX1     YRKRFGMKPY TSFQELTGEK EMAAELEELY GDIDALEFYP GLLLEKCHPN
  CanineCOX3     YRKRFGMRPY MSFQELTGEK EMAAELEELY GDIDALEFYP GLLLEKCHPN
  CanineCOX1     YRKRFGMRPY MSFQELTGEK EMAAELEELY GDIDALEFYP GLLLEKCHPN
  RabbitCOX1     YRKRFGLKPY ASFQELTGET EMAAELEELY GDIDALEFYP GLLLEKCQPN
   MouseCOX1     YRKRFGLKPY TSFQELTGEK EMAAELEELY GDIDALEFYP GLLLEKCQPN
     RatCOX1     YRKRFGLKPY TSFQEFTGEK EMAAELEELY GDIDALEFYP GLMLEKCQPN
 RainbowCOX1     YRKRFNLKPY TSFSDFTGEE EMARELEELY GDIDALEFYP AIMLEKTRPN
   BrookCOX1     YRKRFNLKPY TSFSDFTGEE EMARELEELY GDIDALEFYP ALMLEKTRPN
   ZfishCOX1     YRKRFNLKPY TSFAELTGEQ EMSKELEELY GHIDAMEFYP ALLLEKTRPG
    SharkCOX     YRKKLGLTPY KSFQELTGER EVAARLEKLY GHIDAMEFYP ALLLEAPNKN
   HumanCOX2     YRKRFMLKPY ESFEELTGEK EMSAELEALY GDIDAVELYP ALLVEKPRPD
  CanineCOX2     YRKRFRLKPY TSFEELTGEK EMAAGLEALY GDIDAMELYP ALLVEKPRPD
   HorseCOX2     YRKRFRLTPY KSFEELTGEK EMAAELEALY GDIDAMELYP ALLVEKPRPD
  RabbitCOX2     YRKRFLLKPY ESFEELTGEK EMAAELEALY GDIDAVELYP ALLVERPRPD
GuineaPigCOX2    YRKRFLMKPY TSFEELTGEK EMAAGLEALY GDIDAMELYP ALLVEKPRPD
   SheepCOX2     YRKRFLLKPY ESFEELTGEK EMAAELEALY GDIDAMELYP ALLVEKPAPD
     CowCOX2     YRKRFLVKPY ESFEELTGEK EMAAELEALY GDIDAMEFYP ALLVEKPRPD
   MouseCOX2     YRKRFSLKPY TSFEELTGEK EMAAELKALY SDIDVMELYP ALLVEKPRPD
     RatCOX2     YRKRFSLKPY TSFEELTGEK EMAAELKALY HDIDAMELYP ALLVEKPRPD
    MinkCOX2     YRKRFSVKPY ASFEELTGEK EMAGELKALY QDIDAMELYP ALLVEKPRPD
 ChickenCOX2     YRKRFMLKPF KSFEELTGEK EMAAELEELY GDIDAMELYP GLLVEKPRPG
 RainbowCOX2     YRKRFNMRVY TSFEDLTGET ELAAELESLY GDVDAVELYP GLLVERPRPN
   BrookCOX2     YRKRFNMRAY TSFEDLTGET ELAAELESLY GDVDAVELYP GLLVERPRPN
PhomomallaCOXA   YRRHFGLPAY KSFEEMTGDP KLAAELKEVY GDVNAVDFYV GFFLEKSLPT
GfruticosaCOXA   YRKHFALEPY KSFEELTGDP KMSAELQEVY GDVNAVDLYV GFFLEKGLTT
GfruticosaCOXB   YREHFALEPY KSFEEMTGDP KMAAELQETY GDVNAVDLYV GFFLEKGLTT
  CaninePCOX1a   YRKRFGMRPY MSFQELTGEK EMAAELEELY GDIDALEFYP GLLLEKCHPN


                 551                                                600
   HumanCOX1     SIFGESMIEI GAPFSLKGLL GNPICSPEYW KPSTFGGEVG FNIVKTATLK
   SheepCOX1     SIFGESMIEM GAPFSLKGLL GNPICSPEYW KASTFGGEVG FNLVKTATLK
  CanineCOX3     SIFGESMIEI GAPFSLKGLL GNPICSPEYW KPSTFGGEMG FNMVKTATLK
  CanineCOX1     SIFGESMIEI GAPFSLKGLL GNPICSPEYW KPSTFGGEMG FNMVKTATLK
  RabbitCOX1     SIFGESMIEI GAPFSLKGLL GNPICSPEYW KPSTFGGEVG SNLIKTATLK
   MouseCOX1     SIFGESMIEM GAPFSLKGLL GNPICSPEYW KPSTFGGDVG FNLVNTASLK
     RatCOX1     SLFGESMIEM GAPFSLKGLL GNPICSPEYW KPSTFGGDVG FNIVNTASLK
 RainbowCOX1     AIFGESMVEM GAPFSLKGLL GNPICSPEYW KPSTFGGQTG FDIVNSASLE
   BrookCOX1     AIFGESMVEM GAPFSLKGLL GNPICSPEYW KPSTFGGQTG FDIVNSASLE
   ZfishCOX1     AVFGESMVEM GAPFSLKGLM GNPICSPDYW KPSTFGGKTG FDIVNSATLK
    SharkCOX     SIFGESMVEM GAPFSLKGLM GNPICSPDYW KPSTFGGKTG FDIVNTATFE
   HumanCOX2     AIFGETMVEV GAPFSLKGLM GNVICSPAYW KPSTFGGEVG FQIIINTASIQ
  CanineCOX2     AIFGETMVEM GAPFSLKGLM GNPICSPDYW KPSTFGGEVG FKIINTASIQ
   HorseCOX2     AIFGETMVEL GAPFSLKGLL GNPICSPDYW KPSTFGGEVG FKIINTASIQ
  RabbitCOX2     AIFGESMVEM GAPFSLKGLM GNPICSPNYW KPSTFGGEVG FKIVNTASIQ
GuineaPigCOX2    AIFGETMVEM GAPFSLKGLM GNPICSPHYW KPSTFGGEVG FQIVNTASIQ
   SheepCOX2     AIFGETMVEA GAPFSLKGLM GNPICSPEYW KPSTFGGEVG FKIINTASIQ
     CowCOX2     AIFGETMVEA GAPFSLKGLM GNPICSPEYW KPSTFGGEVG FKIINTASIQ
   MouseCOX2     AIFGETMVEL GAPFSLKGLM GNPICSPQYW KPSTFGGEVG FKIINTASIQ
     RatCOX2     AIFGETMVEL GAPFSLKGLM GNPICSPQYW KPSTFGGEVG FRIINTASIQ
    MinkCOX2     AIFGETMVEI GAPFSLKGLM GNPICSPDYW KPSHFGGEVG FKIINTASIQ
 ChickenCOX2     AIFGETMVEI GAPFSLKGLM GNTICSPEYW KPSTFGGKVG FEIINTASLQ
 RainbowCOX2     AVFGETMVEM GAPYSLKGLL GNPICSPEYW MPSTFGGSVG FDILNTASLE
   BrookCOX2     AVFGETMVEM GAPYSLKGLL GNPICSPEYW MPSTFGGSVG FDIVNTASLE
PhomomallaCOXA   SPFGITMIAS GAPYSLRGLL SNPVSSPTYW KPSTFGGEVG FDIVKTASVD
GfruticosaCOXA   SPFGITMIAF GAPYSLRGLL SNPVSSPTYW KPSTFGGDVG FDMVKTASLE
GfruticosaCOXB   SPFGITMIAF GAPYSLRGLL SNPVSSPTYW KPSTFGGDIG FDMVKTASLE
  CaninePCOX1a   SIFGESMIEI GAPFSLKGLL GNPICSPEYW KPSTFGGEMG FNMVKTATLK
```

ASA-acetylated serine

Active site valine/isoleucine

FIG. 2F

membrane) where the peroxidase active site is located and where heme is bound. Coordination of the heme is via an iron-histidine bond involving His388 in sheep COX-1. (All numbering hereafter uses sheep COX-1 as reference.). Other important interactions between the protoporphyrin also occur, and specific amino acids that may function in coordinating PGG$_2$ have been identified (Malkowski et al., 2000; Thuresson et al., 2001). The geometry of heme binding leaves a large portion of one side of the heme exposed in the open cleft of the peroxidase active site for interaction with PGG$_2$ and other lipid peroxides.

Glycosylation site (COX2)

```
                601                                              650
    HumanCOX1   KLVCLNTKT. CPYVSFRVP. DASQDDGPAV ERPSTEL... ..........
    SheepCOX1   KLVCLNTKT. CPYVSFHVP. DPRQEDRPGV ERPPTEL... ..........
   CanineCOX3   KLVCLNTKT. CPYVSFRVP. DPHQDGGPGV QRPSTEL... ..........
   CanineCOX1   KLVCLNTKT. CPYVSFRVP. DPHQDGGPGV ERPSTEL... ..........
   RabbitCOX1   KLVCLNTKT. CPYVSFRVP. RSSGDDGPAA ERRSTEL... ..........
    MouseCOX1   KLVCLNTKT. CPYVSFRVP. DYPGDDGSVL VRRSTEL... ..........
      RatCOX1   KLVCLNTKT. CPYVSFRVP. DYPGDDGSVF VRPSTEL... ..........
   RainbowCOX1  RLVCLNTNW. CPYVAFNVP. PAGQEEPP.. RKQSTEL... ..........
    BrookCOX1   RLVCLNTNW. CPYVAFNVP. PAGQEPPP.. RKQSTEL... ..........
    ZfishCOX1   KLVCLNTKW. CPYVSFHTP. PSDYK.PQ.. RTSHGEL... ..........
     SharkCOX   KLICLNVKK. CPYVGFHVPY NVDNDYEREK GKPSTEL... ..........
    HumanCOX2   SLICNNVKG. CPFTSFSVPD PELIKTVTIN ASSSRSGLDD INPTVLLKER
   CanineCOX2   SLICNNVKG. CPFTAFSVQD GQLTKTVTIN ASSSHSGLDD INPTVLLKER
    HorseCOX2   SLICNNVKG. CPFTAFSVQD PQLSKAVTIN ASASHSGLDD VNPTVLLKER
   RabbitCOX2   SLICNNVKG. CPFTSFNVPD PQLTKTVTIN ASASHSRLED INPTVLLKGR
 GuineaPigCOX2  SLICNNVKG. CPVTAFNLPD PQLAKTVTIN ASASHSRLED LSPTVLLKGR
    SheepCOX2   SLICSNVKG. CPFTSFSVQD AHLTKTVTIN ASSSHSGLDD INPTVLLKER
      CowCOX2   SLICSNVKG. CPFTSFSVQD THLTKTVTIN ASSSHSGLDD INPTVLLKER
    MouseCOX2   SLICNNVKG. CPFTSFNVQD PQPTKTATIN ASASRSRLDD INPTVLIKRR
      RatCOX2   SLICNNVKG. CPFASFNVQD PQATKTATIN ASASHSRLDD INPTVLIKRR
     MinkCOX2   SLICNNVKG. CPFTAFSVQD PQLTKTVTIN GSSSHSGLDD INPTVLLKER
  ChickenCOX2   KLICNNVKG. CPFTAFHVLN PEPTE.ATIN VSTSNTAMED INPTLLLKEQ
  RainbowCOX2   RLVCNNVKGS CPMVSFQVPD .FLRAFESAS VNTSEAHLSD MNPGVLFKER
    BrookCOX2   RLVCSNVKGS CPMVSFQVPD .FLRAFESAS VNTSEAHLRG MNPGVVFKER
PhomomallaCOXA  KLFCRNIAGD CPLVTFTVPD EIAREARRNL AANIKDEL.. ..........
 GfruticosaCOXA KLFCQNIAGE CPLVTFTVPD DIARETRKVL EA..RDEL.. ..........
 GfruticosaCOXB KLFCQNIAGE CPLVTFTVPD GIARETRKVL SA..KDEL.. ..........
  CaninePCOX1a  KLVCLNTKT. CPYVSFRVPD PH.QDGGPGV QRPSTEL... ..........
```

```
                651
    HumanCOX1   ....
    SheepCOX1   ....
   CanineCOX3   ....
   CanineCOX1   ....
   RabbitCOX1   ....
    MouseCOX1   ....
      RatCOX1   ....
   RainbowCOX1  ....
    BrookCOX1   ....
    ZfishCOX1   ....
     SharkCOX   ....
    HumanCOX2   STEL
   CanineCOX2   STEL
    HorseCOX2   STEL
   RabbitCOX2   STEL
 GuineaPigCOX2  STEL
    SheepCOX2   STEL
      CowCOX2   STEL
    MouseCOX2   STEL
      RatCOX2   STEL
     MinkCOX2   STEL
  ChickenCOX2   SAEL        ER retention signal
  RainbowCOX2   TSEL
    BrookCOX2   TLEL
PhomomallaCOXA  ....
 GfruticosaCOXA ....
 GfruticosaCOXB ....
  CaninePCOX1a  ....
```

FIG. 2G

b. Cyclooxygenase Active Site. The cyclooxyenase active site is a long, narrow, dead-end channel of largely hydrophobic character whose entrance is framed by the four amphipathic helices of the membrane binding domain. The channel extends approximately 25 Å into the globular catalytic domain and is on average about 8 Å wide (Picot et al., 1994). However, significant narrowing of the channel is observed where arginine 120, one of only two ionic residues found in the COX active site, protrudes into the channel and forms a hydrogen bonded network with glutamate 524 (the other ionic residue in the channel) and tyrosine 355. Arginine 120 is essential for binding substrates and carboxylate-containing NSAIDs in COX-1. In contrast, this residue is unessential in binding substrate in COX-2 (Rieke et al., 1999), where it also appears to be
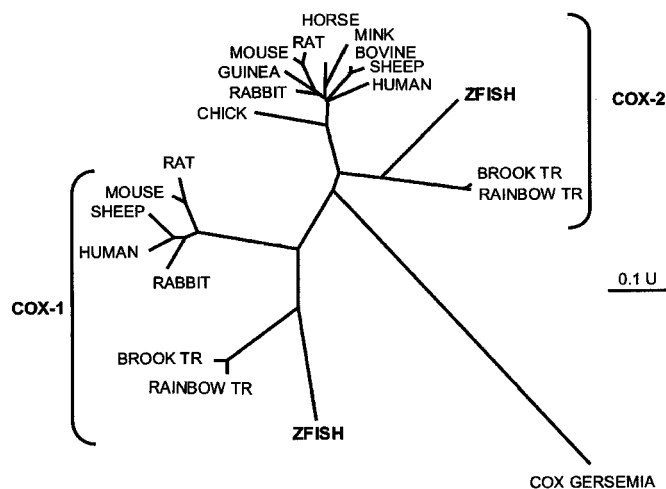
FIG. 3. Dendrogram showing phylogenetic relationships of COX isozymes. Neighbor-joining methodology was used to calculate genetic distances, and tree topology was confirmed by parsimony analysis. Courtesy of Tilo Grosser, Ellina Cheskis, Michael A. Pack and Garret A. FitzGerald.

nonessential in coordinating carboxylate-containing NSAIDs (Greig et al., 1997).

The upper portion of the channel, or catalytic pocket, contains tyrosine 385 that forms a tyrosyl radical, abstracts hydrogen from the pro-S side of carbon 13 of AA, and creates an activated arachidonyl radical that undergoes the cyclization/oxygenation reaction shown in Figs. 6 and 7. Also in the hydrophobic pocket is Ser530, which is transacetylated by aspirin. The hydroxyl of serine 530, together with valine 349, appears to be essential in governing the stereochemistry of oxygen attack at carbon 15 in the production of PGG_2 (Schneider et al., 2002); however, its acetylation prevents abstraction of hydrogen from AA in COX-1 by sterically preventing AA from binding productively in the active site (Rowlinson et al., 2000). In contrast, abstraction of hydrogen does occur in acetylated COX-2, but cyclization of the arachidonyl radical and formation of the endoperoxide does not occur, yielding 15-R-hydroxyeicosotetraenoic acid (15R-HETE) rather than PGH_2 (Holtzman et al., 1992).

A crucial structural difference between the active sites of COX-1 and COX-2 is a substitution of isoleucine 523 in COX-1 for a valine in COX-2 (Figs. 2 and 8). This single difference opens a hydrophobic outpocketing in COX-2 that can be accessed by some COX-2-selective drugs (Kurumbail et al., 1996). There are other changes in residues that are near but do not line the COX active site, so-called second shell residues, that result in subtle changes and a slightly enlarged COX-2 active site relative to COX-1 (Kurumbail et al., 1996; Luong et al., 1996).

The evolutionary conservation of an enlarged cyclooxygenase active site in COX-2 relative to COX-1 may be essential to the recognition of bulkier substrates by COX-2. Anandamide (arachidonylethanolamide) and 2-arachidonylglycerol are endocannabinoids that are ef-

ficiently oxidized by COX-2 to endocannabinoid-derived prostanoids (Kozak et al., 2002, 2003). COX-2 utilizes these bulkier substrates as efficiently as arachidonic acid, and the resulting endoperoxide can be utilized by downstream isomerases (Kozak et al., 2002). The function of these prostanoid-like oxidized endocannabinoids is unknown but may represent new biological roles of COX-2. The endocannabinoid analog methandamide up-regulates COX-2 expression, further linking this enzyme with metabolism of endocannabinoids (Gardner et al., 2003; Ramer et al., 2003)

The catalytic reactions involved in AA cyclooxygenation to form PGG_2 are shown in Figs. 6 and 7. Elegant studies done collaboratively by the laboratories of Garavito and Smith (Malkowski et al., 2000; Thuresson et al., 2001) have succeeded in defining the productive structure of COX-1 with its substrate AA as well as with eicosapentaenoic and linoleic acids (Malkowski et al., 2001). COX-1 binds AA in an extended L shape, its carboxylate forming both a salt bridge with the guanidinium group of arginine 120 and also a hydrogen bond with tyrosine 355. The remainder of the fatty acid makes more than 50 mostly hydrophobic interactions with 19 amino acid residues, which position substrate for hydrogen abstraction and facilitate conversion to PGG_2 rather than to HETEs (Thuresson et al., 2001). Two molecules of oxygen for the bisoxygenation reaction and hydroperoxidation reaction that yield the endoperoxide and hydroperoxide moieties, respectively, have been postulated to diffuse into the COX active site from the direction of the membrane, thus resulting in the observed fact that attack of oxygen at carbon 11, to eventually result in the PGG_2 endoperoxide, occurs from the opposite or anterofacial orientation from that of hydrogen abstraction at carbon 13.

At the carboxy terminus of the catalytic domain of COX-1 and COX-2 are modified versions of the KDEL sequence that act as a signal for retention of proteins in the endoplasmic reticulum (Song and Smith, 1996). Additionally, COX-2 has an 18-amino acid sequence located next to this retention signal (Fig. 2G). This structure, which is not found in COX-1, is not fixed in crystallographic studies, and its function is unknown.

The above structural features are consistent with localization of COX isozymes inside the lumen of the ER, a fact that is further supported by numerous studies using fluorescence and immuno-electron microscopy (Morita et al., 1995; Ren et al., 1995; Song and Smith, 1996; Liou et al., 2000); however COX-1 has been found by Weller and colleagues to be localized to lipid bodies in leukocytes and other cells (Bozza et al., 1996). Lipid bodies in these cells are rapidly formed following treatment with platelet-activating factor (PAF), nonesterified fatty arachidonate, or other fatty acids (Bozza et al., 1997; Bozza and Weller, 2001) and are induced in endothelial cells by hypoxia (Scarfo et al., 2001). Unlike the ER, which contains a lumen, the structure of lipid bodies

```
Rice α-oxy   ----------MGSGLFKPRVHPDLRDVFSKMSFFDKIGFLFIHAFDKRNLWHKVPVPIGLLYLNTRRTLLEKYNLLAVGRSSHGALFDPKEFLYRTEDGKYNDPHNAEAG
A.Thaliana   ----MKVITSLISSILLKFIHKDFHEIYARMSLLDRFLLLIVHGVDKMVPWHKLPVFLGLTYLEVRRHLHQQYNLLNVGQTPTGIRFDPANYPYRTADGKFNDPFNEGVG
Tobacco      MSLVMASLKNLLLSPLRGFIHKDFHDIFERMTLLDKLFFLIVHFVDKLNLWHRLPVFLGLLYLGARRHLHQEYNLINVGKTPVGVRSNPADHPYRTADGKYNDPFNEGAG
hCOX-1       -----MSRSLLLRFLLFLLLLPPLPVLLADPGAPTPVNPCCYYPCQHQGICVRFG-LDRYQCDCTRTGYSGPNCTIPGLWTWLRNSLRPSPSFTHFLLTHGRWFWEFVN

Rice α-oxy   KPKHLFWGETWSRLINRNELMSPDPFVVATKLLAREYKDTGKQFNILAAAWIQFMVHDWMDHMEDTGQIGITA----------PKEVANECPLKSFKFHPTKELPTNSD
A.Thaliana   S-QNSFFGRNCPPVDQKSKLRRPDPMVVATKLLGRKKFIDTGKQFNMIAASWIQFMIHDWIDHLEDTHQIELVA----------PKEVASKCPLSSFRFLKTKEVPTGFF
Tobacco      S-ELSFFGRNMLPVDQHNQLKKPDPMVVATKLLARRNVVDTGKQFNMIAASWIQFMIHDWIDHLEDTKQIELRA----------PEEVASQCPLKSFKFFKTKEIPTGFY
hCOX-1       ATFIREMLMRLVLTVRSNLIPSPPTYNSAHDYISWESFSNVSYYTRILPSVPKDCPTPMGTKGKKQLPDAQLLARRFLLRRKFIPDPQGTNLMFAFFAQHFTHQFFKTSG

Rice α-oxy   GIKIGHYNIRTAWWDGSAVYGNNEERAEKLRTYVDGKLVIG-----------DD-GLLLHKENGV------ALSGDIRNSWAGVSILQALFVK**EHNAVCDA**IKE**E**HPNLS
A.Thaliana   EIKTGSQNIRTPWWDSSVIYGSNSKTLDRVRTYKDGKLKIS-----------EETGLLLHDEDGL------AISGDIRNSWAGVSALQALFIK**EHNAVCDA**LKD**E**DDDLE
Tobacco      EIKTGHLNRRTPWWDGSAIYGSNAEVLKKVRTFKYGKLKLS-----------AD-GLLEIDENGK------IISGDVRNTWAGLSALQALFVQ**EHNSVCD**VLKK**E**YPELE
hCOX-1       KMGPGFTKALGHGVDLGHIYGDNLERQYQLRLFKDGKLKYQVLDGEMYPPSVEEAPVLMHYPRGIPPQSQMAVGQEVFGLLPGLMLYATLWLH**EHN**RVCDLLKA**E**HPTWG
                                                                                                      *  **

Rice α-oxy   **DE**ELYRYAK**L**VTSAVIAKVHTIDWTVELLKTKTMRAAMRANWYGLLGKKIKDTFGHIGGPILGGLVGLKKPPNHGVPYSLTEEFTSV**Y**RM**HSLIP**STLKLRDPTGQPDAN
A.Thaliana   **DE**DLYRYARL**V**TSAVVAKIHTIDWTVQLLKTDTLLAGMRANWYGLLGKKFKDSFGHAGSSILGGVVGMKKPQNHGVPYSLTEDFTSV**Y**RM**HSLLP**DQLHILDIDDDVPGTN
Tobacco      **DE**DLYRHARL**V**TSAVIAKVHTIDWTVELLKTDTLLAGMRANWYGLLGKKFKDTFGHVGGSILGGFVGMKKPENYGVPYSLTEEFTSV**Y**RM**HQLLP**DNLQLRNIDATPGPN
hCOX-1       **DE**QLFQTTR**L**ILIGETIKIVIEEYVQQL---SGYFLQLKFDPELLFGVQFQ-----------------------YRNRIAMEFNHL**Y**HW**HPLMP**DSFK-----------

Rice α-oxy   NSPPCLEDIDIGEMIGLKGEEQLSKIGFEKQALSMGYQACGALELWNYPSFF**RN**LIPQNLD**G**TNRSDRIDLAA**A**LEVY**RD-RE**RSVPRY**N**EF**R**RRLFLI**P**IKSWEDLTSDK
A.Thaliana   KSLPLIQEISMRDLIGRKGEETMSHIGFTKLMVSMGHQASGALELMNYPMWL**RD**IVPHDPNG**Q**ARPDHVDLAA**A**LEIY**RD-RE**RSVPRY**N**EF**R**RSMFMI**P**ITKWEDLTEDE
Tobacco      KSLPLTNEIPMEDLIGSKGEENLARIGFTKQMVSMGHQACGALELWNYPMWM**RD**LIPQDVD**G**TDRPDHVDLAA**A**LEIY**RD-RE**RSVARY**N**EF**R**RGMLQI**P**ISKWEDLTDDE
hCOX-1       --------------VGSQEYSYEQFLFNTSMLVDYGVEAL-------VDAFS**RQ**IAGRIGG**G**RNMDHHILHVA**A**VDVIRES**RE**MRLQPF**N**EY**R**KRFGMK**P**YTSFQELVGEK

Rice α-oxy   DAIETIRAI**Y**GDDVEKLDLLVGLMAE**KK**IKGFAIS**E**TAFNIFILMASRRLEADRFFTSNF-NEETYTKK**G**MQWVKTTEGLRDVINRHYPEITAKWMKSSSAFSVWDADY-
A.Thaliana   EAIEVLDDV**Y**GDGDVEELDLLVGLMAE**KK**IKGFAIS**E**TAFYIFLIMATRRLEADRFFTSDF-NETIYTKK**G**LEWVNTTESLKDVIDRHYPDMTDKWMNSESAFSVWDSPPL
Tobacco      EVINTLREV**Y**GDDVEELDLMVGMAAE**KK**IKGFAIS**E**TAFFIFLIMASRRLEADRFFTSNY-NEETYTKK**G**LEWVNTTESLKDVLDRHYPEMTEKWMNSSSAFSVWDSSPE
hCOX-1       EMAAELEEL**Y**G-DIDALEFYPGLLL**E**KCHPNSIFGE**S**MIEIGAPFSLKGLLGNPICSPEYWKPSTFGGEVGFNIVKTATLKKLVCLN-TKTCPYVSFRVPDASQDDGPA

Rice α-oxy   --------------
A.Thaliana   TKNPIPLYLRIPS-
Tobacco      PHNPIPLYFRVPPQ
hCOX-1       VERPSTEL------
```

FIG. 4. Comparison of plant PIOX enzymes from rice, *Arabbidopsis thaliana*, and tobacco with human COX-1. Some important conserved sequences are shown in bold. The conserved tyrosine that functions as a tyrosyl radical and the conserved histidine that forms the proximal ligand to heme are denoted by one and two asterisks, respectively.
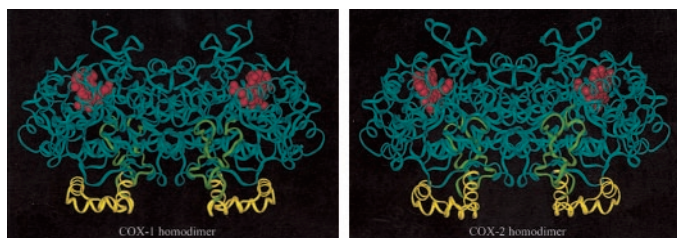
FIG. 5. Crystallographic structures of ovine COX-1 (left) and murine COX-2 (right) homodimers. The crystallographic structure of ovine COX-1 was taken from Protein Data Bank file 1PRH and the murine COX-2 structure was from file COX5 of the same source. Functional domains: 1) membrane binding domain (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. Glycosyl residues are not shown.

is less defined and may contain a central core of neutral lipids surrounded by a monolayer of phospholipid, which is thought to be derived from the cytosolic side of the ER bilayer (Murphy and Vance, 1999). In addition to containing COX-1, lipid bodies have also been shown to be rich in other lipid-metabolizing enzymes (Bandeira-Melo et al., 2001).

In addition to lipid bodies, COX-1 has been localized to unusual filamentous structures in endothelial ECV304 cells (Liou et al., 2000), and COX-2 was localized to caveolin-1-containing vesicles in bovine arterial endothelial cells treated with phorbol ester (Liou et al., 2000) or human fibroblasts treated with either phorbol ester or IL-1 (Liou et al., 2001). COX-1 and COX-2 have been
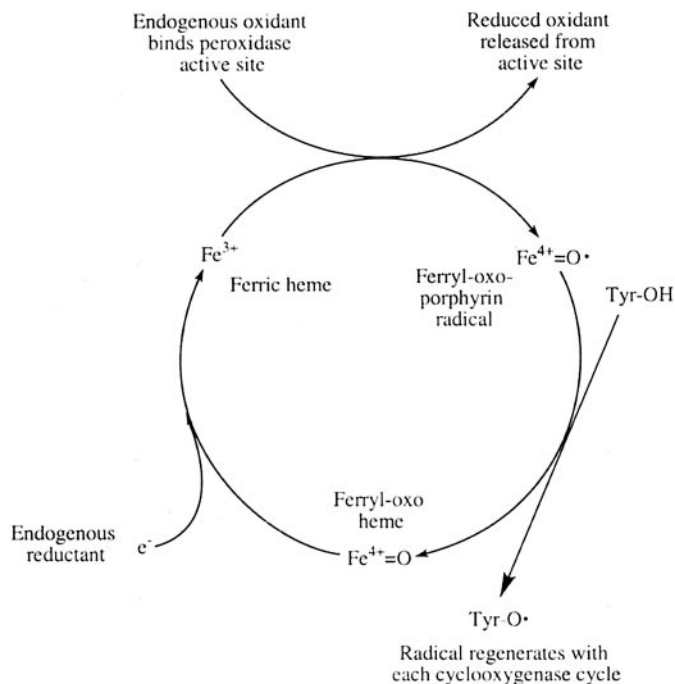
FIG. 6. Priming of COX enzymes by formation of a tyrosyl radical. Nascent COX enzymes are inactive and must be primed by binding and oxidizing an endogenous oxidant (e.g., a lipid peroxide). In the process, a ferryl-oxo-porphyrin radical is generated that abstracts an electron from tyrosine 385 to produce the critical radical needed for cyclooxygenase activity. This radical is regenerated with each cyclooxygenase cycle (see Fig. 7). The resulting ferryl-oxo heme is reduced to resting state ferric heme by endogenous reductants.
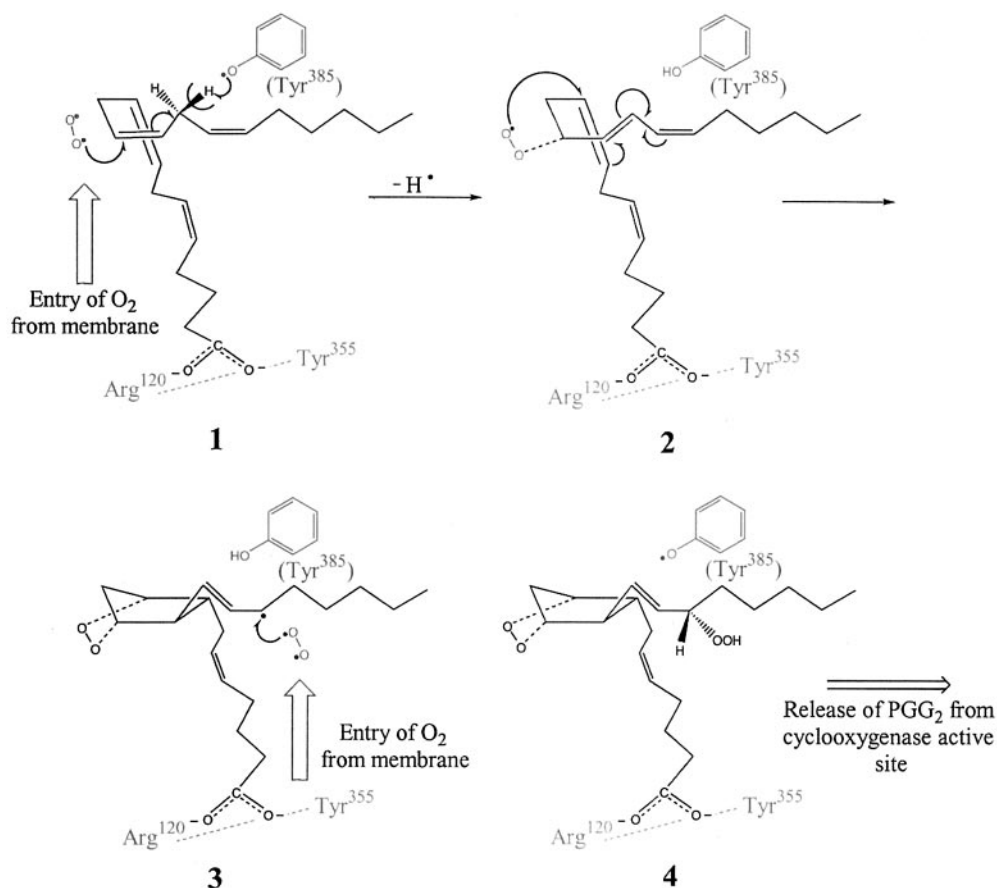
FIG. 7. Catalytic steps in cyclooxygenation of arachidonic acid. 1) Arachidonic acid is bound productively in the COX active site in an extended L-shape placing carbon 15 in the correct orientation for abstraction of the pro S hydrogen by tyrosyl radical 385. 2) The arachidonyl radical localizes at carbons 11 and 9 where oxygen attacks from the anterofacial side to form an endoperoxide. 3) the radical localized at carbon 15 is attacked by a second molecule of oxygen. Reabstraction of hydrogen from the tyrosine 385 produces hydroperoxyl-containing PGG$_2$ and regenerates the tyrosyl radical.

identified by a number of laboratories to traffic within the nucleus following a variety of stimuli (Coffey et al., 1997; Parfenova et al., 1997; Neeraja et al., 2003). How extraluminal COX isozymes might structurally and enzymatically differ from their intraluminal counterparts or result in differential targeting of prostaglandins (e.g., to the nucleus) is currently unknown.

E. Post-Translational Modification of Cyclooxygenases

COX-1 is glycosylated at three asparagines, whereas COX-2 may be glycosylated at up to four asparagines. (Nemeth et al., 2001; also see Fig. 2 for positions). Glycosyl moieties are of the high mannose type. Glycosylation of asparagine 410 in COX-1 is essential for COX and POX activities, probably by promoting proper protein folding (Otto et al., 1993). This suggests that glycosylation is cotranslational rather than post-translational; however, once COX-1 has folded, this glycosyl residue can be removed by endoglycosidase treatment with little to no effect on enzyme activity. COX-2 is frequently observed in multiple glycosylated states that in Western blots produce a characteristic COX-2 doublet or triplet banding pattern. Three distinct glycoforms of COX-2
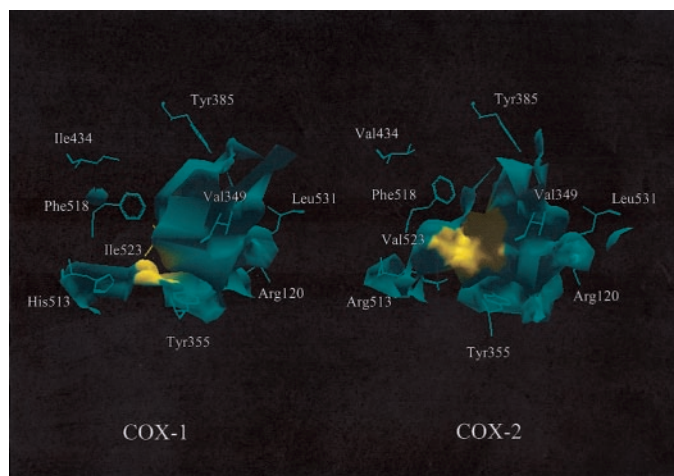
FIG. 8. Contour of COX-1 and COX-2 cyclooxygenase active sites. The solvent-accessible surfaces of the COX-1 and COX-2 active sites, with important surrounding amino acid residues, are shown. Highlighted in yellow on the left is the effect of isoleucine 523 on COX-1, which produces two prominent "bulges" into the active site. These bulges prevent aromatic substituents of some COX-2-specific drugs from accessing the leftward side pocket shown. The effect of valine 523 in COX-2, highlighted in yellow on the right, does not create these bulges and allows access of coxibs into this pocket.

have recently been characterized by mass spectrometry (Nemeth et al., 2001) The function of these COX-2 glycosylation states is unknown, but changes in glycosylation have been found in one case to accompany cell transformation by a tumor virus (Evett et al.,1993).

F. Variants of Cyclooxygenase Isoenzymes

Recently it has become clear that the transcriptome and proteome is significantly larger than the genome. Much of the discrepancy is due to alternative splicing. The first COX-1 splice variant was identified by Diaz in 1992 from a cDNA clone that contained the complete coding region for human lung COX-1; however, the cDNA contained an in-frame removal, due to alternative splicing, of the last 111 base pairs encoded by exon 9. This deletion eliminated the *N*-glycosylation site at residue 409, which had previously been shown by others to be essential for proper folding of the enzyme and for enzyme activity. Differential expression of this variant relative to COX-1 was observed following treatment of human lung fibroblasts with transforming growth factor-β, IL-1β, TNFα, serum, and phorbol esters. Human myometrium was found to express this transcript at low levels that do not change during parturition (Moore et al., 1999).

A second COX-1 variant, which lacks exon 1 and instead contains part of intron 2, was identified (Kitzler et al., 1995) in a rat tracheal cell line (EGV-6). This transcript was expressed at low levels; however, more than 90% of the COX-1 transcripts in this cell line are in this variant form. Primary rat tracheal epithelial cells and fibroblasts were also found to contain the variant transcript, but at only 1% of the level of COX-1 mRNA. Because this transcript lacks exon 1, which contains the initiating codon for translation, it has been considered to encode a nonsense COX protein (Kitzler et al., 1995). Interestingly, however, studies of the rat gastrointestinal tract show differential expression of this variant relative to COX-1 in aging stomach (Vogiagis et al., 2000). Moreover, expression of this variant was elevated in colorectal tumors, and its expression was reduced following treatment with NSAIDs (Vogiagis et al., 2001).

Very recently, brain-specific splice variants have been identified in dogs (Chandrasekharan et al., 2002). One of these, termed by the authors COX-3, consists of the COX-1 mRNA that retains intron-1. Intron-1 is small in all mammalian COX-1 genes thus far characterized. In dogs, it is 90 nucleotides in length and represents an in-frame insertion into the portion of the COX-1 open reading frame encoding the N-terminal hydrophobic signal peptide. The COX-3 variant produces protein containing the encoded intron-1 sequence when expressed in insect cells. The protein possesses reduced prostaglandin synthesis activity relative to COX-1, but analgesic/antipyretic drugs such as acetaminophen and dipyrone preferentially inhibit this activity. Evolutionary comparisons show that intron-1 is of similar size in all

species but is not always in frame as in canines. For example, it is out of frame in humans and rodents and would require additional mechanisms such as the use of alternative splice sites, ribosomal frameshifting, or RNA editing to make a functional protein (Chandrasekharan et al., 2002; Dinchuk et al., 2003; and Simmons, 2003).

Other COX-1 splice variants recently identified encode PCOX-1 (partial COX-1) proteins (Chandrasekharan et al., 2002). PCOX-1 variants exhibit in-frame deletion of exons 5 through 8. This deletion results in the removal of 219 amino acids from the catalytic domain corresponding to amino acids 119–337 in COX-1. Two forms of PCOX-1 are known, PCOX-1a and PCOX-1b. PCOX-1a contains intron-1 whereas this sequence is removed by splicing in PCOX-1b.

The deleted portion of PCOX-1 proteins contains structural helices HE, H1, H2, H3, H5, and part of H6, which constitute part of the cyclooxygenase and peroxidase catalytic sites. Consequently, PCOX-1 proteins do not make prostaglandins (Chandrasekharan et al., 2002); however, the critical proximal ligand to heme is not deleted and, therefore, PCOX-1, like their distant relatives PIOX and linoleate diol synthase, may be fatty acid oxidases or isomerases. It is important to note that the intron/exon placements in mammalian COX-1 and COX-2 genes are strictly conserved except for intron-1 in COX-1. COX-2 genes lack this intron. Therefore, it is possible that a PCOX-2 protein exists that would be analogous to PCOX-1b; however, a PCOX-2a could not exist because COX-2 lacks the equivalent of intron-1 in COX-1. Exons 2 through 5 and 7 in COX-1 and exons 2 through 4 and 6 in COX-2 genes all have the potential of producing in-frame deletions if excised during pre-mRNA splicing. Simultaneously skipping exons 6 and 8 in COX-1 or exons 5 and 7 in COX-2 transcripts also produces in-frame deletions. Thus many different splice variants of COX-1 and COX-2 can be generated by exon skipping that produce proteins that potentially contain a heme binding site.

In addition to the above splice variants that affect the coding region of COX-1, a number of alternatively polyadenylated transcripts are known. COX-1 in some human cells and tissues (e.g., endothelial cells) is expressed as three transcripts of 2.8, 4.5, and 5.2 kb (Hla, 1996). The 2.8-kb transcript encodes COX-1 and is the most abundant of these mRNAs. The 4.5-kb transcript has been poorly characterized. The 5.2-kb transcript arises by read-through of the consensus polyadenylation site and termination at another consensus termination site that is approximately 2.7 kb downstream (Plant and Laneuville, 1999). The 5.2-kb transcript was expressed at highest levels in human bladder and colon where its level exceeded that of the 2.8-kb transcript. A 5.2-kb COX-1 mRNA in cerebral cortex, other regions of the forebrain, heart, and muscle can contain all or part of intron-1 and is the human analog of the COX-3 mRNA in dog (Chandrasekharan et al., 2002). In megakaryocytes,

all three transcripts can be induced to different extents by mitogens such as phorbol esters (Plant and Laneuville, 1999). COX-1 in NIH3T3 cells is expressed as two transcripts of 2.8 and >7.0 kb in size (Evett et al., 1993). The 2.8-kb transcript encodes COX-1 and is greater than 10 times the abundance of the >7.0-kb transcript, which has been poorly characterized. At least some of the >7.0-kb transcript contains intron-1 and is analogous to the 5.2-kb intron-1-containing transcript in humans (unpublished data). COX-1 variants are summarized in Fig. 9A, and COX-3 and PCOX-1a variants are diagrammed in Fig. 9B.

COX-2 is expressed in many organisms as three alternatively polyadenylated transcripts of 4.2, 3.8, and 2.2 kb in size (Fig. 10). The 3.8- and 2.2-kb transcripts arise from polyadenylation at cryptic nonconsensus sites containing the sequence AUUAAA (Ristimaki et al., 1996; Evanson, 2002). Noncoordinated expression of these transcripts has been observed (Evanson, 2002). For example, rat spermatogonial cells contain primarily a 2.8-kb COX-2 transcript, and COX-2 in these cells was found to localize primarily within the nucleus (Neeraja et al., 2003). Thus alternative 3′-untranslated regions may serve to direct subcellular locations of COX isoenzymes.

In addition to variant COX mRNAs, which potentially produce COX or PCOX proteins with altered or expanded biological function, is the issue of mutations and epigenetic (e.g., CpG methylation; Deng et al., 2002) changes in COX genes or regulatory regions that may be involved in disease states. Numerous COX-1 and COX-2 single nucleotide polymorphisms (SNPs) have been identified, and a more complete discussion of them has been done by Ulrich et al. (2002) and Cipollone and Patrono (2002). Silent and nonsilent SNPs have been identified in COX coding regions, and SNPs of unknown function have also been identified in COX introns, untranslated regions, and upstream regulatory regions (Fritsche et al., 2001; Cipollone and Patrono, 2002; Ulrich et al., 2002; Halushka et al., 2003; Konheim and Wolford, 2003).

Because of the central role that COX-1 and COX-2 play in physiological and pathophysiological processes such as inflammation and cancer, it is anticipated that SNPs in COX genes may result in altered susceptibility to diseases. Although the genetic/epidemiological data are at present limited, early studies suggest this to be the case. Lin et al. (2002) associated a Val511Ala polymorphism found in some African Americans with a potential decreased susceptibility to colon cancer (odds ratios 0.56 and 0.67 in two separate study populations). Other SNPs found in the COX-2 promoter region and in intron-6 have been associated with a higher prevalence of type 2 diabetes mellitus in Pima Indians (Konheim and Wolford, 2003). Patients heterozygous for two single nucleotide changes in the COX-1 gene (A842G/C50T) demonstrated greater inhibition of platelet COX activity

by aspirin (Halushka et al., 2003). Finally, a SNP (−756 G>C) in the COX-2 gene promoter has been associated with lower promoter activity. Patients carrying this allele had lower C-reactive protein levels 1 to 4 days after coronary artery bypass graft surgery (Papafili et al., 2002). Thus, future studies of COX variants and mutants are likely to yield new and exciting insights into the roles of COX gene products.

G. Cyclooxygenase Activation by Endogenous Compounds

Endogenous radicals are required to activate newly made COX holoenzymes to form the enzymatically crucial tyrosyl radical at Tyr385. The precise identity of the endogenous activator(s) is presently unknown. Lipid peroxides and peroxynitrite have both been implicated as the oxidants that oxidize ferric heme (FeIII) to a ferryl-oxo (FeIV) protoporphyrin radical. Significant debate has focused on how this activation occurs and whether COX enzymes need to be activated after each cyclooxygenation catalytic cycle. However, the preponderance of evidence suggests that oxidation of tyrosine 385, with concomitant reduction of the protoporphyrin radical to FeIV heme generates the Tyr385 radical. The catalytic cycle, in which this crucial radical is formed, and the relationship of Tyr385 radical formation to POX and COX activities is illustrated in Fig. 6. Current data suggest that, once made, the radical is typically regenerated with each catalytic event (as shown in Figs. 6 and 7), and thus, the COX active site needs only one initial activation to be fully catalytically active. This model predicts that, under some circumstances, the peroxidase active site can act independently of the COX active site, a phenomenon that occurs when the COX site is inhibited by NSAIDs (Mizuno et al., 1982). However, this model also predicts that the COX site requires the heme of the peroxidase active site for its initial activation and only thereafter can act independently of the peroxidase active site. Consistent with this model has been the creation of site-directionally derived mutants of COX that possess only the peroxidase or cyclooxygenase activities of COX (Goodwin et al., 2000). A lag time in the activation of COX activity was observed in these POX mutants, suggesting that these mutant enzymes, and perhaps other naturally occurring enzymes such as PIOXs, require only sufficient POX activity to activate the enzyme. Such enzymes would be expected to exhibit delayed kinetics of activation compared with COXs, which have much more efficient POX active sites. Structural characteristics of COX isoenzymes have recently been reviewed in greater detail elsewhere (Garavito et al., 2002).
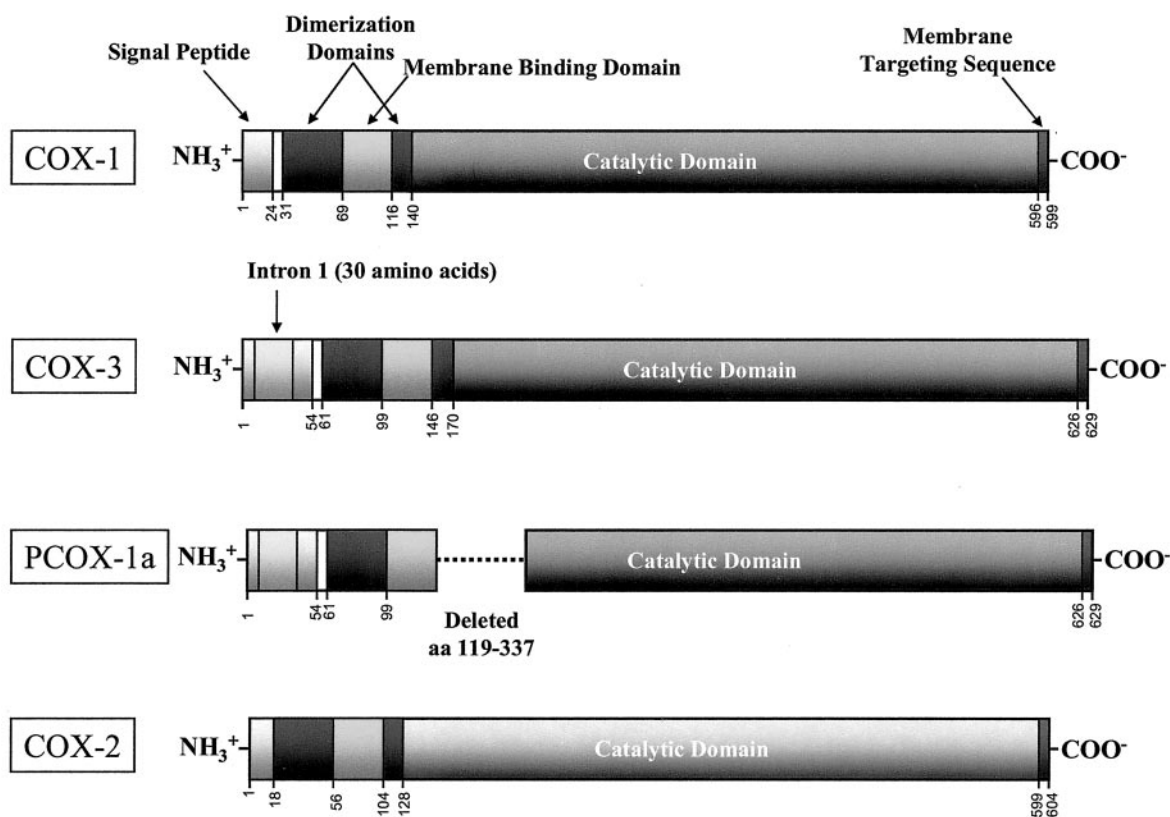
H. Enzyme Autoinactivation

COX-1 and COX-2 show different reactivities to lipid substrates (Chen et al., 1999). COX-2 but not COX-1 has been reported to exhibit positive cooperativity in AA

COX-1 and Known Variants
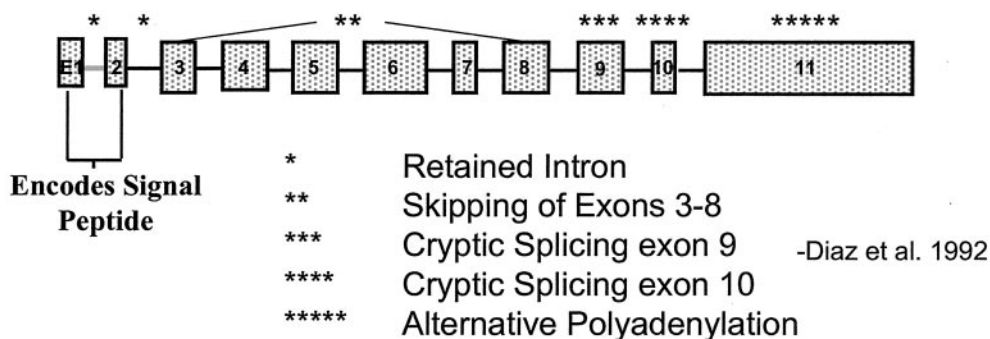
FIG. 9. COX-1 and known variants. A, structure of the COX-1 gene (length of introns not drawn to scale). Retention of intron-1 or parts thereof (denoted by one asterisk) gives rise to canine COX-3 and is found in some canine PCOXs. Retention of part of intron-2 is found in some human COX-1 transcripts (unpublished data). This intron is analogous to intron-1 in COX-2 transcripts that can be retained in a signal transduction-responsive fashion in chicken embryo fibroblasts (Xie et al., 1991). Skipping of exons 3–8 (denoted by 2 asterisks) can produce a PCOX protein. Use of a cryptic splice site in exon 9 (denoted by three asterisks) produces an inactive COX variant in humans (Diaz et al., 1992). Cryptic splicing has been detected in exon 10 (denoted by four asterisks; unpublished data) in humans resulting in a truncated open reading frame. Alternative polyadenylation (denoted by five asterisks) produces COX-1 transcript ranging from 2 to 6 through >7 kb in size. In humans and rodents, retention of intron-1 is frequently coupled to alternative polyadenylation. Alternative polyadenylation is also observed in COX-2 transcripts. B, diagram of the functional domains of COX-1 and COX-2 and the effects of alternative splicing in the insertion of intron-1 in canine COX-3 and PCOX-1b and deletion of 5 to 8 in canine PCOX-1a.

binding (Swinney et al., 1997). COX-2 has also been shown to be more catalytically efficient in oxidizing some 18-carbon chain fatty acids and eicosapentaenoic acid than COX-1 (Laneuville et al., 1995). Both enzymes show relatively short catalytic life spans (less than 1–2 min) when exposed to exogenous AA at concentrations that approach V_{max}. One explanation for this phenomenon has been that these isozymes generate arachidonyl peroxides and other reactive species such as malondialdehyde, which could attack the enzyme inactivating it. More likely, however, is that in the course of the catalytic cycle the Tyr385 radical or tyrosyl radicals at positions other than 385 result in internal protein crosslinking.

I. Synthetic Cyclooxygenase Inhibitors—Nonsteroidal Anti-Inflammatory Drugs

NSAIDs have been prominent analgesic/anti-inflammatory/antipyretic medications since 1898 when aspirin was first marketed. COX-2-selective drugs were introduced in 1999. All NSAIDs act as inhibitors of the cyclooxygenase active site of COX isozymes. Important mechanistic differences in the actions of individual NSAIDs with the COX active site are complex (Llorens et al., 2002).

1. Aspirin. Of the NSAIDs in medical use, only aspirin is a covalent modifier of COX-1 and COX-2. The crystallographic studies of Garavito and colleagues (Picot et al., 1994; Loll et al., 1995) demonstrated why this drug so efficiently acetylates serine 530 of COX-1. Like other NSAIDs, aspirin diffuses into the COX active site through the mouth of the channel and traverses up the channel to the constriction point formed by Arg120, Tyr355, and Glu524. At this point in the channel, the carboxyl of aspirin forms a weak ionic bond with the side chain of Arg 120, positioning aspirin only 5 Å below Ser530 and in the correct orientation for transacetylation (Loll et al., 1995). Because the catalytic pocket of the channel is somewhat larger in COX-2 than in COX-1, orientation of aspirin for attack on Ser530 is not as good, and transacetylation efficiency in COX-2 is reduced. This accounts for the 10- to 100-fold lowered sensitivity to aspirin of COX-2 in comparison to COX-1.

2. Competitively Acting Nonsteroidal Anti-Inflammatory Drugs. Other NSAIDs besides aspirin inhibit COX-1 and COX-2 by competing with AA for binding in the COX active site. However, NSAIDs significantly differ from each other in whether they bind the COX active site in a time-dependent or independent fashion.
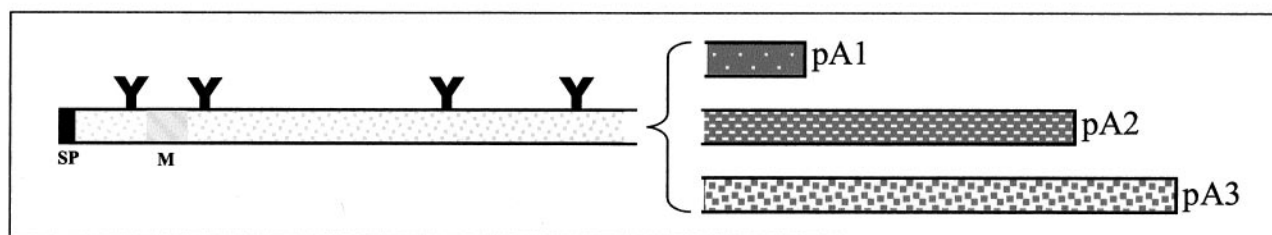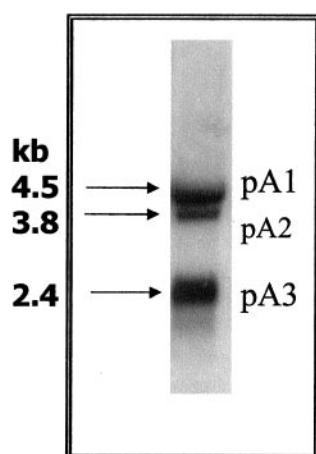
FIG. 10. Alternative polyadenylation variants of COX-2. Upper inset: Northern blot of COX-2 mRNA in human A541 cells treated with cytokines and 500 μM diclofenac demonstrating at least three COX-2 polyadenylation variants (pA1–pA3). Lower inset: diagram representing the difference in length of the 3′-untranslated region of these variants.

a. Time Dependence. NSAIDs differ dramatically with regard to how quickly they productively bind in the COX active site and how quickly they come out of the COX channel (Marnett and Kalgutkar, 1998). Some NSAIDs have very rapid on and off rates, such as ibuprofen (Selinsky et al., 2001). Such drugs do not show time dependence. They inhibit COX activity essentially instantaneously after addition of the NSAID, and they readily wash out of the COX active site when the NSAID is removed from the environment of the enzyme. In contrast, many NSAIDs such as indomethacin and diclofenac are time-dependent. They require typically seconds to minutes to bind the COX active site. Once bound, however, these drugs typically have low off-rates that may require hours for the NSAID to wash out of the active site. Time-dependent NSAIDs compete very poorly with AA in instantaneous assays of COX activity.

Time-dependent NSAIDs bind the COX active site first in a loose interaction and then in a productive tight complex. The rate-limiting step in drug binding is the formation of the tight binding conformation of the NSAID within the COX channel. Of particular importance to this second step in NSAID binding is the constriction point created by the hydrogen bonding network of Arg120, Tyr355, and Glu524 and the proposed difficulty for some NSAIDs to traverse it. A plausible scenario is that time-dependent NSAIDs likely require conformational heterogeneity in the constriction site caused by molecular breathing of the polypeptide to enter into the upper portion of the catalytic channel. One open state of the COX-2 enzyme has been identified crystallographically (Luong et al., 1996). An open state of the COX-1 enzyme that allows NSAIDs to pass the constriction point is likely to be transient since crystallographic studies show no difference in COX-1 conformation bound to time-dependent or nondependent NSAIDs (Selinsky et al., 2001).

Once having passed through the constriction site into the catalytic pocket, carboxyl-containing NSAIDs form a salt bridge between the carboxylate of the NSAID and the guanidinium moiety of Arg120 in COX-1 (Loll et al., 1995; Mancini et al., 1995). The ionic bond formed, however, is stronger for competitively acting NSAIDS than for aspirin. Hydrophobic interactions between the aromatic ring(s) of NSAIDs and the hydrophobic amino acids lining the channel further stabilize binding. The sum of these interactions results in tight binding of many NSAIDs at the constriction point of the channel, where they totally block entry of AA. Cocrystallization studies have been performed for flurbiprofen and COX-1 and COX-2 as well as indomethacin and COX-1, which define the precise binding interactions of carboxyl-containing NSAIDs in the COX binding site (Loll et al., 1994, 1996; Picot et al., 1994).

b. Selective Cyclooxygenase Inhibitors. Celecoxib (Celebrex) and rofecoxib (Vioxx) were marketed in 1999 as the first NSAIDs developed as selective COX-2 inhib-

itors. Other NSAIDs including meloxicam (Mobic), nimesulide, and etodolac (Lodine), which were marketed earlier in Europe or the United States as safer NSAIDs, were found after the discovery of COX-2 to be preferential inhibitors of this enzyme (Fig. 11). Currently, second generation COX-2 inhibitors, such as valdecoxib (Bextra; Smith and Baird, 2003) and etoricoxib (Hunt et al., 2003) are in use or are coming to market as are other COX-2-selective agents such as lumiracoxib (Ding and Jones, 2002). NS398 is a particularly important COX-2 inhibitor that is not in clinical use but is commercially available and, therefore, is widely used in pharmacology studies. Celecoxib and rofecoxib are diaryl compounds containing a sulfonamide and methylsulfone, respectively, rather than a carboxyl group (Fig. 11). Each of these compounds is a weak time-independent inhibitor of COX-1, but a potent time-dependent inhibitor of COX-2. Like time-dependent carboxyl-containing NSAIDs, time dependence for celecoxib and rofecoxib requires these compounds to enter and be stabilized in the catalytic pocket (Gierse et al., 1999). However, because these drugs lack a carboxyl group, stabilization of binding for both of these drugs does not require Arg120. Instead, a sum of hydrophobic and hydrogen bonding interactions stabilizes binding. Of particular importance is penetration of the sulfur-containing phenyl ring into the hydrophobic outpocketing in the COX-2 catalytic pocket shown in Fig. 8 (Kurumbail et al., 1996).

The structural basis for NS398 selectivity toward COX-2 is unclear, since its sulfonamide moiety is coordinated in the COX active site by ion pairing, just like carboxyl moieties in nonselective NSAIDs (Marnett and Kalgutkar, 1998).

3. Analgesic/Antipyretic Drugs. Acetaminophen (paracetamol in the United Kingdom) and dipyrone (Fig. 12) are important pain and fever relievers that lack anti-inflammatory activity. Acetaminophen is used primarily in North America and Western Europe whereas dipyrone is used extensively in Mexico, South America, Eastern Europe, and Africa. Although older than aspirin and used extensively for decades, acetaminophen has no certain mechanism of action. Flower and Vane (1972) proposed a central action for acetaminophen of inhibiting COX activity in brain. Indeed, neither acetaminophen nor dipyrone is acidic and both agents cross the blood-brain barrier well, but acetaminophen is a poor inhibitor of purified COX enzymes (Ouellet and Percival, 2001). Marginal inhibition of COX-1 can be achieved by performing inhibition studies at low arachidonate levels in the presence of low oxidant tone (Ouellet and Percival, 2001). Even under these conditions COX-2 was not inhibited at physiological concentrations. In whole cells, COX inhibition by acetaminophen has been observed in microglia (Fiebich et al., 2000; Greco et al., 2003), platelets, and leukocytes (Sciulli et al., 2003). Oates et al. (Boutaud et al., 2002) showed that in human umbilical vein endothelial cells in culture, acetaminophen inhibits
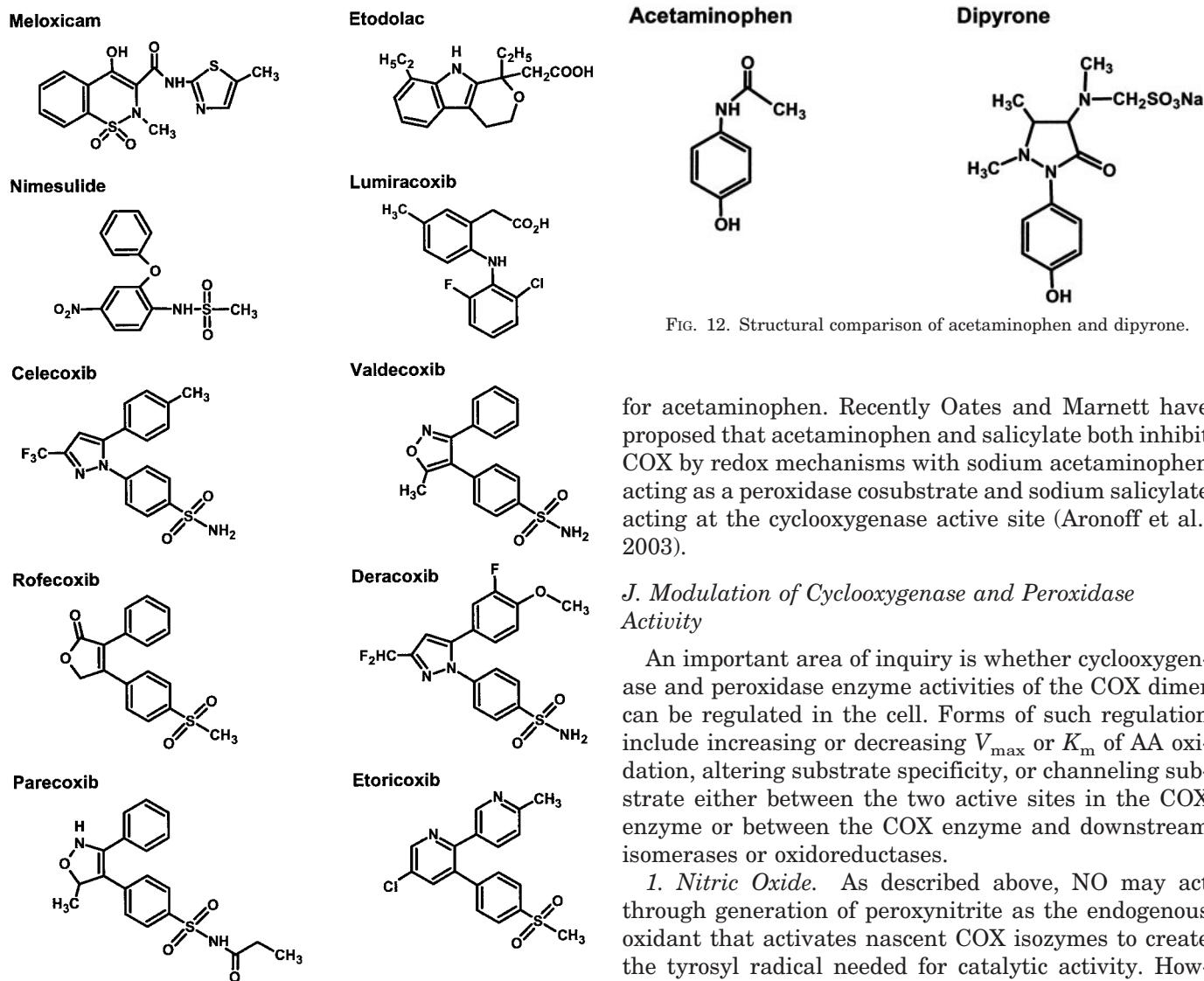
Meloxicam

Etodolac

Acetaminophen

Dipyrone

FIG. 12. Structural comparison of acetaminophen and dipyrone.

Nimesulide

Lumiracoxib

Celecoxib

Valdecoxib

Rofecoxib

Deracoxib

Parecoxib

Etoricoxib

FIG. 11. Structural comparison of selected commercially available COX-2 selective inhibitors.

for acetaminophen. Recently Oates and Marnett have proposed that acetaminophen and salicylate both inhibit COX by redox mechanisms with sodium acetaminophen acting as a peroxidase cosubstrate and sodium salicylate acting at the cyclooxygenase active site (Aronoff et al., 2003).

J. Modulation of Cyclooxygenase and Peroxidase Activity

An important area of inquiry is whether cyclooxygenase and peroxidase enzyme activities of the COX dimer can be regulated in the cell. Forms of such regulation include increasing or decreasing V_{max} or K_m of AA oxidation, altering substrate specificity, or channeling substrate either between the two active sites in the COX enzyme or between the COX enzyme and downstream isomerases or oxidoreductases.

1. Nitric Oxide. As described above, NO may act through generation of peroxynitrite as the endogenous oxidant that activates nascent COX isozymes to create the tyrosyl radical needed for catalytic activity. However, NO has been reported in different studies to increase (Salvemini et al., 1993) or decrease (Swierkosz et al., 1995) COX-2 activity, a phenomenon that has been reviewed elsewhere (Salvemini, 1997). For the most part, how NO functions to modulate catalytic activity is unknown. Modulation of activity by NO could occur by regulating other enzymes, including binding of NO to heme or nitration of cysteines or tyrosines via formation of peroxynitrite. Direct binding to heme is unlikely in COX-1 since NO has been demonstrated to have a low affinity ($K_d \approx 1$ mM) for ferric heme in this isozyme (Tsai et al., 1994).

Consistent with a role for NO in regulating COX-2 is that inducible NO synthase is frequently coregulated with this enzyme (Swierkosz et al., 1995). In vitro studies have suggested cross-talk (defined as codependence of both activities to a biological phenomenon) between COX and NO; however, selective COX-2 and inducible NO synthase inhibitors in rats failed to find cross-talk in vivo in rats exposed to endotoxin, which induces both enzymes in monocytes and tissues (Hamilton and Warner, 1998). Recently, Abramson and colleagues

COX-2 with an IC$_{50}$ of 66 μM, well within the therapeutic range in humans. It is unclear what factors may make COX susceptible to inhibition by acetaminophen in these whole cells, although changes in oxidant tone have been proposed (Boutaud et al., 2002). Recently, Chandrasekharan et al. (2002) identified a COX-1 variant, COX-3, that was sensitive to inhibition by acetaminophen and dipyrone in whole insect cells expressing the protein. The variant was identified in dog brain and may represent a central target of analgesic/antipyretic drugs.

Salicylate has analgesic, antipyretic, and anti-inflammatory activity, but unlike aspirin, is a poor inhibitor of COXs in vitro. In this regard, it resembles acetaminophen. Mitchell et al. (1997) found that salicylate does inhibit COX activity when substrate concentrations are maintained at low levels, similar to the findings of Ouellet and Percival (2001) and Oates and colleagues (2000)

(Clancy et al., 2000) have reported that NO activates COX-1 in J774.1 and mouse fetal lung fibroblasts and inhibits COX-2 in the same cell types. NO inhibited COX-2 by nitration of Tyr385. The mechanism by which COX-1 is stimulated by NO was not determined; however, the finding of a direct mechanism of NO action through nitration of Tyr385 suggests a potentially important role of NO synthase in regulating COX enzymes. In addition to modulation of COX activities by NO, NO levels may be influenced by COX through consumption of NO, which can act as a reducing cosubstrate for COX (O'Donnell et al., 2000).

2. Substrate Channeling and Enzyme Coupling. The present model for PGH_2 synthesis requires PGG_2 to exit the mouth of the COX channel and to diffuse by random motion to a POX site on the same or a neighboring enzyme for reduction to PGH_2. There is no obvious channel within the COX monomer through which PGG_2 could travel from the COX to the POX site. Few studies have addressed the concept of channeling PGG_2 from the COX to the POX active site.

Of current interest is whether COX isozymes are coupled with upstream phospholipases and downstream synthases to cause selective production of specific eicosanoid isomers. Many examples of coupling have been reported (e.g., Kampfer et al., 2003), but no universal coupling relationships between COXs and phospholipases or isomerases have been fully defined. Functional coupling of phospholipase A2S and COXs has been recently reviewed (Murakami et al., 2002a). Coupling depends on cell types and stimulation status of cells. Coupling also may involve intracellular translocation of isomerases (Ueno et al., 2001) or distinct subcellular locations of COXs (Schade et al., 2002). Cytosolic prostaglandin E synthase has been reported to be predominantly coupled to COX-1 and membrane-associated prostaglandin E synthase to COX-2 (Murakami et al., 2002b). Wu and colleagues (Liou et al., 2000) have shown that PGI synthase colocalizes with COX-1 and basal levels of COX-2 in bovine endothelial cells grown in 10% fetal bovine serum; however, PGI synthase did not colocalize with COX-2 induced with phorbol 12-myristate13-acetate in the same cells. The mechanism by which substrate might be channeled between enzymes that are colocalized is presently undefined.

3. Cyclooxygenase-Binding Proteins. Of current interest is whether other proteins may bind to and regulate COX isozymes. The apoptosis- and autoimmune disease-associated protein nucleobindin (Nuc) was shown to bind COX-1 and COX-2 fragments in the yeast 2-hybrid system. Amino acids 381–500 represented the shortest fragment of COX needed to bind Nuc. Binding between Nuc and COX was further substantiated by antibody competition experiments and other studies (Ballif, 1996); however, coimmunoprecipitation studies of COX-1 and COX-2 with Nuc expressed in insect cells showed that only unglycosylated or hypoglycosylated

forms of COX-2 bind tightly to Nuc (unpublished data), suggesting that Nuc does not tightly bind to glycosylated, enzymatically active forms of COX. Instead, glutathione *S*-transferase-pull-down assays suggest that NEFA (DNA-binding EF-hand acidic amino acid-rich region) (Barnikol-Watanabe et al., 1994), a protein closely related to Nuc, may be protein that binds to glycosylated COX-1 and COX-2.

II. Pharmacological Actions of Cyclooxygenase Isozyme-Generated Prostanoids

A. Prostaglandin Receptors

Prostaglandin receptors are designated by the letter "P" and a prefix of "D", "E", "F", "I", or "T" to signify preference for prostaglandins D, E, F, I, or thromboxane, respectively. To date, four subtypes of EP receptors have been identified, EP1–EP4.

In addition to classical prostanoids that act via plasma membrane-derived G-protein-coupled receptors, several COX products such as PGJ_2, 15-deoxy-^{12,14}-PGJ_2 (15d-PGJ_2) and PGA_2 can activate nuclear receptors of the PPAR class (Forman et al., 1995). Although it is not clear whether these classes of compounds are generated under physiological conditions and thus act as physiologically relevant inducers of PPARγ receptors, they are stimulators of this nuclear receptor pathway (Forman et al., 1995). Recent studies show that 15d-PGJ_2 is produced from the COX-2 pathway (Shibata et al., 2002). 15d-PGJ_2 is found in chronic inflammatory exudates of animal models during the late resolution phase (Gilroy et al., 1999). In this study, the authors showed that treatment with COX-2 inhibitors inhibited the appearance of 15d-PGJ_2, suggesting that it is produced from the COX-2 pathway (Gilroy et al., 1999). Recent studies show that in addition to stimulating the PPARγ receptors, these nuclear-acting prostanoid ligands inhibit the IκB kinase activity and thereby block the NFκB transcription factor pathway (Rossi et al., 2000). Indeed, treatment of vascular endothelial cells and ECV304 bladder cancer cells resulted in cellular apoptosis that requires the PPARγ activity, suggesting that nuclear-acting prostanoids may act to down-regulate angiogenesis (Bishop-Bailey and Hla, 1999). Indeed, PPARγ-activating prostanoids, such as 15d-PGJ_2 induce synoviocyte apoptosis and inhibit the development of adjuvant-induced arthritis in animal models (Kawahito et al., 2000). These data raise the possibility that the COX pathway may induce anti-angiogenic effects by nuclear-acting prostanoids.

B. Inflammation

Both PGE_2 and PGI_2 have been found in the synovial fluid from knee joints of arthritic patients (Higgs et al., 1974; Brodie et al., 1980; Bombardieri et al., 1981). In the rat model of carrageenan-induced paw edema, PGE_2 is the major PG involved in inflammation and pain,

since antibodies to PGE_2 inhibit both symptoms in this rat model (Mnich et al., 1995; Portanova et al., 1996). Carrageenan-induced hyperalgesia in the rat paw was also reversed by administration of SC58635 (now known as celecoxib), a selective COX-2 inhibitor, demonstrating that PGE_2 synthesis by the COX-2 enzyme is responsible for inflammatory symptoms in this animal model (Zhang et al., 1997). PGI_2 has also been detected in inflammatory lesions, and there may well be species differences, because inflammation is completely suppressed in mice in which the IP receptor for PGI_2 has been deleted (Murata et al., 1997). It is likely, therefore, that both PGE_2 and PGI_2 contribute to the development of inflammatory erythema and pain (Higgs et al., 1978).

Unexpectedly, in COX-2 gene-deleted mice the inflammatory response was not affected. However, only an acute inflammatory response was tested, which almost certainly involved COX-1 rather than COX-2 (Langenbach et al., 1995). The cyclopentenone PGs may be anti-inflammatory since 15-deoxy-PGJ_2 seems to resolve carrageenan-induced pleural inflammation in rats (Gilroy et al., 1999; Kawahito et al., 2000; Rossi et al., 2000). This anti-inflammatory activity may be mediated, at least in part, by inhibition of $I\kappa B$ kinase (Straus et al., 2000).

C. Pain

Prostaglandin E_2 does not cause pain when applied to an unprotected blister base on a human forearm, but greatly potentiates the pain induced by pain-producing mediators such as bradykinin or histamine (Ferreira, 1972). Therefore, Ferreira concluded that the pain-producing action of inflammatory mediators such as bradykinin or histamine was increased when PGs sensitized chemical receptors on primary afferent nerve terminals. PGs are therefore hyperalgesic. To produce its hyperalgesic action, PGE_2 released during the inflammatory response or by other trauma, lowers the activation threshold of tetrodotoxin-resistant sodium channels on sensory neurons (England, 1996). PGI_2 rather than PGE_2 may be involved in short-lasting hyperalgesia since it was more potent than PGE_2 in producing hyperalgesia in the rat and dog models (Ferreira et al., 1978). PGI_2 is mainly responsible for the stretching response to an intraperitoneal (i.p.) injection of zymosan in mice (Doherty et al., 1987), and IP receptor-deficient mice showed greatly reduced nociceptive responses to i.p. administration of dilute acetic acid (Murata et al., 1997). The stretching response to acetic acid is mediated mainly by COX-1, since it is abolished in COX-1$^{-/-}$ mice (Ballou et al., 2000). Although the major PG involved in the stretching response is PGI_2, stretching responses to acetic acid or phenylbenzoquinone are reduced by 50% in mice with a deleted EP1 receptor. This provides evidence that PGE_2 as well as PGI_2 mediates nociceptive responses to these hyperalgesic agents (Stock et al.,

2001). Thus, both PGE_2 and PGI_2 can sensitize nociceptors on sensory nerve terminals to painful stimulation.

Several studies, however, suggest that agonists for the IP receptor can activate sensory neurons in the absence of any other nociceptive stimuli. For example, the stable prostacyclin analogs, carbaprostacyclin and iloprost, produce stretching responses when injected i.p. into mice (Doherty et al., 1987; Berkenkopf and Weichman, 1988; Akarsu et al., 1989). In addition, PGI_2 and cicaprost increased spontaneous activity and mechanically evoked discharges of articular mechanonociceptors in the rat ankle joint arthritis model (Birrell et al., 1991; McQueen et al., 1991). Infusions of iloprost or cicaprost into patients suffering from vascular occlusive disease cause pain at the infusion site and headaches are a frequent side effect of this treatment (Shindo et al., 1991). A high density of IP receptors have been found on sensory neurons. Binding sites for [^{3}H]iloprost were observed in the rat dorsal root ganglion and the dorsal horn of the spinal cord (Matsumura et al., 1995; Pierce et al., 1995). In the mouse dorsal root ganglion, almost 40% of neurons demonstrated binding for IP receptor mRNA (Oida et al., 1995). IP receptors in sensory neurons are linked to the activation of adenyl cyclase and phospholipase C and can thus modulate the activity of ion channels and neurotransmitter release through activation of protein kinases A and C (Hingtgen et al., 1995; Smith et al., 1998b).

D. Fever

Fever is caused by PGE_2 released by inflammatory mediators from endothelial cells lining the blood vessels of the hypothalamus (Cao et al., 1998). Bacterial LPS from infecting organisms, or circulating IL-1, stimulate the expression of COX-2 and of PGE synthase in endothelial cells that constitute the blood-brain barrier (Ek et al., 2001; Samad et al., 2001). PGE_2 generated by PGE synthase diffuses out of the endothelial cells into the organum vasculosum lamina terminalis (OVLT) region of the hypothalamus, which is responsible for controlling fever. The pyretic action of PGE_2 is mediated by the EP3 receptor, since mutant mice lacking this receptor do not develop fever after administration of PGE_2, IL-1, or LPS (Ushikubi et al., 1998). Pyrexia-producing PGE_2 is formed by COX-2 for selective COX-2 inhibitors, such as rofecoxib, abolish fever in several species, including humans (Li et al., 1999; Schwartz, 1999), and LPS fails to raise the core temperature of COX-2$^{-/-}$ mice (Li et al., 1999). Although COX-2 is primarily involved in the fever response to LPS, source components of this response are dependent on COX-1 (Zhang et al., 2003)

E. Immune System

Mouse macrophages stimulated with inflammatory mediators to induce COX-2 release PGE_2 and PGI_2, whereas stimulated human monocytes and macrophages secrete large amounts of PGE_2 together with

TXA_2 (Tripp et al., 1985; Fels et al., 1986). Neutrophils make moderate amounts of PGE_2, whereas mast cells produce almost exclusively PGD_2 (Stenson and Parker, 1983). No prostanoids appear to be made by lymphocytes, although both COX-1 and COX-2 have been detected in these cells (Pablos et al., 1999). Release of PGE_2 by macrophages may act as a negative feedback control mechanism, reducing further activation through increase of cAMP thus resulting in inhibition of immune function.

PGE_2 also inhibits IL-2 and interferon γ (IFNγ) production from T lymphocytes (Betz and Fox, 1991) and IL-1 and TNFα release from macrophages (Kunkel et al., 1986a,b, 1988); however, immature cells of the immune system are stimulated by PGE_2. For example, PGE_2 induces immature thymocytes and B lymphocytes to differentiate and acquire the functional characteristics of mature cells (Parker, 1986).

It has been suggested that PGE_2 produced by tumor cells accounts for the depression of the immune system associated with cancer. Large amounts of PGs are produced by certain tumor cells (Bennett et al., 1977), which induce a generalized state of immunodeficiency (Plescia et al., 1975). This immunosuppression was prevented in tumor-bearing mice by inhibitors of PG synthesis such as indomethacin (Pollard and Luckert, 1981). Treatment of rheumatoid arthritis with aspirin-like drugs leads to inhibition of PG formation and thus to removal of the immunosuppressant effect of these eicosanoids. Removal of immunosuppression may be one of the factors responsible for the cancer-inhibiting action of the NSAIDs. Another consequence of removing the suppression of immune processes by PGs may be the enhancement of cartilage breakdown seen with NSAIDs in vitro and in vivo (Pettipher et al., 1988; Desa et al., 1989).

F. Gastrointestinal Tract

PG synthesis can be demonstrated to occur in every part of the gastrointestinal tract. In rat tissues, using vortex generation, the rank order of PG synthesis, as determined by bioassay techniques, was greatest in gastric muscle and forestomach, followed by gastric mucosa, colon, rectum, ileum, cecum, duodenum, jejunum, and esophagus (Whittle and Salmon, 1983). PGE_2 contracts gastrointestinal smooth muscle through stimulation of smooth muscle EP1 receptors.

Prostanoids are "cytoprotective" in the gastrointestinal tract, as strikingly demonstrated by the finding in rat that gastric damage induced by topical application of strong acids, hypertonic solutions or ethanol, could be reduced by coadministration of various PGs (Robert et al., 1967, 1979; Miller, 1983). The mechanism of the cytoprotective action is complex and depends on a combination of several mechanisms.

1. Both PGE_2 (acting on the EP3 receptor) and PGI_2 (acting on the IP receptor) reduce secretion of gastric acid, even histamine-stimulated acid secretion, by the parietal cells of the stomach. This action is species-dependent since PGI_2 is more active than PGE_2 in anesthetized rat, conscious dog, and monkey, whereas PGE_2 is a more potent inhibitor of acid secretion in the stomach of the anesthetized dog (Robert et al., 1977, 1979; Gerkens et al., 1978; Whittle et al., 1978; Konturek et al., 1980; Shea-Donohue et al., 1982).

2. Intravenous infusions of PGE_2 or PGI_2 exert a direct vasodilator action on the gastric mucosa (Konturek et al., 1980). Increase in gastric mucosal blood flow is obviously beneficial in maintaining the functional integrity of the gastric tissue (Whittle et al., 1978).

3. PGE_2 is synthesized by epithelial and smooth muscle cells in the stomach, and intragastric administration of PGE_2 to humans stimulates the release of viscous mucus (Johansson and Kollberg, 1979), which could play a defensive role against mucosal injury (Allen and Garner, 1980) by gastric acid. Other than providing a physical barrier, mucus may act to create an unstirred layer of secreted bicarbonate on the epithelium (Bahari et al., 1982) and hence help to neutralize hydrogen ions diffusing back from the lumen into the mucosa. PGE_2 stimulates bicarbonate secretion via the EP3 receptor, thus application of acid induces more severe damage to the stomach mucosa in $EP3^{-/-}$ mice than in wild-type animals (Takeuchi et al., 1999).

Most surprising has been the finding that animals without the COX-1 gene did not spontaneously develop stomach ulcers (Langenbach et al., 1995). This has been explained by postulation of an adaptation process whereby increased production of nitric oxide or calcitonin gene-related peptide may have taken over the cytoprotective role of the absent PGs. An alternative explanation is that both COX-1 and COX-2 may be required for gastrointestinal mucosal defense. $COX-1^{-/-}$ or $COX-2^{-/-}$ mice were more susceptible to colonic injury with dextran sodium sulfate than wild-type mice, but the administration of a selective COX-2 inhibitor exacerbated the mucosal injury with dextran sodium sulfate in $COX-1^{-/-}$ mice (Morteau et al., 2000). Similarly, neither the selective COX-1 inhibitor, SC-560, nor the selective COX-2 inhibitor, celecoxib, administered to rats produced gastric damage, even though SC-560 reduced both gastric PGE_2 synthesis and gastric blood flow; however, the combination of SC-560 with celecoxib resulted in gastric erosions in all rats. Celecoxib, but not SC-560, increased leukocyte adherence to the vascular endothelium of the gastrointestinal microcirculation. Thus, it appears that inhibition of the activity of both COX-1 and COX-2 is required to produce gastric damage (Wallace et al., 2000). This work was confirmed by Gretzer et al. (2001) who showed that cotreatment of rats with SC-560 and the COX-2-selective inhibitor, rofecoxib, induced severe gastric lesions.

G. Cardiovascular System

Various prostanoids are secreted by vascular cells, including PGI_2, PGE_2, and $PGF_{2\alpha}$, among others. In addition, cells in the vascular wall respond to various prostanoids (FitzGerald et al., 1983). The major prostanoid secreted by endothelial cells is PGI_2, as the prostacyclin synthase enzyme is enriched in this cell type. This prostanoid binds to the IP receptors on vascular smooth muscle cells and inhibits vascular contraction (FitzGerald et al., 1983). The IP receptor couples to the G_s protein and increases intracellular cAMP concentrations, thus antagonizing the contractile agonists and inhibiting the mitogen-activated protein kinase pathway (FitzGerald and Patrono, 2001). In platelets, the IP receptor signaling antagonizes the aggregation response and thus inhibits thrombosis. PGI_2 synthesis by the COX pathway is important in normal control of vascular homeostasis and thrombosis. Interestingly, an unexpected role of PGI_2 in the control of the inflammatory process was elucidated by the deletion of the IP receptor in knockout mice (Murata et al., 1997). This is probably related to the ability of PGI_2 to induce vascular relaxation, which is important in the increased blood flow that occurs during inflammation. PGE_2 and $PGF_{2\alpha}$, in contrast to PGI_2 can induce either vasoconstriction or vasorelaxation, depending on the vascular bed (FitzGerald et al., 1983). These effects are mediated by specific expression of the respective receptor subtypes on the vascular smooth muscle cells (FitzGerald et al., 1983). These findings indicate that the products of the COX pathway mediate complex and critical homeostatic interactions in the vessel wall.

PGE_2 can also potently relax vascular smooth muscle contributing to the characteristic vasodilatation (via the EP2 receptor) leading to the erythema seen in acute inflammation (Solomon et al., 1968). This increases blood flow through inflamed tissues and thus augments the extravasation of fluid, facilitating edema formation (Williams and Peck, 1977). EP2 receptors generally mediate arterial dilatation and are also involved in salt-sensitive hypertension. An infusion of PGE_2, normally hypotensive, raised blood pressure in $EP2^{-/-}$ mice, whereas $EP2^{-/-}$ mice fed a high salt diet became hypertensive. There was no change in systolic blood pressure of control animals on a high salt diet (Kennedy et al., 1999).

Blood platelets contain only COX-1, which converts AA to the potent pro-aggregatory and vasoconstrictor eicosanoid TXA_2, the major COX product formed by platelets. TXA_2 has a half-life at body pH and temperature of 30 s, degrading to inactive TXB_2 (Needleman et al., 1976). It was proposed in 1976 that PGI_2 and TXA_2 represent the opposite poles of a homeostatic mechanism for regulation of hemostasis in vivo (Moncada et al., 1976). Stimulation of the TP receptor on platelets leads to their aggregation and $TP^{-/-}$ mice have greatly pro-

longed bleeding times, demonstrating the importance of TXA_2 in hemostasis (Murata et al., 1997; Thomas et al., 1998). TP receptors are coupled through regulatory G proteins to increased intracellular phosphoinositol hydrolysis. Antagonists for the TP receptor on platelets are of interest to inhibit platelet aggregation and prevent further thrombosis after myocardial infarction. TXA_2 also causes contraction of all vascular and airway smooth muscle by stimulating the TP receptor. As expected, blood platelets of mice with a nonfunctional COX-1 gene did not aggregate to AA (Langenbach et al., 1995).

Normal endothelial cells and vascular smooth muscle cells express COX-1 (Hla and Neilson, 1992); however, COX-2 was identified as a shear stress-inducible gene in vascular endothelial cell cultures (Topper et al., 1996). These data suggest that vascular endothelial cells express COX-2 in response to normal blood flow. Indeed, recent studies in human volunteers after administration of COX-2 inhibitors suggest that total body prostacyclin synthesis (as measured by the quantitation of urinary metabolites) is contributed significantly by the COX-2 isoenzyme (McAdam et al., 1999). Immunocytochemical studies, however, suggest that COX-2 expression in normal vessels (both large and small) is negligible to undetectable, whereas that of COX-1 is readily detectable (Crofford et al., 1994; Schonbeck et al., 1999). These observations suggest that COX-1 is highly expressed whereas COX-2 is expressed at a much lower level in the normal vascular tissues. In contrast, high levels of COX-2 are detected in activated and proliferating vascular tissues, for example angiogenic microvessels, atherosclerotic lesions, and inflamed tissues (Sano et al., 1992).

Normal production of prostacyclin is critical for vessel tone control and inhibition of thrombosis (FitzGerald et al., 1983). This is because PGI_2/TXA_2 balance is critical. After ingestion of aspirin, platelet thromboxane synthesis and vascular prostacyclin synthesis are inhibited rapidly; however, nucleated vascular cells recover their ability to synthesize prostacyclin rapidly, estimated to be ~6 h (Jaffe and Weksler, 1979). In contrast, thromboxane synthesis is inhibited for a much longer period, since platelets lack the ability to resynthesize COX via de novo protein synthesis (Jaffe and Weksler, 1979). Thus, aspirin is a first line of defense against thrombotic and vaso-occlusive vascular diseases (Marcus et al., 1981). Whether COX-2 inhibition presents a risk for thrombotic events requires further study (Mukherjee et al., 2001a).

Atherosclerotic lesions occur in large vessels like the carotid and the coronary arteries. Plaques are classified as stable and unstable plaques, depending on the degree of lipid accumulation and inflammation in the pathologic tissue (Lusis, 2000). Unstable plaques have high levels of metalloproteinase production, contain more activated foam cells, and have lipid accumulation in the

lesion. They are highly prothrombotic and lead to rupture, culminating in the occlusion of the vessel (Lusis, 2000). COX-2 expression was found to be elevated in human atherosclerotic plaques (Schonbeck et al., 1999). Monocytic foam cells and vascular endothelial cells, express high levels of COX-2 in these lesions (Schonbeck et al., 1999). It is likely that oxidized low-density lipoprotein components, such as lysophosphatidyl choline, as well as cytokines and growth factors found in the lesions induce the COX-2 gene expression. The role of COX-2 in these lesions in not well understood. Studies with COX-2 null mice and COX-2 inhibitors are required to better define the causal role (if any) of COX-2 in atherosclerosis and other vascular pathologies.

Recently there is renewed interest in the role of COX products in the formation of new vessels, a process commonly referred to as angiogenesis (Hla et al., 1993). Early studies by Gullino showed that PGE_2 is a potent inducer of angiogenesis in the corneal models of angiogenesis (Ziche et al., 1982); however, the mechanisms involved are not well understood since PGE_2 does not potently stimulate endothelial cell migration, proliferation, and morphogenesis. In addition, various in vivo studies indicate that NSAIDs inhibit angiogenesis in various in vivo models (Majima et al., 1997). Such studies were inconclusive since the doses of drugs used to inhibit angiogenesis are much higher than the doses required to inhibit COX-1 or -2 activity. It is known that high concentration of NSAIDs have effects independent on COX enzyme activity (Marx, 2001). Various studies reported that PGE_2 is a potent inducer of vascular endothelial cell growth factor (VEGF) expression in rheumatoid synovial fibroblasts and osteoblasts (Harada et al., 1994; Ben-Av et al., 1995). These data suggest that PGE_2 can induce angiogenesis indirectly by up-regulating VEGF expression of stromal cells. The receptor subtype involved in VEGF expression is not known, but cAMP increases may be involved, suggesting that EP2 and/or EP4 subtypes may be critical (Ben-Av et al., 1995).

The induction of angiogenesis by the COX-derived PGE_2 may be potentially involved in colon cancer (Hansen-Petrik et al., 2002). Tsujii et al. (1998) reported that enhanced COX-2 expression in colon cancer cells modulates the angiogenic behavior of endothelial cells in the coculture system. The authors showed that secretion of angiogenic factors, such as VEGF and fibroblast growth factor, is modulated by COX-2 overexpression (Tsujii et al., 1998). The mechanistic details of how COX-2 regulates angiogenic growth factor expression secretions are not well understood. Furthermore, the DuBois laboratory has recently extended these findings and showed that tumor angiogenesis and growth of explanted tumors are reduced in the COX-2 null mice; suggesting that host COX-2 induction by the tumor cells contributes to tumor angiogenesis and ultimately the growth of the tumors (Williams et al., 2000a). These observations, cou-

pled with the findings that COX-2 is overexpressed in the angiogenic lesions, suggest that COX-2 inhibitors may possess an antiangiogenic effect in various pathologic conditions such as rheumatoid arthritis and solid tumors; however, effective inhibition of angiogenesis should lead to disease modification in rheumatoid arthritis, an effect that is not observed in chronic clinical use of NSAIDs and COX-2 inhibitors (Crofford, 2000). Thus, the role of COX-2 and its products in angiogenesis in various pathological contexts is unclear at present.

The effects of COX-1 and -2 on angiogenesis are assumed to be due to the actions of secreted prostanoids that act in an autocrine and/or paracrine manner; however, the ability of the peroxidase activity to utilize various reducing equivalents may also contribute to those effects (Ohki et al., 1979). Some in vitro and in vivo studies have raised the possibility that NSAID-treated COX enzymes as well as active site mutants of the COX enzymes that fail to synthesize prostanoids induce effects on transfected cells (Narko et al., 1997; Trifan et al., 1999). Although these studies are suggestive, definitive involvement of the peroxidase activity of the COX enzyme in various physiological phenomena dependent on COX-1 or -2 expression is not yet demonstrated.

Although the mechanistic studies have yielded a multitude of possibilities of the COX pathway to regulate angiogenesis, unequivocal evidence that COX enzymes regulate angiogenesis is lacking at present. Clearly, angiogenesis during embryo development does not require the COX pathway since the vascular system develops normally in both COX-1- and COX-2-deficient mice (Langenbach et al., 1999a). However, embryo implantation defects seen in COX-2$^{-/-}$ female mice may be related to the effect of prostanoid induction of angiogenesis in the uterine implantation site. In this system, the PPARδ nuclear receptor is essential and induction can be restored by carbaprostacyclin, a pharmacological agonist of the PPARδ receptor (Lim et al., 1997); however, various NSAIDs inhibit angiogenesis in models of angiogenesis, such as the sponge and corneal model (Majima et al., 1997). Further studies using both selective antagonists, enzyme inhibitors, as well as receptor gene null mice are required to establish the role of the COX enzymes in angiogenesis in vivo.

In contrast, prostanoids are required for maintenance of an open ductus and for its closure in the postnatal period (Heymann et al., 1976; Loftin et al., 2001). The transition from maternal to fetal circulation is followed by the closure of the ductus, which separates the arterial and venous systems and thereby allows for efficient pulmonary blood flow (Loftin et al., 2001). Premature closure of the ductus leads to abnormal pulmonary pressure and lung dysfunction. Clinical studies have shown that indomethacin treatment produces closure of the ductus, suggesting that prostanoids mediate this process (Segi et al., 1998; Loftin et al., 2001). Indeed, COX-1 and -2 double null homozygous mice develop neonatal

circulatory failure due to the failure to close the ductus arteriosus (Loftin et al., 2001). This phenotype was also seen in EP4 null mice, suggesting that PGE_2 signaling via the EP4 receptor is involved (Loftin et al., 2001); however, the role of other prostanoids such as thromboxane A_2 is also implicated (Ushikubi et al., 2000; Loftin et al., 2001). These data indicate the essential nonredundant function of prostanoids in vascular development and remodeling in the neonatal period (Ushikubi et al., 2000). Whether similar mechanisms operate in pathological vascular remodeling is not known.

H. Kidney

The cortex of normal kidneys produces mainly PGE_2 and PGI_2 with very small amounts of TXA_2 (Farman et al., 1987). The renal medulla produces mostly PGE_2 for which it has a synthetic capacity approximately 20 times that of the cortex (Zusman and Keiser, 1977). Urinary PGE_2 levels are generally regarded as reflecting production of PGE_2 by the kidneys (Patrono and Dunn, 1987). PGE_2 and PGI_2 have vasodilator actions in the kidney, and intrarenal infusions of these PGs increase renal blood flow. PGs are also natriuretic, inhibiting tubular sodium reabsorption, and in the thick ascending limb of the loop of Henle, they reduce chloride transport. Glomerular epithelial and mesangial cells have the synthetic capacity to form both PGI_2 and PGE_2. These prostanoids are therefore uniquely situated to influence renal blood flow, glomerular filtration rate, and the release of renin. PGI_2 and PGE_2 synthesized in the renal cortex are important stimulators of renin release (Osborn et al., 1984). PGI_2 formed by COX-2 in mesangial cells may directly stimulate renin secretion since upregulation of COX-2 has been observed in the macula densa following salt deprivation (Harris et al., 1994).

Different nephron segments synthesize a distinctive spectrum of AA metabolites that behave as either modulators or mediators of the actions of hormones on tubular function (Carroll et al., 1991; Omata et al., 1992). Studies on rabbit medullary cells in the thick ascending limb of Henle's loop (mTALH) revealed that the principal pathway of AA metabolism in this segment of the nephron is via cytochrome P450 (P450) and not COX-1 (Schwartzman et al., 1985). Thus, the major P450-derived AA products synthesized by the rabbit mTALH are 19- and 20-hydroxyeicosatetraenoates (HETEs) and 20-COOH HETE, a metabolite of 20-HETE (Omata et al., 1992); however, some COX-2 protein is also expressed constitutively in unstimulated mTALH cells and COX-2 expression increases after treatment with TNF or phorbol 12-myristate 13-acetate (Ferreri et al., 1999). In addition, the products of P450 interact with TNF formed by mTALH cells and with angiotensin II to regulate ion transport in cells of the mTALH (Ferreri et al., 1998).

Maintenance of kidney function in animal models of disease states and in patients with congestive heart failure, liver cirrhosis, or renal insufficiency is dependent on vasodilator PGs. These patients are, therefore, at risk of renal ischemia when PG synthesis is reduced by NSAIDs. Synthesis of PGE_2 is mainly by COX-1, although as mentioned above, there are discrete cells in the macula densa that contain constitutive COX-2 (Harris et al., 1994; Harris, 1996). Prostacyclin, made by constitutive COX-2 may drive the renin-angiotensin system (Harris, 1996). This rapidly evolving field is reviewed by Schneider and Stahl (1998).

FitzGerald's group (Catella-Lawson et al., 1999) compared the renal effects of the nonselective COX inhibitor indomethacin with those of the selective COX-2 inhibitor rofecoxib and with placebo in healthy older adults over 2 weeks of treatment. Both active regimes were associated with a transient but significant decline in urinary sodium excretion during the first 72 h. The glomerular filtration rate (GFR) was decreased by indomethacin but not changed significantly by rofecoxib. Thus, acute sodium retention by NSAIDs in healthy adults is mediated by inhibition of COX-2, whereas depression of GFR is due to inhibition of COX-1. The urinary excretion of the PGI_2 metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$ was decreased by both rofecoxib and indomethacin, but not by placebo (Catella-Lawson et al., 1999). The implication of this is that prostacyclin is synthesized in endothelial cell by COX-2 rather than COX-1. COX-2 is possibly continuously induced by the shear stress on the arterial wall, rather than being present constitutively (Topper et al., 1996).

Young male and female COX-2 gene-deficient mice showed arrested development of the kidneys (Dinchuk et al., 1995; Morham et al., 1995). Rodent kidneys develop fully only after birth, and COX-2 appears to be important in this process. Failure to develop mature kidneys shortened the life span of the COX-2 gene null mice to approximately 8 weeks. This retardation of renal cortical development could be mimicked in mice and rats by chronic administration of a selective COX-2 inhibitor to the mother during pregnancy and to the pups until weaning (Kömhoff et al., 2000). Failure to develop mature kidneys shortens the life span of COX-2 null mice to approximately 2 weeks in some genetic backgrounds; however, it may be possible to overcome this developmental defect by cross-breeding the original COX-2$^{-/-}$ C57BL/6 mice with a DBA/1 strain (Ballou et al., 2000). The animals of this mixed strain live a full life span, and their kidneys appear to develop normally (Laulederkind et al., 2002). This suggests the potent effects of modifier genes on COX-2 regulation of kidney development.

I. Lungs

PGs have potent actions on bronchiolar tone and on the diameter of the pulmonary blood vessels. The airways of most species, including humans, contract to $PGF_{2\alpha}$, TXA_2, and PGD_2, whereas PGE_2 and PGI_2 are weak bronchodilators. PGD_2 and $PGF_{2\alpha}$ potently constrict the airways in asthmatic patients and potentiate

the constrictor responses to other spasmogens (Hardy et al., 1984; Fuller et al., 1986). The concentrations of PGD_2 and $PGF_{2\alpha}$ in bronchoalveolar lavage fluid of asthmatic subjects were 10-fold higher than in control nonasthmatic and atopic individuals (Liu et al., 1990). Excretion of the stable metabolite of TXA_2 increases after allergen challenge (Sladek et al., 1990). Thus, raised levels of bronchoconstrictor PGs in the lungs may contribute to allergic bronchospasm during asthmatic attacks. Pulmonary blood vessels are constricted by $PGF_{2\alpha}$ and TXA_2, but in some species they dilate to PGE_2. Prostacyclin is a potent vasodilator of the pulmonary circulation in humans and other species.

Blood levels of prostacyclin increase 15- to 20-fold in anesthetized patients with artificial ventilation. This endothelium-derived prostacyclin is well placed to function as a local vasodilator and to prevent the formation of microthrombi (Bakhle and Ferreira, 1985). PGI_2 may be important in regulating pulmonary vascular tone during chronic hypoxia. Overexpression of prostacyclin synthase (PGIS) in lung epithelium of transgenic mice prevented development of pulmonary hypertension after exposure to hypobaric hypoxia (Geraci et al., 1999), whereas lungs of patients with severe pulmonary hypertension expressed lower levels of PGIS than those of control subjects (Tuder et al., 1999).

Mediators of inflammation such as bradykinin, histamine, and 5-hydroxytryptamine release PGs from lung tissue. Histamine releases $PGF_{2\alpha}$ from human lung fragments by stimulating H1 receptors. Lungs of asthmatics produce more histamine than normal lungs, which correlates with the greater number of mast cells found in asthmatic lungs (Holgate, 1986). Pro-inflammatory cytokines such as IL-1β and TNFα are present in the inflamed airways of asthmatic patients (Barnes, 1994) and induce COX-2 expression in lung epithelial cells, airway smooth muscle, pulmonary endothelial cells, and alveolar macrophages (Mitchell et al., 1995). In the carrageenan-induced pleurisy model of inflammation, levels of COX-2 in the cell pellets of pleural exudate increased maximally 2 h after the injection of carrageenan (Tomlinson et al., 1994). This was accounted for by induction of COX-2 in 100% of mast cells, in 65% of resident mononuclear leukocytes, and in 8% of extravasated neutrophils present in the exudates (Hatanaka et al., 1996).

Inflammatory stimuli cause differential release of PGs from various regions of the lungs. Human cultured pulmonary epithelial cells stimulated with LPS, IL-1β, TNFα, or a mixture of cytokines synthesize mainly PGE_2 together with smaller amounts of $PGF_{2\alpha}$, PGI_2, and TXA_2. This PG production can be suppressed by dexamethasone (Mitchell et al., 1994). Thus, PGE_2 is the main product of COX-2 induced in lung epithelial cells (Springall et al., 1995; Newton et al., 1997) and in vitro studies in animals suggest that epithelial PGE_2 may protect against bronchoconstriction induced by bradyki-

nin, tachykinins, and endothelin (Frossard et al., 1989, 1990; Battistini et al., 1990; Goldie et al., 1990; Devillier et al., 1991). Moreover, evidence in COX-2 null mice suggests that COX-2-produced PGE_2 prevents chemically induced pulmonary fibrogenesis (Bonner et al., 2002). Endogenous PGE_2 may therefore be bronchoprotective in asthma and other pulmonary conditions and may act as an endogenous anti-inflammatory factor (Pavord and Tattersfield, 1995). Aspirin-induced asthma may be triggered by increased release of leukotrienes from inflammatory cells caused by removal of the inhibitory influence of PGE_2, a major product of COX-2 in airways (Szczeklik, 1988, 1995; Kuitert et al., 1996).

The true role of PGs in asthma is unclear. The nonselective NSAIDs have very little effect on airway function in most patients with asthma except to make the disease worse in aspirin-sensitive asthmatics. Perhaps the actions of the bronchoconstrictor PGs are counterbalanced by the protective dilator action of PGE_2. It remains to be seen whether selective COX-2 inhibitors will be beneficial in allergic asthma; however, mast cells, by producing PGD_2 in response to an allergic challenge (Lewis and Austen, 1981), may have a pathological role in allergic asthma. Disruption of the gene encoding the DP receptor in ovalbumin-sensitized mice (Matsuoka et al., 2000) prevented the infiltration of cytokine-responsive cells into the lungs. This suggests that PGD_2 released from mast cells stimulates production of cytokines and chemokines by an action on DP receptors, which leads to recruitment of inflammatory cells into the lungs.

A physiological role has been proposed for constitutive COX-2 in the lung, for it was found in noninflamed rat lungs (Ermert et al., 1998). COX-2 is present in vascular smooth muscle cells of normal rat lungs as well as in lung macrophages and mast cells. Arachidonic acid perfused through isolated rat lungs forms TXA_2 and causes vasoconstriction, which is blocked dose dependently with selective COX-2 inhibitors, suggesting a physiological role for COX-2 in the regulation of pulmonary blood flow (Ermert et al., 1998).

J. Reproduction

Human seminal fluid contains high concentrations of several PGs including PGE_2, PGE_1, PGE_3, and $PGF_{2\alpha}$ (Samuelsson, 1963), which perhaps function to relax corporeal smooth muscle. These PGs may also facilitate conception by stimulating contractions of the cervix, fallopian tubes, and uterus. Prostaglandin E_1 given by injection into the corpus cavernosum has been used as a treatment for impotence.

The EP2 receptor is important for in vivo fertilization, as EP2$^{-/-}$ mice ovulated normally but the ova failed to become fertilized (Tilley et al., 1999). Because of these problems with ovulation and fertilization, EP2$^{-/-}$ female mice give birth to unusually small litters (Hizaki et al., 1999), emphasizing the importance of PGE_2 in reproductive processes. PGE_2 is involved in ripening of the

cervix prior to labor and can itself induce labor at any stage of pregnancy. It is made by COX-1 and COX-2, as is $PGF_{2\alpha}$, in the pregnant uterus, fetal membranes, and umbilical cord. COX-2 mRNA in the amnion and placenta increases considerably immediately before and after the start of labor (Gibb and Sun, 1996).

$PGF_{2\alpha}$ is involved in reproductive processes such as ovulation, luteolysis and parturition. In most species, except primates and humans, $PGF_{2\alpha}$ activity made by the induction of COX-2 is required for commencement of parturition, since mutant mice lacking the gene for the FP receptor, are unable to give birth. The action of $PGF_{2\alpha}$ on the FP receptor of the corpus luteum induces luteolysis, which terminates progesterone production, and hence triggers parturition, for, in the absence of progesterone, the uterus becomes more sensitive to oxytocin (Sugimoto et al., 1997). Analogs of $PGF_{2\alpha}$ are, in fact, used to synchronize estrus and to produce luteolysis in farm animals. In humans, the induction of COX-2 in the amniotic membranes and uterine wall at parturition leads to the synthesis of $PGF_{2\alpha}$ and PGE_2, which contract the smooth muscle to expel the fetus (Allport and Bennett, 2001).

Normally, PGs acting on the EP4 receptor maintain the patency of the ductus arteriosus before birth, yet most $EP4^{-/-}$ mouse neonates die within 72 h after birth. Histological examination of these animals showed that the ductus arteriosus remained open. Presumably, in the absence of the EP4 receptor, PGs are no longer involved in maintaining a patent ductus arteriosus. Other mechanisms have taken over the role of PGs, but no means exist for the termination of their action at birth. Thus, normal function of the EP4 receptor is essential to mediate neonatal adaptation of the cardiovascular system (Nguyen et al., 1997; Segi et al., 1998). Moreover, some fetuses of COX-1 null mice and all neonates born to homozygous COX-1/COX-2 null animals did not survive, most likely because their ductus arteriosus remained patent after birth (Langenbach et al., 1995).

Deletion of the COX-2 gene in female mice resulted in infertility because they did not ovulate (Lim et al., 1997). Thus, COX-2 appears to be essential for ovulation in mice. Ovulation was restored in these animals by treatment with PGE_2 or IL-1β (Davis et al., 1999) demonstrating the role of PGE_2 in ovulation. The role of IL-1β in restoring ovulation of $COX-2^{-/-}$ mice requires further clarification.

K. Brain and Spinal Cord

COX-1 is found in neurons throughout the brain but it is most abundant in the forebrain (Yamagata et al., 1993; Breder et al., 1995), where PGs may be involved in complex integrative functions such as control of the autonomic nervous system and in sensory processing. COX-2 mRNA is induced in brain tissue and cultured glial cells by pyrogenic substances such as LPS, IL-1, or TNF (Breder and Saper, 1996; Cao et al., 1996, 1998). Low levels of COX-2 protein and COX-2 mRNA have been detected in neurons of the forebrain without previous stimulation by pro-inflammatory stimuli (Yamagata et al., 1993; Breder et al., 1995; Cao et al., 1995). These "basal" levels of COX-2 are particularly high in neonates and are probably induced by nervous activity. Intense nerve stimulation, leading to seizures, induces COX-2 mRNA in discrete neurons of the hippocampus (Marcheselli and Bazan, 1996), whereas acute stress raises levels in the cerebral cortex (Yamagata et al., 1993). COX-2 mRNA is also constitutively expressed in the spinal cord of normal rats and may be involved with processing of nociceptive stimuli by releasing PGE_2 (Beiche et al., 1996; Yaksh and Svensson, 2001). The antihyperalgesic action of NSAIDs is mediated by inhibition of constitutive spinal COX-2 but not COX-1 (Yaksh and Svensson, 2001). Endogenous fever-producing PGE_2 is thought to originate from COX-2 induced in endothelial cells lining the blood vessels of the hypothalamus (Cao et al., 1996) by circulating LPS or IL-1 (Ek et al., 2001).

PGE_2 is synthesized in the human brain as well as PGD_2, which has a more limited distribution. Large amounts of PGD_2 are found in the brains of mammals (Narumiya et al., 1982; Ogorochi et al., 1984) and in mast cells but practically nowhere else. In addition to PGD_2 itself, PGD synthase and 15-hydroxy-PGD_2 dehydrogenase (which metabolizes PGD_2) have been identified in mammalian brains (Watanabe et al., 1980; Tokumoto et al., 1982). In young rodents, PGD synthase is localized in neurons, whereas in adult animals it is mainly restricted to oligodendrocytes (Urade et al., 1987). The reason for this selective distribution and the significance of PGD_2 in the brain is unknown. DP receptors have been found in the brain as well as in some vascular smooth muscle and blood platelets. They are coupled to adenylate cyclase through a G_s protein and stimulation results in formation of cyclic AMP.

PGD_2 and PGE_2 have opposing actions in sleep and temperature regulation. Microinjections of PGD_2 into the preoptic area of the rat brain induces normal sleep (Ueno et al., 1982a), whereas PGE_2 infused into the region of the posterior hypothalamus causes wakefulness (Hayaishi, 1991). Similarly, administration of PGD_2 lowers body temperature (Ueno et al., 1982b), and PGE_2 has a pyretic action (Milton and Wendlandt, 1971). It is interesting that patients with systemic mastocytosis fall deeply asleep after periods of production of large amounts of PGD_2 by their mast cells (Roberts et al., 1980).

III. Regulation of Expression of Cyclooxygenase-1 and -2

Prostaglandin research underwent a dramatic paradigm shift in the early 1990s in that the regulation of COX enzyme levels was recognized as a major control point in the biosynthesis of prostanoids (Bailey et al., 1985; Albrightson et al., 1985). This is in contrast to the

studies a decade ago, when the phospholipase step was considered the major control point (Hirata et al., 1987). For example, anti-inflammatory effects of glucocorticoids were thought to be primarily due to the induction of phospholipase inhibitory proteins called lipocortins, a hypothesis that is now largely abandoned. Although early studies by Murota (Chang et al., 1980), Needleman, and Bailey (Bailey et al., 1985) pointed out the regulatory mechanisms of induction of COX enzyme by hormones, cytokines, and growth factors, many studies attributed the effects of inducers of prostanoid synthesis to the induction of phospholipases (Hirata et al., 1987). The cloning efforts of COX-1 and -2, studies on the expression of COX genes in cultured cells, and the demonstration that COX expression levels are correlated with inflammatory disease phenotype in animal studies as well as human tissues played a role in this paradigm shift of prostanoid synthesis regulation (Hla et al., 1986; DeWitt and Smith, 1988; Kujubu et al., 1991; Xie et al., 1991; Sano et al., 1992).

The genes for COX-1 and -2 from the mouse and human were isolated by various laboratories (Fletcher et al., 1992; Kraemer et al., 1992; Appleby et al., 1994). These data show that the COX-1 gene is encoded by a large (>28 kb) gene, containing 11 exons. The promoter structure of the COX-1 gene suggested that GC-rich, SP1-like elements are involved in transcription of this gene (Kraemer et al., 1992). The expression of the COX-1 gene is generally constitutive and ubiquitous and relatively little is known about promoter elements that control transcription (Kraemer et al., 1992; Smith and DeWitt, 1996). COX-1 expression is induced during cell quiescence and differentiation in endothelial and mast cells, respectively (Smith and DeWitt, 1996). Enhancer elements that confer such regulation have not been defined.

The COX-2 gene is compact (~8 kb), consistent with the notion that immediate-early genes are transcribed from small genes (Fletcher et al., 1992; Appleby et al., 1994). The exon/intron organization is nearly identical between the two genes; however, the COX-1 gene contains an extra intron, intron-1, that participates in alternative splicing to give rise to COX-3 and PCOX proteins (Chandrasekharan et al., 2002). The polyadenylation sites for the human COX-2 gene have been mapped, and two functional sites were identified, which gives rise to a small 2.8-kb and a large 4.6-kb mRNA isoform (Ristimaki et al., 1996). The large transcript is found in higher abundance, suggesting that the downstream polyadenylation site is used preferentially (Ristimaki et al., 1996).

The promoter region of the COX-2 gene has been characterized extensively. Early studies on serum and v-*src* regulation of mouse COX-2 transcription indicated that the cAMP response element is critical (Xie et al., 1994). Furthermore, activation of mitogen-activated protein kinase pathways (such as extracellular signal-regulated kinase and c-Jun NH_2-terminal kinase) results in the

modulation of transcription factor activity that works through the cAMP response element site (Herschman et al., 1997). In addition, other investigators have found that CREB/NF-IL-6 elements, NFκB sites either alone or in concert with each other, are critical for maximal induction of transcription by cytokines, growth factors, oncogenes, and tumor promoters (Sirois and Richards, 1993; Crofford et al., 1997; Herschman et al., 1997). In addition, PPARγ response elements, Ets sites, and PEA sites have also been identified recently as functional enhancer elements (Smith et al., 2000). The role of these inducible enhancer elements in COX-2 transcription in vivo has not been characterized.

Early studies on the effects of serum, cytokines, and glucocorticoids on the regulation of COX-2 expression identified that mRNA stability of COX-2 is also likely to be a major regulatory step (Evett et al., 1993; Ristimaki et al., 1994). Indeed, multiple copies of the AUUUA element, first identified by Shaw and Kamen (1986) were present in the 3′-UTR of the COX-2 gene (Appleby et al., 1994). Moreover, the 3′-UTR of the COX-2 gene contains several sequence stretches that are conserved between diverse species (Ristimaki et al., 1996). Furthermore, dexamethasone treatment of synovial fibroblasts involves dramatic destabilization of cytokine-induced COX-2 mRNA (Ristimaki et al., 1996). Interestingly, the large transcript isoform decays with faster kinetics than the shorter isoform, suggesting that multiple elements in the 3′-UTR cooperate in the mRNA degradation processes (Ristimaki et al., 1996). Morrison and colleagues also showed that IL-1 induced COX-2 mRNA stability in kidney mesangial cells and that several polypeptide species from IL-1-stimulated cells bound to the COX-2 3′-UTR (Cok and Morrison, 2001; Srivastava et al., 1994). These observations were confirmed by Barnes and colleagues in fibroblasts and synoviocytes (Ridley et al., 1998; Lasa et al., 2000).

Functional studies of mRNA stability were recently conducted to better define the *cis*-elements and *trans*-factors. Fusion of the COX-2 3′-UTR to the luciferase open reading frame reporter gene resulted in the decreased expression of the luciferase expression and mRNA levels (Gou et al., 1998; Cok and Morrison, 2001). Deletion of the proximal AU-rich cluster reversed that effect, consistent with the notion that this region contains mRNA destability elements (Gou et al., 1998; Cok and Morrison, 2001). Such chimeric reporter constructs did not respond to IL-1 and dexamethasone in transfected kidney epithelial cells and mesangial cells as well as in colon cancer cells (Gou et al., 1998; Cok and Morrison, 2001). Deletion analysis of these constructs showed that multiple elements in the 3′-UTR regulated expression of the transgenes. Thus, the luciferase reporter system was useful in identifying basal instability of the COX-2 mRNA but was not able to reconstitute the extracellular mediator regulation of mRNA stability.

Using a tetracycline-regulated system for examining *cis*-element, the proximal conserved AU-rich element was identified as a major element involved in cytokine-induced COX-2 mRNA stabilization (Srivastava et al., 1994; Cok and Morrison, 2001). The authors went on to show that p38 stress-activated kinase pathway is critical for cytokine-induced COX-2 mRNA stability, which seems to work, at least in part, through the proximal AU-rich element (Srivastava et al., 1994; Cok and Morrison, 2001). Recently, the same laboratory demonstrated that inhibition of the p38 pathway may account for the ability of dexamethasone to inhibit COX-2 mRNA stabilization (Srivastava et al., 1994; Cok and Morrison, 2001). Murphy and coworkers showed in vascular smooth muscle cells that purinergic stimulation of its G-protein-coupled receptors regulated COX-2 mRNA stabilization through the proximal and distal elements (Xu et al., 2000). These efforts have begun to define the *cis*-elements involved in COX-2 mRNA stability regulation; however, proteins that interact with these elements are only presently being defined. Recently, Sawaoka (2003) identified tristetraprolin as an mRNA destabilizing protein that binds in the region between 3125 and 3432 of the COX-2 mRNA. This protein is proposed to participate in the destabilization of the large COX-2 transcripts. Based on the analogy with other immediate early transcripts, such as c-*myc* and c-*fos*, it is likely that several RNA-binding proteins such as AUF-1 and HuR will be involved. Indeed, recent work showed that HuR binds with high affinity to three distinct sites on the 3′-UTR of COX-2 mRNA and that this binding is necessary for the serum starvation-induced COX-2 expression in MDA-MB-231 mammary cancer cells (Sengupta et al., 2003). How RNA-binding proteins interact with signaling proteins to regulate both basal and regulated RNA degradation is a future challenge in this area. Given that physiologic regulation of COX-2 expression occurs by the inhibition of cytokine-induced mRNA stability, and COX-2 mRNA stability seems to be involved in the exaggerated COX-2 expression observed in tumors, new knowledge in this area may have important therapeutic implications. COX-2 regulation in neoplasia and the role of transcriptional mechanisms and RNA stabilization has recently been reviewed by Dixon (2003).

In addition to regulation of COX-2 expression by transcription and mRNA stability, the chicken COX-2 mRNA is additionally regulated by a unique mechanism of RNA splicing—further underscoring the exquisite control regulating COX-2 expression at the RNA level. In proliferatively quiescent chicken embryo fibroblasts, intron-1 of the COX-2 pre-mRNA is retained even after the mRNA is fully processed (Xie et al., 1991). The consequence of this intron retention is that the mRNA is nonfunctional due to the introduction of a frame shift by insertion of intron-1, which is located immediately following the sequence encoding the signal peptide of COX-2. Addition-

ally, this intron-1-containing transcript is sequestered in the nucleus preventing translation. Upon mitogenic stimulation, COX-2 mRNA becomes fully spliced to allow export from the nucleus and translation of COX-2 (Xie et al., 1991). This unique mechanism governing COX-2 expression requires a series of 18 tandem repeat sequences as well as a unique 3′ splice site that utilizes CU rather than the canonical AG as the dinucleotide at the intron/exon junction (Xie et al., 1991). The creation of variants of COX-1 by retention of all or part of intron-1 as well as skipping of other introns is described above.

Finally, regulation of transport of the COX-2 mRNA may also have functional significance. The transport of COX-2 mRNA from the nucleus to the rough ER requires the CRM1 nuclear export pathway, and as a specific inhibitor of this pathway, leptomycinβ, blocks transport and decreases the expression of COX-2 gene in MDA-MB-231 mammary cancer cells (Jang et al., 2003). Since some RNA-binding proteins such as HuR are also transported by this pathway, it is likely that some RNA/protein interactions regulate proper trafficking of COX-2 mRNA, which has a major impact on COX-2 expression in some systems.

IV. Cyclooxygenase Isozymes in Human Disease

A. Treatment of Inflammatory Diseases

NSAIDs are currently used as first-line therapeutics in the treatment of osteoarthritis (OA), rheumatoid arthritis (RA), systemic lupus erythematosis, and other inflammatory syndromes. In each case, NSAID treatment is palliative rather than disease modifying. NSAIDs reduce inflammation and pain in these syndromes.

In the short period of 8 years after the discovery of COX-2, selective inhibitors of this enzyme were developed for use in RA, OA, and for pain relief. Even before the discovery of COX-2, pharmaceutical companies were searching for anti-inflammatory drugs that would have less damaging effects on the stomach than existing therapies. In the 1980s, these experiments resulted in the development of three drugs with anti-inflammatory activity but with very little inhibitory effect on PG production by the stomach. Nimesulide, etodolac, and meloxicam emerged from preclinical studies as anti-inflammatory compounds with less damaging effects on the stomach than established NSAIDs. After the discovery of COX-2 in 1991 (Kujubu et al., 1991; Xie et al., 1991), these drugs were shown to have a selective inhibitory action on COX-2 compared with COX-1; however, after the cloning of COX-2, inhibitors were designed with an even greater selectivity for COX-2.

Selectivity for the inducible isoform was established by comparing inhibitory potency against COX-1 measured as the IC_{50} with inhibition of COX-2 in isolated enzymes, cultured cells, or in the whole blood assay.

Slightly different measurements of selectivity were obtained in each system, but the relative values between drugs and their order of potency generally remained the same. The most reproducible estimates of selectivity have been obtained by comparing inhibitory potency on recombinant human enzymes or by measuring the selectivity with the human whole blood assay (Patrignani et al., 1994). The latter is considered to resemble most closely the clinical situation in patients taking NSAIDs. Blood proteins are present so that drug binding to protein is accounted for and endogenous human enzymes are used. The inhibitory potency against COX-1 is measured on platelets in clotting blood, whereas potency for inhibition of COX-2 is estimated in blood monocytes previously incubated with endotoxin to induce COX-2. The modified William Harvey whole blood assay (Warner et al., 1999) uses cultured A549 cells, instead of blood monocytes, incubated with IL-1 and added to human blood for estimation of activity against COX-2.

1. Nimesulide. Nimesulide, like etodolac, was developed in the 1980s and has been licensed by Helsinn Healthcare SA to a number of other pharmaceutical companies. It has been sold over the counter in Italy since 1985 and subsequently in many other European and South American countries. Chemically, nimesulide has a sulfone structure and resembles structurally the selective COX-2 inhibitors developed in the 1990s (Fig. 11).

Early experimental studies in rats demonstrated that at anti-inflammatory doses that reduced carrageenan-induced paw edema, nimesulide had no effect on gastric PG levels and did not cause bleeding of the gastric mucosa (Carr et al., 1986; Nakatsugi et al., 1996). Its selective inhibition of COX-2 has been demonstrated in vitro using purified enzymes; the IC_{50} for COX-1 was 5-fold greater than that for COX-2 (Barnett et al., 1994; Cullen et al., 1998). In the human whole blood assay (Patrignani et al., 1997), nimesulide was 20 times more potent in inhibiting COX-2 in monocytes than COX-1 in platelets (IC_{50} for COX-2 = 0.5 μM; IC_{50} for COX-1 = 9 μM). A single oral dose (100 mg) of nimesulide reduced monocyte COX-2 and platelet COX-1 ex vivo by 90 and 50%, respectively; however, this reduction in COX-1 activity may not be sufficient to affect platelet aggregation or bleeding time (Panara et al., 1998). A reduction of 29% in serum TXB_2 levels with nimesulide had no effect on arachidonic acid-induced platelet aggregation in humans in vivo; however, reducing TXB_2 levels by 98% with naproxen prevented arachidonic acid-induced platelet aggregation (Shah et al., 1999). PGE_2 production from gastric biopsies was also reduced by 79% with naproxen and only by 19.5% with nimesulide.

Comparing gastroduodenal damage with nimesulide or naproxen in 36 healthy volunteers showed a highly significant difference in favor of nimesulide ($P < 0.0001$) (Shah et al., 2001). The relatively short half-life of nimesulide (1.8–4.7 h) (Bree et al., 1993; Bernareggi, 1998)

may contribute to its lack of gastrointestinal toxicity, because this would allow PG synthesis in the gastric mucosa to recover before their protective effect has worn off. Estimates of rate ratios for peptic ulcer, gastrointestinal hemorrhage, or perforation placed the incidence for nimesulide below that for diclofenac and naproxen (Menniti-Ippolito et al., 1998). Other epidemiological analyses (Garcia Rodriguez et al., 1998; Porto et al., 1998) have estimated gastric damage with nimesulide to be equal to or worse than with diclofenac. This surprising result may be due to the small numbers of patients in these studies and the wide range of confidence intervals of the estimates (Rainsford, 1999).

The therapeutic efficacy of nimesulide has been demonstrated in clinical trials in at least 55,000 patients for inflammation and pain including OA (Lücker et al., 1994; Huskisson et al., 1999), RA, musculoskeletal inflammation, headache, dysmenorrhea, postsurgical and cancer pain, vascular diseases, upper respiratory tract diseases, and airways inflammation (Bennett, 2001). Its action is rapid in onset because it is effective within 20 to 30 min for pain of oral surgery (Ragot et al., 1993) or in dysmenorrhea (Pulkkinen, 1984, 1993). Of an estimated 200 million patients treated with nimesulide since its introduction in 1985, only 1212 adverse events were reported to Helsinn Healthcare SA (Rainsford, 1999) during 1985 through 1999; however, in March 2002, nimesulide was withdrawn from the Finnish market because of unacceptable hepatoxicity and then in May 2002 it was withdrawn from the Spanish market. Data from the Spanish Pharmacovigilance System reported a higher rate of hepatic injury with nimesulide than with other NSAIDs during the 1990s (Maciá et al., 2002)

At therapeutic doses, nimesulide does not appear to precipitate asthma in NSAID-sensitive asthmatics. This supports the concept that aspirin-induced asthma is caused by inhibition of COX-1 (Bianco et al., 1993; Senna et al., 1996; Gryglewski, 1998; Bennett, 2000). Prolonged treatment with nimesulide has delayed premature labor for up to 34 weeks without serious fetal side effects and resulted in a successful delivery (Sawdy et al., 1997).

A number of actions of nimesulide demonstrated in vitro may contribute to its therapeutic anti-inflammatory effects in vivo. These include, inhibition of neutrophil function (Ottonello et al., 1992; Capecchi et al., 1993; Maffei et al., 1995), reduction of collagenase synthesis (Barracchini et al., 1998), inhibition of histamine action (Berti et al., 1990), and protection of chondrocytes against apoptosis (Bennett, 2001; Mukherjee et al., 2001b).

2. Etodolac. Etodolac has been available for clinical use in Europe and North America for many years. It has a pyranocarboxylic acid structure (Fig. 11), which in a range of preclinical tests showed an anti-inflammatory effect without producing damage to the stomach mucosa

(Jones, 2001). On human recombinant enzymes, etodolac had an 11-fold greater potency for inhibition of COX-2 than for COX-1 and an 8-fold selectivity for COX-2 over COX-1 in the human whole blood assay (Glaser, 1995). This was confirmed in the modified William Harvey whole blood assay in which a 10-fold selectivity for COX-2 over COX-1 was recorded (Warner et al., 1999).

In various randomized double-blind clinical trials in more than 2500 OA patients lasting from 6 to 12 weeks, 600 mg of etodolac daily was equally efficacious as naproxen 1000 mg/day, piroxicam 20 mg/day, diclofenac 150 mg/day, and tenoxicam 20 mg/day (Platt, 1989; Bacon, 1990; Porzio, 1993; Schnitzer and Constantine, 1997). In a 12-week randomized double blind study in RA, 600 mg/day etodolac had comparable efficacy with 20 mg/day piroxicam (Lightfoot, 1997) and in a 6-week randomized trial, 600 mg/day etodolac was equal in efficacy to 100 mg/day indomethacin (Delcambre, 1990). A long-term double blind trial lasting for 3 years with 1446 RA patients, showed that 300 mg/day or 1000 mg/day etodolac had comparable efficacy to 2400 mg/day ibuprofen (Neustadt, 1997). The cumulative incidence of gastrointestinal (GI) ulcers or bleeds over the 3 year period amounted to two patients on each dose of etodolac (0.43% and 0.67%) and nine patients on ibuprofen (4.74%).

The safety of etodolac, particularly its toxicity to the GI tract has been evaluated in 2629 patients enrolled in double blind and open-label clinical trials and in 8334 patients taking etodolac in postmarketing surveillance studies during the past 14 years (Lightfoot, 1991; Schattenkirchner, 1991). The incidence of GI ulceration and bleeding in patients receiving etodolac was 0.42% (11 patients of 2629) in clinical trials and 0.06% (5 patients of 8334) in postmarketing surveillance studies. Studies in healthy volunteers reported that fecal blood loss with etodolac was similar to that seen with placebo, but less than the GI blood loss with aspirin, ibuprofen, naproxen, or indomethacin (Ryder et al., 1983; Salom et al., 1984; Arnold et al., 1985; Lanza and Arnold, 1989; Leese, 1992). A 7-day endoscopic study by Lanza et al. (1987) in 72 healthy volunteers assessed GI erosions with etodolac as no greater than with placebo and significantly less than with ibuprofen, naproxen, or indomethacin. Russell et al. (1991) compared the GI safety of etodolac (300 mg twice daily) and naproxen (500 mg twice daily) in 30 RA patients for 4 weeks. Only 3 of 15 patients receiving etodolac developed upper GI mucosal lesions, whereas 8 of 15 patients treated with naproxen demonstrated lesions of the upper GI tract. Data from the Arthritis, Rheumatism and Aging Medical Information System (ARAMIS) indicates that etodolac (on the basis of data for 88 patient years) and nabumetone (based on data for 221 patient years) are the only two established NSAIDs that are not associated with serious GI bleeds or other significant events requiring hospitalizations.

The renal toxicity of etodolac has been reviewed in 16 double blind open-label clinical trials involving 1382 patients with OA or RA. The trials lasted from 4 to 52 weeks, and patients received 50 to 600 mg/day etodolac. The risk of renal function impairment was no greater among patients treated with etodolac than those receiving placebo (Shand et al., 1986).

3. Meloxicam. Meloxicam also emerged as a potential new drug from a preclinical search for an anti-inflammatory drug with a low propensity to damage the stomach mucosa. It was first registered in 1995 and is now marketed in more than 100 countries for OA, RA, and ankylosing spondylitis. Its chemical structure is that of an enolcarboxamide (Fig. 11) and it has been recognized as a selective COX-2 inhibitor since 1994. The 5-methyl group on the thiazolyl ring of meloxicam can enter the side pocket at the active site of COX-2. Meloxicam has a 100-fold selectivity in favor of COX-2 in microsomal preparations of human recombinant enzymes (Churchill et al., 1996; Pairet et al., 1998) and a 10-fold selectivity for COX-2 in the human whole blood assay (Patrignani et al., 1997; Warner et al., 1999); however, in the modified William Harvey whole blood assay, a 25-fold selectivity for COX-2 over COX-1 has been reported (Warner et al., 1999).

In animal studies, meloxicam had potent anti-inflammatory activity with less inhibition of PGE_2 production in the stomach and kidneys than standard NSAIDs (Engelhardt, 1996). Meloxicam was also more potent in reducing edema of the inflamed rat paw than indomethacin, piroxicam, diclofenac, or naproxen (Engelhardt et al., 1995; Engelhardt, 1996) and produced analgesia in rat and dog models of inflammatory pain (Cross et al., 1997; Laird et al., 1997; Santos et al., 1998). At therapeutic doses of 7.5 or 15 mg, meloxicam did not reduce platelet aggregation or prolong bleeding time ex vivo (Stichtenoth et al., 1997; De Meijer et al., 1999; Panara et al., 1999; Tegeder et al., 1999), although thromboxane formation by platelets was inhibited by 35% after 15 mg of meloxicam. This demonstrated the low inhibitory activity against COX-1 of meloxicam in vivo.

A total of 20,084 patients were treated with meloxicam in early clinical efficacy and safety studies (Degner et al., 2001). These trials showed that meloxicam was as effective as diclofenac or piroxicam (Hosie et al., 1996, 1997; Linden et al., 1996; Goei et al., 1997) and more effective than placebo (Lund et al., 1998) in patients diagnosed with OA. Similarly, trials in patients with RA demonstrated that in doses of 7.5, 15, or 22.5 mg, meloxicam was as effective as comparator NSAIDs in established doses for periods of up to 18 months (Huskisson et al., 1994, 1996; Wojtulewski et al., 1996; Lemmel et al., 1997).

Two large-scale, prospective, multicenter trials, the Meloxicam Large-Scale International Study Safety As-

sessment (MELISSA) and the Safety and Efficacy Large-Scale Evaluation of COX-Inhibiting Therapies (SELECT) compared 7.5 mg/day meloxicam with 100 mg of slow release diclofenac (MELISSA) or 20 mg/day piroxicam (SELECT) for 28 days in OA patients. In the MELISSA (Hawkey et al., 1998) trial during which 4635 patients were treated with meloxicam and 4688 patients with diclofenac, fewer GI adverse events were recorded for meloxicam-treated patients (13%) than for patients receiving diclofenac (19%; $P < 0.001$). There were 5 patient days of hospitalization in patients on meloxicam compared with 121 with diclofenac. In the SELECT trial (Dequeker et al., 1998), comparing 4320 patients treated with meloxicam to 4336 patients receiving piroxicam, both drugs had equal efficacy, but the incidence of GI adverse events was lower in the meloxicam than in the piroxicam group (10.3% versus 15.4%; $P < 0.001$). Sixteen patients had perforations, ulcerations, or bleeding (PUBs) of the upper GI tract in the piroxicam group compared with seven in the meloxicam group (relative risk piroxicam: meloxicam = 1.4). A systematic review of published clinical trials covering all treatments showed significant reductions in risk of GI adverse events, withdrawals due to GI adverse events, and dyspepsia by 36%, 41%, and 27% for meloxicam relative to comparator NSAIDs (Schoenfeld, 1999).

A controlled postmarketing study by Degner et al. (2000) assessed the efficacy and GI tolerability of meloxicam during 6 months under prescribing conditions. Results from 2530 patients receiving meloxicam were compared with 1996 patients treated with diclofenac, ibuprofen, piroxicam, or indomethacin. Fewer GI adverse reactions were reported for meloxicam-treated patients than for those receiving other NSAIDs (1.8% versus 3.2%; $P = 0.003$). Lanes et al. (2000), however, estimated the baseline risk of an upper GI event in the United Kingdom to be higher among meloxicam users than in patients treated with other NSAIDs, since patients who received meloxicam were characterized by a recent history of dyspepsia, gastritis, peptic ulcer, or treatment with acid-suppressing drugs. A prescription-monitoring study involving 19,087 patients in the United Kingdom reported that upper gastrointestinal adverse events occurred more frequently in patients with a past history of gastrointestinal disorders (Martin et al., 2000).

4. *Celecoxib.* Celecoxib (Celebrex) was developed by Monsanto/Searle after the cloning of COX-2 in 1991, specifically for its inhibitory activity against COX-2, when it became clear that inhibition of COX-1 resulted in removal of protective PGs and injury to the gastric mucosa. The sulfonamide group on celecoxib (Fig. 11) binds into the side pocket within the channel that forms the active site of COX-2 and confers COX-2-selectivity on celecoxib and its analogs. In the initial stages of its action, celecoxib inhibits COX-2 competitively, but this competitive inhibition becomes converted into an irre-

versible, slow, time-dependent inhibition; however, celecoxib weakly inhibits COX-1 by a simple, easily reversed competitive inhibition (Copeland et al., 1994; Gierse et al., 1995).

In a range of in vitro assays, celecoxib demonstrated 155- to 3200-fold selectivity for COX-2 over COX-1 (Gierse et al., 1999). The selectivity measured by the human whole blood assay and by the modified William Harvey whole blood assay has been much lower than these estimates (Warner et al., 1999). One explanation may be that the time-dependent element is missing from the previous in vitro assays, so they provide an artificially high estimate of selectivity. It has also been suggested that a more relevant evaluation of the potential in vivo GI toxicity can be provided by measuring the percentage of inhibition of COX-1 at drug concentrations that inhibit COX-2 activity by 80%. This is the amount of COX-2 inhibition exerted by anti-inflammatory drugs at therapeutic doses (Warner et al., 1999). The higher the COX-1 inhibitory activity at these drug concentrations, the greater the likelihood of damage to the gastric mucosa.

In rat models of inflammation and pain, such as adjuvant-induced arthritis or carrageenan-induced paw edema, celecoxib effectively reduced paw swelling and responses to noxious stimuli at ED_{50} doses of 0.37 to 34.5 mg/kg given orally (Seibert et al., 1994; Penning et al., 1997; Tindall, 1999; Smith et al., 1998a). In addition, celecoxib in doses up to 200 mg/kg (Penning et al., 1997) did not produce gastric damage in rats. On the basis of extensive clinical trials, celecoxib was registered by the U.S. Food and Drug Administration (FDA) in December of 1998 for therapeutic use in OA and RA and, since 2001, acute pain.

a. Clinical Efficacy. The efficacy of celecoxib in OA was established in several double blind clinical studies lasting up to 12 weeks that included patients with OA of the knee and hip. Celecoxib in doses of 50 to 200 mg b.i.d. was compared with placebo, 500 mg of naproxen b.i.d., 75 mg of diclofenac b.i.d., or 800 mg ibuprofen t.i.d. (Bensen et al., 1999; Geis et al., 1999b; Lefkowith et al., 2000a,b; Williams et al., 2000b). Celecoxib was as effective as therapeutic doses of conventional NSAIDs and the most effective dose of celecoxib in OA was found to be 200 mg/day in a once daily regimen. A 12-week study in 1093 patients and a 6-week trial in 688 patients demonstrated that celecoxib was as effective as naproxen or diclofenac in relieving signs and symptoms of OA of the knee (Bensen et al., 1999). A total of 1061 patients with OA of the hip were studied for 12 weeks while receiving celecoxib, naproxen, or placebo. Celecoxib (100 or 200 mg b.i.d.) was as effective as naproxen and more efficacious than placebo (Geis et al., 1999a).

Celecoxib also demonstrated equal efficacy to therapeutic doses of naproxen or diclofenac in relieving the symptoms of RA. The maximum effective dose of celecoxib in RA was found to be 200 mg b.i.d. (Emery et al.,

1999; Simon et al., 1999). In a 12-week study comparing the efficacy and tolerability of 100 to 400 mg of celecoxib b.i.d. with 500 mg of naproxen b.i.d. in 1149 patients (Simon et al., 1999), all doses of celecoxib were equally effective and as efficacious as naproxen in reducing the symptoms of RA.

The analgesic effects of celecoxib were confirmed in patients undergoing tooth extraction or orthopedic surgery (Lefkowith, 1999; Gimbel et al., 2001). A double blind study of post orthopedic pain in 418 patients compared 200 mg of celecoxib with 10 mg of hydrocodone/ 1000 mg of paracetamol. Both treatments were effective in producing analgesia with fewer doses of celecoxib needed to ease the pain (Gimbel et al., 2001).

b. Gastrointestinal Safety. Since more than 100,000 patients in the United States experience PUBs every year as a result of taking NSAIDs and 16,500 die from ulcers and bleeding (Fries et al., 1991; Singh et al., 1996; Fries, 1999; Singh and Triadafilopoulos, 1999), it was essential to establish that the selective COX-2 inhibitors would cause less GI mucosal toxicity than comparable nonselective COX inhibitors. To assess GI safety rigorously, doses which were two and four times the maximum clinically effective doses for RA and OA were evaluated. The incidence of upper GI ulceration with celecoxib compared with standard NSAIDs and placebo was investigated endoscopically in 2089 patients with OA and RA during trials lasting up to 24 weeks (Emery et al., 1999; Simon et al., 1999; Gimbel et al., 2001). Celecoxib treatment even at twice the usual dose used for RA and four times the usual dose used for OA did not cause more GI ulcers than placebo. A 12-week study in 1149 patients with RA showed that naproxen produced more ulcers than even the highest dose of celecoxib (400 mg b.i.d.) (Simon et al., 1999). When 200 mg of celecoxib b.i.d. was compared with 75 mg of diclofenac b.i.d. for 24 weeks in 430 patients, more gastroduodenal ulcers were detected in patients treated with diclofenac (15%) than in patients treated with celecoxib (4%) (Emery et al., 1999).

Ulcer complications manifested as perforation, bleeding, and gastric outlet obstruction (POBs) are more clinically relevant than endoscopically observed ulcers. A *meta*-analysis of upper GI events in patients with RA or OA enrolled in 14 studies recorded less frequent GI events with celecoxib than with conventional NSAIDs. Fourteen controlled studies lasting 2 to 24 weeks compared 6376 patients receiving celecoxib with 2768 patients receiving standard doses of naproxen, diclofenac, or ibuprofen. Of the eleven serious upper GI events recorded, nine were in patients treated with conventional NSAIDs and two were among patients treated with celecoxib. The risk of serious upper GI events related to conventional NSAIDs was 1.68% greater than if the patients had received celecoxib. In addition, celecoxib treatment was not associated with a greater risk of GI adverse events than placebo (Goldstein et al., 2000).

The Celecoxib Long-Term Arthritis Safety Study (CLASS) determined the incidence of upper GI ulcer complications among arthritis patients receiving celecoxib or conventional NSAIDs for 6 months (Silverstein et al., 2000). A supratherapeutic dose of celecoxib (400 mg b.i.d.) was compared with therapeutic doses of ibuprofen (800 mg t.i.d.) and diclofenac (75 mg b.i.d.) in approximately 8000 patients (Silverstein et al., 2000). Anti-thrombotic doses of aspirin were allowed during the study. For all patients, the annualized incidence rates of upper GI ulcer complications alone or combined with symptomatic ulcers for celecoxib compared with conventional NSAIDs were 0.76% versus 1.45% ($P = 0.092$) and 2.08% versus 3.54% ($P = 0.023$); however, the incidence rates of ulcer complications alone or combined with symptomatic ulcers for celecoxib compared with standard NSAIDs in patients simultaneously taking aspirin were 2.01% versus 2.12% ($P = 0.92$) and 4.70% versus 6.00% ($P = 0.49$). These data indicate that administration of anti-thrombotic doses of aspirin concomitantly with celecoxib reduces the upper GI safety conferred by the selective COX-2 inhibitor. Further analysis of the CLASS study extended to 12 months, reported that the frequency of ulcer complications with celecoxib, whether or not aspirin was administered at the same time, became no different from those recorded in patients receiving diclofenac. Paradoxically, patients taking cardioprotective doses of aspirin with celecoxib experienced a greater incidence of complicated ulcers than patients receiving aspirin and ibuprofen (FDA Celecoxib Hearing July 2, 2001).

c. Absence of Other Adverse Events. Celecoxib does not affect platelet function, indicating a lack of inhibition of COX-1 in platelets (Geis et al., 1999b; Leese et al., 2000). Platelet aggregation, bleeding time, and serum TXB_2 concentrations were not altered by supratherapeutic doses of celecoxib, whereas treatment with naproxen increased bleeding time, decreased platelet aggregation, and reduced serum TXB_2 levels (Leese et al., 2000).

Celecoxib may demonstrate less renal toxicity than conventional NSAIDs. In a study in healthy elderly subjects lasting for 10 days, celecoxib had no effect on GFR, whereas naproxen reduced GFR by 6% on day 1 of the study and by 9% on day 6 (Catella-Lawson et al., 1999).

It has been suggested that inhibition of endothelial COX-2, which synthesizes prostacyclin, may result in over-activity of prothrombotic TXA_2 produced by platelets and an increase in thromboembolic cardiovascular events (McAdam et al., 1999). The CLASS trial demonstrated that celecoxib treatment was not associated with any greater incidence of cerebrovascular accidents or myocardial infarction than conventional NSAIDs (Silverstein et al., 2000). The incidence of hepatic adverse events was higher among patients treated with conventional NSAIDs such as diclofenac, naproxen, or ibuprofen than with celecoxib (Maddrey et al., 2000).

5. Rofecoxib. Rofecoxib (Vioxx) was developed by Merck Frosst in the early 1990s. Having cloned and purified human COX-2 from a recombinant baculovirus system (Cromlish et al., 1994), the Merck Frosst group set up various in vitro and in vivo assays to search for compounds which would be selective inhibitors of COX-2. Compounds with a methylsulfonyl group in the para position of a phenyl ring proved to be potent and selective COX-2 inhibitors (Fig. 11). The methylsulfonyl group fits into the side pocket of the channel, which forms the catalytic site of COX-2 and provides better selectivity but less bioavailability than the sulfonamide series on which celecoxib is based. It also avoids the possible allergic reactions, such as skin rashes, which are typical of sulfonamides. A lactone ring was then incorporated into the molecule to improve the oral bioavailability. The resulting compound had the best overall profile and was designated as rofecoxib or Vioxx.

Rofecoxib was a potent and selective inhibitor of COX-2 in a large number of in vitro assays including PGE_2 production by osteosarcoma cells, selectively expressing COX-2 and PGE_2 production by U937 cells expressing only COX-1 (Wong et al., 1997). It was 77 times more potent in inhibiting purified human COX-2 than human COX-1. Its in vitro activity in the human whole blood assay demonstrated a 36-fold selectivity for COX-2 compared with the 8-fold selectivity estimated for celecoxib. Oral administration of 1 g of rofecoxib in humans had no effect on TXA_2 production ex vivo from platelets in the COX-1 whole blood assay (Ehrich et al., 1999). Inhibition of platelet COX-1 and monocyte COX-2 was also assessed ex vivo in 25 patients treated for 7 days with 50 mg/day rofecoxib or 50 mg of diclofenac t.i.d. Both drugs caused more than 90% suppression of COX-2 whereas COX-1 activity was not affected by rofecoxib but reduced by more than 50% by diclofenac (Patrignani et al., 2000).

Rofecoxib was essentially equipotent to indomethacin in rat models of pain and inflammation such as carrageenan-induced paw edema, paw analgesia, pyresis, and adjuvant arthritis (Chan et al., 1999; Prasit et al., 1999). The low incidence of GI damage with rofecoxib was demonstrated in both animal and human studies. For example, oral dosing of 300 mg/kg rofecoxib to rats for 14 days did not produce GI lesions, whereas a single dose of 3 mg/kg indomethacin caused obvious gastric damage. In the fecal ^{51}Cr excretion assay, red blood cells are labeled with ^{51}Cr and the amount of ^{51}Cr recovered in the feces provides a measure of GI permeability. A daily oral dose of 200 mg/kg rofecoxib for 5 days in either rats or squirrel monkeys did not show any ^{51}Cr leakage whereas a single dose of indomethacin or diclofenac at 10 mg/kg caused a significant increase in ^{51}Cr excretion (Prasit et al., 2001). This lack of GI toxicity of rofecoxib was confirmed in human studies using ^{51}Cr as a measure of GI integrity (Sigthorsson et al., 2000) or by

endoscopic examination of the upper GI tract for PUBs (Laine et al., 1999; Langman et al., 1999).

Rofecoxib was approved by the FDA and launched in the United States in May of 1999 for treatment of acute pain as well as osteoarthritic pain. It has now also been registered in many European countries, Canada, and Australia. In extensive clinical trials, rofecoxib was shown to possess anti-inflammatory and analgesic activity equal to nonselective NSAIDs (Morrison et al., 1999a,b; Cannon et al., 2000; Day et al., 2000) but with much greater GI safety and tolerability (Laine et al., 1999; Langman et al., 1999; Lanza et al., 1999; Hunt et al., 2000; Sigthorsson et al., 2000).

a. Clinical Efficacy. Rofecoxib reduces fever that is largely mediated by induction of COX-2. In a trial including 94 febrile patients with viral infections, rofecoxib effectively counteracted the fever (Schwartz et al., 1999).

The analgesic efficacy of rofecoxib was assessed in several studies of postoperative dental pain and in postorthopedic surgical pain. In studies of dental pain, doses of 12.5 to 500 mg of rofecoxib were more effective than placebo, and 50 mg of rofecoxib provided pain relief equal to 400 mg of ibuprofen but of longer duration. In a trial comparing rofecoxib with naproxen and placebo, 50 mg of rofecoxib, recognized as the maximum effective dose, gave an analgesic efficacy better than placebo and similar to 550 mg of naproxen (Morrison et al., 1999a). In another dental pain study, 50 mg of rofecoxib, 400 mg of ibuprofen, or 200 mg of celecoxib were more effective in relieving pain than placebo, and rofecoxib and ibuprofen were more effective than celecoxib (Malmstrom et al., 1999).

In patients with postorthopedic surgical pain following hip or knee replacement, 50 mg of rofecoxib provided pain relief similar to 550 mg of naproxen but with a longer duration of action (Reicin et al., 2001). In 127 patients with primary dysmenorrhea, rofecoxib (25 or 50 mg) provided analgesia similar to that demonstrated by naproxen (550 mg) (Morrison et al., 1999b).

Extensive clinical trials in OA patients demonstrated the effectiveness of rofecoxib compared with the nonselective NSAIDs normally used to treat OA. Doses of 12.5 and 25 mg of rofecoxib were found to provide optimal efficacy during 6 weeks of treatment (Ehrich et al., 1997, 1999) and to be more effective than placebo (Truitt et al., 1999; Day et al., 2000; Saag et al., 2000). In phase III clinical trials, treatment with rofecoxib (12.5 or 25 mg/day) for 1 year was as effective as treatment with ibuprofen (800 mg t.i.d) or diclofenac (50 mg t.i.d.) (Cannon et al., 2000; Saag et al., 2000).

A 6-week trial of rofecoxib in RA patients showed that 43.8% of patients improved with 25 mg/day and 49.7% responded to 50 mg/day. Both doses of rofecoxib were more effective than placebo (Schnitzer et al., 1999). Rofecoxib was approved for RA in 2002.

b. Gastrointestinal Safety. Information on GI safety of rofecoxib has been obtained from clinical efficacy trials in approximately 5400 patients with RA, OA, and acute pain receiving doses of rofecoxib ranging from 5 to 1000 mg (Cannon et al., 2000; Day et al., 2000; Saag et al., 2000). Regular users of NSAIDs suffer a 1 to 4% annual incidence of PUBs (Singh, 1998) and one-third of PUB-related mortality in patients over 65 years is probably due to the use of NSAIDs (Griffin et al., 1991).

During studies conducted in healthy human volunteers, rofecoxib (25 or 50 mg/day) produced less change in intestinal permeability than indomethacin (50 mg t.i.d.) and effects similar to placebo (Sigthorsson et al., 2000). Fecal blood loss in healthy subjects receiving rofecoxib (25 or 50 mg/day) for 4 weeks was less than with ibuprofen (800 mg t.i.d.) and no more than with placebo (Hunt et al., 2000). An endoscopic study in 170 healthy subjects (Lanza et al., 1999) showed that rofecoxib (250 mg/day for 7 days) produced fewer GI mucosal lesions than ibuprofen (2400 mg/day) or aspirin (2600 mg/day) and no more damage than placebo. In two other endoscopic trials lasting 6 months, OA patients receiving rofecoxib (25 or 50 mg/day) manifested a lower incidence of gastroduodenal ulcers than a comparator group treated with ibuprofen (2400 mg/day) (Laine et al., 1999).

A combined analysis of PUBs among 5435 OA patients (Langman et al., 1999) in eight clinical trials lasting 12 months showed a lower incidence of PUBs in the rofecoxib group than in the group treated with all other NSAIDs with a relative risk of 0.45 ($P < 0.001$) (Langman et al., 1999). In addition, fewer patients discontinued treatment with rofecoxib due to GI adverse events than patients treated with nonselective NSAIDs.

The Vioxx Gastrointestinal Outcomes Research Study (VIGOR), carried out in approximately 8000 patients with RA, compared upper GI events during treatment with rofecoxib (50 mg/day) or naproxen (500 mg b.i.d.) for 9 months (Bombardier et al., 2000). The primary end points recorded were the rates of gastroduodenal perforation or obstruction, upper GI bleeding, or symptomatic gastroduodenal ulcers. The rates of these complicated events were 0.6 per 100 patient-years with rofecoxib and 1.4 per 100 patient-years with naproxen (relative risk, 0.4; 95% confidence limits, 0.2 to 0.8; $P = 0.005$); however, the incidence of myocardial infarction was higher among patients in the rofecoxib group than among those in the naproxen group (0.4% versus 0.1%; relative risk, 0.2; 95% confidence limits, 0.1 to 0.7) even though the mortality rate from cardiovascular causes was similar in the two groups. It has been proposed that inhibition of COX-2, which synthesizes PGI_2 in vascular endothelial cells, increases the risk of myocardial infarction after treatment with rofecoxib (McAdam et al., 1999). Alternately, naproxen may demonstrate an anti-platelet effect through its potent COX-1-inhibiting action and reduce the incidence of heart attacks.

c. Renal Safety. NSAIDs can alter renal function by reducing GFR, renal blood flow, and sodium and potassium excretion. These actions result in fluid retention, hypertension, edema, and hyperkalemia. Since COX-2 is constitutively expressed in the kidney its selective inhibition may cause adverse renal effects. In a placebo-controlled study in healthy human subjects, rofecoxib (50 mg q.i.d.) and indomethacin (50 mg t.i.d) decreased urinary sodium excretion during the first 72 h of treatment. The GFR was not altered by rofecoxib but reduced by indomethacin (Catella-Lawson et al., 1999). Both rofecoxib and indomethacin reduced GFR in elderly patients with diminished renal function (Swan et al., 1999) or in elderly subjects administered a sodium restricted diet (Swan et al., 2000). Thus, under certain conditions, inhibition of COX-2 may produce effects on renal function similar to the nonselective NSAIDs.

6. Second Generation Cyclooxygenase-2-Selective Inhibitors. Valdecoxib (Bextra), the successor to celecoxib, was recently approved in the United States for treatment of the signs and symptoms of OA, RA, and menstrual pain (Talley et al., 2000). In the human whole blood assay, it is more selective for COX-2 than celecoxib having a COX-2 selectivity ratio of 30, compared with a ratio of 7.6 for celecoxib (Riendeau et al., 2001). In a dose of 10 mg/day, valdecoxib gave 24 h of pain relief in arthritic patients. Trials in more than 5000 patients have shown a similar efficacy to standard NSAIDs but with much reduced side effects. Pfizer has also filed for FDA approval for parecoxib, an injectable pro-drug of valdecoxib for the treatment of acute pain associated with surgery or trauma (Cheer and Goa, 2001).

The successor to rofecoxib, etoricoxib (Arcoxia), has been approved for marketing in Mexico and Europe but not yet in the United States. Its selectivity ratio in the human whole blood assay was 106 compared with selectivity for COX-2 of 35 for rofecoxib (Riendeau et al., 2001).

B. Neoplastic Disease

Prostanoids may be involved in the pathogenesis of cancers. Early studies have recognized that growth factors, tumor promoters, and oncogenes induce prostanoid synthesis (Levine, 1981). It is now recognized that such effects are due to the induction of COX-2 in various cell types (Dubois et al., 1998). Early studies also pointed out that metabolism of AA via the COX pathway is enhanced in various human tumors, compared with the nontumorigenic counterparts (Levine, 1981). For example, mammary tumors secrete high levels of PGE_2 compared with the normal adjacent mammary tissue (Karmali et al., 1983). The functional role of such a finding has not been clear; however, several theories have been proposed on the role of tumor-derived prostanoids. For example, induction of angiogenesis, induction of tumor cell proliferation, suppression of immune response, and inhibition of cell death (Taketo, 1998a,b). Early work on

animal models of tumorigenesis, indicated that NSAIDs profoundly inhibit colon and breast tumors induced by carcinogens in rodents (Reddy et al., 1987). These data, although correlative, strongly suggested that products of the COX pathway may participate in carcinogen-induced tumorigenesis.

These evidences were further supported by epidemiological studies of humans who use aspirin and other NSAIDs chronically; it was found in various studies that incidence of various cancers, including, colon, intestinal, gastric, breast, and bladder cancers, were reduced up to 40 to 50% (Thun et al., 2002). These large-scale epidemiologic studies strongly suggested that the COX pathway is involved in the cancer chemopreventive activity of NSAIDs in the GI tract (Baron, 2003).

Aspirin and sulindac are the best studied NSAIDs with regard to chemoprevention and induction of tumor regression in the colon/rectum, respectively; however, all NSAIDs may share these properties (Smalley et al., 1999). Epidemiologic evidence implicates both COX-1 and COX-2 in the chemopreventive roles of NSAIDs. Aspirin, which is a preferential COX-1 inhibitor, has recently been shown in three randomized placebo-controlled trials to be "moderately effective" in preventing the appearance of sporadic colorectal adenomas in patients with a history of these tumors (Baron et al., 2003; Benamouzig et al., 2003; Sandler et al., 2003). Significantly, doses of only 80 to 325 mg/per day, which would be expected to inhibit primarily COX-1, were sufficient to prevent adenoma incidence by 45%, although there was some variation in the degree of effect between studies (Huls et al., 2003). COX-2 selective drugs celecoxib and rofecoxib have also been shown to reduce adenoma incidence and to evoke tumor regression in patients with familial polyposis (Phillips et al., 2002; Higuchi et al., 2003). Duodenal adenomas, which are otherwise untreatable, show some reduction by treatment with celecoxib (Phillips et al., 2002).

Many studies have examined the expression of COX-1 and -2 in tumor tissues from various cancers. Early studies by Eberhart et al. (1994), Kargman et al. (1995), and Sano et al. (1995) showed that COX-2 is overexpressed in ≥80% of colorectal cancer tissues. Interestingly, epithelial cells as well as inflammatory cells and stromal cells express this enzyme. In contrast, COX-2 expression is detectable but lower in adjacent normal tissues. COX-1 isoenzyme is expressed in both normal and tumor tissue. This finding has been repeatedly confirmed in other tumors such as pancreas, skin, gastric, bladder, lung, head and neck, among others (Thun et al., 2002). These studies suggested that COX-2 may play a role in tumor formation and/or maintenance.

After the development of the COX-2 inhibitors, they were employed in animal models of carcinogenesis (Kawamori et al., 1998; Harris et al., 2000). They were also shown to be effective in reducing the incidence of carcinogen-induced tumors; however, nonselective in-

hibitors of COX and derivatives of some NSAIDs that do not inhibit COX enzyme activity (for example, sulindac sulfone) were also effective, raising questions about the requirement of COX enzymes in tumorigenesis (Marx, 2001).

Various other lines of evidence also cast a doubt on the causal role of COX enzymes in tumorigenesis. Aspirin and salicylates, albeit at high doses, were shown to inhibit the IκB kinase pathway, suggesting that additional targets exist for the NSAIDs (Yin et al., 1998). In addition, sulindac sulfone was shown to inhibit the PPARδ pathway, which was induced by APC gene deletion (He et al., 1999); however, recent studies indicate that PPARδ is not required for sulindac-induced epithelial cell apoptosis, which occur at very high nonphysiological levels of this drug (Park et al., 2001). In addition, NSAIDs at high doses inhibited oncogene-induced transformation of mouse embryonic fibroblasts derived from COX-1 and -2 double null embryos (Zhang et al., 1999). Furthermore, neither COX-1 nor COX-2 acted as classical oncogenes in cellular models of transformation (Narko et al., 1997; Trifan et al., 1999). Indeed, overexpression of the COX enzymes was associated with cellular growth arrest in many cell types (Narko et al., 1997; Trifan et al., 1999). Together, these studies have suggested that overexpression of COX enzymes in tumors may not be simply acting as oncogenes in tumor development.

Oshima et al. (1996) provided definitive evidence that COX-2 is required for intestinal tumorigenesis in the ApcΔ^{715} deletion mouse model neoplasia. This mutation results in the truncation of the APC tumor suppressor gene, which regulates the level and activity of the β-catenin protein. Enhanced levels of β-catenin results in transcriptional activation of various growth regulatory genes, for example, c-myc, cyclin D1 via the TCF/LEF family of transcriptional regulators. Thus, ApcΔ^{715} deletion mice develop intestinal polyps with a very high penetrance (Taketo, 1998a). Deletion of the COX-2 gene in these mice resulted in gene dose-dependent reduction in polyps. In addition, COX-2 inhibitor also reduced polyps in these mice. These data strongly suggested that COX-2 expression is required for intestinal tumorigenesis (Oshima et al., 1996); however, the expression of COX-2 was detected in the stromal tissues of the small intestine and not in the epithelial compartment, suggesting that it may function in a paracrine manner to regulate epithelial cell transformation. Therefore, an endocrine role for COX-2-derived prostanoids cannot be ruled out from this study. Thus, these critical experiments provided strong evidence that COX-2 is required for intestinal tumorigenesis. Langenbach's laboratory showed that deletion of the COX-1 gene also attenuated polyp formation (Langenbach et al., 1999b; Chulada et al., 2000). These data further suggest that both COX-1 and -2 may be important in tumorigenesis. In addition, in carcinogen-induced tumor initiation and promotion

model in skin papilloma formation also exhibited similar requirement for both COX-1 and -2 gene expression (Langenbach et al., 1999b). Although papilloma numbers were decreased in both COX-1 and -2 deleted mice, the phenotype of the skin polyps was distinct. For example, papillomas formed in COX-2$^{-/-}$ mice showed increased cellular apoptosis, increased differentiation of keratinocytes, and formed small elongated polyps. In contrast, COX-1-deficient animals formed larger dome-topped polyps and did not show differences in keratinocyte apoptosis and differentiation (Langenbach et al., 1999b). Takeda and colleagues (2003) have recently advanced the postulate that COX-1 expressed in intestinal stromal cells provides basal expression of PGE_2 sufficient for polyps to grow to 1 mm, whereafter COX-2 and microsomal PGE_2 synthase are induced to support further polyp growth and development of tumor vasculature. These studies indicate that both COX-1 and -2 enzymes play complex roles in tissue homeostasis and participate in multiple nodes of the tumorigenesis process.

The question of whether COX-2 overexpression is sufficient to induce tumorigenesis was recently addressed (Liu et al., 2001). Liu et al. overexpressed the human COX-2 gene in the mammary glands of transgenic mice using the mouse mammary tumor virus promoter. This promoter is highly selectively expressed in the mammary epithelium and is hormonally induced. Thus, expression of mouse mammary tumor virus-linked transgenes is induced in the mammary glands during pregnancy and lactation. Transgenic mice expressing the human COX-2 gene exhibited high levels of COX-2 mRNA, protein, and enzymatic activity in the mammary glands, particularly during pregnancy and lactation. The expression of exogenous COX-2 gene did not influence the expression of COX-1 gene in the mammary glands of transgenic mice. The COX-2 transgenic mice showed precocious mammary gland differentiation, which was characterized by premature expression of the β-casein gene and premature lobuloalveolar development in the virgin animals. These effects were reversed by the administration of the COX inhibitor indomethacin, suggesting that they are mediated by secreted prostanoids. The COX-2 transgenic mice underwent normal pregnancy and lactation in the first cycle. Mammary gland involution was delayed, which was associated with decreased apoptosis of the mammary epithelial cells. These findings were in concert with decreased apoptosis observed in COX-2 overexpressing epithelial cells. After repeated cycles of pregnancy and lactation, however, the COX-2 transgenic mice developed tumors in the mammary glands (Liu et al., 2001). The histology of the mammary tumors indicated that invasive, metastatic tumors of both alveolar and ductal histotypes were observed. In addition, the tumors were focal in nature, suggesting that COX-2 overexpression as well as other mutagenic events were required to fully transform the

mammary epithelium. COX-2-induced tumors continued to express COX-2 and contained lower levels of the apoptotic regulatory proteins Bax and Bcl-X$_L$ as well as containing higher levels of the anti-apoptotic protein Bcl-2 (Liu et al., 2001). These data suggest that ectopic overexpression of COX-2 gene is sufficient to transform the mammary gland after repeated cycles of pregnancy and lactation (Liu et al., 2001). These data support the notion that unregulated expression of COX-2, perhaps induced by carcinogenic stimuli or other tumor promoters is an important contributor of tumorigenesis. Recent transgenic overexpression studies in which COX-2 was targeted to the skin of transgenic mice also support this concept (Neufang et al., 2001). In this study, hyperplasia of the epidermis and abnormal sebaceous gland differentiation was observed, although spontaneous skin tumors did not develop (Neufang et al., 2001). Recent work from the same group showed that carcinogen-induced skin papilloma formation is enhanced in COX-2TG mice, suggesting that COX-2 acts as a potent tumor progression factor in the skin (Marks et al., 2003)

Even though the effect of COX-2 to induce tissue changes that ultimately lead to tumorigenesis is beginning to be appreciated, the mechanisms involved are not well understood. Many in vitro studies, however, support the notion that COX-2 overexpression inhibits apoptosis and that tumor angiogenesis is induced as well (Dubois et al., 1998; Tsujii et al., 1998). Tsujii and DuBois showed that overexpression of COX-2 in intestinal epithelial cells resulted in enhanced E-cadherin expression and decreased apoptosis, when cells are induced with butyrate (Tsujii and DuBois, 1995). Later studies from the same laboratory indicated that PGE_2 stimulation of these cells results in cell survival due to the phosphatidylinositol 3-kinase/Akt pathway. In addition, epithelial cell motility and invasive behavior were also induced (Sheng et al., 2001). The ability of COX-2 overexpression to inhibit apoptosis was observed in other epithelial cells and neuronal PC-12 cells (Chang et al., 2000) but not in ECV-304 bladder carcinoma cells (Narko et al., 1997). Coupled with the finding that apoptosis of mammary epithelial cells are altered in COX-2 transgenic mice (Liu et al., 2001), regulation of apoptosis may be an important event in COX-2-induced tissue changes that lead to tumorigenesis.

In addition, induction of angiogenesis by the COX-2 (and the COX-1 pathway) may contribute to the development of tumors (Hla et al., 1993; Ben-Av et al., 1995; Tsujii et al., 1998). Angiogenesis is regulated by a plethora of factors, the balance of which is thought to be critical. As discussed above, COX-2 expression and secretion of prostanoids such as PGE_2 may induce angiogenesis during cancer development; however, the reason why normal tissue that produces PGE_2 abundantly via the COX-1 pathway fails to induce angiogenesis is not understood. The enhanced production of COX-2, together with other changes that occur during cancer de-

velopment, may contribute to the overall balance of the angiogenic response during tumor development. Further studies are needed to better define the molecular mechanisms involved in COX-2-induced tumorigenesis.

Evidence that NSAIDs reduce cancer incidence and evoke tumor regression in the GI tract has been extensively reviewed elsewhere (Dannhardt and Kiefer, 2001; Dempke et al., 2001), as has overexpression of COX-2 in epithelial cancers in humans (Howe et al., 2001; Kalgutkar and Zhao, 2001; Tsuji et al., 2001; Dixon, 2003). The antineoplastic activity of NSAIDs is likely multifactorial, but the induction of apoptosis is essential to the ability of these drugs to cause tumor regression and may also be integral to their ability to prevent tumor growth. Induction in experimentally induced tumors in rodents of tumor regression has been observed in several studies (Oshima et al., 1996; Chiu et al., 1997; Ritland and Gendler 1999; Jacoby et al., 2000). Giardello has also reported an increase in apoptosis in the carcinomas of the colon/rectum following 3 months of treatment with sulindac (Pasricha et al., 1995). Currently the mechanism(s) by which NSAIDs induce apoptosis in tumors of the GI tract, and perhaps elsewhere, is unknown. Cyclooxygenase and noncyclooxygenase pathways that have been proposed to govern NSAID-induced apoptosis have been reviewed elsewhere (Moore and Simmons, 2000; Simmons and Wilson, 2001).

C. Alzheimer's Disease

Epidemiologic evidence indicates that NSAID use is associated with a lower incidence or risk of AD (McGeer et al., 1996; Hendrie, 1997). An inverse relationship is seen between NSAID use (particularly aspirin) and AD incidence in case-controlled studies of patients who have osteoarthritis, rheumatoid arthritis, or who use NSAIDs for other purposes. A similar inverse correlation between NSAID use and AD was seen in a co-twin control study of 50 elderly twins with AD onset separated by 3 years or more (Breitner et al., 1995). Both decreased risk of AD among NSAID users as well as a decreased risk of AD with increased duration of NSAID use was found in the prospective Baltimore Longitudinal Study of Aging (Stewart et al., 1997). In this study, 1686 participants were followed for 15 years, and participants 55 and older were assessed for AD. A decrease in cognitive decline was also associated with NSAID use in the 1-year longitudinal Rotterdam Study (Andersen et al., 1995); however, another longitudinal study, The Medical Research Council Treatment Trial of Hypertension in Older Adults, found that increased beneficial cognitive effects among NSAID users compared with controls was not evident in AD patients over 74 (Prince et al., 1998). A fourth longitudinal study also found no beneficial cognitive effect of aspirin or other NSAIDs in AD patients with a mean age of 80 (Henderson et al., 1997). Together these studies suggest that the window of efficacy for NSAID use may precede the age of 75; however, in a

recent case-control study of specific dementias including AD, vascular, and other dementias in patients 75 and older, a strong inverse correlation between NSAID usage and the presence of AD, but not other dementias, was observed (Broe et al., 2000). This study suggests that a lack of effect observed in older patients may be due to confounding neurodegenerative conditions in elderly people that are not affected by NSAIDs.

A number of excellent reviews have recently explored the complex and, as yet, unclear roles that COX isozymes play in AD (O'Banion, 1999; McGeer, 2000, and Pasinetti, 2001). This disease exhibits a strong inflammatory component initiated and/or exacerbated by fibrillar β-sheet β-amyloid deposits (Halliday et al., 2000). Proinflammatory cytokines, acute phase proteins, prostaglandins, and other mediators of inflammation are elevated in and around the senile plaques present in AD brains. COX-2 has been reported to be increased in the cortex of AD brains (Oka and Takashima, 1997; Kitamura et al., 1999); however, it is important to note that COX-2 is also normally expressed in neurons of the neocortex and hippocampus and appears to be preferentially expressed in glutamatergic pyramidal neurons (Kaufmann et al., 1996; Yasojima et al., 1999; Ho et al., 2001). Within these neurons it shows a perinuclear subcellular localization typical of other cells but also may be found accumulated in dendrites and particularly in dendritic spines (Kaufmann et al., 1996, Thore et al., 1998). Pasinetti and colleagues (Ho et al., 2001) have recently correlated increased COX-2 expression in the CA2 and CA3 subdivision of the hippocampal pyramidal layer with increasing dementia through mild to severe stages. Increased COX-2 in the CA1 subdivision was evident only in severe stage AD.

The normal function of COX-2 in brain neurons is unknown, and it is unclear whether long-term use of COX-2 inhibitors will have a physiological effect through inhibition of this function. COX-2 can be induced in neurons, microglia, and astrocytes by a variety of neurotoxic stimuli including hypoxia and excitotoxins, such as kainic acid (Adams et al., 1996; Marcheselli and Bazan, 1996; Tocco et al., 1997; Tomimoto et al., 2000). The current debate is whether COX-2 induction after neuronal insult serves to protect against cell death or promote apoptosis in the expressing neuron itself or in neighboring neurons. Data using animal models and in vitro systems support both protective (Kunz and Oliw, 2001) and pro-apoptotic roles (Iadecola et al., 2001). Increased neuronal PG synthesis resulting from increased COX-2 may evoke increased levels of other proinflammatory agents produced by astroglial cells and cause neuronal cell death. This is consistent with the finding that NSAIDs result in a decrease in activated microglial cells in AD patients and in the Tg2576 mouse, which is predisposed to an AD-like syndrome (Lim et al., 2000; Hoozemans et al., 2001). Ibuprofen treatment in these mice decreases concentrations of inflammatory mediators

such as IL-1β, slows β-amyloid plaque deposition, and decreases the number of dystrophic neurites (Lim et al., 2000). These findings suggest that COX isozymes influence the rate of β-amyloid secretion or deposition as well as function in any inflammatory process that results from plaque formation; however, NSAIDs used in both human and animal studies described above were nonselective toward COX enzymes or COX-1 preferential and suggests that COX-1 and COX-2 are important in the pathogenesis of AD. Aspirin is a strongly COX-1 preferential NSAID and a small (44 patients), double blind, placebo-controlled clinical trial of the nonselective drug indomethacin showed reduction in the rate of cognitive decline in AD patients (Rogers et al., 1993). Diclofenac administered with misoprostol showed no statistically significant effects in an even smaller study although a trend to prevention was seen (Scharf et al., 1999).

Several lines of evidence suggest that COX-2 is important in AD. COX-2 is clearly induced following various neurotoxic stimuli as mentioned above. Furthermore, elevated COX-2 is also found in other neurodegenerative diseases such as in the spinal cord of patients with sporadic amyotrophic lateral sclerosis and in the spinal cords of transgenic mice that exhibit an amyotrophic lateral sclerosis-like syndrome (Almer et al., 2001). Genetic deletion of COX-2 in laboratory animals decreases susceptibility to ischemic brain injury and N-methyl-D-aspartate-mediated neurotoxicity (Iadecola et al., 2001), and COX-2 overexpression in transgenic mice increases susceptibility to β-amyloid-induced neurotoxicity (Kelley et al., 1999). A COX-2 selective NSAID reduces focal ischemic brain injury in a rat model, suggesting a role for COX-2 in stroke and a possible role of COX-2-selective inhibitors in stroke treatment (Govoni et al., 2001); however, pretreatment of mice with NS398 leads to markedly increased neuronal cell death in the hippocampus and increased mortality following kainate treatment. This latter finding suggests that inhibition of COX-2 induced by excitotoxins may be neuroprotective, but that inhibition of constitutive COX-2 expression may be deleterious in the event of a seizure (Baik et al., 1999).

In addition to determining the specific roles that COX-1 and COX-2 play in AD, other important issues remain to be addressed with regard to the action of these drugs in this disease. First, dose levels that maximally evoke a protective effect in AD need to be determined for NSAIDs. Limited data suggest that low dose NSAID treatment may be as effective as high doses (Broe et al., 2000). If prevention of AD requires inhibition of both COX-1 and COX-2 as present data suggests, appropriate dose levels may be difficult to establish due to NSAID-induced gastric toxicity; however, if low doses are required, the establishment of regimens with acceptable toxicity levels for most people should be feasible. Second, it will be important to determine whether aspirin or competitively acting NSAIDs more effectively reduce risk of AD. Most studies have detected a preventive effect of aspirin in AD, but some studies comparing aspirin with competitively acting NSAIDs have shown the latter to be better at reducing risk of AD (Stewart et al., 1997). Third, the cells directly involved in the NSAID-induced protection mechanism need to be determined. NSAIDs may inhibit the inflammatory component of AD through inhibiting COX isozymes in neurons or glial cells. Alternatively, if low doses of NSAIDs maximally reduce AD, the important target for anti-AD therapy may be cells of the vascular system such as platelets or endothelial cells.

V. Cyclooxygenase Isozymes and the Future

Twelve plus years of research on the COX-2 isozyme has yielded three widely marketed drugs (celecoxib, valdecoxib, and rofecoxib) developed for COX-2 selectivity that appear to possess all of the analgesic, antipyretic, and anti-inflammatory activity of the older nonselective NSAIDs. More drugs are on the horizon. It is important to note, however, that COX-2-selective drugs are not more effective than noncompetitive NSAIDs—thus the term "superaspirins" should be avoided.

The results of long-term treatment with COX-2-selective drugs, particularly rofecoxib as tested in the VIGOR trial, is suggestive that these NSAIDs do possess lower gastrotoxicity than nonselective NSAIDs, but the finding of an elevated incidence of myocardial infarction (MI) in this study raises the question of whether we know all of the side-effects that may be associated with these drugs. Studies during the next decade will reveal whether this increase in MIs is real or is a statistical aberration. If the MI effect is real, further studies will define whether cardiovascular risk is idiosyncratic to rofecoxib or is to be expected of all selective COX-2 inhibitors.

Historically, NSAIDs have been used palliatively to treat inflammation, pain, and fever. People who could tolerate these drugs experienced transient relief from their symptoms; however, if isozyme-specific NSAIDs exhibit reduced gastrotoxicity, these new drugs have the potential of being used even more widely and perhaps as important chemopreventive agents. This is particularly promising with regard to decreasing the incidence of cancer of the gastrointestinal tract and Alzheimer's disease. Additionally, the fact that NSAIDs can cause tumor regression may allow future use of these drugs as adjunctive treatments in cancer. The finding of variants of COX may provide new roles for the COX genes. Thus the future may hail exciting new uses for this historically old class of drugs.

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