PREFACE

The revelation that aspirin and aspirin-like compounds have notable antineoplastic properties has revolutionized cancer research. *COX-2 Blockade in Cancer Prevention and Therapy* chronicles the evidence and presents exciting new opportunities for the use of cyclooxygenase-2 (COX-2) blockade in the prevention and treatment of cancer. The text is divided broadly into five areas. First, an historical overview documents the scientific discovery of COX-2 and the pharmaceutical development of nonsteroidal anti-inflammatory drugs (NSAIDs) designed for selective COX-2 inhibition. The process by which essential polyunsaturated fatty acids (PUFAs) stimulate prostaglandin biosynthesis and cancer development, and its interruption by COX-2 inhibition, is elucidated. This is followed by a section on the epidemiology of NSAIDs and cancers of the colon and breast, and other anatomic sites. These chapters reflect significant cancer protection owing to the regular use of common NSAIDs such as aspirin and ibuprofen. A section on animal models of carcinogenesis presents comprehensive evidence that general NSAIDs inhibit a variety of malignant neoplasms in vivo, and highlights recent findings which show that COX-2 blocking agents produce striking chemopreventive effects against colon cancer and breast cancer as well as other malignancies. Genetic models are presented confirming the critical role of COX-2 in carcinogenesis. Section IV then discusses the molecular biology of COX-2 *vis-à-vis* the role of COX-2 and, to a lesser extent, COX-1, in modulating a number of important processes in molecular carcinogenesis such as mutagenesis, cell division, angiogenesis, cell differentiation, and apoptosis. Autocrine and paracrine mechanisms of carcinogenesis are addressed, as well as COX-dependent and COX-independent effects of NSAIDs. Finally in Section V, clinical applications of selective NSAIDs are discussed that are immediately relevant to cancer prevention and control, and future perspectives of utilizing COX-2 blocking agents are projected, which may help reduce the burden of cancer. The comprehensive nature of *COX-2 Blockade in Cancer Prevention and Therapy* makes it an important reference text for applied cancer research and provides a general basis for extended research and development on the antineoplastic properties of COX-2 blockers.

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2 Historical Aspects of COX-2

Cloning and Characterization of the cDNA, Protein and Gene

Harvey R. Herschman, PhD

CONTENTS

1. INTRODUCTION

The *prostanoids* and the *leukotrienes* are the two major subclasses of the bioactive compounds known as *eicosanoids.* These hormones are derived from C_{20} fatty acids. Although a variety of polyunsaturated fatty acids (PUFAs) can serve as precursors to the eicosanoids, the bulk of the prostanoids and leukotrienes are derived from arachidonic acid (AA). Precursor AA does not exist free in cells; AA is present in membrane bound glycerophospholipids. When cells receive an appropriate stimulus, either a secretory or

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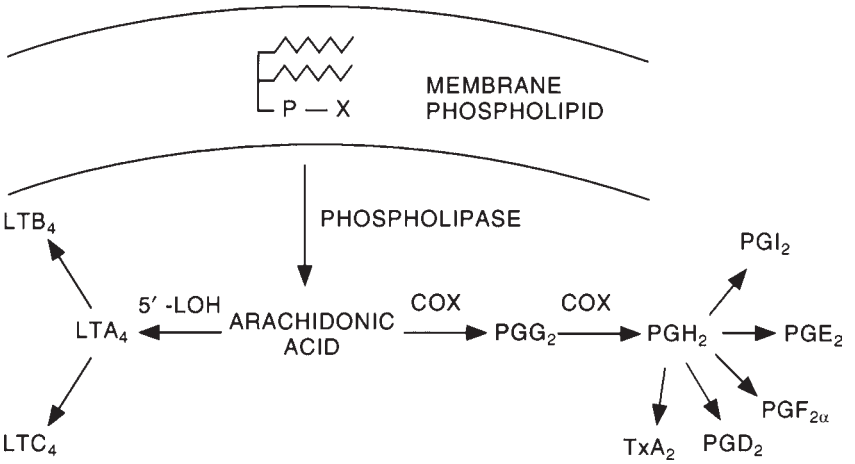

Fig. 1. The pathways of leukotriene and prostanoid biosynthesis. PLA_2, phosopholipase A_2; COX, cyclooxygenase; 5'-LOH, 5' lipoxygenase; PG, prostaglandin; Tx, thromboxane; LT, leukotriene.

a cellular phospholipase is activated to cleave AA from the membrane phospholipid pool (Fig. 1). The free AA liberated by ligand-stimulated phospholipase activation can then serve as substrate for the formation either of prostanoids or leukotrienes. The prostaglandin synthase/cyclooxygenase (COX) enzyme carries out a two-step reaction. In the first step, AA is subjected to a *bis* oxygenation COX reaction that results in the formation of prostaglandin G_2 (PGG_2). This COX reaction is rapidly followed by a hydroperoxidase reaction, occurring at a distinct site on the prostaglandin synthase/COX enzyme, to convert PGG_2 to PGH_2. PGH_2 is the common intermediate for the synthesis of the various prostaglandins (e.g., PGE_2, $PGF_{2\alpha}$, PGD_2, etc.), the prostacyclins, and the thromboxanes. The specific nature of the prostaglandins produced in various cell types depends on the presence of specific prostaglandin synthases (e.g., prostaglandin E_2 synthase, prostaglandin D_2 synthase, etc.); each of these enzymes uses as substrate the common PGH_2 produced by (COX) from free AA. Alternatively, the free AA released by ligand-activated phospholipases can serve as substrate for the lipoxygenase pathway, leading to the formation of the leukotrienes.

2. THE ROLE OF PROSTAGLANDINS IN NORMAL PHYSIOLOGY AND PATHOPHYSIOLOGY

Experimental and clinical studies have demonstrated that the prostaglandins play major roles in a number of biological processes, including thermoregulation, platelet aggregation, wound healing, luteinization, ovulation, parturition, water balance, glomerular filtration, and hemostasis. However, the pharmacologic blocking of prostaglandin production has provided us with probably the greatest insight into the role of these hormones. In 1971, Vane *(1)* reported that the pharmacologic effects of aspirin resulted from its ability to prevent prostaglandin production. Aspirin, and all the commonly used nonsteroidal anti-inflammatory pharmaceuticals, exert their pharmacologic effects by inhibiting COX activity and blocking prostaglandin production. The analgesic, antipyretic and anti-inflammatory effects of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) therefore

suggest major roles for prostaglandins in pain perception, thermoregulation, and chronic inflammatory illnesses such as arthritis, asthma, and inflammatory bowel disease. Aspirin is used to prevent cardiovascular disease, suggesting a role for prostaglandins in heart and blood vessel biology. The ulcerogenic effects of NSAIDs suggest a role for prostaglandins in epithelial cell physiology in the gut, whereas excessive prostaglandin production has been has associated with bone resorption. In the context of this book, perhaps the most notable effect is on the frequency of colon cancer; moderate doses of aspirin reduce both morbidity and mortality because of colon cancer by nearly 50% *(2)*. More recently, as discussed in other chapters, COX activity and prostaglandin production have been suggested to play a role in other types of cancers.

3. COX ACTIVITY IS FOUND IN NEARLY ALL CELLS

COX activity is nearly ubiquitous. When extracts are prepared from almost all tissues or cells, the ability to convert AA to prostaglandins is a nearly universal property. Following ligand stimulation, secretory or cytoplasmic phospholipases are activated and release AA from membrane phospholipids. Because COX activity is present in nearly all tissues, the rate-limiting step in the synthesis of prostaglandins was thought to be the activation of phospholipases to release free AA; constitutive COX present in cells should convert the newly synthesized AA substrate to PGH_2. The cell-type specific prostaglandin synthases should then convert the PGH_2 to the appropriate prostanoid.

4. PURIFICATION AND CLONING OF COX FROM SHEEP SEMINAL VESICLES

Because of its intense importance to the pharmaceutical industry, the purification and characterization of COX has been a major goal of protein chemists. Early studies demonstrated that the enzyme is found as a homodimer of two ~70,000 Dalton subunits localized to the endoplasmic reticulum. Sheep seminal vesicles have served as the richest source of the enzyme for purification and characterization *(3–5)*. In 1988, three laboratories *(6–8)* used amino acid sequence data from peptides isolated from sheep seminal vesicle COX to create oligonucleotide probes. Lambda phage cDNA libraries prepared from ram seminal vesicle RNA preparations were then screened to isolate cDNA clones for the COX message. All three laboratories reported the cloning of a COX cDNA that predicts a messenger RNA of approx 2.8 kb encoding a protein of 576 amino acids. Using the sequence data from the ovine COX cDNA, orthologs from murine *(9)* and human *(10,11)* cDNA libraries were subsequently cloned and sequenced.

5. NOTHING IS SIMPLE; EVIDENCE FOR LIGAND AND ONCOGENE-INDUCED COX ACCUMULATION

Because 1) ligand activation is necessary to activate the phospholipases to release AA from phospholipid membrane stores and 2) COX activity is present in nearly all unstimulated cells, it appeared that the regulation of ligand-induced prostaglandin synthesis was quite straightforward. Substrate for the COX would be limiting; ligand stimulation would activate phospholipase to release AA. The constitutive COX present in most tissues, present in excess, would convert the free AA to PGH_2. The cell type-specific enzymes present in various tissues would then convert the PGH_2 to the final prostanoid product(s). However,

a number of studies suggested that ligand-stimulated prostanoid synthesis is accompanied by an increase in COX activity in addition to activation of phospholipase activity.

5.1. Transcriptional and Translational Inhibitors Suggested that Ligand and Oncogene-Induced COX Activity is the Result of New Gene Expression

Whiteley and Needleman *(12)* demonstrated that conditioned medium from mononuclear cells [presumably containing interleukin 1 (IL-1)] could stimulate the release of PGE_2 from human dermal fibroblasts. Moreover, the ligand-stimulated prostaglandin production could be blocked by concomitant incubation with actinomycin D, an inhibitor of transcription, or with cycloheximide, an inhibitor of translation. Microsomal COX prepared from the stimulated fibroblasts had a threefold increase in the V_{max}, but no change in the Michaelis constant (Km) when compared to control cells. These data suggest that ligand stimulation increases COX activity in cells in a transcriptionally and translationally dependent fashion.

When murine 3T3 fibroblasts are treated with platelet derived growth factor (PDGF), they accumulate PGE_2 over a 4 h period *(13)*. Simultaneous addition of cycloheximide, an inhibitor of protein synthesis, with PDGF can block the long-term accumulation of PGE_2. Moreover, if the COX activity in the cells is first covalently inactivated by aspirin treatment, the PDGF treated cells can recover and synthesize prostaglandins within 3 h. Habenicht et al. *(13)* suggest that "PDGF stimulates prostaglandin synthesis by direct effects on the prostaglandin-synthesizing enzyme system…"

Treatment of the murine osteoblastic cell line MC3T3-E1 with epidermal growth factor (EGF) also leads to PGE_2 production. After a "lag phase" of 1–2 h, PGE_2 synthesis peaks at about 3 h. EGF-stimulated PGE_2 synthesis is almost completely blocked by cycloheximide or by actinomycin D *(14)*. Following EGF treatment, microsomal preparations from MC3T3-E1 cells have increased COX activity. The authors conclude that their results "…suggested an EGF-mediated induction of COX."

5.2. Antibodies to Sheep Seminal Vesicle COX Confirmed Ligand and Oncogene Induced Synthesis of COX in Cultured Cells

Treatment of human umbilical vein endothelial cells and bovine aortic endothelial cells with interleukin 2 (IL-2) stimulates the production of PGI_2 *(15)*. Both cycloheximide and actinomycin D can prevent the ligand-induced accumulation of prostacyclin. Using an affinity purified antibody to sheep seminal vesicle COX, Frasier-Scott et al. *(15)* demonstrated, by Western blotting, an increase in immunoreactive COX 4 h after ligand stimulation. They conclude, from their data, "…that IL-2 induces *de novo* synthesis of PGH synthase."

Like EGF *(14)*, epinephrine can stimulate PGE_2 production in MC3T3-E1 murine osteoblasts *(16)*. Inhibitors of transcription and translation were able to block the hormone-induced accumulation of prostaglandin. When microsome preparations were assayed for COX activity, an increase in COX activity was observed in microsomes from epinephrine-treated cells. The increased COX activity demonstrated in the microsomes from epinephrine-treated cells could be immunoprecipitated with antibody to COX protein. These authors also conclude that the ligand-induced increase in prostaglandin production is dependent on induction of COX synthesis.

Human dermal fibroblasts treated with IL-1 synthesize increased levels of prostaglandin *(17)*. The Needleman laboratory, using an antibody to sheep seminar vesicle COX, demonstrated increased incorporation of radioactive methionine into immunoprecipitable COX following IL-1 stimulation. Using both N-terminal sequencing and endoglycosidase H treatment of the labeled, immunoprecipitated product from IL-1 stimulated cells, the authors concluded that the COX produced from IL-1 stimulated fibroblasts was similar to the native sheep COX use to prepare the antiserum, and suggested that "the IL-1 effect is mediated mainly, if not solely, via induction of COX synthesis." In a subsequent paper, Raz et al. *(18)* demonstrated that the IL-1 induced synthesis of immunoprecipitable COX in these human dermal fibroblasts is inhibited both by actinomycin D at early times and by concomitant administration of dexamethasone. They concluded that "…glucocorticoids exert their effect via a newly synthesized protein, causing a profound translational control of PG synthase synthesis."

PGE_2 and $PGF_{2\alpha}$ play important steps in the process of ovulation. When preovulatory follicles from the rat are stimulated with luteinizing hormone (LH), there is a dramatic induction of COX protein, as measured by immunoblotting *(19)*. When granulosa cells were prepared from LH-stimulated follicles, induced expression of immunoreactive COX was observed in these cells. Co-incubation with the transcriptional inhibitor α-amanitin blocked the LH induction of immunoreactive COX in the granulosa cells. Wong et al. *(19)* also examined their LH-treated cultures by Northern blot for the level of the 2.8 kb COX message *(6)* and found no change in the level of this mRNA, despite the substantial increase in immunoprecipitable COX protein. The authors concluded that the increased levels of COX protein in LH treated cells "…may not involve increased transcription of the PGS gene. Or, if increased transcription of PGS gene does occur, it is rapid and coupled to cotranslational degradation of the message."

Needleman's group extended their work from human dermal fibroblasts *(17,18)* to human blood monocytes, and demonstrated that endotoxin-treated cells produce "prodigious amounts" of prostaglandins and thromboxanes *(20)*. Endotoxin also stimulated substantial increases in microsomal COX activity and the accumulation of radioactively labeled protein immunoprecipitated by their anti-COX antiserum. Endotoxin-induced accumulation of prostaglandins, increased COX activity and increased COX synthesis were all inhibited by dexamethasone. The authors suggest that cells "…may contain two pools of COX, each with a differential sensitivity to endotoxin or dexamethasone."

Han et al. *(21)* in a search for molecular alterations induced by oncogene expression, used "giant two-dimensional gel electrophoresis" to examine radioactively labeled proteins of chicken embryo fibroblasts (CEF) transformed with a temperature-sensitive *v-src* gene, the oncogene of Avian Sarcoma Virus. After shifting the cells to the permissive temperature and labeling with radioactive methionine, the cellular proteins were separated by the two-dimensional gel electrophoresis procedure and the gel was subjected to autoradiography. Remarkably, these investigators tentatively identified a radioactive doublet on their gels as COX, based on the molecular mass, isoelectric point, and subcellular distribution of the labeled protein. They demonstrated that the protein induced by oncogene activation could be immunoprecipitated with anti-COX antibodies. Moreover, the oncogene-induced induction of COX synthesis was blocked by glucocorticoid administration. The authors suggested that oncogene stimulation could bring about transformation by causing "…persistent changes in the expression of genes normally induced only transiently during passage from the G_o stage of the cell cycle."

6. EVIDENCE FOR A SECOND, INDUCIBLE COX GENE

When pulmonary epithelial cells isolated from sheep trachea were cultured and exposed to serum, the level of PGE_2 was dramatically increased when compared to PGE_2 produced by control cells *(22)*. A dramatic increase in COX specific activity was also observed in the serum-treated cells. When radioactively labeled COX was precipitated from control and serum-treated cells, using the antiserum prepared by Raz et al. *(17)*, a 12-fold increase in labeling of immunoprecipitable COX was observed in the serum-stimulated population. To determine whether a corresponding increase in COX message was present, northern blots of RNA from control and serum-stimulated pulmonary epithelial cells were probed with the 2.8-kb cDNA for sheep seminal vesicle COX *(7)*. At high stringency, rather than an increase in this message, a slight *decrease* in the level of the 2.8-kb message occurred in the serum-stimulated cells. However, when similar northern blots were hybridized at a lower stringency, a 4.0-kb crossreacting message was seen. In contrast to the signal present at 2.8 kb, the 4.0-kb band hybridizing with the cDNA probe increased in the serum-treated pulmonary epithelial cells. Rosen et al. *(22)* suggested that "…the 4.0 kb mRNA species may be derived from a distinct COX related gene and that it may encode for a protein with COX activity."

Needleman's group extended their study of the roles of endotoxin and glucocorticoid in COX synthesis to in vivo studies *(23)*. Peritoneal macrophages isolated from endotoxin treated mice showed a substantial increase both in COX activity and in the amount of radioactive, immunoprecipitable COX labeled in cell culture with radioactive methionine. Dexamethasone blocked the induction of prostaglandin production and COX synthesis in macrophages isolated from endotoxin-treated mice. In contrast, glucorticoid administration did not substantially modulate basal prostaglandin production or COX levels in untreated cells. Masferrer et al. *(23)* suggested that the two functionally distinct COX enzymes, differentially regulated by glucocorticoids, may arise through the expression of different COX genes.

7. CLONING THE INDUCIBLE COX

Although intense interest in the regulation of COX gene expression and its role in prostaglandin synthesis had developed in the late 1980s, the cloning of the inducible COX gene occurred in three laboratories whose primary interests were not in the area of eicosanoid metabolism or biology. Each of the laboratories that isolated a cDNA for the inducible COX had, as their primary interest, the regulation of gene expression by growth factors and/or oncogenes.

Ray Erikson et al. had, for many years, been investigating the molecular changes induced by the v-src oncogene that lead to oncogenic transformation in chick embryo fibroblasts. As one approach to this problem, Simmons et al. *(24)* cloned a set of cDNAs for "immediate early genes" whose mRNA levels were increased soon after CEF cells expressing a temperature-sensitive *v-src* gene were shifted to the "permissive" temperature. Xie et al. *(25)* sequenced one of these genes *CEF-147*. The *CEF-147* message is 4.1 kb long and contains an open reading frame that encodes a protein with 59% homology to the sheep COX cDNA. Xie et al. *(25)* renamed *CEF-147* as *miPHS*$_{ch}$, for "mitogen-inducible $PGS_{chicken}$". The authors pointed out significant differences at the protein and mRNA levels between $miPHS_{ch}$ and the sheep seminal vesicle COX message and predicted protein product, and suggested that $miPHS_{ch}$ "may be a new form of the enzyme.." They observed

a number of 5'-AUUUA-3' sequences characteristic of rapidly degraded messages in the 3'-untranslated region of the miPHS$_{ch}$ message. In concluding their report, Xie et al. *(25)* remark "… our data imply possible homology between Rosen's 4.0 kb mRNA and the CEF-147-encoded 4.1 kb mRNA, suggesting the existence of two forms of PGHS." However, because no orthologue of the sheep seminal vesicle COX had been cloned from chickens, Xie et al. *(25)* could not conclusively determine whether miPHS$_{ch}$ is the orthologue of the ovine/murine/human COX encoded by the 2.8 kb message or is the product of a distinct gene.

My own laboratory was, at the time, interested in the nature and role of genes whose transcription is induced when mitogens stimulate nonproliferating, G_o-arrested cells to re-enter the cell cycle *(26,27)*. We treated resting, G_o murine 3T3 fibroblasts with the mitogen/tumor promoter tetradecanoyl phorbol acetate (TPA) and prepared a lambda phage cDNA library from cells shortly after stimulation. The library was differentially screened with cDNAs from control and TPA-treated cells, to isolate cDNAs for TPA Induced Sequences, or TIS genes *(26)*. One of the TIS cDNAs, TIS10, was subsequently sequenced and striking homology with the murine COX encoded by the 2.8 kb message *(28)* was observed. Because we could directly compare the sequences of the murine 2.8 kb COX message and the induced TIS10 4.0 kb message, and their predicted open reading frames, we could conclude without any ambiguity that the 2.8 kb message and the 4.0 kb message encoded distinct, but similar proteins that are the products of two separate genes. The TIS10 message, which also contained multiple 5'-AUUUA-3' sequences, could be rapidly and transiently induced in murine fibroblasts not only by TPA, but also by EGF, forskolin and serum *(28)*. From these data, we could conclude unequivocally that "TIS10…..Encodes a Novel Prostaglandin Synthase/COX Homologue *(28)*." From this point on, I will refer to the "constitutive COX" encoded by the 2.8 kb message as COX-1 and to the "inducible COX" encoded by the 4.0 kb message as COX-2.

Using "giant two-dimensional gel electrophoresis *(21)*," O'Bannion et al. *(29)* demonstrated induction of an immunoprecipitable COX molecule induced by the *v-src* oncogene and by serum treatment of murine fibroblasts. When Northern blots were probed with a cDNA for the 2.8 kb message, a 4.0 kb message was observed in serum-treated cells at lowered stringency—a result similar to that of Rosen et al. *(22)*. O'Bannion et al. *(29)* screened a cDNA library and sequenced a small fragment of one of their clones. They concluded, from sequence comparison data, that the 4.0 kb message induced by *v-src* and serum encodes a COX-related protein. The following year O'Bannion et al. *(30)* cloned a full length cDNA for COX-2, and confirmed our predicted amino acid sequence *(28)*, with the exception of a single amino acid. Ryseck et al. *(31)* also cloned a COX-2 cDNA from a mitogen-induced murine fibroblast cDNA library and demonstrated its induction by PDGF, EGF, cAMP, and TPA. Using sequence information from the murine COX-2 cDNA, the human *(32,33)*, and rat *(34,35)* COX-2 cDNAs were subsequently cloned.

8. COMPARING THE COX-1 AND COX-2 GENES

The COX-1 gene was cloned from both human *(36)* and murine *(37)* cDNA libraries. The genomic sequence that encodes the COX-1 2.8 kb message is approx 22 kb for both species (Fig. 2). Both the murine and human COX-1 genes consist of eleven exons and ten introns. The intron-exon borders for murine and human COX-1 are completely conserved, and the intron sizes are quite similar for the two species.

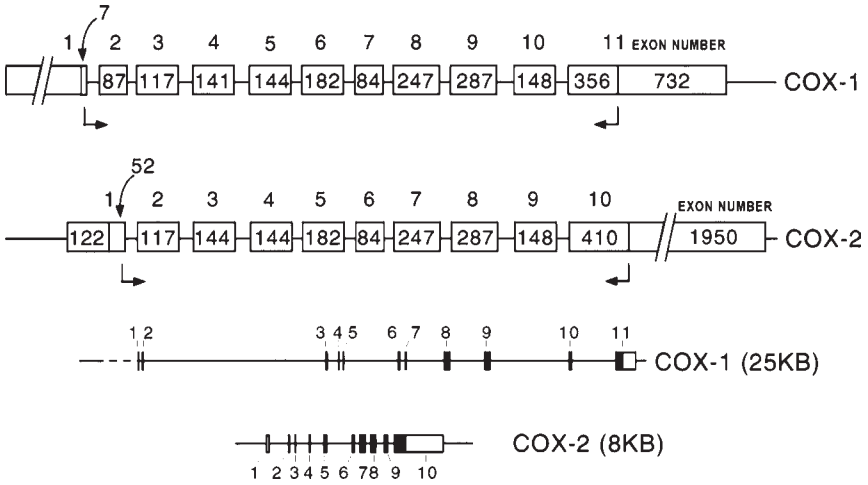

Fig. 2. Exon-intron structure of the COX-1 and COX-2 genes. The (upper panel) shows the number of nucleotides in each exon. The open reading frames are shown by the arrows below the genes. The (lower panel) shows the introns and exons of the COX-1 and COX-2 genes drawn to scale.

We cloned the murine COX-2 gene and characterized its structure (Fig. 2). The COX-2 gene is much smaller than the COX-1 gene; the genomic sequence that encodes the COX-2 4.0 kb message is approx 8 kb long *(38)*. The murine COX-2 consists of only ten exons and nine introns. COX-1 has an hydrophobic leader sequence in its N-terminal region that is encoded by an exon that is missing in the COX-2 gene. All other exons and introns for the COX-1 and COX-2 transcription unit are similar; the sites of splicing are similar—with the exception of the distal (C-terminal) 3' exon. The 3' untranslated region of the COX-2 message is substantially longer than that of the COX-1 message and contains multiple copies of the AUUUA sequence that confers message instability. The chicken COX-2 gene, cloned by Simmons et al. *(39)* also contains only ten exons and is approx 8–9 kb in length. Like the murine *(38)* and chicken genes *(39)* the human COX-2 gene is similarly 8.3 kb in length and is composed of ten exons and nine introns *(40,41)*. Both in the mouse *(31,42)* and in the human *(40)* the COX-1 and COX-2 genes map to distinct chromosomes. Unlike their coding regions, the regulatory regions of the COX-2 and COX-1 genes proximal to the start site of transcription bear essentially no sequence similarity. However, the regulatory regions of the human and murine COX-2 genes share substantial sequence similarity and putative *cis*-acting transcription factor binding sites.

9. THE STRUCTURE AND FUNCTION OF THE COX-1 AND COX-2 GENE PRODUCTS

It was, of course, essential to demonstrate that message derived from the presumptive COX-2 cDNA does, in fact, encode for a functional COX/hydroperoxidase. The initial paper describing the cloning of the murine COX-2 cDNA demonstrated sequence homology between COX-1 and COX-2 *(28)*, but did not demonstrate COX-2 enzymatic activity. When a plasmid expressing the murine COX-2 coding region was transiently expressed

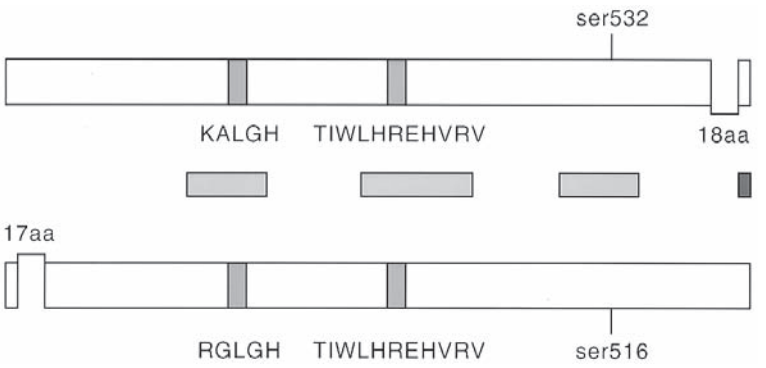

Fig. 3. The COX-1 and COX-2 proteins. The aspirin-sensitive serine acetylation site is identified in each open reading frame. The 17 amino acid deletion of the N-terminal region of COX-2 and the 18 amino acid deletion of the COX-1 proteins are indicated by gaps. The regions of greatest amino acid similarity are indicated by the solid bars between the two proteins. The solid bars shown within the proteins are the axial (TIWLREHNRV) and distal (KALGH/RGLGH) heme binding sites.

in COS cells, microsomal COX and hydroperoxidase activities were substantially elevated; control cells exhibited no activity *(38)*. Expression of the murine COX-2 in insect cells, using a baculovirus vector, led to substantial prostaglandin production *(31)*. The subsequent use of recombinant COX-1 and COX-2 provided the platform to identify the lead compounds and the subsequent derivatives that have now found their way into the pharmaceutical marketplace as the COX-2 inhibitors *Celebrex* and *Vioxx*.

At the amino acid sequence level, COX-1 and COX-2 share nearly 80% amino acid sequence similarity. The amino terminal sequence of COX-1 has a highly hydrophobic 17 amino acid sequence that is not present in COX-2 (Fig. 3). In contrast, there is an 18 amino acid sequence present in the COX-2 C-terminal region that is not present in COX-1. Many of the important amino acids implicated in COX function are conserved between COX-1 and COX-2, including the TIWLREHNRV and RGLGF sequences thought to be the axial and distal heme binding sites, the serine residue (at 516 in COX-2 and 530 in COX-1), which is the site of aspirin acetylation, and a tyrosine (371 in COX-2 and 385 in COX-1) essential for COX activity. A number of potential N-glycosylation sites are conserved between COX-1 and COX-2. The greatest difference in the sequences between COX-1 and COX-2 are in a region that was subsequently found to be the membrane binding domain(s) of the molecules.

The crystal structures of the ovine COX-1 *(43)* and the murine and human COX-2 proteins *(44,45)* have been solved. The structures of the human and murine COX-2 molecules are essentially indistinguishable, and nearly superimposable on the ovine COX-1 structure (the C-terminal tails of the molecules, where the COX-2 18 amino acid insertion occurs, are not resolved in the crystal structures). The amino terminal domains of both COX-1 and COX-2 contain sequences resembling the EGF molecule. The membrane binding domains follow the EGF domains in both COX-1 and COX-2. The C-terminal domains of COX-1 and COX-2 include the catalytic sites. Small variations in the size and shape of the active sites of COX-1 and COX-2 account for the structural basis of differential inhibition of COX-2 vs COX-1 by COX-2 specific inhibitors such as *Vioxx* and *Celebrex*.

The review by Smith et al. *(46)* describes more fully the structural characteristics of the COX-1 and COX-2 proteins, and provides models for 1) the association of the two COXs with cellular membranes, 2) the nature of the accessibility of fatty acids and NSAIDs to the enzyme active sites, 3) mechanisms of catalysis in the two enzymes, 4) kinetic comparisons of the two enzymes, and 5) the structural differences that may account for the ability of COX-2 specific inhibitors to gain preferential access to the COX-2 active site. Because this chapter is charged with presenting the historical aspects of COX-2 molecular and cell biology and, more significantly, the author is out of his depth in areas of protein structure and enzyme catalysis, readers are referred to Smith et al. *(46)* for a review and others like it for a more comprehensive and expert discussion of structure, catalysis and kinetics of the COX-1 and COX-2 enzymes.

10. THE COX PARADOX

The discovery of COX-2 raises an interesting and as yet unsolved paradox. In fact, it is a paradox that was essentially ignored in much of the subsequent literature on COXs that appeared for several years following the discovery of COX-2. The question is as follows: "If activation of phospholipases to release AA from membrane phospholipids is the rate-limiting step in providing substrate for COX following ligand stimulation, and if most cells express COX-1 constitutively, why do cells need COX-2?" One would expect the constitutive COX-1 enzyme, present in most cells, to convert to prostaglandin the AA released from membrane phospholipids by ligand-activated phospholipases. From this paradox follows a second question: "If COX-1 is present in cells, and can convert AA released from membrane phospholipids to prostaglandins, why do COX-2 inhibitors "work"? Why do COX-2 inhibitors prevent prostaglandin production in ligand-stimulated cells that contain constitutive COX-1?" This could now be considered the "four billion dollar (annually) question," because sales of *Celebrex* and *Vioxx* are expected to reach this level in 2001.

To address this question, we wanted to specifically inhibit the synthesis of COX-2 in ligand-stimulated cells and ask whether prostaglandin expression was inhibited. Antisense oligonucleotides specific for COX-2 mRNA were used to block the ligand-induced expression of COX-2 in both mitogen (TPA or PDGF)-stimulated murine fibroblasts and endotoxin-stimulated murine macrophages *(47)*. Immunofluorescence analysis showed that COX-2 protein expression was prevented and that COX-1 protein levels were unaffected by COX-2 antisense oligonucleotides. However, the COX-2 antisense oligonucleotides (but not random or sense oligonucleotides) blocked the ligand-stimulated production of PGE_2 in both fibroblasts and macrophages. To rule out the possibility that the antisense COX-2 oligonucleotides had some effect on COX-1 enzyme activity, exogenous AA was provided to all cells (control, ligand-induced, ligand-induced + COX-2 antisense oligonucleotides, ligand-induced + random oligonucleotides and ligand-induced + sense oligonucleotides), and the ability of the cultured cells to produce prostaglandins from *exogenous* AA was determined. All the cultures produced substantial levels of PGE_2; cells in which expression of COX-2 was blocked by COX-2 antisense oligonucleotides were capable of producing prostaglandin from exogenous AA, using constitutive COX-1.

One other possibility remained; perhaps the COX-2 antisense oligonucleotide—in addition to blocking COX-2 expression—could block the mitogen or endotoxin activation of phospholipase, preventing the release of AA. However, when we measured release of mem-

brane-bound AA in response to mitogen treatment of fibroblasts or endotoxin treatment of macrophages, the presence of COX-2 antisense oligonucleotides *enhanced* the ligand-induced accumulation of free AA in the cells and medium. *AA released from membrane lipid stores by ligand stimulation of fibroblasts or macrophages is not available to constitutive COX-1; ligand-induced COX-2 expression is essential for ligand-induced prostaglandin production (47).*

If COX-2 antisense oligonucleotides, which block the synthesis of COX-2 but not of COX-1, can prevent ligand-induced prostaglandin production in cells that contain COX-1, then perhaps COX-2 specific inhibitors—that block the enzymatic activity of COX-2, but not COX-1—might similarly prevent ligand-induced prostaglandin production in cells that contain COX-1. When NS-398, the first COX-2 specific inhibitor *(48)*, became available, we tested this hypothesis. NS-398 is able to block the production of PGE_2 from endogenous membrane AA stores in mitogen-treated murine fibroblasts. In contrast, NS-398 is unable to prevent conversion of exogenous AA to prostaglandin *(49)*. *The AA released by ligand stimulation cannot be converted to prostaglandin PGH_2 by constitutive COX-1; COX-2 induction is required for the conversion to PGH_2 of AA released by ligand stimulation.*

Although this observation regarding access of endogenous AA to prostaglandins by COX-1 versus COX-2 provides the rationale for a blockbuster pharmaceutical, the molecular/mechanistic basis for this difference in the ability of COX-1 and COX-2 to convert endogenous AA to prostaglandin still is not clear. Why is endogenous AA, released from membrane stores by ligand activation of phospholipase, not accessible to COX-1?

10.1. Subcellular Localization is not Likely to Account for the Difference in Arachidonic Accessibility of COX-1 and COX-2

One simple potential explanation is that differential cellular compartmentation of COX-1 and COX-2 accounts for the difference of COX-1 vs COX-2 accessibility for endogenous AA. Although initial immunofluorescence data suggested that differences in subcellular localization of COX-1 and COX-2 might, indeed, account for the difference in AA accessibility *(50)*, subsequent immunofluorescent *(47,51)* and immunogold electron microscopy (EM) *(51)* demonstrated that differential subcellular localization is not likely to be the explanation.

10.2. Temporal Distinctions in the Expression of COX-1 and COX-2 can Account for Differential Utilization of AA in Activated Mast Cells

In most cells, COX-1 is present prior to ligand-stimulation and COX-2 expression and accumulation are induced by ligand stimulation. For fibroblasts, endothelial cells, macrophages, epithelial cells, etc., the bulk of the ligand-induced prostaglandin occurs following COX-2 expression, and the paradox discussed above needs to be considered. In mast cells, following activation by antigen-dependent aggregation of IgE receptors, PGD_2 is released in a biphasic fashion *(52,53)*. Using glucocorticoid inhibition, aspirin inactivation and COX-1 or COX-2 specific inhibitors, the initial burst of PGD_2 production in activated mast cells has been shown to be due to COX-1, whereas the delayed production of PGD_2 results from COX-2 activity induced as a result of IgE receptor aggregation *(52, 53)*. In this special case, it is clear that temporal separation of COX-1 and COX-2 expression accounts for the distinction in temporal production of prostaglandin.

10.3. Concentration-Dependent Differences in AA Utilization by COX-1 and COX-2 Have Been Described In Vivo and in Cultured Cells

Antisensense inhibition experiments *(47)* and transcellular prostaglandin studies *(54)* suggest that COX-1 can preferentially utilize exogenous arachidonic acid and that COX-2 can preferentially utilize endogenous AA. When COX-1 and COX-2 were ectopically expressed, following retroviral infection, COX-2 was able to convert exogenous arachidonic acid to prostaglandin more effectively that COX-1 *(55)*. In contrast, when phorbol esters were used to stimulate endogenous AA release, COX-1 was more effective in prostaglandin production, again suggesting that "PGHS1 and PGHS2 preferentially utilize different pools of arachidonic acid."

Swinny et al. *(56)*, using recombinant COX-1 and COX-2, demonstrated positive cooperativity for AA as substrate for COX-1, whereas no cooperativity was observed for COX-2. Thus, at low AA concentrations (below 0.5 μM) COX-2 was more active than COX-1; in contrast, at higher AA concentrations (above 2.5 μM) COX-1 was more active than COX-2. The authors suggest that one consequence of this difference in cooperativity would be the preferential utilization of AA by COX-2 vs COX-1 under conditions where substrate concentration is limiting. Using a continuous assay for COX activity, Chen et al. *(57)* confirmed the positive cooperativity of the COX-1 enzyme. Studies with cells stably transfected with COX-1 and COX-2 expression vectors support this argument; COX-1 is more effective than COX-2 when high concentrations of AA are provided. Conversely, COX-2 is more effective than COX-1 when low concentrations of exogenous AA are provided *(58)*.

10.4. Why Does COX-1 Exhibit Positive Co-Operativity for AA as Substrate?

Both COX-1 and COX-2 COX activity require initiation by peroxide (for a review of these studies, *see ref. 57*), resulting from reaction of the peroxidase with heme in the peroxidase site, leading to formation of a tyrosyl radical in the COX active site. Chen et al. *(57)* suggest that PGG_2, which is a hydroperoxide, can participate in a feedback loop that initiates COX activity in previously latent enzyme. This feedback loop is stronger in COX-2 than in COX-1 *(59)*. Using kinetic simulations, Chen et al. *(57)* concluded that "…a positive cooperative response to arachidonate is a consequence of the complex feedback activation loop." These authors conclude that "…the difference between the two PGHS isoforms in the degree of COX cooperativity can be simply explained by the difference in the efficiency of the hydroperoxide feedback loops in PGHS-1 and PGHS-2."

10.5. Differential Coupling of Upstream (Phospholipase) and Downstream (Prostaglandin Synthases) to COX-1 and COX-2 may also Modulate Prostaglandin Production in Cells

The literature has seen a proliferation in the identification of new secretory and cytoplasmic phospholipases and in the identification of alternative prostaglandin synthases that convert PGH_2 to prostaglandins. An extensive and currently confusing literature exists on the preferential coupling of secretory and cytoplasmic phospholipases with COX-1 and COX-2. Without attempting to review this literature, it is clear 1) that COX-1- or COX-2-preferential utilization of AA produced by alternative phospholipases has been

demonstrated and 2) that the specific phospholipase-to-COX pathways vary from cell-type to cell-type, making generalizations about such coupling difficult. Very recently, distinct PGE_2 synthases that couple preferentially to COX-1 and COX-2 have been described *(60–63)*. The picture that is emerging is one of ligand-induced activation of specific phospholipases that preferentially provide AA to one of the COX isoforms, which then preferentially pass the PGH_2 intermediate to a coupled prostaglandin synthase for formation of the final prostanoid. Whether kinetically or physically separated, these "channeled" biosynthetic pathways can be thought of as "eicosasomes"—functional enzyme "complexes" that synthesize specific prostanoids in response to ligand stimulation.

11. REGULATION OF COX-2 GENE EXPRESSION

Prior to the cloning of COX-2, a variety of studies suggested that synthesis of COX mRNA, protein and activity might play an essential role in ligand-induced prostaglandin production in a variety of cells *(12–21)*. All the initial reports of COX-2 cDNA cloning were the result of paradigms that examined differences in gene expression following growth factor or oncogene activation in fibroblasts *(25,28,29)*. Since these initial reports, dozens of stimulatory agents have been shown to induce COX-2 expression in an extraordinary variety of cells. Simply providing a table of all the cell types in which COX-2 can be induced and the agents that have been demonstrated to elicit elevated COX-2 mRNA and/or protein would take several pages; readers are referred to previous tabulations *(46,49)*.

We know, from the initial cloning exercise *(25,28,29)*, that increased COX-2 mRNA levels occur in fibroblasts stimulated by growth factors and oncogenes. Since that time, a multitude of experiments have shown that COX-2 induction by endotoxins, inflammatory cytokines, hormones, neurotransmitters, depolarization, radiation, free-radical generators, and stressors can induce COX-2 mRNA and/or protein accumulation in a wide range of appropriate target cells.

In the context of this set of manuscripts, which focus on the role of COX-2 in cancer, the mechanisms by which growth factors and oncogenes elicit elevated COX-2 are probably the most relevant. In my own laboratory, we initially confined our efforts to the mechanisms of COX-2 induction by growth factors (serum and PDGF) and oncogenes (*v-src*). Use of COX-2 promoter-luciferase chimeric reporter gene constructs demonstrated that *v-src (64,65)* and PDGF *(66)* induced expression from the COX-2 gene was due, at least in part, to ligand-stimulated transcriptional activation. Among the factors to consider in ligand-dependent transcriptional activation of gene expression in general, and COX-2 expression in particular, are 1) the *cis*-acting regulatory elements of the responsive gene, 2) the transcription factors activated by the signal transduction pathways, and 3) the signal transduction pathway(s) activated by ligand-receptor interactions or by oncogenes.

11.1. Cis-*Regulatory Elements of the COX-2 Gene that Regulate COX-2 Expression*

Sequencing the murine COX-2 regulatory region suggested a number of putative transcription response sequences, including an E-Box, a cyclic AMP response element (CRE), NF-IL6 sites, nuclear factor-κB (NFκB) sites, etc. *(38)*. The human COX-2 gene shares many of these same potential regulatory elements *(40,41)*. By using luciferase reporter genes with mutations in the various putative COX-2 *cis*-regulatory elements, we

were able to demonstrate a critical role for the CRE in both *v-src (65)* and PDGF *(66)* induction of COX-2 gene expression. The CRE site of the murine and human COX-2 promoter has subsequently been shown to play a major role in the regulation of COX-2 gene expression by a number of ligands, in a variety of cell types *(46)*. Surprisingly, the rat COX-2 gene does not share a CRE with the human and murine COX-2 genes. Instead, it appears that—in the rat—the E-Box may play a role in COX-2 gene regulation *(67)*.

Since the initial characterization of the *cis*-acting regions of the COX-2 gene that play major roles in *v-src* and PDGF induction were carried out, the COX-2 promoter of the human, rat, murine and chicken COX-2 genes have been subjected to an enormous number of studies; summarizing these many studies would be well beyond both the limited space and my own organizational capabilities. NFκB regulation of COX-2 gene expression has been implicated in well over 50 studies. However, mutational analysis of the COX-2 promoter to demonstrate a role for NFκB at appropriate sites has been demonstrated in only a few instances *(68,69)*.

We demonstrated, by mutational analysis, that NF-IL6 sites of the COX-2 promoter play a role in endotoxin-treated COX-2 induction macrophages *(70)*, activated mast cells *(71)* and ligand-stimulated osteoblasts *(72)*. Mutational analysis has also demonstrated a role for NF-IL6 sites in TNFα treated *(68)* and fluid shear stressed *(73)* osteoblasts, endotoxin treated vascular endothelial cells *(74)* and macrophages *(75)* and IL-1β stimulated chondrocytes *(76)*, amnion cells *(77)* and endothelial cells *(78)*. A variety of additional *cis*-acting regulatory regions of the COX-2 gene have been suggested to play roles in ligand-induced activation. However, the CRE and NF-IL6 sites have been the best characterized by mutational analysis.

11.2. Transcription Factors that Modulate COX-2 Gene Expression

Probably the biggest surprise in our early analysis of the pathways of COX-2 induction by oncogenes and growth factors came in our identification of the transcription factor acting at the COX-2 CRE. We had assumed that the active factor would be the cyclic AMP Response Element Binding protein, or CREB. CREB can, indeed, bind to the CRE of the murine COX-2 gene *(64)*. However, cotransfection experiments with plasmids expressing wild-type CREB, *c-JUN* and chimeric transcription factors demonstrated that *c-JUN* plays the primary role in activation of the COX-2 gene in murine fibroblasts treated with PDGF *(66)* or stimulated by *v-src* expression *(65)*. Subsequent to these early experiments, we found that activation of *c-JUN* plays a major role in COX-2 induction in endotoxin-treated macrophages *(70)*, activated mast cells *(71)* and ligand-stimulated osteoblasts *(72)*.

Several laboratories have demonstrated that C/EBP proteins can bind to regions of the COX-2 gene, and have implicated C/EBP in COX-2 induction by mutational analysis of the COX-2 NF-IL6 regions (vida supra). We used cotransfection experiments of COX-2 reporter genes with constitutively active and dominant-negative versions of the C/EBP transcription factors to demonstrate their roles, at the NF-IL6 elements of the COX-2 gene, in COX-2 induction in endotoxin-treated macrophages *(70)*, activated mast cells *(71)*, and ligand-stimulated osteoblasts *(72)*. Cotransfection experiments have also implicated C/EBP proteins in COX-2 induction in IL-1β treated osteoblasts *(79)*, IL-1β treated chondrocytes *(76)*, and endotoxin treated vascular endothelial cells *(74)*.

Probably the most controversial of the transcription factors proposed to regulate the COX-2 gene is NFκB. Many studies suggest that NFκB plays a role in the activation of

the COX-2 gene, whereas an alternative set of reports suggests that NFκB does not play a role in COX-2 expression. Because it is clear that some of these reports may reflect differences in cell types and/or inducers, there are several contradictory reports for the same inducer in the same cell type. For example, Mestre et al. *(75)* and Huang et al. *(80)* report that NFκB mediates COX-2 expression in endotoxin-stimulated RAW 264.7 murine macrophages, whereas we were unable to show a role for NFκB in this same system *(70)*. It seems likely that NFκB plays a role in a subset of COX-2 induction pathways.

11.3. The Signal Transduction Pathway(s) Activated by Ligand-Receptor Interactions or Oncogenes that lead to COX-2 Induction

A variety of ligand-activation paradigms resulting in COX-2 gene expression converge on activation through the COX-2 CRE via the *c-Jun* transcription factor. We initially characterized the pathway to *c-JUN* activation by *v-src* and by PDGF treatment in murine fibroblasts. Activation of c-JUN suggest a Ras-mediated pathway that proceeds via a *Raf* and/or mitogen activated protein (MAP) kinase-mediated phosphorylation cascade to Jun kinase. Use of dominant-negative and activated *Ras* and signaling protein kinase mutants demonstrated that both *v-src* induction *(65)* and PDGF induction *(66)* of COX-2 are mediated by *Ras* activation and subsequent *Raf* and MEKK mediated kinase cascades. We have subsequently demonstrated that this same pathway is active in activated murine mast cells *(71)* and ligand-stimulated osteoblasts *(72)*. Additional studies, by an ever-increasing number of laboratories studying the regulation of COX-2 gene expression, have used pharmacologic inhibitors, overexpression of signaling molecules and inhibition by dominant-negative constructs to demonstrate roles for the ERK 1/2, p38, and c-JUN MAP kinases in COX-2 induction by a wide variety of ligands *(81–86)*. The references cited here are meant to be a sampling, and are by no means comprehensive.

Ras has been implicated as an intermediate in ligand-induced COX-2 gene expression by a number of investigators *(87–89)*. As discussed extensively in other chapters in this collection, activation of Ras and overexpression of COX-2 are correlated in a number of solid tumors. *It seems likely that deregulation of the Ras/Raf/MAPKK/ERK and Ras/MEKK1/JNKK/JNK/c-Jun signaling pathway are likely to be responsible for the elevated COX-2 expression observed in so many different tumors.*

12. CONCLUSIONS AND REFLECTIONS

When we first cloned TIS10/COX-2 *(28)* we had difficulty in the lab spelling "COX" and "prostaglandin." We knew essentially none of the prior literature, and only a handful of the "players." In only a decade, COX-2 went from an hypothetical enzymatic activity to one of the most important genes in fields as diverse as inflammation, reproduction, neurodegenerative diseases, angiogenesis, and cancer. In the brief period of time from the cloning of the COX-2 cDNA, the COX-2 protein has made the transition from a potential drug target to the basis for a four billion dollar per year pharmaceutical industry. For many of the investigators now working on COX-2, including myself, this gene was causal in the transition from fundamental cellular studies in areas such as cell cycle, mitogenesis, virology, and regulation of gene expression to considerations of clinical relevance and animal models of disease. The discovery of COX-2 initiated a convergence of molecular and cellular biology, pharmacology, preclinical studies, animal models of disease, clinical trials, and the development of effective therapeutics. On the horizon are likely

to be important elucidations for the role of aberrant COX-2 expression in cancer biology, neurodegenerative diseases, and acute and chronic inflammatory diseases, as well as new roles for COX-2, both in normal physiology and pathophysiology. It has been quite a decade, personally, professionally, and practically.

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REFERENCES

1. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol* 1971; 231:232–235.
2. DuBois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, van de Putte LBA, Lipsky PE. Cyclooxygenase in biology and disease. *FASEB J* 1998; 12:1063–1073.
3. Hemler M, Lands WEM, Smith WL. Purification of the cyclooxygenase that forms prostaglandins. Demonstration of two forms of iron in the holoenzyme. *J Biol Chem* 1976; 251:5575–5579.
4. Miyamoto T, Ogino N, Yamamoto S, Hayaishi O. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J Biol Chem* 1976; 251:2629–2636.
5. VanDerOuderaa FJ, Buytenhek M, Nugteren DH, VanDorp DA. Purification and characterization of prostaglandin synthetase from sheep vesicular glands. *Biochim Biophys Acta* 1977; 487:315–331.
6. DeWitt DL, Smith WL. Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from the complementary DNA sequence. *Proc Natl Acad Sci USA* 1988; 85:1412–1416.
7. Merlie JP, Fagan D, Mudd J, Needleman P. Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). *J Biol Chem* 1988; 263:3550–3553.
8. Yokayama C, Takai T, Tanabe T. Primary structure of sheep prostaglandin endoperoxide synthase deduced from cDNA sequence. *FEBS Lett* 1988; 231:347–351.
9. DeWitt DL, El-Harith EA, Kraemer SA, Andrews MJ, Yao EF, Armstrong RL, Smith WL. The aspirin and heme-binding sites of ovine and murine prostaglandin endoperoxide synthases. *J Biol Chem* 1990; 265:5192–5198.
10. Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. *FASEB J* 1991; 5:2304–2312.
11. Takahashi Y, Ueda N, Yoshimoto T, Yamamoto S, Yokoyama C, Miyata A, et al. Immunoaffinity purification and cDNA cloning of human platelet prostaglandin endoperoxide synthase (cyclooxygenase). *Biochem Biophys Res Commun* 1992; 182:433–438.
12. Whiteley PJ, Needleman P. Mechanism of enhanced fibroblast arachidonic acid metabolism by mononuclear cell factor. *J Clin Invest* 1984; 74:2249–2253.
13. Habenicht AJ, Goerig M, Grulich J, Rothe D, Gronwald R, Loth U, et al. Human platelet-derived growth factor stimulates prostaglandin synthesis by activation and by rapid de novo synthesis of cyclooxygenase. *J Clin Invest* 1985; 75:1381–1387.
14. Yokota K, Kusaka M, Ohshima T, Yamamoto S, Kurihara N, Yoshino T, Kumegawa M. Stimulation of prostaglandin E_2 synthesis in cloned osteoblastic cells of mouse (MC3T3-E1) by epidermal growth factor. *J Biol Chem* 1986; 261:15,410–15,415.
15. Frasier-Scott K, Hatzakis H, Seong D, Jones CM, Wu KK. Influence of natural and recombinant interleukin 2 on endothelial cell arachidonate metabolism. *J Clin Invest* 1988; 82:1877–1883.

16. Kusaka M, Oshima T, Yokota K, Yamamoto S, Kumegawa M. Possible induction of fatty acid cyclooxygenase in mouse osteoblastic cells (MC3T3-E1) by cAMP. *Biochimica et Biophysica Acta* 1988; 972:339–346.
17. Raz A, Wyche A, Siegel N, Needleman P. Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. *J Biol Chem* 1988; 263:3022–3025.
18. Raz A, Wyche A, Needleman P. Temporal and pharmacological division of fibroblast cyclooxygenase expression into transcriptional and translational phases. *Proc Natl Acad Sci USA* 1989; 86:1657–1661.
19. Wong WYL, DeWitt DL, Smith WL, Richards JS. Rapid induction of prostaglandin endoperoxide synthase in rat preovulatory follicles by luteinizing hormone and cAMP is blocked by inhibitors of transcription and translation. *Mol Endocrinology* 1989; 3:1714–1723.
20. Fu JY, Masferrer JL, Seibert K, Raz A, Needleman P. The induction and suppression of prostaglandin H2 synthase (cyclooxygenase) in human monocytes. *J Biol Chem* 1990; 265:16,737–16,740.
21. Han JW, Sadowski H, Young DA, Macara IG. Persistent induction of cyclooxygenase in $p60^{v\text{-}src}$-transformed 3T3 fibroblasts. *Proc Natl Acad Sci USA* 1990; 87:3373–3377.
22. Rosen GD, Birkenmeier TM, Raz A, Holtzman MJ. Identification of cyclooxygenase-related gene and its potential role in prostaglandin formation. *Biochem Biophysical Res Commun* 1989; 164:1358–1365.
23. Masferrer JL, Zweifel BS, Seibert K, Needleman P. Selective regulation of cellular cyclooxygenase by dexamethasone and endotoxin in mice. *J Clin Invest* 1990; 86:1375–1379.
24. Simmons DL, Levy DB, Yannoni Y, Erikson RL. Identification of a phorbol ester-repressible *v-src*-inducible gene. *Proc Natl Acad Sci USA* 1989; 86:1178–1182.
25. Xie W, Chipman JG, Robertson DL, Erikson RL, Simmons DL. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. *Proc Natl Acad Sci USA* 1991; 88:2692–2696.
26. Lim RW, Varnum BC, Herschman HR. Cloning of tetradecanoyl phorbol ester induced "primary response" sequences and their expression in density-arrested Swiss 3T3 cells and a TPA nonproliferative variant. *Oncogene* 1987; 1:263–270.
27. Herschman HR. Primary response genes induced by growth factors and tumor promoters. In: Richardson CC, Abelson JN, Meister A, Walsh CT, eds. *Annual Review of Biochem*, vol. 60, Annual Reviews, Inc., Palo Alto, CA, 1991, pp. 281–319.
28. Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. *J Biol Chem* 1991; 266:12,866–12,872.
29. O'Banion MK, Winn VD, Young DA. cDNA cloning and functional activity of a glucocorticoid-regulated inflammatory cyclooxygenase. *Proc Natl Acad Sci USA* 1992; 89:4888–4892.
30. O'Banion MK, Sadowski HB, Winn V, Young DA. A serum-and glucocorticoid-regulated 4-kilobase mRNA encodes a cyclooxygenase-related protein. *J Biol Chem* 1991; 266:23,261–23,267.
31. Ryseck RP, Raynoschek C, Macdonald-Bravo H, Dorfman K, Mattei M, Bravo R. Identification of an immediate early gene, *phs-B*, whose protein product has prostaglandin synthase/cyclooxygenase activity. *Cell Growth Differ* 1992; 3:443–450.
32. Hla T, Neilson K. Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci USA* 1992; 89:7384–7385.
33. Jones DA, Carlton DP, McIntyre TM, Zimmerman GA, Prescott SM. Molecular cloning of human prostaglandin endoperoxide synthase type II and demonstration of expression in response to cytokines. *J Biol Chem* 1993; 268:9049–9054.
34. Kennedy BP, Chan CC, Culp SA, Cromlish WA. Cloning and expression of rat prostaglandin endoperoxide synthase (cyclooxygenase)-2 cDNA. *Biochem Biophys Res Commun* 1993; 197:494–500.
35. Feng L, Sun W, Xia Y, Tang WW, Chanmugam P, Soyoola E, et al. Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch Biochem Biophys* 1993; 307:361–368.
36. Yokoyama C, Tanabe T. Cloning of human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. *Biochem Biophys Res Commun* 1989; 165:888–894.
37. Kraemer SA, Meade EA, DeWitt DL. Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5'-flanking regulatory sequences. *Arch Biochem Biophys* 1992; 293: 391–400.
38. Fletcher BS, Kujubu DA, Perrin DM, Herschman HR. Structure of the mitogen-inducible TIS10 gene and demonstration that the TIS10-encoded protein is a functional prostaglandin G/H synthase. *J Biol Chem* 1992; 267:4338–4344.
39. Xie W, Merrill JR, Bradshaw WS, Simmons DL. Structural determination and promoter analysis of the chicken mitogen-inducible prostaglandin G/H synthase gene and genetic mapping of the murine homolog. *Arch Biochem Biophys* 1993; 300:247–252.

40. Kosaka T, Miyata A, Ihara H, Hara S, Sugimoto T, Takeda O, et al. Characterization of the human gene (PTGS2) encoding prostaglandin-endoperoxide synthase 2. *Eur J Biochem* 1994; 221:889–897.
41. Appleby SB, Ristimäki A, Neilson K, Narko K, Hla T. Structure of the human cyclooxygenase-2 gene. *Biochem J* 1994; 302:723–727.
42. Ping XW, Warden C, Fletcher BS, Kujubu DA, Herschman HR, Lusis AL. Chromosomal organization of the inducible and constitutive prostaglandin synthase/cyclooxygenase genes in mouse. *Genomics* 1993; 15:458–460.
43. Picot D, Loll PJ, Garavito RM. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature* 1994; 367:243–249.
44. Kurumbail RG, Stevens AM, Gierse JK, McDonald JJ, Stegeman RA, Pak JY, et al. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* 1996; 384:644–648. *Erratum in Nature* 1997; 385:555.
45. Luong C, Miller A, Barnett J, Chow J, Ramesha C, Browner MF. Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. *Nat Struct Biol* 1996; 3:927–933.
46. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. *Annu Rev Biochem* 2000; 69:145–182.
47. Reddy ST, Herschman HR. Ligand-induced prostaglandin synthesis requires expression of the TIS10/PGS-2 prostaglandin synthase gene in murine fibroblasts and macrophages. *J Biol Chem* 1994; 269: 15,473–15,480.
48. Futaki N, Yoshikawa K, Hamasaka Y, Arai I, Higuchi S, Iizuka H, Otomo S. NS-398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen Pharmacol* 1993; 24:105–110.
49. Herschman HR. Prostaglandin synthase 2. In: *Biochim Biophys Acta*, vol. 1299. Elsevier, 1996, pp. 125–140.
50. Regier MK, DeWitt DL, Schindler MS, Smith WL. Subcellular localization of prostaglandin endoperoxide synthase-2 in murine 3T3 cells. *Arch Biochem Biophys* 1993; 301:439–444.
51. Spencer AG, Woods JW, Arakawa T, Singer II, Smith WL. Subcellular localization of prostaglandin endoperoxide H synthases-1 and -2 by immunoelectron microscopy. *J Biol Chem* 1998; 273:9886–9893.
52. Kawata R, Reddy ST, Wolner B, Herschman HR. Prostaglandin synthase 1 and prostaglandin synthase 2 both participate in activation-induced PGD_2 production in mast cells. *J Immunol* 1995; 155:818–825.
53. Murakami M, Bingham CO III, Matsumoto R, Austen KF, Arm JP. IgE-dependent activation of cytokine-primed mouse cultured mast cells induces a delayed phase of prostaglandin D2 generation via prostaglandin endoperoxide synthase-2. *J Immunol* 1995; 155:4445–4453.
54. Reddy ST, Herschman HR. Transcellular prostaglandin production following mast cell activation is mediated by proximal secretory phospholipase A_2 and distal prostaglandin synthase 1. *J Biol Chem* 1996; 271:186–191.
55. Chulada PC, Loftin CD, Winn VD, Young DA, Tiano HF, Eling TE, Langenbach R. Relative activities of retrovirally expressed murine prostaglandin synthase-1 and –2 depend on source of arachidonic acid. *Arch Biochem Biophys* 1996; 330:301–313.
56. Swinney DC, Mak AY, Barnett J, Ramesha CS. Differential allosteric regulation of prostaglandin H synthase 1 and 2 by arachidonic acid. *J Biol Chem* 1997; 272:12,393–12,398.
57. Chen W, Pawelek TR, Kulmacz RJ. Hydroperoxide dependence and cooperative cyclooxygenase kinetics in prostaglandin H synthase-1 and -2. *J Biol Chem* 1999; 274:20,301–20,306.
58. Murakami M, Kambe T, Shimbara S, Kudo I. Functional coupling between various phospholipase A_2s and cyclooxygenases in immediate and delayed prostanoid biosynthetic pathways. *J Biol Chem* 1999; 274:3103–3115.
59. Kulmacz RJ, Wang LH. Comparison of hydroperoxide initiator requirements for the cyclooxygenase activities of prostaglandin H synthase-1 and -2. *J Biol Chem* 1995; 270:24,019–24,023.
60. Jakobsson PJ, Thoren S, Morgenstern R, Samuelsson B. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. *Proc Natl Acad Sci USA* 1999; 96:7220–7225.
61. Thoren S, Jakobsson PJ. Coordinate up- and down-regulation of glutathione-dependent prostaglandin E synthase and cyclooxygenase-2 in A549 cells. Inhibition by NS-398 and leukotriene C4. *Eur J Biochem* 2000; 267:6428–6434.
62. Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, et al. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J Biol Chem* 2000; 275:32,783–32,792.

63. Tanioka T, Nakatani Y, Semmyo N, Murakami M, Kudo I. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. *J Biol Chem* 2000; 275:32,775–32,782.
64. Xie W, Fletcher BS, Andersen RD, Herschman HR. *v-src* induction of the TIS10/PGS2 prostaglandin synthase gene is mediated by an ATF/CRE transcription response element. *Mol Cell Biol* 1994; 14:6531–6539.
65. Xie W, Herschman HR. *v-src* induces prostaglandin synthase 2 gene expression by activation of the c-Jun N-terminal kinase and the c-Jun transcription factor. *J Biol Chem* 1995; 270:27,622–27,628.
66. Xie W, Herschman HR. Transcriptional regulation of prostaglandin synthase 2 gene expression by platelet derived growth factor and serum. *J Biol Chem* 1996; 271:31,742–31,748.
67. Morris JK, Richards JS. An E-box region within the prostaglandin endoperoxide synthase-2 (PGS-2) promoter is required for transcription in rat ovarian granulosa cells. *J Biol Chem* 1996; 271:16,633–16,643.
68. Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. *J Biol Chem* 1995; 270:31,315–31,320.
69. Schmedtje JF Jr, Ji Y-S, Liu WL, DuBois RN, Runge MS. Hypoxia induces cyclooxygenase-2 via the NF-*k*B p65 transcription factor in human vascular endothelial cells. *J Biol Chem* 1997; 272:601–608.
70. Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. *J Biol Chem* 2000; 275:6259–6266.
71. Reddy ST, Wadleigh DJ, Herschman HR. Transcriptional regulation of the cyclooxygenase-2 gene in activated mast cells. *J Biol Chem* 2000; 275:3107–3113.
72. Wadleigh DJ, Herschman HR. Transcriptional regulation of the cyclooxygenase-2 gene by diverse ligands in murine osteoblasts. *Biochem Biophys Res Commun* 1999; 264:865–870.
73. Ogasawara A, Arakawa T, Kaneda T, Takuma T, Sato T, Kaneko H, et al. Fluid shear stress-induced cyclooxygenase-2 expression is mediated by C/EBP beta, CREB, and AP-1 in osteoblastic MC3T3-E1 cells. *J Biol Chem* 2001; 276:7048–7054.
74. Inoue H, Yokoyama C, Hara S, Tone Y, Tanabe T. Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. *J Biol Chem* 1995; 270:24,965–24,971.
75. Mestre JR, Mackrell PJ, Rivadeneira DE, Stapleton PP, Tanabe T, Daly JM. Redundancy in the signaling pathways and promoter elements regulating cyclooxygenase-2 gene expression in endotoxin-treated macrophage/monocytic cells. *J Biol Chem* 2001; 276:3977–3982.
76. Thomas B, Berenbaum F, Humbert L, Bian H, Bereziat G, Crofford L, Olivier JL. Critical role of C/EBPdelta and C/EBPbeta factors in the stimulation of the cyclooxygenase-2 gene transcription by interleukin-1beta in articular chondrocytes. *Eur J Biochem* 2000; 267:6798–6809.
77. Potter S, Mitchell MD, Hansen WR, Marvin KW. NF-IL6 and CRE elements principally account for both basal and interleukin-1 beta-induced transcriptional activity of the proximal 528bp of the PGHS-2 promoter in amnion-derived AV3 cells: evidence for involvement of C/EBP beta. *Mol Hum Reprod* 2000; 6:771–778.
78. Kirtikara K, Raghow R, Laulederkind SJ, Goorha S, Kanekura T, Ballou LR. Transcriptional regulation of cyclooxygenase-2 in the human microvascular endothelial cell line, HMEC-1: control by the combinatorial actions of AP2, NF-IL-6 and CRE elements. *Mol Cell Biochem* 2000; 203:41–51.
79. Harrison JR, Kelly PL, Pilbeam CC. Involvement of CCAAT enhancer binding protein transcription factors in the regulation of prostaglandin G/H synthase 2 expression by interleukin-1 in osteoblastic MC3T3-E1 cells. *J Bone Miner Res* 2000; 15:1138–1146.
80. Hwang D, Jang BC, Yu G, Boudreau M. Expression of mitogen-inducible cyclooxygenase induced by lipopolysaccharide: mediation through both mitogen-activated protein kinase and NF-kappaB signaling pathways in macrophages. *Biochem Pharmacol* 1997; 54:87–96.
81. Guan Z, Buckman SY, Miller BW, Springer LD, Morrison AR. Interleukin-1 beta-induced cyclooxygenase-2 expression requires activation of both c-Jun NH2-terminal kinase and p38 MAPK signal pathways in rat renal mesangial cells. *J Biol Chem* 1998; 273:28,670–28,676.
82. Subbaramaiah K, Chung WJ, Dannenberg AJ. Ceramide regulates the transcription of cyclooxygenase-2. Evidence for involvement of extracellular signal-regulated kinase/c-Jun N-terminal kinase and p38 mitogen-activated protein kinase pathways. *J Biol Chem* 1998; 273:32,943–32,949.
83. McGinty A, Foschi M, Chang YW, Han J, Dunn MJ, Sorokin A. Induction of prostaglandin endoperoxide synthase 2 by mitogen-activated protein kinase cascades. *Biochem J* 2000; 352 Pt 2:419–424.

84. Yang T, Huang Y, Heasley LE, Berl T, Schnermann JB, Briggs JP. MAPK mediation of hypertonicity-stimulated cyclooxygenase-2 expression in renal medullary collecting duct cells. *J Biol Chem* 2000; 275:23,281–23,286.
85. Subbaramaiah K, Hart JC, Norton L, Dannenberg AJ. Microtubule-interfering agents stimulate the transcription of cyclooxygenase-2. Evidence for involvement of ERK1/2 AND p38 mitogen-activated protein kinase pathways. *J Biol Chem* 2000; 275:14,838–14,845.
86. Chen CC, Sun YT, Chen JJ, Chang YJ. Tumor necrosis factor-alpha-induced cyclooxygenase-2 expression via sequential activation of ceramide-dependent mitogen-activated protein kinases, and IkappaB kinase ½ in human alveolar epithelial cells. *Mol Pharmacol* 2001; 59:493–500.
87. Sheng H, Williams CS, Shao J, Liang P, DuBois RN, Beauchamp RD. Induction of cyclooxygenase-2 by activated Ha-ras oncogene in Rat-1 fibroblasts and the role of mitogen-activated protein kinase pathway. *J Biol Chem* 1998; 273:22,1203–22,127.
88. Sheng H, Shao J, Dixon DA, Williams CS, Prescott SM, DuBois RN, Beauchamp RD. Transforming growth factor-beta1 enhances Ha-ras-induced expression of cyclooxygenase-2 in intestinal epithelial cells via stabilization of mRNA. *J Biol Chem* 2000; 275:6628–6635.
89. Slice LW, Bui L, Mak C, Walsh JH. Differential regulation of COX-2 transcription by Ras- and Rho-family of GTPases. *Biochem Biophys Res Commun* 2000; 276:406–410.