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CYCLOOXYGENASES: Structural, Cellular, and Molecular Biology

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■ **Abstract** The prostaglandin endoperoxide H synthases-1 and 2 (PGHS-1 and PGHS-2; also cyclooxygenases-1 and 2, COX-1 and COX-2) catalyze the committed step in prostaglandin synthesis. PGHS-1 and 2 are of particular interest because they are the major targets of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin, ibuprofen, and the new COX-2 inhibitors. Inhibition of the PGHSs with NSAIDs acutely reduces inflammation, pain, and fever, and long-term use of these drugs reduces fatal thrombotic events, as well as the development of colon cancer and Alzheimer's disease. In this review, we examine how the structures of these enzymes relate mechanistically to cyclooxygenase and peroxidase catalysis, and how differences in the structure of PGHS-2 confer on this isozyme differential sensitivity to COX-2 inhibitors. We further examine the evidence for independent signaling by PGHS-1 and PGHS-2, and the complex mechanisms for regulation of PGHS-2 gene expression.

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INTRODUCTION

This review focuses on prostaglandin endoperoxide H synthases-1 and 2 (PGHS-1 and 2), which catalyze the committed step in prostanoid synthesis (1–4); the generic names for these isozymes are cyclooxygenase-1 and 2 (COX-1 and COX-2). PGHS-1 and PGHS-2 are of particular interest because they are the major targets of nonsteroidal anti-inflammatory drugs (NSAIDs) including aspirin and ibuprofen and the new COX-2 inhibitors Celebrex and Vioxx and because they are involved in a range of pathologies that for PGHS-1 includes thrombosis (5, 6) and for PGHS-2 includes inflammation, pain, and fever (7–9), various cancers (10–14), and Alzheimer's disease (15).

Prostanoids are members of a large group of hormonally active, oxygenated C_{18}, C_{20}, and C_{22} fatty acids collectively known as eicosanoids that are derived from ω3 (n-3) and ω6 (n-6) polyunsaturated fatty acids and include (*a*) prostanoids formed through cyclooxygenase pathways; (*b*) leukotrienes (16, 17), lipoxins (18), hepoxilins (19), and monohydroxy fatty acids (20) produced via lipoxygenase pathways; (*c*) epoxy and dihydroxy fatty acids formed by cytochrome P450s (21); and (*d*) isoprostanes (22, 23), isoleukotrienes, and other peroxidized fatty acid products (24) that are formed nonenzymatically.

Arachidonic acid (20:4 n-6) is the major prostanoid precursor. The biosynthesis of prostanoids involves a three-step sequence (Figure 1) of stimulus-initiated hydrolysis of arachidonate from glycerophospholipids involving secretory, cytoplasmic or both types of phospholipase A_2 ($sPLA_2$, $cPLA_2$) (25); oxygenation of arachidonate, yielding prostaglandin endoperoxide H_2 (PGH_2) by PGHSs; and conversion of PGH_2 to the most important biologically active end products, PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), or TxA_2 (thromboxane A_2) via specific synthases (26–29) (Figure 1). The resulting products then exit the cells via a carrier-mediated process (30) to activate prostanoid G protein–linked prostanoid receptors (31–33), or in some cases may interact with nuclear receptors (34).

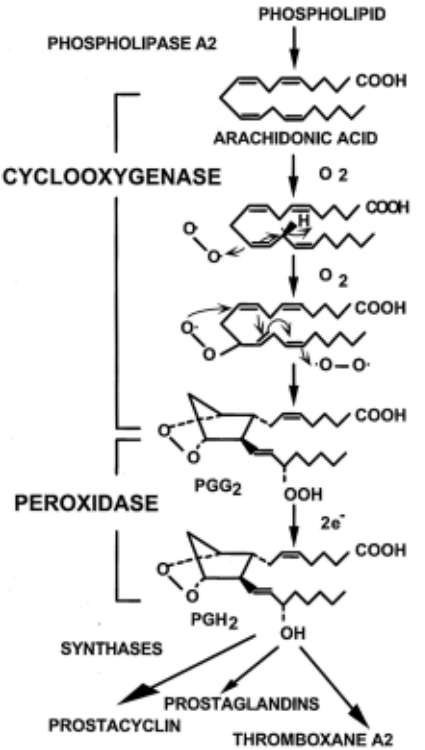

Figure 1 Biosynthetic pathway for the formation of prostanoids derived from arachidonic acid.

PGHS STRUCTURE AND CATALYSIS

Overview of Peroxidase and Cyclooxygenase Catalysis

PGHS-1 and 2 are considered to be the constitutive and inducible PGHSs, respectively. Both enzymes catalyze a cyclooxygenase (*bis*-oxygenase) reaction in which arachidonate plus two molecules of O_2 are converted to PGG_2 (prostaglandin G_2) and a peroxidase reaction in which PGG_2 is reduced to PGH_2 by two electrons (Figures 1 and 2). These two reactions occur at distinct but structurally and functionally interconnected sites (Figure 3). The peroxidase reaction occurs at a heme-containing active site located near the protein surface. The cyclooxygenase reaction occurs in a hydrophobic channel in the core of the enzyme. In vitro, the peroxidase activity can operate independently of the cyclooxygenase (e.g. when the cyclooxygenase site is occupied by an NSAID) (35) or during ongoing cyclooxygenase catalysis (36). In contrast, the cyclooxygenase reaction is peroxide-dependent (37) and requires that the heme group at the peroxidase site undergo a two-electron oxidation (38). The identity of the agent that initiates the

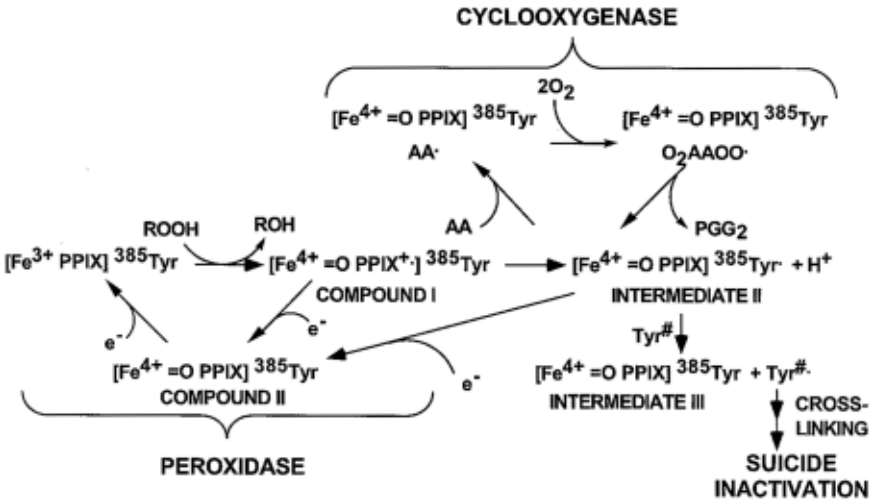

Figure 2 Cyclooxygenase and peroxidase catalysis and suicide inactivation by PGHSs. Fe^{3+} PPIX, ferric iron protoporphyrin IX (heme); ROOH, alkyl hydroperoxide; ROH, alcohol; AA, arachidonic acid; Fe^{4+}=O PPIX, oxyferryl heme. Compound I, an oxyferryl group (Fe(IV)=O) plus a protoporphyrin IX radical cation; intermediate II, an oxyferryl group plus a neutral protoporphyrin IX plus a Tyr385 tyrosyl radical; compound II, an oxyferryl group plus a neutral protoporphyrin IX; intermediate III, a spectral intermediate of unknown structure but perhaps involving a heme group with a protein radical located on an amino acid sidechain other than Tyr385.

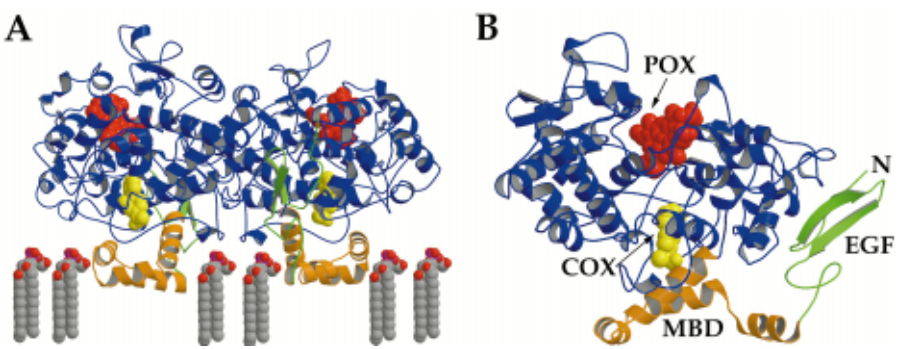

Figure 3 Structure of ovine prostaglandin endoperoxidase H synthase-1 (oPGHS-1). (*A*) Ribbon diagram of the oPGHS-1 homodimer with flurbiprofen (*yellow*) bound within the cyclooxygenase active site and the protein interdigitated into one face of the lumenal surface of the membrane bilayer. The positions of the three major folding domains are indicated: epidermal growth factor (EGF; *green*), membrane binding domain (MBD; *gold*) and globular catalytic domain (*blue*); as are the peroxidase and cyclooxygenase active sites (64). The heme is shown in *red*. (*B*) Ribbon drawing of the oPGHS-1 monomer with flurbiprofen bound, indicating the locations of the peroxidase (POX) and cyclooxygenase (COX) active sites and the EGF and membrane binding (MBD) domains. The color scheme is the same as in (*A*).

heme oxidation in vivo is not known, but peroxynitrite may serve as the physiological heme oxidant in some cells (e.g. macrophages) (3). In vitro, hydroperoxides contaminating commercial fatty acid preparations oxidize the heme groups of a small fraction of PGHS molecules, and the remaining PGHS molecules are then activated autocatalytically by newly generated PGG_2. The cyclooxygenase activity of PGHS-2 is activated at about 10-fold lower concentrations of hydroperoxide than PGHS-1 (39–41). As discussed below, this difference in hydroperoxide dependency permits PGHS-2 to function independently of PGHS-1 in cells expressing both isoforms (41, 42).

A branched-chain, mechanistic model that explains the necessity for peroxidase heme oxidation for cyclooxygenase catalysis was first proposed by Ruf and coworkers (Figure 2) (43). Initially, a peroxide reacts with the heme group. A two-electron oxidation occurs, yielding compound I and an alcohol derived from the oxidizing peroxide (38, 43–45). Compound I next undergoes an intramolecular reduction by a single electron traveling from Tyr385 along the peptide chain to the proximal heme ligand, His388, and finally to the heme group. [The numbering of amino acids begins at the N terminus of ovine PGHS-1 (oPGHS-1) with methionine as residue number 1. The N-terminal residue of oPGHS-1 is Ala25; homologous residues of PGHS-2 are numbered in parallel with oPGHS-1, e.g. the actual Arg106 of hPGHS-2 is numbered as Arg120.] The result is intermediate II, an oxyferryl group plus a neutral protoporphyrin IX plus a Tyr385 tyrosyl radical (43, 44, 46, 47). [Compound I can be reduced directly to heme by certain arylsulfides (47a).] Alternatively, compound I can undergo a one-electron reduction by an exogenous electron donor, yielding compound II (45). The visible spectra of compound II and intermediate II are the same; electron paramagnetic resonance spectroscopy is necessary to distinguish between these species (47). Importantly, although not indicated in Figure 2, the tyrosyl radical–containing species can continue to cycle through the peroxidase reaction independently from cyclooxygenase turnover (36). When the cyclooxygenase site is occupied by an appropriate fatty acid substrate such as arachidonate, the tyrosyl radical of intermediate II initiates the cyclooxygenase reaction by abstracting the 13pro*S* hydrogen atom to yield an arachidonate radical (48) (Figures 1 and 2). The fatty acid radical then reacts with molecular O_2 (a diradical) to produce an 11-hydroperoxyl radical, which in turn forms a C-11 to C-9 endoperoxide moiety that cyclizes, reacts with a second O_2 molecule, and ultimately produces PGG_2.

Unresolved issues remain regarding the PGHS mechanism (49), but the branched chain model explains much of what we know. The most compelling evidence for the branched chain mechanism is that PGG_2 can accumulate during catalysis even in the presence of peroxidase-reducing cosubstrates (50). In a pure branched chain mechanism, the tyrosyl radical of intermediate II, once formed, would cycle continuously (Figure 2). In fact, removal of hydroperoxides after catalysis has been initiated (e.g. upon addition of glutathione peroxidase plus reduced glutathione) stops the cyclooxygenase reaction in midstream (45, 51, 52).

Thus, cyclooxygenase catalysis requires the ongoing presence of hydroperoxides, presumably to regenerate compound I. The continuous need for hydroperoxides implies that intermediate II is reduced to compound II (and back to heme) at a rate that competes effectively with the rate of abstraction of the hydrogen atom from arachidonate by intermediate II (Figure 2). Overall, the coupling between the peroxidase and cyclooxygenase reactions involves a "leaky" branched chain mechanism. Indeed, the pathway from intermediate II to compound II may be important in preventing untoward accumulation of enzyme radicals, particularly when substrate is not being provided to the enzyme.

Suicide Inactivation

Confounding the interpretation of kinetic and mechanistic data on the peroxidase and cyclooxygenase reactions of PGHSs is the phenomenon of suicide inactivation. Both the peroxidase and the cyclooxygenase activities are inactivated during catalysis by mechanism-based, first-order processes (2, 53, 54). That is, PGHS-1 or -2 peroxidase or cyclooxygenase activities fall to zero within 1–2 min even in the presence of sufficient substrates. In Figure 2, peroxidase and cyclooxygenase suicide inactivation are depicted as common events involving intermediate III (53), although this point is not resolved. For example, peroxidase inactivation is independent of the nature of the oxidizing peroxide (53), whereas cyclooxygenase inactivation appears to depend on the nature of the fatty acid substrate (37), and thus apparently on the nature of the peroxide. Suicide inactivation originates with a reaction intermediate (53). This intermediate does not directly involve an arachidonate-derived radical because the rate of covalent attachment of fatty acid to PGHS during catalysis is 30 times slower than that of suicide inactivation (55, 56). As depicted in Figure 2, suicide inactivation likely proceeds from intermediate II (53) and involves the formation of a tyrosyl radical other than the Tyr385 radical. Consistent with this concept are findings that protein tyrosyl radicals in oPGHS-1 can be localized to tyrosines other than Tyr385 (57–59), and that an intermediate III has been detected in association with peroxidase inactivation (53). The nature of the protein modification(s) resulting in suicide inactivation has not been defined. Interestingly, an H386A oPGHS-1 mutant undergoes suicide inactivation at $<3\%$ of the rate of native enzyme but exhibits 35% of the native cyclooxygenase activity (60; WL Smith, unpublished data). Studies with this mutant should provide further insight into suicide inactivation. It should be noted that the rates of both peroxidase and cyclooxygenase suicide inactivation are slowed markedly by peroxidase-reducing cosubstrates (36, 53, 61). Reducing cosubstrates may bias the rate of conversion of intermediate II to compound II versus intermediate III. Suicide inactivation is an interesting chemical phenomenon, but its biological relevance is unclear. In general, the amounts of PGHSs are in excess of substrate and bursts of prostanoid production by cells do not lead to major losses in PGHS activity.

PGHS Structure

The primary structures of PGHS-1 and 2 from numerous species are known (1). Both isoforms contain signal peptides of varying lengths. Mature, processed PGHS-1 contains 576 amino acids; the mature form of PGHS-2 contains 587 amino acids. There is a 60%–65% sequence identity between PGHS-1 and 2 from the same species and 85%–90% identity among individual isoforms from different species. The major sequence differences between PGHS isoforms occur in the membrane binding domains (62, 63). A unique difference between PGHS-1 and 2 is 18 amino acids inserted 6 residues in from the C terminus of PGHS-2 that are not present in PGHS-1. The function of this insert is not established but may mark PGHS-2 for rapid proteolysis or provide a signal for subcellular trafficking; elimination of this cassette by deletion mutagenesis has no apparent effect on PGHS-2 catalysis (WL Smith, unpublished data).

Figures 3*A* and *B* are ribbon diagrams showing the overall three-dimensional structure of oPGHS-1 (64). The structures of human (65) and murine (66) PGHS-2 are virtually superimposable on the oPGHS-1 structure. PGHSs are homodimers both functionally (67) and structurally, but the reason that dimerization is necessary for catalysis is unknown. Each monomer consists of three structural domains: an epidermal growth factor (EGF) domain of 50 amino acids at the N terminus, a neighboring membrane binding domain (MBD) of about 50 amino acids, and a large C-terminal globular catalytic domain with about 460 amino acids (64–66). The structures of the C-terminal tails (17 amino acids in PGHS-1 and 35 amino acids in PGHS-2 following Pro583) are not distinguishable crystallographically. The EGF domain forms a portion of the dimer interface. A C69S mutation in oPGHS-1, which disrupts one of the EGF disulfide bonds, lacks activity; thus an intact EGF domain is essential for folding (WL Smith, unpublished data). The MBDs of PGHSs contain four short, consecutive, amphipathic α helices, the last of which, helix D, merges into the catalytic domain. Hydrophobic and aromatic residues protrude from these helices and away from the hydrophilic surface of the catalytic domain to create a hydrophobic patch that interacts with the one face of the underlying bilipid layer (64). These helices also surround an opening through which fatty acid substrates and NSAIDs are believed to enter the cyclooxygenase active site. The MBDs of PGHS-1 and 2 represent a novel mechanism for membrane association; squalene cyclase has recently been found to have a structurally similar domain (68). The globular catalytic domain closely resembles that of myeloperoxidase but with a hydrophobic channel protruding into the core of this domain (64). The upper half of the tunnel is the cyclooxygenase active site and can bind fatty acid substrates and NSAIDs. PGHS-1 and 2 contain C-terminal KDEL-like sequences that target PGHSs to the endoplasmic reticulum and the associated nuclear envelope (69). Both enzymes are present on the lumenal surfaces of the ER and of the inner and outer membranes of the nuclear envelope (70–72). PGHS-2 appears to be relatively more concentrated within the nuclear envelope (71), raising the intriguing possibility that products formed via

this isoform may have greater access to the nucleoplasm to affect nuclear events, perhaps via nuclear receptors (34). PGHS-1 is N-glycosylated at three sites (73). PGHS-2 is variably glycosylated at two to four sites, leading to the formation of doublets or sometimes triplets on SDS-PAGE (73). N-Glycosylation of oPGHS-1 is required for enzyme folding (73), which has created problems in producing large quantities of this isoform. This contrasts with successes with expressing PGHS-2 in baculovirus systems (74). The PGHS structures contain several water channels, including a branched channel that extends from the cyclooxygenase site near Gly533 to the dimer interface. It is not clear if the water channels are simply structural or play some direct role in catalysis (e.g. as conduits for proton flow).

PEROXIDASE CATALYSIS

Peroxidase Active Site Structure and Peroxidase Catalysis

The structures of the peroxidase active sites of PGHSs (Figures 3 and 4) are similar to those of other heme peroxidases (64, 75). Moreover, the peroxidase reactions catalyzed by PGHSs involve typical compound I and II peroxidase intermediates (Figure 2) (38, 43–45) and have kinetic properties (45) similar to those of other heme peroxidases. There are two significant differences between the peroxidase activities of PGHS-1 and 2. First, PGHS-1 catalyzes a two-electron reduction of hydroperoxidase substrates almost exclusively, whereas PGHS-2 catalyzes 60% two-electron and 40% one-electron reductions (38). Second, the rate of formation of compound II from compound I is much faster with PGHS-2 (45).

Figure 4 shows the structure of the peroxidase active site of oPGHS-1 with PGG_2 modeled into the site. His388 is the proximal (fifth) heme ligand; one imidazole nitrogen bonds to the ferric iron and the other imidazole nitrogen hydrogen bonds via a water molecule to Tyr504 (64–66; RM Garavito, unpublished data). The latter arrangement contrasts with other peroxidases in which the proximal histidine forms an ionic bond with glutamate (75). The distal histidine, His207, is predicted to be important in deprotonation of the hydroperoxide substrate and subsequent protonation of the incipient alkoxide ion to form the alcohol during generation of compound I (Figures 2 and 4) (38). Gln203 is also important in catalysis, although its function has not been resolved (38). Mutations of Gln203, His207, or His388 lead to a reduction or elimination of peroxidase activity (38, 60).

Substrate Specificities

In vitro, the peroxidase activities of PGHSs can reduce a variety of peroxides [e.g. H_2O_2, 15-hydroperoxyeicosatetraenoic acid (15-HPETE)], but both isozymes show a preference for secondary alkyl hydroperoxides and the physiologically

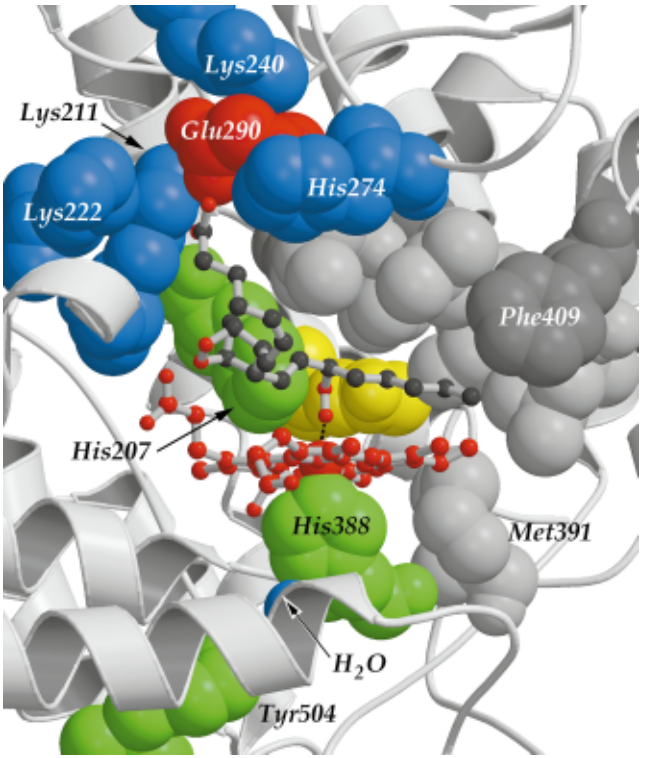

Figure 4 Model of the peroxidase active site of oPGHS-1 with PGG_2 bound. The protein structure is from Picot et al (64) with PGG_2 manually modeled into the peroxidase site. The heme group is in *red* with the hydroperoxide group of PGG_2 (*red*) interacting with the iron. The ball-and-stick figure in *gray* is PGG_2 with its carboxyl group in *red* surrounded by polar residues. The area around the carboxyl group of PGG_2 has three basic charges, including one that is not compensated for and that could interact with the negatively charged carboxyl group of PGG_2. The nonpolar portion of PGG_2 is in a hydrophobic trough made up of valines and leucines (*gray*) with Phe409 near the ω end of PGG_2.

important substrate is PGG_2 (38, 45). This is difficult to rationalize structurally because the PGHS peroxidase sites are open to solvent in crystal structures (64–66). However, interactions between the carboxylate group of PGG_2 and basic residues at one end of the peroxidase site and the ω end of PGG_2 with hydrophobic residues may facilitate binding (Figure 4). Cyanide binds reasonably tightly to oPGHS-1 and inhibits peroxidase (and cyclooxygenase) catalysis but the slightly larger azide does not (76; WL Smith, unpublished data). The low affinity of N_3^- for PGHSs is surprising because there is no "ceiling" over the peroxidase active site in the crystal structure. The physiological reducing cosubstrate(s) for PGHS peroxidases is not known. Common reductants found in high concentrations in

cells such as ascorbate (77) and reduced glutathione are inefficient substrates (78).

Consistent with crystallographic evidence, magnetic circular dichroism, electron paramagnetic resonance, visible, and resonance (r) Raman spectroscopy of resting oPGHS-1 (76, 79; WL Smith, unpublished data) indicate that it is mainly in the high-spin ferric heme form with water occupying the distal (sixth) coordination position; lesser amounts of enzyme (~20%) are in a low-spin form in which an imidazole (His207) replaces water (79). Raman spectroscopy indicates that His388 of oPGHS-1 is more acidic in character than corresponding histidines in other peroxidases (RM Garavito, unpublished data). In myeloperoxidase the proximal histidine is bonded directly to an asparagine (75). This difference in acidity of the proximal histidine is not reflected in peroxidase turnover numbers, which are comparable to those of other heme peroxidases (78), but it may facilitate the formation of intermediate II in cyclooxygenase catalysis (Figure 2). The acidity of the proximal histidine may also account in part for the relatively low K_d for the binding of heme to oPGHS-1 (~1 μM); the K_d for the binding of heme to PGHS-2 is estimated to be even lower based on the relative ease with which apo-PGHS-2 is formed. Although Fe^{3+}-protoporphyrin IX is the natural heme ligand, Mn^{3+}-heme, but not other heme forms (80), will substitute, albeit inefficiently, in peroxidase catalysis by PGHSs (81–84).

PGHS-1 catalyzes primarily a standard two-electron reduction of hydroperoxides involving heterolytic cleavage of the peroxide group (Figure 2); in contrast, PGHS-2 catalyzes both one- and two-electron reductions (38). The abilities of native and mutant PGHS-2 to catalyze heterolytic cleavage of peroxides correlate with their abilities to catalyze the cyclooxygenase reaction; this is consistent with compound I being the precursor of intermediate II (Figure 2) (38). It is unclear if there is any biological significance to PGHS-2–catalyzed homolytic cleavage of hydroperoxides, and there is no obvious structural reason why PGHS-2 tends to produce homolytic cleavage.

Peroxidase Kinetics

Peroxidase activities of PGHS-1 and 2 have been characterized by examining rates of oxidation of reducing cosubstrates such as guaiacol (38), 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) (38), and tetramethylphenylenediamine (TMPD) (85); by examining rates of formation of peroxidase heme spectral intermediates (compounds I and II) by stop-flow spectrophotometry (47); and by analyzing differential product formation from hydroperoxides that undergo one- or two-electron oxidations (86). The kinetic constants for compound I (oxyferryl heme radical cation) and compound II/intermediate II (oxyferryl heme) associated with heterolytic cleavage of alkyl hydroperoxides have been measured for both PGHS-1 and 2. Relatively hydrophobic alkyl hydroperoxides such as 15-HPETE and 5-phenyl-4-pentenyl-1-hydroperoxide (PPHP) exhibit about

10-fold higher secondary rate constants for formation of compound I ($\sim 2 \times 10^7$ mol^{-1} s^{-1}) versus soluble peroxides such as ethylhydroperoxide (45) and have lower apparent K_m values (~ 10 μM versus 300 μM for H_2O_2) for the peroxidase reaction as measured by rates of oxidation of reducing cosubstrates (38). Although the second order rate constants k_1 for compound I formation with alkyl hydroperoxides are approximately the same for both isozymes ($\sim 2 \times 10^7$ M^{-1} s^{-1}), the first-order rate constant for the conversion of compound I to compound II/intermediate II is considerably more rapid for PGHS-2 (45). This partly accounts for the fact that for PGHS-2, intermediate II is formed more rapidly and at lower peroxide concentrations. There is no obvious structural explanation for this property.

CYCLOOXYGENASE CATALYSIS AND INHIBITION

Similarities and Differences Between PGHS-1 and 2

PGHS-1 and 2 have very similar cyclooxygenase active site structures, catalytic mechanisms, products, and kinetics. There are, however, two structural differences between the two isozymes that have important pharmacological and biological consequences. First, the cyclooxygenase active site of PGHS-2 is larger and more accommodating than that of PGHS-1. This size difference has been exploited in developing COX-2–specific NSAIDs. Second, although the gross kinetic properties (e.g. K_m, V_{max}) of PGHS-1 and 2 are nearly identical, PGHS-1, but not PGHS-2, exhibits negative allosterism at low arachidonate concentrations; this difference may permit PGHS-2 to compete more effectively for newly released arachidonate when the isozymes are expressed in the same cell.

Cyclooxygenase Active Site Structure

PGHS-1 and 2 monomers each contain a 25-Å hydrophobic channel that originates at the membrane binding domain (MBD) and extends into the core of the globular domain (64–66) (Figure 3). The MBD forms the mouth and the first half of the channel and allows arachidonate and O_2 to enter directly from the apolar compartment of the lipid bilayer. The NSAID binding site involves the upper half of this channel from Arg120 to near Tyr385 (Figure 5). There are as yet no published crystal structures of PGHS complexed with fatty acids (WL Smith, unpublished data; R Kurumbail, personal communication); however, it is clear that the NSAID binding site corresponds to the cyclooxygenase active site. NSAIDs are competitive cyclooxygenase inhibitors (1, 87); arachidonate is located within the channel in apo-PGHS-2 (R Kurumbail, personal communication); and as discussed below, several amino acids composing the upper half of the channel are uniquely important in cyclooxygenase catalysis. Twenty-four residues line the hydrophobic cyclooxygenase active site with only one difference between the isozymes—Ile at position 523 in PGHS-1 and Val at position 523 in PGHS-2. (Amino acids lining the hydrophobic cyclooxygenase active site channel include

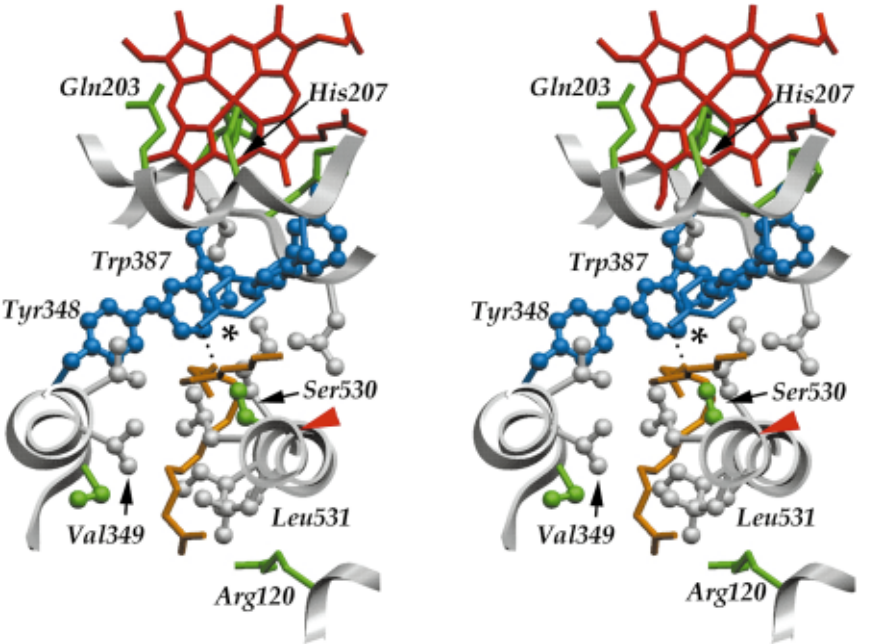

Figure 5 Stereo diagram of the L-conformation of arachidonate (*orange*) bound in the cyclooxygenase active site of oPGHS-1. This model is based on crystallographic data for apo-PGHS-2 (R Kurumbail, personal communication), chemical evidence for an appropriate structure for oxygenation and cyclization of arachidonate to PGG_2, positioning of Tyr385 (*) aligned (•••••) with the 13-pro*S* hydrogen of arachidonate, and interaction between the carboxylate of arachidonate and the guanido group of Arg120 as discussed in the text. The *red arrowhead* denotes Gly533.

Leu117, Arg120, Phe205, Phe209, Val344, Ile345, Tyr348, Val349, Leu352, Ser353, Tyr355, Leu359, Phe381, Leu384, Tyr385, Trp387, Phe518, Ile/Val523, Gly526, Ala527, Ser530, Leu531, Gly533, Leu534.) Only three of the channel residues are polar (Arg120, Ser353, and Ser530). Ser530 is the site of acetylation by aspirin (88–90), and Arg120 binds to the carboxylate groups of fatty acids and many NSAIDs (91–94).

The cyclooxygenase active site of PGHS-2 is about 20% larger and has a slightly different shape than that of PGHS-1 (65). This difference in active site size and shape is due to three amino acid differences between PGHS-1 and 2: Ile523 to Val523 in the first shell of the active site, and Ile 434 to Val434 and His513 to Arg513 in the surrounding second shell. The other major structural difference is in the position of helix D, the last of the four helices of the MBD. In PGHS-2 this helical segment is cantilevered upward to provide a larger opening in the MBD, and as a consequence, Arg120 is displaced (64, 65). As detailed below, these various differences influence inhibitor binding and provide for more substrate flexibility in the PGHS-2 cyclooxygenase site.

Substrate Positioning and Turnover in the Cyclooxygenase Site

An L-shaped model of arachidonate in the cyclooxygenase active site based on inferences from crystallographic studies, mutagenic analysis of active site residues, and consideration of the chemistry of the arachidonate to PGG_2 conversion is presented in Figure 5. It is likely that 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, and 20:5n-3 would be positioned similarly to permit removal of the n-8 hydrogen but that 18:3n-3 would be aligned for abstraction of the n-5 hydrogen (95, 96). Key features of the model are appropriate positioning of (*a*) the carboxylate of arachidonate to interact with Arg120 (64–66, 91–94); (*b*) the 13pro*S* hydrogen of arachidonate for abstraction by a Tyr385 radical (97–99; R Kurumbail, personal communication); (*c*) the carboxylate and ω halves of the arachidonate molecule for formation of an endoperoxide bridge between C-11 and C-9 (R Kurumbail, personal communication); and (*d*) the ω end of arachidonate in a side pocket of the channel that ends at Gly533 (96, 100; WL Smith, unpublished data).

oPGHS-1 synthesizes primarily PGG_2, but it also produces significant amounts of 11-hydroxy-(5*Z*, 8*Z*, 12*E*, 13*Z*)-eicosatetraenoic acid (11*R*-HETE), 15-hydroxy-(5*Z*, 8*Z*, 11*Z*, 13*E*)-eicosatetraenoic acid (15*R*-HETE), and 15*S*-hydroxy-(5*Z*, 8*Z*, 11*Z*, 13*E*)-eicosatetracnoiacid (15*S*-HETE) (WL Smith, unpublished data). Analysis of the kinetics of the formation of these products suggests that arachidonate can exist in four slightly different but catalytically competent conformers in the cyclooxygenase site and that each product is derived from a different conformer (WL Smith, unpublished data). With human PGHS-2 (hPGHS-2), which forms PGG_2, 11*R*-HETE, and 15*S*-HETE but not 15*R*-HETE (99), there are apparently three catalytically competent arachidonate conformers. Formation of PGG_2, 11*R*-HETE, 15*R*-HETE, and 15*S*-HETE all likely involve abstraction of the 13pro*S* hydrogen (99, 99a; WL Smith, unpublished data).

Overall, the hydrophobic residues in the cyclooxygenase active site of PGHS-1 function to position arachidonate in a conformation that optimizes production of PGG_2, and they contribute little to substrate binding (WL Smith, unpublished data). For example, substitution of active site hydrophobic residues generally causes less than a 10-fold change in K_m values (100; WL Smith, unpublished data). The most critical residue for arachidonate binding in PGHS-1 is Arg120, which forms an ionic linkage with the carboxylate of arachidonate (91, 93). The R120Q mutant of oPGHS-1 has a K_m for arachidonate that is 500–1000 times higher than that observed with native oPGHS-1 (91). In contrast, the R120Q mutant of hPGHS-2 has the same K_m as native hPGHS-2, indicating that the hydrophobic residues lining the PGHS-2 channel must play a relatively more significant role in substrate binding (92).

Besides Arg120, several residues lining the cyclooxygenase site have been studied in some detail in both isozymes including Val349 (WL Smith, unpublished data), Tyr355 (101, 102), Ser530 (88–90, 99, 100, 103), and Gly533 (96, 100). There is also information available on Tyr348 (57, 58, 101), Trp387 (104; WL

Smith, unpublished data), Phe518 (WL Smith, unpublished data), and Leu531 of oPGHS-1 (100; WL Smith, unpublished data). Val349 is thought to be situated between the carboxylate and ω halves of arachidonate (Figure 5) and appears to be important primarily in positioning arachidonate for conversion to PGG_2 (WL Smith, unpublished data). The major product formed by both V349A oPGHS-1 and V349A hPGHS-2 mutants is 11*R*-HETE (WL Smith, unpublished data). Apparently, the favored arachidonate conformer in these mutants is one in which C-9 is mispositioned with respect to C-11 such that after the initial hydrogen abstraction and O_2 insertion at C-11, the reaction aborts to form 11*R*-hydroperoxyeicosatetraenoic acid (11*R*-HPETE). Tyr355 lies on the opposite side of the channel from Arg120 and governs the stereospecificity of PGHSs toward NSAIDs (91) and may play a role in the negative allosteric effect of arachidonate in PGHS-1 (102). Ser530 is the site of acetylation by aspirin (88–90). Acetylated PGHS-2 forms significant amounts of 15*R*-hydroperoxyeicosatetraenoic acid (15*R*-HPETE) whereas acetylated PGHS-1 forms no products; however, an S530T mutant of PGHS-1 does form 15*R*-HPETE (WL Smith, unpublished data). Ser530 is important in positioning C-13 with respect to the Tyr385 radical (99). Studies with Gly533 mutants of oPGHS-1 and hPGHS-2 suggest that this residue neighbors the ω methyl group of arachidonate bound in the cyclooxygenase site (96, 100; WL Smith, unpublished data)

Cyclooxygenase Kinetics

Cyclooxygenase kinetics are complex and the reader is referred to several sophisticated papers on this topic (42, 47, 54, 105, 106). Three points are of general interest. The first is that the K_m values are the same for both microsomal and solubilized, purified enzymes, suggesting that the membrane-bound and purified enzyme forms behave very similarly. Interestingly, PGHS-1 and PGHS-2 have nearly identical cyclooxygenase turnover numbers ($\sim$3500 mol/min of arachidonate per mole of dimer) (74, 107) and apparent K_m values for arachidonate ($\sim$5 μM) and other fatty acid substrates (74, 95, 107–109) and O_2 ($\sim$5 μM) (110, 111; WL Smith, unpublished data). They also have similar rates of suicide inactivation ($t_{1/2} \sim 30$ s). The second point is that the K_m values probably approximate the binding affinities for the substrates. This supposition is based on the observations that the K_i and K_m values are similar to one another for poor substrates such as eicosapentaenoic acid that behave as both inhibitors (e.g. of arachidonate oxygenation) and substrates (92, 106, 109; WL Smith, unpublished data), and that the K_m values for most substrates are below the critical micelle concentration of oleic acid (112). The third point, which we expand on here, is the unusual negative allosteric regulation of PGHS-1 (42, 102, 105, 113), which is not seen with PGHS-2. This difference, as discussed below, may be relevant to the preferential oxygenation of arachidonate by PGHS-2 in intact cells at relatively low substrate concentrations (41, 113). Both microsomal and purified PGHS-1 exhibit negative allosterism at arachidonate concentrations between 50 nM and 1 μM such that when equivalent amounts of the two

isozymes are functioning in this substrate range, the amount of product formed via PGHS-1 is less than 25% of that formed via PGHS-2 (105); intact cells expressing both isozymes also show preferential utilization of exogenous arachidonate via PGHS-2 (113). This is important because this concentration of substrate is in the range likely to be available under many conditions in vivo. Moreover, negative allosteric regulation of PGHS-1 can explain how PGHS-2 can operate independently of PGHS-1. Interestingly, this negative allosterism is not observed in the presence of excess hydroperoxide (which activates the peroxidase), suggesting that hydroperoxide concentrations may be key to regulating the relative activities of PGHS-1 and PGHS-2 in vivo (42).

Cyclooxygenase Inhibition by NSAIDs

NSAID interactions with the cyclooxygenase active sites of PGHS-1 and 2 have been studied extensively, and a number of crystal structures of NSAID/PGHS complexes are available (64–66). Because there are quite recent, excellent reviews of this topic (3, 114), we will not address NSAID inhibition extensively here. There are, however, three general points to be made. First, there are two classes of NSAIDs: (*a*) classical (pre-1995) NSAIDs and (*b*) COX-2 inhibitors. All classical NSAIDs can inhibit both PGHS-1 and 2 but in general bind more tightly to PGHS-1 (115). Of course, COX-2 inhibitors exhibit selectivity toward PGHS-2 (3, 114). Second, while all NSAIDs compete with arachidonate for binding to the cyclooxygenase active site, each NSAID exhibits one of three kinetic modes of inhibition (1–3, 114): (*a*) rapid, reversible binding (e.g. ibuprofen); (*b*) rapid, lower affinity reversible binding followed by time-dependent, higher affinity, slowly reversible binding (e.g. flurbiprofen), or (*c*) rapid, reversible binding followed by covalent modification (acetylation) of Ser530 (e.g. aspirin). The structural basis for time-dependent inhibition is not well defined and may be different for different drugs. The kinetic differences in NSAID inhibition have made simple comparisons of drug interactions with PGHS-1 versus PGHS-2 difficult, particularly in vitro. And finally, all COX-2 inhibitors cause time-dependent inhibition of PGHS-2 but not PGHS-1.

Classical NSAIDs Versus COX-2 Inhibitors

About 1% of chronic users of nonsteroidal anti-inflammatory drugs (NSAIDs), typically those with arthritis or other chronic inflammatory diseases, develop ulcers or other serious gastrointestinal complications each year. These ulcers result from inhibition of prostaglandin synthesis by PGHS-1, the predominant cyclooxygenase isozyme in the stomach lining. Because of the widespread use of NSAIDs, these toxicities are one of the most prevalent drug-associated health risks. COX-2 selective NSAIDs have little effect on PGHS-1, but potently inhibit PGHS-2, the principal isozyme responsible for production of inflammatory prostaglandins, Thus, COX-2 selective inhibitors are proving to have the same anti-inflammatory,

anti-pyretic, and analgesic activities as nonselective inhibitors NSAIDs, with little or none of the gastrointestinal side-effects.

The mechanism for a differential inhibition by classical and COX-2 NSAIDs can be rationalized to some extent based on differences between the cyclooxygenase active sites of PGHS-1 and 2. Substitution of Ile523 in PGHS-1 with Val523 in PGHS-2 results in the presence of a small side pocket adjacent to the active site channel, appreciably increasing the volume of the PGHS-2 active site (65, 66). This change is compounded by the substitution of Ile434 in PGHS-1 with Val434 in PGHS-2, within the second shell of amino acids surrounding the cyclooxygenase active site. The Ile to Val substitution at position 434 outside the PGHS-2 catalytic center further increases the effective size of the active site channel by enhancing the local mobility of side chains within the side pocket. The combination of these two differences at positions 523 and 434 in PGHS-2 causes a movement of Phe518 that further increases the size of the side pocket (Figure 6). The larger main channel combined with the extra nook increases the volume of the PGHS-2 NSAID binding site by about 20% over that in PGHS-1 (65, 66). This extra size is a structural feature exploited by COX-2 inhibitors. When access to this side pocket is restricted in PGHS-2 by mutating Val523 to an isoleucine, PGHS-2 is no longer differentially sensitive to these inhibitors, and conversely, a I523V mutation in

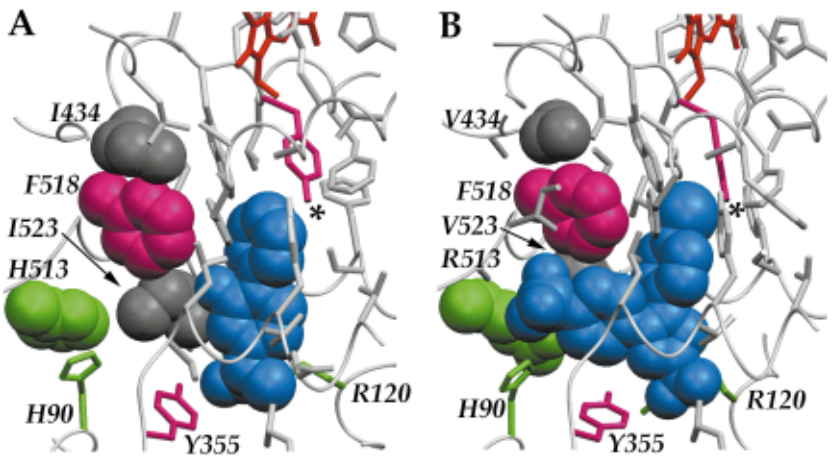

Figure 6 NSAID binding to the cyclooxygenase active sites of PGHS-1 versus PGHS-2. (*A*) Flurbiprofen bound in the COX active site channel in ovine PGHS-1 (64). Residues Ile434, His513, Phe518, and Ile523 shown as *space-filling*; Arg120 (*green*) extends behind flurbiprofen to its carboxylate. (*B*) The COX-2 inhibitor SC-588 bound in the COX active site channel of mouse PGHS-2 (66). Residues Val434, Arg513, Phe518, and Val523 shown as *space-filling*; the phenylsulfonamide group of SC-588 extends into the side pocket made accessible by Val523 (*barely visible behind the drug*) and interacts with Arg513. Access to the side pocket is made easier by the I434V change in PGHS-2, which allows Phe518 to move out of the way when drugs bind in this pocket.

PGHS-1 increases its affinity for COX-2 inhibitors (116–118). The larger effective size of the central channel in the PGHS-2 may also preferentially reduce steric and ionic crowding by the charged Arg120 in PGHS-2 and thus may enhance the binding of nonacidic NSAIDs by this isozyme. Finally, the substitution of His513 in PGHS-1 with Arg513 in PGHS-2 alters the chemical environment of the side pocket (116). This substitution results in a stable positive charge being placed at the center of this pocket, which can interact with polar moieties entering the pocket (66). For example, Arg513 appears to interact with the 4-methysulfonyl or 4-sulfonamoylphenyl substituents of diarylheterocyclic COX-2 inhibitors and give rise to the time-dependent inhibition displayed by this class of inhibitors (116). I523V/H513R PGHS-1 interacts with COX-2 inhibitors much as PGHS-2 does (66).

Other mechanisms for drug binding and isozyme selectivity in the PGHS isozymes also exist. For example, the zompirac class of inhibitors may require the presence of Arg513 to exhibit tight binding and time-dependent inhibition, although Arg513 does not interact directly with these inhibitors (65). In this case, Arg513 forms hydrogen bonds with Glu524 and Tyr355 at the base of the NSAID binding pocket, causing a change in the conformation of Arg120. This structural perturbation is significant enough to alter the structure of the mouth of the active site (65). Although the physical basis for the tighter binding is not completely clear, inhibition by these compounds does not actually involve binding to the side pocket in PGHS-2.

Time-Dependent Inhibition by NSAIDs

The new COX-2 inhibitors exhibit PGHS-2 selectivity because they inhibit this isoform by a time-dependent, pseudoirreversible mechanism, whereas they inhibit PGHS-1 by a rapid, competitive, and reversible mechanism (3, 114). The practical result of this mixed mode of inhibition is that when the blood concentration of a PGHS-2–selective NSAID is below that required for half-maximal inhibition (IC_{50}) of PGHS-1, the activity of PGHS-1 will be minimally affected by the inhibitor, while PGHS-2 becomes effectively inactivated. The physical basis for time-dependent inhibition of the COX isozymes is not well understood, and indeed seems to vary among inhibitor classes, and even between drugs within a given class. For example, flurbiprofen inhibits PGHS-1 by a time-dependent, pseudoirreversible mechanism, but ibuprofen, a close chemical relative, does not (87). For most acidic time-dependent NSAIDs, interaction with Arg120 appears to be required (91, 93). As mentioned earlier, time-dependent inhibition of PGHS-2 by COX-2 inhibitors containing sulfonamide or methylsulfoxide moieties apparently arises from their interaction with Arg513 (116). Curiously, inhibition by the methylsulfoxide inhibitor NS-398 appears to depend on interaction with Arg120 but not with Arg513. NS-398 binds in the PGHS-2 active site similarly to acidic inhibitors and inhibits the R120E and R120Q PGHS-2 mutants only competitively (92, 94).

SEPARATE BIOSYNTHETIC AND SIGNALING PATHWAYS FOR PGHS-1 AND PGHS-2

Segregated Signaling by PGHS-1 and PGHS-2

Studies of knockout mice that do not express either PGHS-1 or PGHS-2 (119), as well as analyses conducted with isozyme selective inhibitors (114) have established that each PGHS isozyme subserves unique physiological functions. Moreover, selective synthesis of prostanoids via PGHS-2 has been observed in cells that also express PGHS-1 (25, 120–124), indicating that the PGHS-1 and 2 biosynthetic pathways are segregated within cells. A key question is why two PGHS isozymes are necessary. We suspect the answer is that having metabolically distinct PGHS-1 and 2 biosynthetic pathways permits products derived from the two pathways to be channeled to different downstream signaling targets. More specifically, we hypothesize that prostanoids arising via PGHS-2 can signal uniquely through a nuclear pathway in addition to signaling through G protein–linked receptors, whereas products derived via PGHS-1 probably signal only through cell surface receptors.

An obvious factor permitting PGHS-1 and 2 to signal independently is differential gene expression (2). PGHS-1 is expressed constitutively and generally produces prostanoids acutely in response to hormonal stimuli to fine-tune physiological processes requiring instantaneous, continuous regulation (e.g. hemostasis) (6); in contrast, PGHS-2 is inducible and typically produces prostanoids that mediate responses to physiological stresses such as infection and inflammation (125, 126), hypotonicity (127), hyperpolarization in nerve cells (128, 129), and mechanical or shear stress (130, 131). In addition to differential expression, metabolic separation of PGHS-1 and 2 may also depend on subtle differences in kinetic properties, subcellular compartmentation, and interactions with phospholipases and synthases (Figure 2).

Mechanisms for Segregated Signaling by PGHS-1 and PGHS-2

Although there are no gross differences in the subcellular locations of PGHS-1 and 2 (72), PGHS-2 is more highly concentrated in the nuclear envelope (71), which may contribute to the segregation of PGHS-1 and 2 systems. Nonetheless, because PGHS-1 and 2 have similar V_{max} and K_m values with arachidonate and because arachidonate diffuses freely within cells, it was difficult to envision how separate biosynthetic systems could coexist. The breakthrough observation in this area was made by Swinney and coworkers (105); as outlined above, negative allosteric regulation of PGHS-1 permits PGHS-2 to oxygenate low concentrations of arachidonate ($<1\ \mu$M) up to four times more efficiently (42, 105). Thus, simply limiting intracellular substrate concentrations effectively directs arachidonate to the PGHS-2 pathway. For example, arachidonate added at concentrations as low as 1 μM stimulates prostanoid synthesis by intact cells expressing PGHS-2, whereas

concentrations in excess of 10 μM must be added to stimulate prostanoid synthesis in cells expressing only PGHS-1 (132).

The kinetic model for segregating PGHS-1 and 2 pathways explains the patterns of PGHS-2–dependent prostanoid synthesis in cultured cell lines (25, 120) and primary cultures of macrophages (121, 122) and mouse bone marrow–derived mast cells (BMMC) (123, 124). Stimulating these cells with growth factors, hormones, cytokines, or lipopolysaccharide (LPS) results in a biphasic production of prostanoids: (*a*) an initial burst of PGHS-1–dependent synthesis that lasts 10–30 min followed 2–12 h later by (*b*) a delayed phase of slow synthesis that is coincident with and dependent on PGHS-2 induction. Delayed-phase prostanoid production typically lasts 6–24 hrs and is characterized by a slow, prolonged synthesis of prostanoids probably associated with low intracellular arachidonate concentrations.

Although low arachidonate concentrations favor prostanoid synthesis via PGHS-2, high concentrations favor PGHS-1. For example, high concentrations of arachidonate (>25 μM) produced about fivefold more PGE_2 by cells expressing PGHS-1 than by cells expressing PGHS-2 (132). Preferential channeling through PGHS-1 also occurs in cells expressing both isozymes simultaneously. The production of both PGD_2 in BMMC following immunoglobulin E (IgE) activation (133) and of PGE_2 in ionophore-stimulated rat fibroblasts proceed through PGHS-1 (120). Ionophore activates Ca^{2+}-dependent phospholipases nonspecifically and would produce relatively high concentrations of free arachidonate; activation of IgE receptors in mast cells likely has similar effects. The mechanism for enhanced metabolism of arachidonate by PGHS-1 probably does not depend solely on kinetic properties because the K_m and V_{max} values for the two isozymes are nearly identical (1). Preferential synthesis via PGHS-1 probably relies more on the relative levels of expression; in many cells PGHS-2 levels typically do not reach more than 20–30% that of constitutive PGHS-1 (134).

Coupling of Phospholipases to PGHS-1 and PGHS-2

Three phospholipase A_2 (PLA_2) forms are primarily involved in agonist-stimulated arachidonate release: type IV cytoplasmic (c)PLA_2 and types IIa and V secreted (s)PLA_2 enzymes (132, 135). There appears to be no general pattern of coupling between specific PLA_2s and specific PGHS isoforms. Indeed, each PLA_2 could theoretically provide arachidonate for both PGHS-1 and PGHS-2, and they do so when coexpressed in engineered HEK 293 cell lines (132). Moreover, there is no direct physical interaction between the PLA_2s and PGHSs; cPLA_2 is a cytoplasmic enzyme, the sPLAs are secreted, and both PGHSs are present in the ER lumen. While numerous observations suggest that specific PLA_2s provide arachidonate selectively to PGHS-1 versus PGHS-2, this coupling probably depends primarily on coincidental expression of PLA_2s and PGHSs and not a unique physical or metabolic interaction between individual PLA_2s and PGHSs.

Coupling of PLA_2s to PGHSs also seems to depend on the amplitude of the activation of individual PLA_2s caused by different stimuli. For example, in

engineered HEK 293 cells (132), acute bradykinin-dependent activation of $cPLA_2$ or type IIa or V $sPLA_2$ leads to PGE_2 production via both PGHS-1 and PGHS-2. However, when the same cells are subjected to a delayed-phase type of activation by serum plus interleukin-1β (IL-1β) products are formed only via PGHS-2. Apparently, bradykinin activates PLA_2s robustly, and serum and IL-1β activate them only modestly.

There is a hierarchy to the activation of PLA_2s involving a particular dependence on $cPLA_2$. Initial activation of $cPLA_2$ is essential for both acute and delayed phases of arachidonate release. Early-phase (ionophore-stimulated) and late-phase (LPS-stimulated) PGE_2 is abrogated in peritoneal macrophages from cPLA-knockout mice (136, 137), and similarly, PGD_2 synthesis resulting from activation of IgE receptors (early-phase) and cytokine receptors (late-phase) is absent in mast cells from these animals. The physical properties of $cPLA_2$ make it an ideal candidate to regulate prostanoid synthesis. $cPLA_2$ preferentially utilizes arachidonate containing phospholipids, and this enzyme can be activated by any signaling pathway that results in increases in intracellular Ca^{2+} or activation of the MAP kinases (138). It is not known how $cPLA_2$ activation leads to eicosanoid production. $cPLA_2$ could provide the bulk of arachidonate used in early or late prostaglandin synthesis, or $cPLA_2$ could simply act as a catalyst to stimulate secretion (or synthesis) of $sPLA_2$s necessary for early or late prostanoid synthesis. Each of these scenarios has been observed. In $P388D_1$ cells (139, 140), MMC-34 mast cells (123), and fibroblasts (141), acute prostanoid synthesis requires $sPLA_2$. Because experiments with $cPLA_2$ knockout mice indicate that activation of $cPLA_2$ is needed for ligand-induced prostanoid synthesis (136, 137), arachidonate released by $cPLA_2$ in these cells may simply act as a trigger to stimulate exocytosis and activation of $sPLA_2$. In isolated BMMC (142), the $P388D_1$/MAB clonal line (25), and rat 3Y1 fibroblasts (120), $cPLA_2$ provides the bulk of arachidonate for acute eicosanoid synthesis, while $sPLA_2$ provides substrate for delayed-phase synthesis. In the MC3T3-E1 cell line and in primary cultures of mouse osteoblasts, $cPLA_2$ provides arachidonate for both acute and delayed-phase prostanoid synthesis (143, 144).

Finally, it should be noted that activation of $cPLA_2$ and the release of arachidonate can also somehow initiate de novo synthesis of proteins required for delayed signaling. cPLA activation is required for induction of type V $sPLA_2$ in $P388D_1$ and mast cells (25, 142), and type IIa $sPLA_2$ in rat fibroblasts (120). Failure of this activation mechanism has been observed with mast cells from $cPLA_2$ knockout mice where cytokines do not induce PGHS-2 expression or delayed-phase synthesis of PGD_2 (124); however, mast cells stimulated with cytokines together with arachidonate or PGE_2 do express PGHS-2, although they later do not synthesize PGD_2. Thus, early activation of $cPLA_2$ in mast cells triggers signaling that leads to the coordinate expression of PGHS-2 and the $sPLA_2$ responsible for late-phase eicosanoid synthesis. The observation that arachidonate can restore PGHS-2 expression, but not PGD_2 synthesis, in mast cells from $cPLA_2$-deficient mice suggests that both $cPLA_2$ and type V $sPLA_2$ are involved in delayed-phase PGD_2 production in these cells.

Coupling the PGHSs to the Terminal Prostaglandin Synthases

Because prostanoids interact with individual receptors that each elicit specific biological activities, differential signaling by PGHS-1 and PGHS-2 could result from selective coupling to the terminal isomerases or reductases that convert PGH_2 to the signaling prostanoids (Figure 2). PGHS-2 appears to couple preferentially to PGE synthase, PGI_2 synthase, or both. For example, there is a coordinate induction of both PGHS-2 and PGE synthase in A549 cells following IL-1 stimulation (29, 145) and in BMMC after tumor necrosis factor alpha (TNFα) stimulation (146). Resident peritoneal macrophages switch from forming a mixture of PGI_2, TxA_2, PGD_2, and 12*S*-hydroxy-5z,8E,10E-heptadecatrienoic acid (HHT) to producing primarily PGE_2 and PGI_2 when PGHS-2 is induced by LPS (121, 122). Coupling of PGHS-2 to PGI_2 synthase has also been observed in the cardiovascular system (147, 148). Secretion of prostacyclin, but not thromboxane metabolites, is reduced by 75%–80% in volunteers given anti-inflammatory doses of Celebrex and Vioxx. These experiments suggest that PGHS-2 is the primary source of vascular and renal prostacyclin. PGHS-2–derived PGI_2 signaling is also important for embryo implantation in mouse uteri (149, 150). PGHS-2 is elevated at sites of implantation in mice uteri (149), and ova do not implant in PGHS-2 knockout mice (150). PGI_2 synthase and PGHS-2 expression is coincident at sites of implantation (34), and the addition of synthetic analogs of PGI_2 allows implantation to proceed in PGHS-2–deficient mice. No PGI_2 cell surface receptors are detected at sites of implantation. Rather, PGI_2 signaling appears to be mediated through the nuclear PPARδ receptor (34). If confirmed, this would be the first established example of a common prostanoid mediating a physiological event via a nuclear receptor. Nuclear signaling could provide a mechanism for the unique function of PGHS-2 in cancer.

Preferential coupling of terminal synthases to PGHS-2 could be achieved by coordinating their expression with that of PGHS-2, provided that arachidonate levels are maintained below those necessary for PGHS-1 activity. This is likely what happens with PGE_2 synthase and PGHS-2 in A549 cells (29). Alternatively, selective expression of the PGHSs with specific synthases, such as PGHS-1 and TxA_2 synthase in platelets, could ensure specific coupling. Protein-protein interactions, differences in kinetic properties, or localization of the terminal prostaglandin synthases may also result in selective coupling, but no direct evidence for these mechanisms exists.

REGULATION OF PGHS-1 AND PGHS-2 EXPRESSION

Regulation of PGHS-1 Expression

Because PGHS-1 is expressed constitutively in most tissues, and expression levels of this enzyme do not vary greatly in adult animals, it has been difficult to study transcriptional regulation of the PGHS-1 gene. Nevertheless, PGHS-1 is

preferentially expressed at high levels in selected cells and tissues, including endothelium, monocytes, platelets, renal collecting tubules, and seminal vesicles, indicating that it is developmentally regulated. As might be expected, expression of PGHS-1 increases in cell lines that undergo differentiation and thus mimic developmental processes [reviewed in (1)]. Unfortunately, these cell systems have not proved useful for promoter analysis. Endothelial cells, which express constitutively high levels of PGHS-1, have been employed with moderate success to examine transcriptional regulation of this gene (151). The PGHS-1 gene has a TATA-less promoter that contains multiple start sites for transcription (152, 153). Two elements have been identified that contribute to constitutive expression of PGHS-1 in human umbilical vein endothelial cells (HUVEC) (153). Gel shift assays have demonstrated that Sp1 *cis*-regulatory elements in the human PGHS-1 promoter, at positions −111/−105 and −610/−604, bind the *trans*-activating Sp1 protein. Deletion of either site leads to a reduction of about 50% in basal transcription, and deletion of both sites results in a reduction of about 75%. To date these Sp1 sites are the only *cis*-acting elements documented to regulate transcription of PGHS-1.

Regulation of PGHS-2 Expression

Examination of the cells and tissues in which PGHS-2 is expressed or can be induced and of the various factors and conditions that stimulate expression of this enzyme (Table 1) allows some general conclusions to be drawn about regulation of PGHS-2. The PGHS-2 gene is particularly responsive to and most commonly elevated by growth factors and mediators of inflammation such as IL-1, TNFα, LPS, and 12-O-tetradecanoylphorbol 13-acetate (TPA); moreover, glucocorticoids and anti-inflammatory cytokines suppress PGHS-2 expression. Selective inhibitors of PGHS-2 have confirmed that this enzyme plays a critical role in inflammation, pain, and fever (154). Regulation by growth factors suggests that PGHS-2, like immediate early genes, such as c-*fos* and c-*myc*, also plays some general role in mitogenesis, and perhaps a specialized role in wound repair. The obligatory contribution of PGHS-2 to the development of cancers of the colon further supports a role for this enzyme in control of cell growth separate from that of PGHS-1 (155, 156).

Inducible or constitutive expression of PGHS-2 also occurs in specialized cell types or tissues where PGHS-2 plays specific functions in individual biological processes. These include reproduction (34, 150), immunity (157), renal physiology (158), neurotransmission (159), bone resorption (160), and pancreatic secretion (161) (Table 1). Factors that regulate PGHS-2 expression in these cells and tissues are specific for the physiological processes and tissues involved. For example, PGHS-2 expressed in granulosa cells can be induced by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (166, 167), while in the kidney, expression of PGHS-2 in the macula densa depends on lumenal salt concentrations (168). Thus, expression of PGHS-2 in many specialized cell types appears to be

TABLE 1 Regulators of PGHS-2 expression[a]

Cell type	Inducer	References
Human 1483 oral epithelial cells	Benzo[α]pyrene, EGF, TPA, retinoids ($\downarrow$)[b], caeffeic acid phenyl ester ($\downarrow$)	(199–202)
Murine embryo (10)1 fibroblasts	p53	(203)
Human 184b5 mammary epithelial cells	TPA, ceramide, peroxisome proliferators, NSAIDs, Reservatol ($\downarrow$)	(38, 180, 183)
Human colonocytes	Transformation	(13)
Human carcinomas	Transformation	(12, 204, 205)
Murine mammary epithelial cells	Wnt-1, v-Src, v-Ras	(206, 207)
Colon adenoma HT-29 cells	Invasive bacteria	(208)
Human SK-GT-4 adenocarcinoma	Bile acids, Curcumin ($\downarrow$)	(188)
Rat IEC-6 intestinal epithelial cells	TPA, IL-1β	(209)
Rat RIE-1 intestinal epithelial cells	TGFα	(210, 211)
Rat bronchial epithelial cells	TPA, EGF	(212)
Bovine endothelial cells	25-Hydroxycholesterol	(213)
Human vascular endothelial cells	IL-1β, TPA, LPS, TNF	(214)
Human rheumatoid synovial endothelial cells	IL-1	(215)
Murine liver cells	Peroxisome proliferators	(216)
MDCK dog kidney cells	TCDD	(217)
Rat kidney macula densa	High salt	(168)
Rat mesangial cells	IL-1β, serotonin, endothelin	(218–220)
Pancreatic islet cells	IL-1, constitutive	(161)
Osteoclasts	Parathyroid hormone	(221)
Murine and human osteoblast	EGF, norepinephrine, serum, TGFβ, forskolin, TGFα, IL-1α	(222–228)
Murine neonatal calvariae	Parathyroid hormone, PGE_2, IL-1α, bFGF	(229)
Murine 3T3 fibroblasts	TPA, EGF, forskolin, serum, PDGF, v-Src, dexamethasone ($\downarrow$)	(194, 230, 231)
Murine C127 fibroblasts	Serum, v-Src, PDGF	(232)
Murine embryo fibroblasts	TPA, PDGF	(233)
Chick embryo fibroblasts	v-Src, PDGF, serum, TPA	(234)
Human embryo fibroblasts	IL-1β	(235)

(*continued*)

TABLE 1 (*Continued*)

Cell type	Inducer	References
Rat fibroblasts	Ha-Ras	(191)
Human keratinocytes	UV-B irradiation	(193)
Murine RAW 264.7 macrophages	LPS, INF-gamma	(236)
Murine peritoneal macrophages	LPS, dexamethasone (↓)	(237–239)
Murine tumor-derived macrophages	LPS	(236)
Human monocytoid U937	Platelet microparticles	(240)
Human monocytes	IL-1β, TPA, Con-A, IL-10 (↓), IL-4 (↓)	(232, 241–243)
Human alveolar macrophages	LPS	(134)
Rat vascular smooth muscle cells	Serum	(244)
Rat aortic smooth muscle cells	Serum, PDGF, EGF, ECGF, FGF, thrombin, TPA	(245)
Rat cardiomyocytes	H_2O_2	(187)
Human peritoneal mesothelial cells	IL-1β, TNFα	(246)
Human rheumatoid synoviocytes	IL-1β, TPA	(247–249)
Rat ovarian granulosa cells	FSH, LH	(166, 167)
Human amnion cells	Spontaneous labor	(250, 251)
Murine decidual cells	Endotoxin	(252, 253)
Amniotic WISH cells	Hypotonic stress	(127)
Central nervous system neurons	Depolarization, dexamethasone (↓)	(128, 129)
Murine bone marrow–derived mast cells	(Kit ligand + IL-10 + IL-1β), dexamethasone (↓)	(133, 142, 254)
Murine mast cell lines	(IgE + αIgE), Ca^{2+} ionophore	(255, 256)
Murine bone marrow–derived mast cells	(IgE + αIgE), Ca^{2+} ionophore	(255, 256)

[a]Updated from Reference 257.

[b](↓) Indicates effectors that inhibit expression of PGHS-2.

Abbreviations not used in the text are TGFα, transforming growth factor alpha; TCDD, 2,3,7,8-tetrachlorodibernzo-[1,4]-dioxin; bFGF, basic fibroblast growth factor; Con-A, conavalin A; ECGF, endothelial cell growth factor.

differentially sensitive to stimuli that regulate the unique physiological activities of each tissue.

Signal Transduction Pathways Linked to Transcriptional Activation of the PGHS-2 Gene It is difficult to identify specific signaling pathways for all of the many activators and different conditions that lead to transcriptional activation of the PGHS-2 gene. However, for the inflammatory agents, such as TNFα, IL-1β, TPA, and LPS, and for growth factors, a number of shared or convergent

pathways are likely regulate transcription of PGHS-2. These include NFκB and C/EBP, two common signaling pathways in inflammatory response (169, 170), as well as one or all of three mitogen-activated protein kinase (MAPK) cascades: ERK1/2, JNK/SAPK, and p38/RK/Mpk2 (138). Each of these signaling pathways has been shown to contribute or be solely required for increased expression of PGHS-2 in one or more cultured cell systems that have been studied (Tables 2 and 3).

Regulation of PGHS-2 Expression by the NFκB Signaling Pathway NFκB signaling has been implicated variously in the expression of PGHS-2 stimulated by TNFα, hypoxia, endothelin, and IL-1β in osteoblastic cells (171), synoviacytes (172, 173), epithelial cells (174, 175), endothelial cells (176), and hepatocytes (177) (Table 2). Each of these effectors, as well as LPS, can activate the NFκB signaling pathway (170, 178), and the PGHS-2 promoter contains two consensus sequences for the *cis*-acting regulatory sequences that are recognized by the NFκB family of transcription factors (Table 4). Evidence that activation of NFκB is required for induction by these treatments, and is not simply coincidental, includes experiments that show inhibition of PGHS-2 expression by antisense oligonucleotides for the p65 protein (172, 173), by decoy oligonucleotides containing the NFκB binding motif, by expression of negative-dominant IκB mutants (175), and by use of salicylate and MG-132, two inhibitors of the IκB kinase (177). Electrophoretic mobility shift assays (EMSAs) have also demonstrated binding of p50 and p65, two of several proteins that can form NFκB complexes (170), to their cognate recognition sequences in PGHS-2 promoters (171, 173, 176, 179). Furthermore, transfection experiments with reporter plasmids have demonstrated that mutations in the NFκB *cis*-regulatory sites attenuate transcriptional activation of the PGHS-2 promoter in response to TNFα stimulation (171). Together these provide convincing evidence that NFκB regulates PGHS-2 expression in response to the appropriate activators in specific cell types.

Regulation of PGHS-2 Expression by MAPK Signaling Pathways Among the major signaling pathways employed by growth factors, and by oncogenes including v-*src* and v-*ras*, are the MAPK signaling cascades (138). Inflammatory stimuli, including IL-1β, TNFα, and LPS, as well as the phorbol ester TPA (180), can all activate the MAPK pathways (138, 170, 181). Activation of these kinase pathways has been shown to be required for ligand-induced and oncogene-dependent expression of PGHS-2 in a variety of cultured cell lines, and in monocytes ex vivo (Table 3). Dependence on kinase signaling for PGHS-2 expression has been demonstrated by overexpressing active kinases, or conversely by using dominant negative mutant kinases (182–184), and more recently by using small molecule inhibitors that selectively block one or more of the ERK1/2, JNK/SAPK, and p38/RK/Mpk2 pathways (184–191).

Lipopolysaccharide LPS-stimulated expression of PGHS-2 in monocytes, as well as release of IL-1, IL-8, and TNFα, is inhibited by the MAP/ERK kinase

TABLE 2 Agents that activate transcription of PGHS-2 via the NFκB signaling pathway

Effector	NFκB	References
TNFα	p50/p65; p50/p50	(175, 177, 258)
Hypoxia	p65	(176)
Il-1β	p50/p65; p50/p50	(173, 179)
Endothelin	p50/p65; p50/p50	(177)

inhibitor U0126 (185). JNK/SAPK and p38/RK/Mpk2 are unaffected by U0126 treatment, suggesting that ERK1/2 may be the primary LPS-activated pathway regulating PGHS-2 expression in these cells. Nevertheless, SB203580, an inhibitor of the p38/RK/Mpk2 pathway, also attenuates PGHS-2 expression in monocytes. However, it does so by destabilizing PGHS-2 mRNA, and has only a small effect on PGHS-2 transcription, suggesting that the p38/RK/Mpk2 pathway may be more important for posttranscriptional regulation of PGHS-2 in LPS-stimulated monocytes (186, 188).

Interleukin-1 Studies examining IL-1–stimulated expression of PGHS-2 indicate that the JNK/SAPK and p38/RK/Mpk2 are the relevant MAPK pathways activated by this cytokine (184, 188–190, 192). The p38 kinase inhibitor SC68376 prevented IL-1–stimulated induction of PGHS-2 in mesangial cells (184, 189, 190),

TABLE 3 Agents that activate transcription of PGHS-2 via the kinase signaling pathways

	Kinase pathways			
Effector	ERK1/2	JUNK/SAPKs	p38/RK/Mpk2	References
IL-1β		X	X	(184, 188–190, 192)
Platelet microparticles	X	X	X	(240)
Smase[a]	X	X	X	(183)
H_2O_2	X			(187)
ras	X			(191)
LPS	X		X	(185, 186)
src		X		(182)
PGDF	X	X		(194)

[a]Sphingomyelinase: releases ceramide from sphingomyelin.

TABLE 4 Protein factors and *cis*-elements involved in transcriptional regulation of PGHS-2[a]

Cell/tissue	Effector	Transcription factors	Responsive elements	References
Murine JWF2 skin carcinoma	Transformation	C/EBPβ, δ; USF-1	NF/IL6; E-box	(131)
Murine mammary epithelial cells	v-Src, v-Ras	ND	ATF/CRE	(206)
Murine NIH3T3 fibroblast	v-Src, serum, PDGF	c-Jun (CREB$\downarrow$)[b]	ATF/CRE	(194, 231)
Murine MC3T3 oesteoblasts	TNFα	p50/p65 C/EBP	NFκB, NF/IL6	(171)
Rat granulosa cells	LH, FSH, GnRH[c]	USF	E-box	(196)
Human vascular endothelial cells	LPS, TPA	C/EBPδ	NF/IL6, ATF/CRE	(195)
Human vascular endothelial cells	Hypoxia	p65	NFκB	(176)
Human mammary epithelial cells	TPA	ND	ATF/CRE	(180)
Human U937 monocytoid cells	TPA	ND	ATF/CRE	(259)

[a]See Footnote b in Table 1.

[b]Overexpression of CREB transcription factor inhibits expression of PGHS-2 by binding to, but not activating transcription from, the PGHS-2 promoter (194).

[c]GnRH, gonadotropin-releasing hormone.

as did the p38 kinase inhibitor SB203580 in HeLa (188) and vascular endothelial cells (192). In mesangial cells, overexpression of dominant negative mutants of JNKK/MKK4, which prevents activation of JNK/SAPK, also attenuated IL-1 dependent PGHS-2 expression (190), suggesting that both the JNK and p38 signaling pathways may be involved.

The p38/RK/Mpk2 signaling pathway is also activated by osmotic stress and UV irradiation (138). Although it has not been demonstrated directly, it is likely that induction of PGHS-2 in human keratinocytes by UV-B irradiation (193) and in WISH cells by hypotonic shock (127) is also mediated by the p38/RK/Mpk2 MAPK pathway.

Growth Factors and Oncogenes Cellular responses to mitogens are generally mediated by sequential activation of receptor tyrosine kinases, Src, Ras, and one or more of the MAPK pathways. It is not unexpected, therefore, that expression of PGHS-2 following stimulation with serum and platelet-derived growth factor

(PDGF), or in response to v-Src or Ha-Ras expression, requires activation of the ERK1/2 and JNK/SAPK pathways (182, 191, 194). Transcriptional activation of the PGHS-2 promoter by v-Src is attenuated by overexpression of dominant negative mutants of either MEKK-1 or JNK, demonstrating a requirement for the JNK kinase cascade for signal transduction by this oncogene (182). Serum- and PDGF-stimulated activation of the PGHS-2 promoter is also inhibited by dominant negative Ras, Rac1, MEKK1, and JNK, as well as dominant negative Raf1 and Erk, implicating both the JNK and ERK cascades in signal transduction by these mitogens (194). Expression of the constitutively active Ha-Ras$^{Val\text{-}12}$ from an IPTG-inducible promoter elevates PGHS-2 expression in rat fibroblasts and intestinal epithelial cells (191). The kinase inhibitor PD 98059, which specifically blocks MEK activity, and thus activation of the ERK1/2 kinases, delays PGHS-2 expression in these cells following induction of Ha-Ras$^{Val\text{-}12}$.

Cis-Regulatory Elements Involved in Transcriptional Control of PGHS-2 Gene Expression Although numerous possible regulatory elements have been identified in the 5′-flanking regions of the PGHS-2 genes, only five have been rigorously demonstrated to regulate transcription: overlapping E-box and ATF/CRE sequences, an NF/IL6 CAAT enhancer binding sequence, and two NFκB binding sites (Table 4 and Figure 7).

Published reports suggest that the most critical of these regulatory sequences is the ATF/CRE, a site that typically is activated by hetero- and homodimers of the c-Fos, c-Jun, and ATF families of bZIP proteins (AP-1) (194), and the cAMP regulatory binding protein (CREB). Mutations in the ATF/CRE sequence attenuate serum-, PDGF-, and Src-stimulated transcription from the mouse PGHS-2 promoter by 70%. The adjacent E-box sequence does not appear to be involved in 3T3 fibroblasts, as mutations in the nonoverlapping portion of this response element had no effect on PGHS-2 transcription. Deletion of the ATF/CRE also effectively abolished TPA-stimulated transcription from a human PGHS-2 promoter transfected into mammary epithelial cells (180). However, TPA and LPS stimulate PGHS-2 expression in human endothelial cells through cooperative activation of both the ATF/CRE and NF/IL6 regulatory elements. Individual mutations in either the ATF/CRE or NF/IL6 sites inhibit transcription modestly (25–40%); mutations in both sites are required for significant attenuation ($\geq$75%) (195).

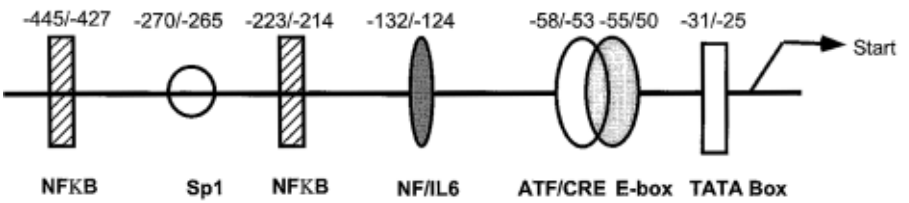

Figure 7 Regulatory elements in the human PGHS-2 promoter. (Adapted from Reference 171.)

In fibroblasts, transcription from the ATF/CRE element is mediated by the c-Jun *trans*-regulatory protein (182, 194), but not by CREB, which binds the promoter but cannot activate (182, 194). Chimeric transcription proteins containing the activator region of c-Jun and the DNA binding region of yeast GAL4 activate transcription from promoters containing GAL4 DNA-binding sites in 3T3 cells stimulated with serum and PDGF (194). Mutation of the c-Jun phosphorylation sites prevents *trans*-activation by the c-Jun/GAL4 chimeras in this system. These experiments show that activation of c-Jun is necessary and sufficient for transcriptional activation of the PGHS-2 promoter, and they provide the most complete signal transduction pathway yet delineated for expression of PGHS-2. Activation of extracellular tyrosine kinase receptors by growth factors leads to sequential activation of Src, Ras, and the ERK1/2 and JNK/SAPK kinase cascades resulting in phosphorylation of c-Jun and transcriptional activation of the PGHS-2 gene. Whether c-Jun forms homodimers or interacts with other bZIP proteins is not yet known.

Although the E-box is not involved in regulation of PGHS-2 expression in mouse fibroblasts and human epithelial cells, it is required for hormonal regulation of PGHS-2 in rat granulosa cells (196) and for the elevated expression of PGHS-2 in the murine carcinoma cell line JWF2 (197). The rat gene does not contain a consensus ATF/CRE sequence, suggesting that regulation of the PGHS-2 gene may be different in this species. In both granulosa and JWF2 cells, the transcriptional regulatory proteins USF-1 bind the E-box sequences and presumably activate transcription. As only CREB and c-Jun were observed to bind the CRE/E-box regions when nuclear extracts from murine fibroblasts were tested, it seems likely that differential utilization of *cis*-regulatory elements in these various cell types may be due to differential expression of *trans*-regulatory factors.

Circumstantial evidence that NFκB regulatory elements play a role in regulation of PGHS-2 has been provided by electrophoretic mobility shift assays (EMSAs) in a number of cells. However, these experiments simply show that NFκB can bind to PGHS-2 NFκB sequences (Table 2). Direct evidence that NFκB activates transcription, as determined using reporter gene constructs containing native and mutated promoter sequences, has only been reported for the MC3T3-E1 osteoblasts and human endothelial cells (171, 176). In murine MC3T3-E1 cells, TNFα-stimulated expression is dependent on two response elements, the distal NFκB site ($-445/-427$ in the human promoter; Table 4) and the NF/IL6 site (Figure 7) (171). EMSA studies with nuclear extracts from this cell line confirmed that both NFκB (p50 and p65) and CEBP proteins bind their cognate regulatory sequences. In hypoxic human endothelial cells (176), induction of PGHS-2 depends on the proximal NFκB site ($-223/-214$; Table 4) and EMSA experiments show binding of p65 (176).

The C/EBPβ and C/EBPδ transcription factors are commonly involved in the regulation of inflammatory responses, and NF/IL6 regulatory elements are frequently found in promoters of acute phase genes (177). This family of transcription factors is activated by most of the inflammatory stimuli that induce PGHS-2

expression. Regulation of C/EBP transcription factors occurs both by increasing their synthesis and by phosphorylation of specific serine and tyrosine residues (177). An NF/IL6 regulatory element is present in the PGHS-2 promoters from all species examined (Figure 7), and C/EBP proteins have been shown to bind to these promoter sequences (Table 4). The C/EBP transcription proteins appear not to work independently but instead to cooperate with USF-1 (131), NFκB (171), and the c-Jun (195) *cis*-regulatory proteins to activate transcription from PGHS-2 promoters.

FUTURE DIRECTIONS

As detailed in this review, there is currently a reasonable understanding of the structures of PGHS-1 and -2. In future studies of the structural biology of these enzymes, it will be particularly important to determine the basis for the allosteric inhibition of PGHS-1 by arachidonate, to determine the structure of the membrane-binding domain of PGHSs in the context of the interaction of these domains with specific membrane lipids and the role of this domain in substrate entry into the cyclooxygenase site and product exit from this site, and to explore aspects of the peroxidase active site, particularly the basis for its specificity toward PGG_2. The longstanding enigma of what structural modification accompanies suicide inactivation also needs to be defined. This work is likely to be enriched by structural studies on evolutionarily related enzymes such as the fungal linoleate diol synthase.

Our understanding of the different biological roles of PGHS-1 and PGHS-2 is only beginning to emerge. The functions of these two isozymes in apoptosis, particularly as it relates to the development of a variety of cancers (e.g. colon, lung, breast), angiogenesis, respiration, inflammation, pain, and reproduction are currently being studied in cultured cells and whole animals using isoform-specific inhibitors and gene knockout mice. On the cell and molecular biology fronts, defining the different biologies associated with PGHS-1 and PGHS-2 will be directed toward understanding the biochemical coupling of phospholipases, G protein-linked and nuclear receptors, and downstream synthases to the two isozymes and the coordinate regulation of expression of genes encoding PGHSs and related receptors, phospholipases, and downstream systhases.

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