Minireviews:

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Minireview

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Prostaglandin Endoperoxide H Synthases (Cyclooxygenases)-1 and -2*

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Prostaglandin endoperoxide H synthases (PGHSs)[1] catalyze the conversion of arachidonic acid and O_2 to PGH_2, the committed step in prostanoid biosynthesis (Fig. 1) (1). Before 1991, only one PGHS had been described, the isozyme now called PGHS-1, COX-1 (for cyclooxygenase-1) or the *constitutive* enzyme. At that time Simmons and Herschman and their colleagues discovered mRNAs whose expression was induced in chicken and mouse fibroblasts in response to *src* and tumor-promoting phorbol esters, respectively, and which encoded proteins having 60% amino acid sequence identity with PGHS-1. Subsequent work has shown that the new protein, called PGHS-2, COX-2 or the *inducible* isoform, is very similar to PGHS-1 in structure but differs substantially from PGHS-1 with respect to its pattern of expression and its biology. The reason for the existence of the two PGHS isozymes is unknown. However, PGHS-1 and -2 are often coexpressed in the same cell and may act as parts of separate prostanoid biosynthetic systems that function somewhat independently to channel prostanoids to the extracellular milieu and the nucleus, respectively.

PGHS-1 and -2 are interesting in the context of both structural biology and enzymology in that they are homodimeric, heme-containing, glycosylated proteins with two catalytic sites. Moreover, the enzymes have a novel mechanism for membrane attachment; they are anchored to one leaflet of the lipid bilayer through the hydrophobic surfaces of amphipathic helices rather than through transmembrane motifs typical of many integral membrane proteins. The isozymes are also important pharmacologically as targets of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) (2). For example, aspirin acts via PGHS-1 to inhibit platelet thromboxane A_2 formation and as a clinical consequence lowers the relative risk for mortality from cardiovascular disease (3). PGHS-2 is the relevant target of NSAIDs acting to inhibit inflammation (4, 5), fever (5), pain (4), and probably colon cancer (6–8). NSAID therapy may even retard the development of Alzheimer's disease (9) although it is not clear which PGHS isozyme may be involved. In this review we compare and contrast PGHS-1 and -2 in the context of the regulation of expression of the two enzymes, the mechanisms of enzyme catalysis, and the biological significance of having two PGHSs.

Regulation of PGHS-1 and PGHS-2 Expression

PGHS-2 is undetectable in most mammalian tissues, but its expression can be induced rapidly (2–6 h) in fibroblasts (10–13), endothelial cells (14), monocytes (15), and ovarian follicles (16) in response to growth factors, tumor promoters, hormones, bacterial endotoxin, and cytokines; in most cases the level of the protein then decreases rapidly. The rapid induction of PGHS-2 mRNA, which can be superinduced by cycloheximide, parallels the expression of c-*fos* leading to the classification of PGHS-2 as an immediate early gene. In fibroblasts, increased expression is due to an increased rate of PGHS-2 gene transcription (11). In other systems, post-transcriptional regulation contributes to the magnitude and duration of PGHS-2 mRNA expression (17). A second notable feature of PGHS-2 expression is that anti-inflammatory steroids (*i.e.* cortisol, dexamethasone) inhibit stimulated expression of PGHS-2 mRNA and protein (18–20) via both transcriptional (11) and post-transcriptional mechanisms (10, 17).

In contrast to PGHS-2, PGHS-1 can be detected in most tissues although not within all cells of a tissue (1). In cultured cells PGHS-1 is typically expressed at constant levels throughout the cell cycle (11). Hence, PGHS-1 has become known as the *constitutive* isozyme, and PGHS-2 is known as the *inducible* isoform. This is actually an oversimplification because PGHS-2 is expressed constitutively in brain (21), testes (22), tracheal epithelia (23), and the macula densa in kidney (24), while PGHS-1 levels change during development (25), and its expression can be down-regulated in endothelial cells in response to acidic fibroblast growth factor (26) and up-regulated in mast cells treated with stem cell factor plus dexamethasone (27).

PGHS-1 and -2 are encoded by separate genes located on different chromosomes. The gene for PGHS-1 is approximately 22 kilobase pairs and contains 11 exons (28). Typical of developmentally regulated "housekeeping" genes, the PGHS-1 gene lacks a TATA box. Virtually nothing is known about the details of the regulation of PGHS-1 gene expression.

The PGHS-2 gene is 8 kilobase pairs in length and contains 10 exons (18). PGHS-2 expression can be induced through multiple signaling pathways involving protein kinases A and C, tyrosine kinases, bacterial endotoxin (lipopolysaccharide), and *src*. Several relevant enhancer sequences have been identified in the PGHS-2 gene promoter. In bovine endothelial cells, a CEBPβ site is responsible for induction of PGHS-2 by lipopolysaccharides and tumor-promoting phorbol ester (29) while the same site is responsible for tumor necrosis factor α-mediated induction of PGHS-2 in MC3T3-E1 cells (30). An E-box sequence is essential for basal and luteinizing hormone- and gonadotrophin-releasing hormone stimulated transcription, and this element binds the upstream stimulating transcription factor (31). A cAMP response element mediates the effect of *src* on PGHS-2 expression in fibroblasts (32). It is not yet known how dexamethasone acts to inhibit PGHS-2 gene expression.

Mechanisms of Enzyme Catalysis

PGHSs catalyze both a cyclooxygenase (*bis*-oxygenase) reaction in which arachidonate is converted to PGG_2 and a peroxidase reaction in which PGG_2 undergoes a two-electron reduction to PGH_2 (Figs. 1 and 2). PGHS-1 and -2 have similar cyclooxygenase turnover numbers (~3500 mol of arachidonate/min/mol of dimer (33, 34)), and the K_m values for arachidonate (~5 μM (34, 35)) and O_2 (~5 μM (36))[2] are about the same for both isozymes. Furthermore, the key residues involved in catalysis are conserved between the isozymes, and the crystal structures of the two isozymes are essentially superimposable (Figs. 3 and 4). There are some subtle differences in peroxide requirements (37–39) and fatty acid substrate specificities (40), but in the overall context of catalytic mechanisms, these proteins appear to be essentially the same.

The cyclooxygenase reaction begins with the rate-limiting abstraction of the (13*S*)-hydrogen from arachidonate to yield an arachidonyl radical (Figs. 1 and 2*B* (41)). This is followed by sequential oxygen additions at C-11 and C-15 to yield PGG_2. Finally, the peroxidase activity reduces the 15-hydroperoxide group of PGG_2 to an alcohol yielding PGH_2. NSAIDs compete directly with arachidonate for binding to the cyclooxygenase site and inhibit

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[1] The abbreviations used are: PGHS, prostaglandin endoperoxide H synthase; oPGHS, ovine PGHS; PG, prostaglandin; NSAID, nonsteroidal anti-inflammatory drug; MBD, membrane binding domain; ER, endoplasmic reticulum; numbering of amino acids for PGHS-1 and -2 begins in each case with the methionine at the translation start site.

[2] L. Hsi and W. L. Smith, unpublished results.

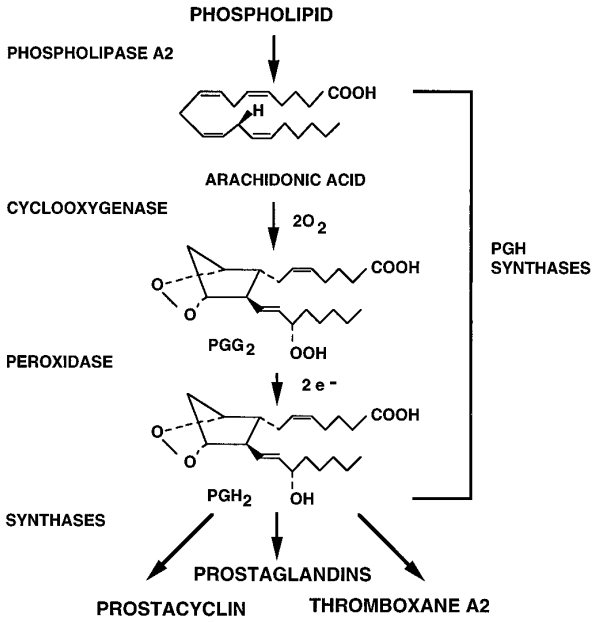

FIG. 1. **Prostanoid biosynthetic pathway.** In response to stimulation of a target cell with a relevant cytokine, growth factor, or circulating hormone, phospholipases are activated and arachidonic acid is hydrolyzed from the *sn*-2 position of membrane phospholipids. Most evidence suggests that this occurs through the action of a phospholipase A_2, either the cytosolic, high molecular weight, Ca^{2+}-dependent cytoplasmic phospholipase A_2 that becomes associated with the cytoplasmic surface of the ER and nuclear envelope and releases arachidonate from these membranes or the nonpancreatic, Ca^{2+}-dependent, Type II phospholipase A_2, which is secreted and acts on phospholipids on the extracellular surface of the plasma membrane (reviewed in Ref. 1). Arachidonate is converted by PGHS-1 or -2 to PGH_2, and then PGH_2 is isomerized to biological active prostanoid products.

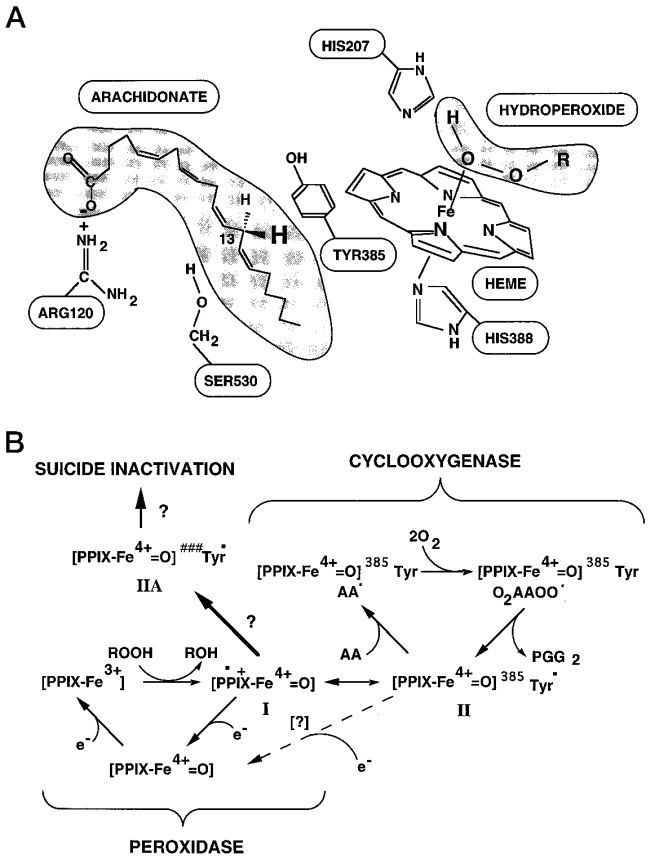

FIG. 2. **Peroxidase and cyclooxygenase catalysis.** *A*, model of the cyclooxygenase and peroxidase active sites of ovine PGHS-1. An alkyl hydroperoxide is shown bound to the heme group at the peroxidase active site, and arachidonate is shown bound to the cyclooxygenase active site. His-388 and His-207 are the proximal and distal heme ligands, respectively. Tyr-385 neighbors the heme group and bound arachidonate and is likely the residue that is converted to a tyrosyl radical and abstracts the (13*S*)-hydrogen from arachidonate, thereby initiating cyclooxygenase catalysis. Ser-530 is the site of aspirin acetylation. Arg-120 is located at the opening of the hydrophobic fatty acid binding channel and is the counterion for the carboxylate group of arachidonate. *B*, the branched chain model for the mechanistic interplay between the cyclooxygenase and peroxidase activities (54, 61). A two-electron oxidation of the heme group of PGHS by a hydroperoxide yields a peroxidase spectral Intermediate I containing an oxyferryl form of iron (Fe(IV)) and a protoporphyrin radical cation (53, 54). The oxidized heme group in turn oxidizes a neighboring tyrosine residue, probably Tyr-385 (60, 63) to yield peroxidase Intermediate II having a tyrosyl radical (56) and an oxyferryl Fe(IV) (53, 54). This protein radical is likely the species that abstracts the (13*S*)-hydrogen from arachidonate. *PPIX*, protoporphyrin IX; *AA*, arachidonic acid.

cyclooxygenase activity but have little effect on peroxidase activity (42, 43). Thus, the cyclooxygenase and peroxidase sites are physically and functionally separate.

A combination of classical protein chemistry (1), UV-visible (44, 45) and epr spectroscopy (46–49), and x-ray crystallography (50) has established that the active sites of PGHS are configured as shown in Fig. 2*A* for ovine PGHS-1 (1). The cyclooxygenase active site is a channel that is lined with hydrophobic residues and protrudes toward the center of the major globular domain of the enzyme (50).

The branched chain mechanism describing the interplay of the cyclooxygenase and peroxidase activities is shown in Fig. 2*B* (45, 51). The cyclooxygenase reaction has an absolute requirement for a hydroperoxide (33). Initially either an alkyl peroxide or peroxynitrite derived from the condensation of nitric oxide and superoxide (39) oxidizes the heme group at the peroxidase active site. The oxidized heme group then oxidizes a neighboring Tyr-385 located in the cyclooxygenase active site (46, 50, 52). The resulting tyrosyl radical abstracts the (13*S*)-hydrogen from arachidonate.

Arg-120 is another key active site residue (Figs. 2*A* and 3). It serves as the counterion for the carboxylate groups of arachidonate and common NSAIDs (50, 53, 54). Tyr-355 lies on the opposite side of the channel from Arg-120 and governs the stereospecificity of PGHSs toward NSAIDs (50, 53).

Serine 530 is the site of acetylation of ovine PGHS-1 by aspirin (Figs. 2*A* and 3 (55)). X-ray crystallographic analysis of the aspirin-acetylated enzyme (55) and studies of mutant proteins in which Ser-530 has been substituted with other residues (56) have established that when serine 530 is acetylated by aspirin the acetyl group protrudes into the cyclooxygenase active site and interferes with arachidonate binding (Fig. 3). Covalent modifications of PGHSs by aspirin cause permanent inactivation of the enzymes. The ability of aspirin to modify PGHS-1 is the basis for the unique, long lived effect of aspirin on platelet activity because circulating platelets, unlike most cells, do not synthesize new PGHS-1.

Aspirin represents a pharmacological extreme in that it causes covalent modification and irreversible inhibition of PGHSs (56, 57). At the other extreme are agents such as ibuprofen, which behave as typical reversible competitive inhibitors (35, 43). Importantly, there are a number of NSAIDs including indomethacin, flurbiprofen, and meclofenamate, which exhibit an intermediate form of inhibitory behavior and which are known as time-dependent, reversible inhibitors (1, 35, 43, 58). Binding of these drugs to PGHSs yields an initial *E*I complex typical of a reversible competitive inhibitor, but this *E*I complex slowly (in seconds to minutes) rearranges to an *E*I* complex from which the drug dissociates very slowly (minutes to hours) (1, 58).

All currently available NSAIDs inhibit both PGHS-1 and -2 and compete with arachidonate for binding to the cyclooxygenase active site (1). These compounds are effective anti-inflammatory agents, but they are also all quite ulcerogenic (2). Many pharmaceutical firms have developed new cyclooxygenase inhibitors that selectively inhibit PGHS-2. These efforts were initially driven by two notions, which subsequently proved to be correct: (*a*) that PGHS-2 is the relevant enzyme in inflammation (4) and (*b*) that PGHS-1 but not PGHS-2 is present in the stomach (59). Indeed, PGHS-2 inhibitors have been reported to be anti-inflammatory and analgesic and to lack gastrointestinal toxicity (4, 5).

All of the PGHS-2-selective agents are time-dependent, reversible inhibitors of PGHS-2 (60–62). Included in this group of agents are DuP697 (62), SC52125 (60), L-745-337 (5), NS398 (63), and meloxicam (2). Curiously, these agents are all relatively poor inhibitors of PGHS-1, and this is probably due to the one amino acid difference between PGHS-1 and -2 within the hydrophobic cyclooxygenase

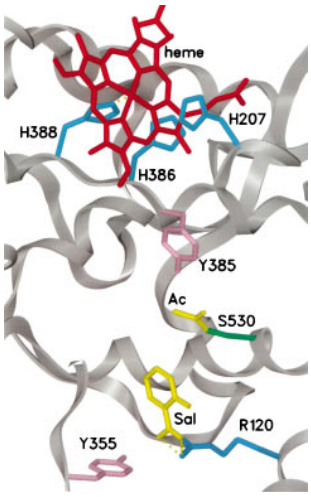

FIG. 3. **Crystal structure of the active site of aspirin-acetylated ovine PGHS-1.** The carboxylate group of salicylic acid is shown interacting with Arg-120 (67).

channel. PGHS-2 has a valine at position 509, while the corresponding amino acid in PGHS-1 is an isoleucine. V509I PGHS-2 behaves somewhat like PGHS-1 in being relatively unresponsive to time-dependent inhibition by most PGHS-2 inhibitors (60, 61).

A final notable feature of cyclooxygenase catalysis is suicide inactivation. Addition of arachidonic acid to preparations of PGHSs results in a rapid but transient burst in O_2 consumption as PGG_2 is formed (33). The rapid fall in oxygenase activity is not due to product inhibition but results from a mechanism-based inactivation of the enzyme (64). On average every cyclooxygenase molecule consumes approximately 400 arachidonate molecules before becoming suicide-inactivated (33). Little is known about the chemical changes that occur during suicide inactivation; an arachidonate metabolite is incorporated into the protein during catalysis but at a substantially slower rate than that of suicide inactivation (65). Suicide inactivation probably involves an intramolecular cross-linking reaction that results from an untoward reaction of a radical intermediate such as Intermediate II (Fig. 2*B*).

Tyrosyl Radicals

Three types of PGHS tyrosyl radical signals have been detected, but no clear relationships have been established between these signals and either enzyme catalysis or suicide inactivation. A broad doublet tyrosyl radical is formed rapidly when oPGHS-1 is incubated aerobically with a hydroperoxide or with arachidonate (46–48, 66), and the peak in doublet signal formation coincides temporally with peak cyclooxygenase activity (48). A broad singlet radical signal appears subsequent to the doublet and is present even after the enzyme is suicide-inactivated. Finally, a broad singlet signal, which is apparently a composite of the broad doublet and narrow singlet signals, occurs when PGHS-1 is incubated with excess substrate or when the enzyme is inactivated by preincubation with indomethacin and then incubated with a hydroperoxide (47, 48, 66, 67). Although the rate of formation of the doublet signal is consistent with its being involved in cyclooxygenase catalysis (48), it is not really clear that this signal represents the expected Tyr-385 radical (Fig. 2*B*). For instance, a Y385F mutant of oPGHS-1, which lacks cyclooxygenase activity, can still form a tyrosyl radical (49). Moreover, an H386A oPGHS-1, which retains 35% of the cyclooxygenase activity of the native enzyme (1), does not form a detectable tyrosyl radical.[2] The fact that H386A oPGHS-1 fails to undergo suicide inactivation and other findings (47, 48) raised the possibility that singlet tyrosyl radical signals are associated with suicide inactivation. However, this concept is inconsistent with our recent studies of an H372A mutant of human PGHS-2, a mutant analogous to H386A oPGHS-1.[2] Like H386A PGHS-1, the H372A PGHS-2 mutant fails to form a tyrosyl radical upon incubation with hydroperoxides, but H372A PGHS-2 does undergo suicide inactivation.

Membrane Localization and Targeting

PGHS-1 and -2 are integral membrane proteins. The interactions of the enzymes with the lipid bilayer involve a unique membrane

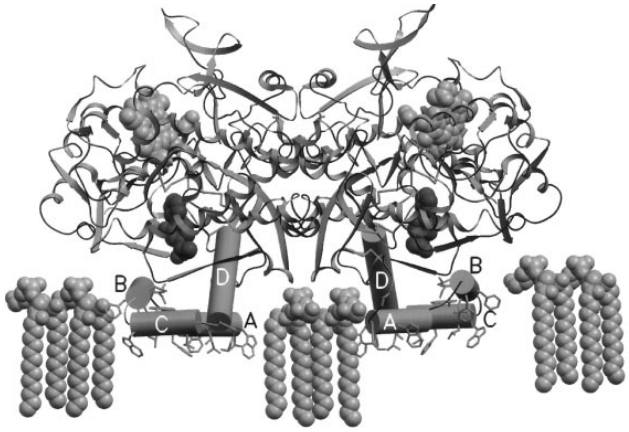

FIG. 4. **Crystal structure of the ovine PGHS-1 dimer highlighting the MBDs (50).**

binding domain (MBD), which was first recognized by analysis of the crystal structure of ovine PGHS-1 (Fig. 4). Each monomer contains an MBD comprised of four short amphipathic helices. In oPGHS-1 the MBD involves residues 70–117. Hydrophobic residues emanating from these helices are thought to interdigitate into only a single leaflet of the lipid bilayer (50). Consistent with this concept labeling experiments with ^{125}I-3-trifluoro-3-(*m*-[^{125}I]iodophenyl)diazirine have indicated that the label becomes associated with a region of the protein that includes the postulated MBD (68). Crystal structures of human and murine PGHS-2 have very recently been reported by Browner[3] and Kurumbail (69) and their colleagues. These two structures, including the MBD, are essentially superimposable with PGHS-1.

Both PGHS-1 and -2 are bound to the lumenal surface of the ER and contiguous *outer* membrane of the nuclear envelope (70). Moreover, both isozymes also appear to be present on the lumenal surface of the inner membrane of the nuclear envelope.[4] PGHS-1 and -2 are initially targeted to the ER via a C-terminal KDEL-type (*i.e.* -(P/S)TEL) of ER retention signal (71). The one obviously unique structural feature of PGHS-2 is that it contains an 18-amino acid cassette near its C terminus. Deletion of this cassette yields a catalytically active enzyme but does not affect the subcellular location of the enzyme suggesting that this cassette is not involved in enzyme targeting.[5]

Biology of the Two PGHS Isoforms

Work on PGHSs during the past 5 years has concentrated on learning about the regulation of enzyme expression and the mechanisms of enzyme catalysis largely in the context of developing PGHS-2-specific, anti-inflammatory agents. A broader biological question is why are there two isozymes, and even more specifically, why do some cells express both isoenzymes? We hypothesize that PGHS-1 and -2 represent at least partially independent prostanoid biosynthetic systems (1). PGHS-1 occurs as part of an ER prostanoid biosynthetic system, which forms prostanoids that act extracellularly as "local" hormones functioning through cell surface, G protein-linked receptors to mediate acute "housekeeping" responses to circulating hormones (*e.g.* in the regulation of renal, gastrointestinal, and platelet functions). PGHS-2 probably has two roles. One role, involving a subpopulation of PGHS-2 colocalizing with PGHS-1 on the lumenal surface of the ER, is to augment the function of PGHS-1 (or to substitute for PGHS-1 in cells lacking this isozyme). The subpopulation of PGHS-2 present on the lumenal surface of the inner membrane of the nuclear envelope may operate as part of a unique nuclear prostanoid biosynthetic system that forms products that act through nucleoplasmic or nuclear membrane targets in association with cell differentiation and replication. There are several general observations that support these concepts. In cells such as murine 3T3 cells, which express PGHS-1 constitutively, maximal induction of PGHS-2 causes, at most, a

[3] M. Browner, personal communication.
[4] A. Spencer, J. Woods, I. Singer, and W. L. Smith, unpublished results.
[5] D. DeWitt and W. L. Smith, unpublished results.

2-fold increase in the net prostanoid biosynthetic capacity of the cell (10, 11). This finding coupled with the fact that the kinetic properties and substrate specificities of the two isozymes are very similar (35, 40) suggests that it is unlikely that PGHS-2 is induced solely to augment PGHS-1. Prostanoid synthesis through PGHS-1 and -2 involves different arachidonate substrate pools and is coupled to different extracellular stimuli and perhaps different phospholipase systems (72, 73). These observations imply that the two enzymes can operate independently. By immunostaining at both the light (74) and electron microscopic[4] levels PGHS-1 and -2 appear to be present in the same subcellular locations. However, PGHS-2 is more concentrated on the nuclear envelope than PGHS-1; moreover, in histochemical staining for enzyme activity in intact cells, the staining attributable to PGHS-1 occurs primarily in the cytoplasm whereas staining attributable to PGHS-2 occurs both in the cytoplasm and over the surface of the nucleus (74). Thus, the two activities appear to function at different locations within the same cells. Finally, as discussed above, the temporal patterns of isozyme expression are such that while PGHS-1 is expressed constitutively, PGHS-2 expression is typically maximal during early stages of cell replication or differentiation.

PGHS-1 generates products that end up outside the cell and function via G protein-linked receptors; this is also true for the subset of PGHS-2 molecules found in the ER (1). However, the unique nuclear activity of PGHS-2 leads to the prediction that there are nuclear prostanoid targets. Peroxisomal proliferator-activated receptor γ has been shown to be activated by PGJ_2 (75). PGJ_2 metabolites also activate the unfolded protein response element (76). We expect that other nuclear targets for other prostanoids will be identified.

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