Competitive Enzymatic Interactions Determine the Relative Amounts of Prostaglandins E₂ and D₂

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ABSTRACT

Prostaglandins (PGs) are a family of cellular messengers exerting diverse homeostatic and pathophysiologic effects. Recently, several studies reported significant increases of PGI₂ and PGF_{2α} after the inhibition of microsomal PGE synthase-1 (mPGES-1) expression, which indicated that PGH₂ metabolism might be redistributed when the PGE₂ pathway is blocked. To address the determinants that govern the relative amounts of PGs, we developed an in vitro cell-free method, based on liquid chromatography-tandem mass spectrometry, to measure the exact amounts of these PGs formed in response to the addition of recombinant isomerases and their selective inhibitors. Our in vitro cell-free assay results were confirmed in cells using bone marrow-derived macrophage. Initially, we determined the in

vitro stability of PGH₂ and noted that there was spontaneous nonenzymatic conversion to PGD₂ and PGE₂. mPGES-1 markedly increased the conversion to PGE₂ and decreased conversion to PGD₂. Reciprocally, the addition of hematopoietic or lipocalin PGD synthase resulted in a relative increase of PGD₂ and decrease of PGE₂. A detailed titration study showed that the ratio of PGE₂/PGD₂ was closely correlated with the ratio of PGE synthase/PGD synthase. Our redistribution results also provide the foundation for understanding how PGH₂ metabolism is redistributed by the presence of distal isomerases or by blocking the major metabolic outlet, which could determine the relative benefits and risks resulting from interdiction in non-rate-limiting components of PG synthesis pathways.

Introduction

Cyclooxygenase (COX) enzymes, also known as PGH₂ synthases, catalyze the oxygenation of arachidonic acid (AA) to PGG₂, followed by the reduction of PGG₂ to PGH₂, which serves as a common substrate for various distal isomerases that generate five distinct primary PGs: PGE₂, PGD₂, PGF_{2α}, PGI₂, and thromboxane A₂ (TXA₂), of which 6-keto-PGF_{1α} and TXB₂ are the main stable nonenzymatic products of PGI₂ and TXA₂, respectively (Fig. 1). These PGs consist of a series

of extracellular and intracellular messengers that produce diverse physiologic effects on pain (Zeilhofer, 2007), inflammation and fever (McAdam et al., 2000), allergy (Pettipher et al., 2007), platelets (FitzGerald, 1991), cardiovascular system (Vane, 1983), cancer growth (Wang et al., 2007), renal function (Hébert et al., 2005), reproduction (Weems et al., 2006), and possibly Alzheimer's disease (McGeer and McGeer, 1999). In many cases, different PGs have counter-regulatory effects. For example, in contrast to PGE₂, PGD₂ in the brain has a role in promoting sleep (Smyth et al., 2009). Furthermore, various PGs have the potential to both promote and counteract inflammatory processes in the body, especially in acute allergic inflammation. Thus, the exact physiologic or pathophysiologic response depends on the relative amounts of biologically active PG species.

After the enzymatic conversion of PGH₂ was reported (Christ-Hazelhof et al., 1976), each PG-specific isomerase was discovered and purified, including PGE synthase, PGD syn-

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ABBREVIATIONS: COX, cyclooxygenase; PG, prostaglandin; mPGES, microsomal PGE synthase; NSAID, nonsteroidal anti-inflammatory drug; LC-MS/MS, liquid chromatography-tandem mass spectrometry; BMDM, bone marrow-derived macrophage; H-PGDS, hematopoietic PGD synthase; L-PGDS, lipocalin PGD synthase; AA, arachidonic acid; TXA₂, thromboxane A₂; TXB₂, thromboxane B₂; LPS, lipopolysaccharide; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; SRM, selected reaction monitoring; TOF, time of flight; HPLC, high-performance liquid chromatography; CAY10526, 4-(benzo[b]thiophen-2-yl)-3-bromo-5-hydroxy-dihydro-furan-2(3H)-one; CAY10589, 2-[4-[[[1,1'-biphenyl]-4-ylmethyl]amino]-6-chloro-2-pyrimidinyl]thio-octanoic acid; HQL-79, 4-(diphenylmethoxy)-1-3-(1H-tetrazol-5-yl)propyl-piperidine; AT-56, 4-(5H-dibenzo[a,d]cyclohepten-5-ylidene)-1-[4-(2H-tetrazol-5-yl)butyl]-piperidine.

Fig. 1. Scheme for the metabolism of AA to form different PGs.

thase, PGF_α synthase, PGI synthase, and TX synthase. Humans express three isoforms of PGE synthase: mPGES-1, mPGES-2, and cytosolic PGE synthase. Whereas mPGES-2 and cytosolic PGE synthase are constitutively expressed *in vivo*, mPGES-1 is of particular interest because it has been shown to be the most potent (Tanikawa et al., 2002) among PGE synthases and is induced by various stimuli including inflammatory signals in various cells and tissues (Guay et al., 2004). CAY10526 [4-(benzo[*b*]thiophen-2-yl)-3-bromo-5-hydroxy-dihydro-furan-2(3*H*)-one; C₁₂H₇BrO₃S] and CAY10589 (2-[[4-[(1,1'-biphenyl)-4-ylmethyl]amino]-6-chloro-2-pyrimidinyl]thio]-octanoic acid; C₂₅H₂₈ClN₃O₂S) are synthetic compounds that have been reported to be selective inhibitors of mPGES-1 (Guerrero et al., 2007; Koeberle et al., 2008). PGD synthase activity is also comprised of two isozymes, H-PGDS and L-PGDS. HQL-79 [4-(diphenylmethoxy)-1-3-(1*H*-tetrazol-5-yl)propyl-piperidine; C₂₂H₂₇N₅O] is a selective H-PGDS inhibitor (Matsushita et al., 1998a,b), and AT-56 [4-(5*H*-dibenzo[*a,d*]cyclohepten-5-ylidene)-1-[4-(2*H*-tetrazol-5-yl)butyl]-piperidine; C₂₅H₂₇N₅] is reported to be a selective L-PGDS inhibitor (Irikura et al., 2009).

Nonsteroidal anti-inflammatory drugs (NSAIDs) competitively interfere with binding of the AA substrate to COX enzymes. Treatment with traditional NSAIDs such as aspirin and ibuprofen decrease PGE₂ biosynthesis by nonselectively inhibiting both COX-1 and COX-2 (Garavito and Mulichak, 2003). However, serious gastrointestinal toxicity (Smyth et al., 2009) occurs at ordinary clinical doses because of their nonselective inhibition of both COX enzymes. There are also unintended consequences of selective inhibition of COX pathway enzymes. For example, rofecoxib and celecoxib are relatively selective for COX-2 and were developed under the assumption that the specific inhibition of COX-2 would be anti-inflammatory and analgesic but lack gastrointestinal toxicity (Garavito and Mulichak, 2003). However, these COX-2-selective inhibitors are associated with an increased risk of hypertension, cardiovascular disease, and stroke (Grosser et al., 2006; Timmers et al., 2007). This is, at least in part, because the inhibition of COX-2 also blocks the production of PGI₂ and alters the balance between the vasoconstrictive

properties of TXA₂ and vasodilatory properties of PGI₂ (Bombardier et al., 2000; Crofford et al., 2000). The recognition of these unintended consequences of individually blocking COX-1 and COX-2 has redirected efforts to examine the effect of interdiction in the individual downstream isomerases of PGH₂, especially on the inhibition of mPGES-1 (Guerrero et al., 2007; Mbalaviele et al., 2010; Rörsch et al., 2010). Because PGH₂ serves as a common substrate for at least five distinctive PGs and PGH₂ itself is extremely unstable and short-lived, it would be premature to consider mPGES-1 as a promising and safe new therapeutic target without further investigation and understanding of the effect of inhibition of mPGES-1 on the entire PG cascade. To address this important issue, we developed an *in vitro* cell-free assay system using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to measure the formation of PGE₂ and PGD₂ and determine the global consequence of the addition of recombinant isomerases and the impact of selective inhibition on the relative production of PGE₂ and PGD₂, which was also examined in cells using BMDM. We hypothesized that there would be a reciprocal relationship between the production of PGE₂ and PGD₂ when inhibiting their specific isomerases that could be biologically important.

Materials and Methods

Animals. For bone marrow experiments, C57BL/6 mice (6–12 weeks old, 25–35 g) were purchased from Harlan (Indianapolis, IN). Mice were housed in a temperature-controlled room with a 12-h light/dark cycle and given standard chow and tap water. All studies involving mice were approved by the Institutional Animal Care and Use Committee and complied with the Animal Welfare Act.

Materials. Ovine COX-1, human recombinant COX-2, human recombinant mPGES-1, human recombinant H-PGDS, human recombinant L-PGDS, GSH, CAY10526, CAY10589, HQL-79, AT-56, AA, PGH₂, PGE₂, PGD₂, PGF_{2α}, 6-keto-PGF_{1α}, TXB₂, PGI₂ (sodium salt), 15-keto-PGF_{2α}, 13,14-dihydro-15-keto-PGE₂, 8-iso PGE₂, d₄-PGE₂, and d₄-PGD₂ were purchased from Cayman Chemical (Ann Arbor, MI). Hemin, epinephrine, Tris base, hydrogen peroxide, citric acid, EDTA, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Monosodium phosphate, disodium

phosphate, hydrochloric acid, and butylated hydroxytoluene were purchased from Thermo Fisher Scientific (Waltham, MA). Formic acid was purchased from EMD Biosciences (San Diego, CA). Purified water was prepared using a Millipore Corporation (Billerica, MA) Milli-Q purification system or an ELGA (Saint Maurice Cedex, France) PURELAB Ultra purification system. Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech (Herndon, VA). All organic solvents were HPLC grade or better and purchased from Thermo Fisher Scientific, and all other chemicals and solvents were American Chemical Society reagent grade, unless stated otherwise.

The In Vitro System. All experiments were carried out using an in vitro system based on a COX functional assay (Cao et al., 2010, 2011). In brief, 20 μ l of 25 mM GSH for mPGES-1 or 20 μ l of 10 mM GSH for H-PGDS and L-PGDS was mixed on ice with 138 μ l of 100 mM Tris-HCl buffer (pH 8.0, 37°C). Next, 20 μ l of Tris-HCl buffer containing mPGES-1, H-PGDS, or L-PGDS was added and incubated on ice for 2 min. A 2- μ l aliquot of enzyme inhibitor in dimethyl sulfoxide was added, and the solution was preincubated at 37°C in an Eppendorf Thermomixer R (Eppendorf North America, New York, NY) for 10 min. Each reaction was initiated by adding 20 μ l of 20 μ M PGH₂ (2 μ M final concentration) in Tris-HCl buffer as substrate and terminated after 30 min by adding 50 μ l of 2 M HCl. In place of PGH₂, 20- μ l aliquots of 20 μ M PGE₂, PGD₂, PGF_{2 α} , 6-keto-PGF_{1 α} , or TXB₂ (final concentration 2 μ M) were added to separate tubes as quantitative controls, which contained the same solution composition but without enzymes. The quantitative controls represented the maximum amounts of each prostaglandin that could be formed if PGH₂ was quantitatively converted to a single product. After measurement using LC-MS/MS, the ratios of the peak areas of each prostaglandin in the experiments to the corresponding quantitative control were determined and expressed as percentages of the maximum theoretical yield.

d₄-PGE₂ and d₄-PGD₂ (10 μ l; 100 ng/ml each in methanol/water, 50:50, v/v) were added as internal standards to correct for sample losses, degradation, or changes in mass spectrometer response. Each sample was extracted using 800 μ l of hexane/ethyl acetate (50:50, v/v), and the organic phase was removed, evaporated to dryness under nitrogen gas, and reconstituted in 100 μ l of methanol/water (50:50, v/v) immediately before quantitative analysis using LC-MS/MS. The peak areas were measured by using Applied Biosystems (Foster City, CA) Analyst software and manually inspected. The curves and data fitting were plotted by using Prism 5 (GraphPad Software Inc., San Diego, CA), and other calculations were carried out using Excel (Microsoft, Redmond, WA) or Numbers (Apple Computer, Cupertino, CA).

Mass Spectrometry. For the quantitative analysis of all prostaglandins except PGH₂, HPLC separations were carried out using a Shimadzu (Columbia, MD) Prominence HPLC system with a Waters (Milford, MA) XTerra MS C₁₈ (2.1 $\times$ 50 mm, 3.5 μ m) analytical column and a 5-min isocratic mobile phase consisting of acetonitrile/aqueous 0.1% formic acid (37:63, v/v) at a flow rate of 200 μ l/min. As shown in Fig. 2, all five derivatives of PGH₂ were resolved to baseline in less than 4 min using these chromatographic conditions. The HPLC system was interfaced to an Applied Biosystems API 4000 triple quadrupole mass spectrometer, which was operated using negative ion electrospray. PGH₂ was measured using LC-MS/MS with an 11-min linear gradient from 33 to 90% acetonitrile in aqueous 0.1% formic acid. Using gradient LC-MS/MS, PGH₂ eluted at a retention time of 7.7 min.

The PGs formed abundant [M-H]⁻ carboxylate ions during negative ion electrospray, which were fragmented using collision-induced dissociation with nitrogen as a collision gas. The collision energy (-24 to -30 V) was optimized for each PG to maximize the formation of product ions for detection using selected reaction monitoring (SRM). Isomeric PGE₂, PGD₂ (Cao et al., 2008), and PGH₂ were

Fig. 2. Negative ion electrospray LC-MS/MS SRM chromatograms showing the detection of 1 μ M PGE₂, PGD₂, PGF_{2 α} , 6-keto-PGF_{1 α} , and TXB₂ standards in methanol/water (50:50, v/v). SRM transitions: 6-keto-PGF_{1 α} , m/z 369 $\rightarrow$ 163 (retention time 1.6 min); TXB₂, m/z 369 $\rightarrow$ 169 (retention time 2.2 min); PGF_{2 α} , m/z 353 $\rightarrow$ 193 (retention time 2.7 min); PGE₂ and PGD₂ m/z 351 $\rightarrow$ 271 (retention times 3.2 and 3.7 min, respectively).

measured using a SRM transition of m/z 351 to m/z 271, and the SRM transition of m/z 353 to m/z 193 was selected for PGF_{2 α} (Dahl and van Breemen, 2010). The SRM transition of m/z 369 to m/z 163 was used for 6-keto-PGF_{1 α} , and the transition of m/z 369 to m/z 169 was used for the measurement of TXB₂. Likewise, the SRM of the transition of m/z 355 to m/z 275 was selected for the internal standards d₄-PGE₂ and d₄-PGD₂ (Cao et al., 2008).

High-resolution negative ion electrospray tandem mass spectra of PGH₂ and its metabolites were acquired using a Waters Synapt G1 quadrupole time of flight (TOF) hybrid tandem mass spectrometer with a Waters Alliance 2690 HPLC system or a Shimadzu ion trap-TOF mass spectrometer with a Prominence HPLC system. HPLC separations were carried out as described above except that the mobile phase consisted of an 11-min linear gradient from 33 to 90% acetonitrile in aqueous 0.1% formic acid.

Cell Culture Assay. Although the in vitro assay provided information regarding biological mechanisms of action, the results might not necessarily reflect in vivo processes or even the situation within a cell. Therefore, the BMDM was used in which mPGES-1 and H-PGDS (L-PGDS) could be selectively inhibited to observe the redistribution of PGH₂ metabolism.

BMDM was isolated from the rear legs of sacrificed C57BL/6 mice. The harvested rear legs were soaked in HBSS containing 2% heat-inactivated FBS under aseptic conditions. The bone marrow cells were obtained by flushing the tibias and femurs using HBSS and cultured in DMEM supplemented with 10% FBS, 10% L929 cell-conditioned medium, 100 U/ml penicillin, and 100 μ g/ml streptomycin. After 72 h of cultivation, the nonadherent cells were removed by changing the medium. Adherent cells were subsequently propagated in culture. Cells were split at day 7 by EDTA (1 mM) and plated at a density of 5×10^5 /ml into six-well plates with L929 cell-conditioned medium. On day 8, the cell culture medium was changed to DMEM containing 1% FBS, penicillin, and streptomycin for 1.5 h before the addition of inhibitors CAY10526, CAY10589, HQL-79, and AT-56 2 h before the LPS (1 μ g/ml) treatment. Cells were incubated with 5% CO₂ humidified air at 37°C. The cell supernatants were collected and stored at -80°C until analysis after 16-h treatment with LPS and different inhibitors. LPS was used to stimulate BMDM to activate COX-2 production and prostaglandin synthesis (Xiao et al., 2008).

Each supernatant was spiked with d₄-PGE₂ and d₄-PGD₂ as internal standards, and citric acid and butylated hydroxytoluene were then added to prevent free radical-catalyzed peroxidation. PGs were extracted using hexane/ethyl acetate. After centrifugation, the upper organic phase was collected and evaporated to dryness. Immediately before analysis using LC-MS/MS, each extract was reconstituted in methanol/water (Cao et al., 2008). Standards for calibration curves and quality-control measurements were prepared by spiking cell culture medium with measured amounts of PGE₂ and PGD₂. These standards were then processed as described above. The concentrations of PGE₂ and PGD₂ in these standards ranged from 0.1 to 1000 ng/ml (Cao et al., 2008).

Statistical Analysis. Samples were run in triplicate, and values are expressed as mean $\pm$ S.D. Statistical significance was assessed using one-way analysis of variance, and *p* values <0.05 were considered to indicate significant differences for all statistical tests.

Results

Validation of Enzyme and PGH₂ Purity. To ensure the purity of each enzyme, ovine COX-1, human COX-2, mPGES-1, H-PGDS, and L-PGDS were incubated with AA and PGH₂ at 37°C for 10 min, and the production of PGs was then determined. Ovine COX-1 and human COX-2 did not increase the formation of PGE₂ or PGD₂ from PGH₂ compared with the control (Fig. 3A), suggesting that neither ovine COX-1 nor human COX-2 was contaminated with PG

TABLE 1

PG formation from PGH₂ incubated with or without GSH and various enzymes

All values are mean $\pm$ S.D. (*n* = 3). GSH at 2.5 mM was used for control and mPGES-1 incubations, whereas 1 mM GSH was used for incubations with H-PGDS and L-PGDS.

GSH Addition	PG Formation	
	PGE ₂	PGD ₂
	%	
Control without GSH	44.3 $\pm$ 3.6	14.9 $\pm$ 1.5
Control with GSH	42.7 $\pm$ 2.9	24.2 $\pm$ 3.1
mPGES-1 without GSH	50.1 $\pm$ 3.3	14.7 $\pm$ 2.1
mPGES-1 with GSH	51.8 $\pm$ 3.4	15.1 $\pm$ 2.0
H-PGDS without GSH	42.4 $\pm$ 4.0	15.7 $\pm$ 3.2
H-PGDS with GSH	20.1 $\pm$ 2.1	48.0 $\pm$ 2.3
L-PGDS without GSH	19.7 $\pm$ 1.6	49.8 $\pm$ 3.1
L-PGDS with GSH	18.5 $\pm$ 2.0	50.8 $\pm$ 3.4

isomerases. In addition, the isomerases mPGES-1, H-PGD, and L-PGDS had no effect on the metabolism of AA in the absence of ovine COX-1 or human COX-2 (Fig. 3B), suggesting that these isomerases were not contaminated with either ovine COX-1 or human COX-2. The 2 μ M PGH₂ solution used in these experiments was also analyzed using LC-MS/MS and was found to already contain 6.1 $\pm$ 1.1% PGE₂, 1.8 $\pm$ 0.7% PGD₂, and 0.2 $\pm$ 0.1% PGF_{2 α} because of nonenzymatic rearrangement.

In Vitro System Optimization. PBS buffer (pH 6.0–8.0, 37°C) and Tris·HCl buffer (pH 7.0–8.0, 37°C) produced similar results during incubations with PGH₂, but Tris·HCl buffer (pH 8.0, 37°C) produced slightly larger enzymatic yields and is recommended by Cayman Chemical. Therefore, Tris·HCl buffer (pH 8.0, 37°C) was used for all subsequent experiments. GSH has been reported to function as an essential cofactor for three isomerases (mPGES-1, H-PGDS, and L-PGDS) (Ouellet et al., 2002; Herlong and Scott, 2006; Hohwy et al., 2008). In our hands, GSH enhanced PGs formation only slightly for mPGES-1 and L-PGDS, and it was not essential for the function of these enzymes. For H-PGDS, however, GSH was indispensable (Table 1). GSH concentrations (0.1–12.5 mM) were varied among three isomerases, but did not affect reaction rates significantly. Thus, according to recommendations of the supplier (Cayman Chemical), 2.5 mM GSH was used for mPGES-1, and 1 mM GSH was used for H-PGDS and L-PGDS. The optimal levels of mPGES-1, H-PGDS, and L-PGDS were approximately 3, 0.1, and 1 unit/ μ l, which was at the upper part of the linear range of the dose-response curve. A preincubation time of 10 min was used before adding the substrate to allow the inhibitors to interact fully with the enzyme and reach maximal inhibition potency (Cao et al., 2010, 2011). A reaction time of 30 min was used for enzymatic conversion of PGH₂ to PGs. A substrate dose-response curve was generated that indicated the production of each PG product after 30 min reached a plateau at approximately 4 μ M substrate. A substrate concentration of 2 μ M corresponded to 50% response and was determined to be the *K_m* of the substrate.

Identification of PGH₂ and Characterization of Unknown Derivatives. During the LC-MS/MS analysis of a PGH₂ standard (monitoring the SRM transition of *m/z* 351 to *m/z* 271), five peaks were observed as shown in Fig. 4. The first two peaks were identified according to comparison with standards as PGE₂ and PGD₂, and the last peak was con-

Fig. 3. Ovine COX-1, human COX-2, mPGES-1, H-PGDS, and L-PGDS were incubated at 37°C for 10 min with substrate PGH₂ (A) or AA (B). PG formation (%) represents the sum of all PGs that were detected relative to the theoretical maximum based on the substrate PGH₂ or AA. All values are the mean $\pm$ S.D. (*n* = 3). *, *p* < 0.05, significance differences in PG formation compared with no enzyme.

Fig. 4. Negative ion electrospray SRM chromatograms of the transition m/z 351 to m/z 271 showing the detection of 2 μ M PGH_2 standard in methanol/water (50:50, v/v) at pH 7 and 4°C and its nonenzymatic derivatives using an 11-min linear gradient from 33 to 90% acetonitrile in aqueous 0.1% formic acid at a flow rate of 200 μ L/min. PGE_2 and PGD_2 (retention times 4.5 and 4.8 min, respectively) were identified by comparison with standards. PGH_2 (retention time 7.7 min) was identified by monitoring its level over 160 min at pH 7 and 4°C. The remaining two peaks (retention times 5.7 and 6.3 min) remain unidentified but were not (by comparison with standards) PGI_2 , 15-keto $\text{PGF}_{2\alpha}$, or 13,14-dihydro-15-keto PGE_2 .

firmed to be PGH_2 because it continued decreasing, whereas other peaks increased over 160 min (Fig. 5). The two remaining peaks (retention times 5.7 and 6.3 min) remain unidentified and are not (by comparison with standards) PGI_2 , 15-keto $\text{PGF}_{2\alpha}$, 13,14-dihydro-15-keto PGE_2 , or 8-iso PGE_2 . In a separate experiment, the half-life of PGH_2 in Tris-HCl buffer at pH 8 and 37°C was determined to be 5 min (Fig. 6).

For additional characterization of the unknown peaks, LC-MS/MS analysis was carried out using high-resolution quadrupole TOF and ion trap-TOF mass spectrometers. All five compounds separated as shown in the chromatogram in Fig. 4 were isomeric with identical elemental compositions of $\text{C}_{20}\text{H}_{32}\text{O}_5$. During tandem mass spectrometry on these mass spectrometers using different collision energies, all five compounds produced similar tandem mass spectra without any characteristic fragmentation that could distinguish them.

In Vitro Nonenzymatic Conversions of PGH_2 . Because PGH_2 is extremely unstable and has a short half-life in aqueous solution, we used LC-MS/MS with SRM to determine the main degradation products of PGH_2 and their relative yields. In the absence of any enzyme, PGH_2 spontaneously converted to PGE_2 , PGD_2 , and a small amount of $\text{PGF}_{2\alpha}$ but no 6-keto- $\text{PGF}_{1\alpha}$ or TXB_2 . Therefore, neither PGI_2 nor TXA_2 was spontaneously produced from PGH_2 . The yields of PGE_2 , PGD_2 , and $\text{PGF}_{2\alpha}$ were approximately 44, 15, and 1.6%, respectively, with a constant ratio among these species of $\sim 3:1:0.1$. We observed an unexpected phenomenon after optimizing the reaction conditions. There was nearly a

Fig. 5. Levels of PGE_2 (A), PGD_2 (B), PGH_2 (E), and two unknown compounds (C and D) formed over 160 min from an initial concentration of 2 μ M PGH_2 in methanol/water (50:50, v/v) at pH 7 and 4°C. The formation of PGE_2 and PGD_2 was constant during the first 50 min and reached a plateau by 70 min (A and B), whereas the amount of PGH_2 continued to decrease over 160 min (E). The curves representing the unknown compounds had inflection points suggesting that these are unstable compounds.

Fig. 6. PGH₂ decomposition curve in Tris-HCl buffer at pH 8 and 37°C (time points 1, 2, 3, 5, 7, 10, 15, 25, and 40 min). Based on this curve, the half-life of PGH₂ was determined to be 5 min.

100% increase in PGD₂ formation from PGH₂ after the addition of 1 or 2.5 mM GSH, and for PGF_{2α}, there was a 600% increase. However, PGE₂ formation was slightly decreased or not affected. Moreover, by adding an equivalent amount of H₂O₂, the effect of GSH addition on PGD₂ and PGF_{2α} was totally eliminated (Fig. 7A), indicating that reactive oxygen species might have a regulatory role in governing the relative amounts of these prostaglandins.

In Vitro Enzymatic Conversions of PGH₂. Because PGE₂ and PGD₂ play significant physiological roles in humans and are predominant products of PGH₂, we examined the roles in their formation of three commercially available human recombinant distal enzymes, mPGES-1, H-PGDS, and L-PGDS. When the highest concentration of mPGES-1 (300 units/μl) was incubated with PGH₂, the yield of PGE₂ increased to 62%, whereas PGD₂ decreased to 3.6% and PGF_{2α} decreased to basal level (Fig. 7A). Likewise, when either the highest concentration of H-PGDS (120 units/μl) or L-PGDS (10 units/μl) was incubated with 2 μM PGH₂, the yield of PGD₂ increased to 78%, whereas the PGE₂ formation decreased to 10.8% and PGF_{2α} decreased to basal level (Fig. 7B).

We next examined the impact of the addition of pharmacologic inhibitors of these downstream enzymes by using the commercially available mPGES-1-selective inhibitors

Fig. 7. PG formation from PGH₂ under different conditions. The initial concentration of PGH₂ was 2 μM, and the GSH and H₂O₂ concentrations were 1 mM for H-PGDS and L-PGDS or 2.5 mM for mPGES-1. mPGES-1, H-PGDS, and L-PGDS are shown in units/μl; CAY10526, CAY10589, HQL-79, and AT-56 are shown in μM. All values are mean ± S.D. (n = 3).

TABLE 2

The effects of mPGES-1 inhibitors CAY10526 and CAY10589, H-PGDS inhibitor HQL-79, and L-PGDS inhibitor AT-56 on conversion of PGH₂ to PGE₂ and PGD₂ by mPGES-1, H-PGDS, and L-PGDS

All values are mean $\pm$ S.D. ($n = 3$).

Inhibitor	PG Formation					
	mPGES-1 (3 units/ μ l)		H-PGDS (0.1 unit/ μ l)		L-PGDS (1 unit/ μ l)	
	PGE ₂	PGD ₂	PGE ₂	PGD ₂	PGE ₂	PGD ₂
	% yield from initial 2 μ M PGH ₂					
No inhibitor	53.5 $\pm$ 3.7	15.1 $\pm$ 0.9	20.3 $\pm$ 2.1	48.1 $\pm$ 3.0	18.2 $\pm$ 2.7	50.1 $\pm$ 2.8
CAY10526, 300 μ M	45.2 $\pm$ 2.5	24.9 $\pm$ 2.0	37.8 $\pm$ 1.8	26.7 $\pm$ 1.3	38.9 $\pm$ 2.6	28.1 $\pm$ 2.4
CAY10589, 100 μ M	44.9 $\pm$ 2.8	25.5 $\pm$ 1.6	40.8 $\pm$ 3.1	24.2 $\pm$ 1.8	40.4 $\pm$ 3.4	23.7 $\pm$ 0.8
HQL-79, 50 μ M	54.4 $\pm$ 2.1	14.6 $\pm$ 1.0	38.3 $\pm$ 2.0	26.4 $\pm$ 1.7	20.4 $\pm$ 1.5	49.1 $\pm$ 1.8
AT-56, 100 μ M	55.1 $\pm$ 3.4	14.7 $\pm$ 1.6	21.2 $\pm$ 1.4	47.3 $\pm$ 2.5	39.8 $\pm$ 2.2	24.1 $\pm$ 1.3

CAY10526 and CAY10589, H-PGDS-selective inhibitor HQL-79, and L-PGDS-selective inhibitor AT-56 in our *in vitro* system. After incubating PGH₂ with mPGES-1 (3 units/ μ l) and 300 μ M CAY10526 or 100 μ M CAY10589, PGE₂ formation decreased to the basal level by 45%, whereas PGD₂ formation increased to 25%, and PGF_{2 α} increased only slightly (Fig. 7A). Incubation with the H-PGDS-specific inhibitor HQL-79 (50 μ M) inhibited approximately 90% of PGD₂ formation (decreased to 26%) in the presence of H-PGDS (0.1 unit/ μ l), whereas PGE₂ formation increased to 38%, and PGF_{2 α} decreased to basal level (Fig. 7B). Likewise, the L-PGDS-specific inhibitor AT-56 (100 μ M) completely prevented PGD₂ formation (decreased to 24%) when incubating PGH₂ with L-PGDS (1 unit/ μ l), whereas PGE₂ formation increased to 40% and PGF_{2 α} decreased to basal level (Fig. 7B). The effects of these inhibitors on other isomerases were also tested. We were surprised to find that CAY10526 and CAY10589 were less specific than expected and also inhibited both H-PGDS and L-PGDS *in vitro*. In contrast, HQL-79 had no inhibitory effect on either L-PGDS or mPGES-1, and AT-56 inhibited neither H-PGDS nor mPGES-1 (Table 2).

In Vitro Redistribution Assay. Because obvious shifts of PGH₂ to other PGs were observed using inhibitors to block their corresponding isomerases, we predicted that there would be redistribution in products by inhibition of isomerases that compete for the substrate PGH₂. We first mixed different volumes of mPGES-1 (3 units/ μ l) and H-PGDS (0.1 unit/ μ l) or L-PGDS (1 unit/ μ l) in the same system. As a result, with more mPGES-1 activity, more PGE₂ and less PGD₂ were produced and vice versa (Fig. 8) in a predictable fashion according to the ratio of PGES to PGDS activity. We also examined the impact of pharmacologic inhibition with HQL-79 and AT-56 to further determine the consequence of redistribution by inhibiting H-PGDS and L-PGDS, respectively (Fig. 9). As expected, by increasing the concentrations of HQL-79 or AT-56, conversions of PGH₂ moved toward the production of PGE₂, whereas the production of PGD₂ decreased. Because our data showed that the purported mPGES-1 inhibitors CAY10526 and CAY10589 also strongly inhibited H-PGDS and L-PGDS, redistribution tests by inhibiting mPGES-1 with these agents did not produce redistribution of PGH₂ metabolism *in vitro*.

Cell Culture Redistribution Assay. CAY10526 and CAY10589 did not produce redistribution of PGH₂ metabolism in BMDM because these agents inhibited not only mPGES-1 but also H-PGDS and L-PGDS. AT-56 had no

Fig. 8. The effects on PGE₂ and PGD₂ formation from 2 μ M PGH₂ of different ratios of mPGES-1 and H-PGDS (A) or mPGES-1 and L-PGDS (B). PGE₂ formation increased when the proportion of mPGES-1 was increased, and PGD₂ formation increased when the proportion of H-PGDS or L-PGDS was increased. All values are expressed as the mean $\pm$ S.D. ($n = 3$). *, $p < 0.05$, significant differences in PGE₂ formation compared with mPGES-1 (0.3 unit/ μ l)/H-PGDS (0.01 units/ μ l) (A) and mPGES-1 (0.3 unit/ μ l)/L-PGDS (0.1 unit/ μ l) (B). #, $p < 0.05$, significant differences in PGD₂ formation compared with mPGES-1 (0.3 unit/ μ l)/H-PGDS (0.01 unit/ μ l) (A) and mPGES-1 (0.3 unit/ μ l)/L-PGDS (0.1 unit/ μ l) (B).

effect on decreasing PGD₂ production, which was not surprising because L-PGDS protein expression was not detectable in BMDM (L. Xiao, unpublished data). Therefore, only H-PGDS was available for inhibition by pharmacologic intervention in BMDM. Similar to the *in vitro* redistribution assay, treatment of BMDM with the H-PGDS inhibitor HQL-79 altered PGH₂ conversion toward the production of PGE₂, whereas the production of PGD₂ decreased (Fig. 10).

Fig. 9. The consequences of adding different concentrations of the H-PGDS-selective inhibitor HQL-79 (A) or L-PGDS-selective inhibitor AT-56 (B) to a system containing equivalent volumes of mPGES-1 (3 units/μl) and H-PGDS (0.1 unit/μl) or L-PGDS (1 unit/μl) incubated with 2 μM substrate PGH₂. All values are expressed as mean ± S.D. (*n* = 3). *, *p* < 0.05, significant differences in PGE₂ formation compared without addition of HQL-79 (A) or without addition of AT-56 (B). PGE₂ formation increased, whereas PGD₂ formation decreased with increasing concentrations of HQL-79 or AT-56. #, *p* < 0.05, significant differences in PGD₂ formation compared without addition of HQL-79 (A) or without addition of AT-56 (B).

Discussion

Previously, we reported that a COX functional assay (Cao et al., 2011) used to screen COX-2-selective inhibitors results in the production of both PGE₂ and PGD₂, which raised the obvious question as to how PGE₂ and PGD₂ are produced in the absence of downstream isomerases. We initially suspected that the COX-2 enzyme was contaminated with certain isomerases or it might have some unknown functions that could catalyze conversion of PGH₂ to PGs. When incubating COX-1 or COX-2 with PGH₂ produced no increase in the production of the PGs, we then redirected our focus to investigating the stability of the intermediate PGH₂. These studies showed that PGH₂ was extremely unstable in aqueous solution in vitro and spontaneously and nonenzymatically converted to PGE₂, PGD₂, and PGF_{2α}, with a constant ratio of ~3:1:0.1 (Fig. 7). This not only provided an explanation for the results in our COX functional assay but also clarified the identities and yields of PGH₂ products. Based on our in vitro findings, it is possible that PGH₂ would also convert to PGs in vivo without any downstream isomerases, which further brings into question the efficacy of inhibiting PGE₂ formation using mPGES-1 inhibitors.

Fig. 10. A, the effect of the H-PGDS-selective inhibitor HQL-79 on the LPS-stimulated production of PGE₂ and PGD₂ from BMDM. Values are the mean ± S.D. (*n* = 3). *, *p* < 0.05, significant differences in PGE₂ formation compared with no HQL-79 addition; #, *p* < 0.05, significant differences in PGD₂ formation compared with no HQL-79 addition. PGE₂ formation increased and PGD₂ formation decreased with increasing concentrations of HQL-79. B, examples of LC-MS/MS analyses of PGE₂ and PGD₂ extracted from BMDM after treatment without or with 100 μM HQL-79.

The pivotal finding of our study is the PGH₂ metabolic redistribution to other forms of PGs by the inhibition of either PGE synthase or PGD synthase (Fig. 7). A possible explanation is that mPGES-1 and H-PGDS compete for the substrate PGH₂ so that inhibition of one enzyme would shunt PGH₂ to the other. To prove this explanation, several different approaches were used. Titration studies (Fig. 8) showed how the conversion of PGH₂ was closely correlated with the relative levels of PGE synthase and PGD synthase. A similar redistribution phenomenon (from PGD₂ to PGE₂) was also observed in vitro (Fig. 9) when using the H-PGDS-selective inhibitor HQL-79 or the L-PGDS-selective inhibitor AT-56, because the major outlet PGD₂ was blocked. Moreover, the BMDM cell culture study (Fig. 10) confirmed the PGH₂ metabolism redistribution in cells by inhibiting H-PGDS. Purported to be mPGES-1-selective inhibitors, CAY10526 and CAY10589 also inhibited PGD synthase and could not be used to study redistribution toward PGD₂. PGH₂ metabolism redistribution also explains the reported increases of PGI₂ and PGF_{2α} by inhibition of mPGES-1 in mice (Guay et al., 2004; Trebino et al., 2005; Mbalaviele et al., 2010; Rörsch et al., 2010).

In the near future, our observations could probably be used to provide therapeutic guidance for diseases involving PGs,

because the redistribution and accumulation of certain types of PGs might be beneficial and useful for treatment. For example, higher levels of PGI_2 and $\text{PGF}_{2\alpha}$ would be favorable because they have anti-inflammatory effects, and more PGD_2 could have anti-inflammatory effects (Smyth et al., 2009). Because various PGs can produce opposing effects, imbalance among them might cause unexpected problems or risks that must not be ignored such as with the use of highly selective COX-2 inhibitors. For instance, an increase of PGI_2 might cause side effects such as low blood pressure in certain patients and even result in certain chronic and fatal diseases that are not easy to observe (Smyth et al., 2009).

We observed moderate inhibition of PGE_2 formation by CAY10526 *in vitro*; however, CAY10526 was reported to be a strong mPGES-1-selective inhibitor (Guerrero et al., 2007). The probable reason (Guerrero et al., 2007) is that CAY10526, which caused a significant reduction in PGE_2 production in the induction phase, did not affect PGE_2 formation in the postinduction phase, which means that CAY10526 selectively inhibited mPGES-1 protein expression.

During the identification of PGH_2 (Fig. 4), two peaks (retention times 5.7 and 6.3 min) remained unidentified but were not (by comparison with standards) PGI_2 , 15-keto $\text{PGF}_{2\alpha}$, 13,14-dihydro-15-keto PGE_2 , or 8-iso PGE_2 . Some possible structures include the reactive γ -keto aldehydes and levuglandins E_2 and D_2 (Boutaud et al., 1999). All of these compounds are isomers of PGH_2 .

GSH has been reported to be an essential cofactor for mPGES-1 (Ouellet et al., 2002), H-PGDS (Hohwy et al., 2008), and L-PGDS (Herlong and Scott, 2006). Our studies confirmed the indispensability of GSH for H-PGDS, but it was not necessary for either mPGES-1 or L-PGDS in our hands (Table 1). This indicated that GSH was not an essential cofactor for mPGES-1 and L-PGDS in our *in vitro* cell-free assay if these two isomerases were not contaminated with GSH. During the nonenzymatic conversion study of PGH_2 , a 600% increase of $\text{PGF}_{2\alpha}$ production and an almost 100% increase of PGD_2 production were found when adding GSH into PGH_2 standard in aqueous solution (Fig. 7A). It has been reported that glutathione transferase increases $\text{PGF}_{2\alpha}$ formation, which results mainly from the glutathione transferase enzymatic catalysis (Burgess et al., 1987). However, other reducing agents, like GSH, can also increase $\text{PGF}_{2\alpha}$ production (Christ-Hazelhof et al., 1976; Nugteren and Christ-Hazelhof, 1980; Burgess et al., 1987), which indicates that this is a nonenzymatic process, possibly by serving as a specific electron donor promoting the conversion of PGH_2 to $\text{PGF}_{2\alpha}$ (Keeting et al., 1987). However, little is known about the increase of PGD_2 by adding GSH, which raised our interest in the undiscovered differences between PGE_2 and PGD_2 . Because both PGE_2 and PGD_2 have the same elemental composition and similar structure, it is very curious that they behave so differently in the presence of GSH or other reagents. These issues are being addressed by our ongoing studies.

In summary, although mPGES-1 inhibitors could be used as alternatives to COX-2 inhibitors to specifically decrease the amount of PGE_2 , they could also cause PGH_2 metabolism redistribution. Thus, it is necessary to have a full understanding of the mechanisms of action and physiological effects of all PGs. The consequences of selective inhibition of one or more prostaglandin isomerases determine possible

subsequent risks. mPGES-1 was found to be functionally coupled with COX-2 (Murakami et al., 2000), which means that mPGES-1 would normally catalyze the transformation of PGH_2 produced by COX-2. Then using one or more inhibitors to partially inhibit COX-2 and strongly inhibit mPGES-1 might be a more reasonable therapeutic approach. Alternatives might include PGE_2 receptor antagonists or high-affinity PGH_2 analogs.

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Authorship Contributions

Participated in research design: Yu, Xiao, Zhao, Christman, and van Breemen.

Conducted experiments: Yu and Zhao.

Contributed new reagents or analytic tools: Xiao, Christman, and van Breemen.

Performed data analysis: Yu.

Wrote or contributed to the writing of the manuscript: Yu, Xiao, Zhao, Christman, and van Breemen.

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