Interactions of 2-O-arachidonylglycerol ether and ibuprofen with the allosteric and catalytic subunits of human COX-2

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Abstract Prostaglandin (PG) endoperoxide H synthase (PGHS)-2, also known as cyclooxygenase (COX)-2, can convert arachidonic acid (AA) to PGH₂ in the committed step of PG synthesis. PGHS-2 functions as a conformational heterodimer composed of an allosteric (E allo) and a catalytic (E cat) monomer. Here we investigated the interplay between human (hu)PGHS-2 and an alternative COX substrate, the endocannabinoid, 2-arachidonoylglycerol ether (2-AG ether), as well as a stable analog, 2-O-arachidonylglycerol ether (2-AG ether). We also compared the inhibition of huPGHS-2-mediated oxygenation of AA, 2-AG, and 2-AG ether by the well-known COX inhibitor, ibuprofen. When tested with huPGHS-2, 2-AG and 2-AG ether exhibit very similar kinetic parameters, responses to stimulation by FAs that are not COX substrates, and modes of inhibition by ibuprofen. The 2-AG ether binds E cat more tightly than E allo, and, thus, can be used as a stable E cat-specific substrate to examine certain E allo-dependent responses. Ibuprofen binding to E allo of huPGHS-2 completely blocks 2-AG or 2-AG ether oxygenation; however, inhibition by ibuprofen of huPGHS-2-mediated oxygenation of AA engages a combination of both allosteric and competitive mechanisms.

Supplementary key words cyclooxygenase-2 • prostaglandin, half-sites • 2-arachidonoylglycerol • arachidonic acid • palmitic acid • ibuprofen

Prostaglandin (PG) endoperoxide synthase (PGHS)-1 and PGHS-2 catalyze the formation of PGH₂ from arachidonic acid (AA) in the committed step of PG biosynthesis (1–4). PGHS-1 and PGHS-2 are considered to be the constitutive and inducible PGHS isoforms, respectively. PGHSs are often called cyclooxygenases (COXs). Both enzymes exhibit a bis-oxygenase or COX activity involved in the formation of the PG endoperoxide G₂ and a peroxidase activity that reduces PG endoperoxide G₂ to PGH₂. COX activities of PGHSs are inhibited by nonsteroidal anti-inflammatory drugs (NSAIDs) that include COX-2-specific inhibitors, sometimes referred to as coxibs (5).

PGHSs are sequence homodimers composed of 72 kDa subunits. Despite the structural symmetry observed in crystal structures, both PGHS isoforms behave in solution as conformational heterodimers. One monomer (E allo) acts as a regulatory allosteric monomer, and the other monomer (E cat) binds heme and functions as the catalytic monomer (6–8). The COX activity of E cat of human (hu)PGHS-2 is allosterically modulated by many common FAs, including saturated and monounsaturated FAs that are not COX substrates [e.g., palmitic acid (PA)] (9, 10), and by some nonspecific NSAIDs, such as flurbiprofen and naproxen (7), that bind preferentially to E allo of huPGHS-2.

Marnett and coworker (3) were the first to demonstrate that the endocannabinoid, 2-arachidonoylglycerol (2-AG), is an alternative substrate that is converted to 2-PGH₂-glycerol by PGHS-2. This latter intermediate can, in turn, be converted to several different 2-prostanoyl-glycerol derivatives. A recent report has indicated that 2-AG binds

Abbreviations: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; 2-AG ether, 2-O-arachidonoylglycerol ether; COX, cyclooxygenase; E allo, allosteric monomer; E cat, catalytic monomer; hu, human; BP, (S)-(+)-ibuprofen; mu, murine; ns, nonsubstrate; NSAID, nonsteroidal anti-inflammatory drug; PA, palmitic acid; PG, prostaglandin; PGHS, prostaglandin endoperoxide H synthase; RP, reverse-phase.

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with higher affinity to E\textsubscript{cat} than E\textsubscript{allo} of murine (mu)PGHS-2 (11); in contrast, AA binds with much higher affinity to E\textsubscript{allo} than E\textsubscript{cat} (7). Interestingly, a nonsubstrate (ns)FA, 13-methyl AA, increases the rate of oxygenation of 2-AG by muPGHS-2 by increasing the \( V_{\text{max}} \) but not the \( K_{\text{cat}} \) toward 2-AG (11).

The 2-AG is unstable and readily rearranges to 1-AG and hydrolyzes to AA and glycerol (12). This instability presents experimental difficulties in studying the interactions of 2-AG with PGHSs. The 2-O-arachidonylglycerol ether (2-AG ether) is a stable analog of 2-AG. In the first part of the present study, we report the characterization of 2-AG ether as a substrate of huPGHS-2. We find that the 2-AG ether behaves very much like 2-AG with huPGHS-2. Because of its stability, 2-AG ether can serve as a surrogate for 2-AG in enzyme studies.

In related work described here, we examined the ability of the commonly used NSAID, (S)-(-)-ibuprofen (IBP), to interact with E\textsubscript{allo} and E\textsubscript{cat} to inhibit huPGHS-2. We confirm results of earlier studies that IBP is an allosteric inhibitor of 2-AG oxygenation (13) and extend this finding to 2-AG ether. We also observed that IBP binding to E\textsubscript{allo} of huPGHS-2 allosterically inhibits AA oxygenation, but does so only incompletely. Complete inhibition involves the binding of IBP to both E\textsubscript{cat} and E\textsubscript{allo} of huPGHS-2.

**EXPERIMENTAL PROCEDURES**

**Materials**

Complete protease inhibitor was from Roche Applied Science. Ni-NTA Superflow resin and Ni-nitrilotriacetic acid were from Qiagen, PA (16:0), oleic acid (18:1α9), stearic acid (18:0), 11-eicosaenoic acid (20:1α9), FLAG peptide, and FLAG affinity resin were from Sigma-Aldrich. AA, 2-AG, and 2-AG ether were from Cayman Chemical (Ann Arbor, MI). Hemin was from Frontier Scientific, Logan, UT. IBP was from Tocris Bioscience. The [1-\(^{14}\)C]AA (1.85 GBq/mmol) was from American Radiolabeled Chemicals. Decyl maltoside, n-octyl-β-D-glucopyranoside, and C\textsubscript{18}E\textsubscript{8} used in protein purification, were purchased from Anatrace (Maumee, OH). BCA protein reagent was from Pierce, Hexane, isopropanol alcohol, and acetic acid were HPLC grade from Thermo Fisher Scientific, Inc. Anti-PGHS-2 antibodies directed against the 18-amino acid insert unique to PGHS-2 were as described (14). Anti-FLAG antibodies were from LifeTein, South Plainfield, NJ. Horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) were from Bio-Rad.

**Expression, purification, and assay of huPGHS-2 variants**

Procedures for the expression and purification of recombinant native huPGHS-2 and mutant huPGHS-2 heterodimer variants from insect cells were as described previously (7, 15). The purity of the recombinant huPGHS-2 was determined by SDS-PAGE and Western blot analysis (9). In most cases, COX activity was determined using measurements of \( O_2 \) consumption with an O\textsubscript{2} electrode (7). One unit of COX activity is defined as 1 \( \mu \)mol of \( O_2 \) consumed per minute at 37°C in the standard assay mixture. The average specific activity of purified huPGHS-2 with 100 \( \mu \)M AA was 40 units per milligram protein. This specific activity is similar to that reported in earlier studies from our laboratory using different lots of purified huPGHS-2 (i.e., within ±5\%) (7, 10, 15).

**COX assays at high enzyme-to-substrate ratios to test for ligand binding to E\textsubscript{allo}**

Briefly, COX assays were performed at high enzyme/[1-\(^{14}\)C]AA ratios in order to quantify [1-\(^{14}\)C]AA binding to E\textsubscript{allo} of huPGHS-2. Unlabeled ligands (e.g., PA, 2-AG, and 2-AG ether) were tested for their abilities to displace unreacted [1-\(^{14}\)C]AA remaining bound to E\textsubscript{allo}. Reaction mixtures (100 \( \mu \)l final volume) containing 1 \( \mu \)M [1-\(^{14}\)C]AA, 0.10–2.0 \( \mu \)M huPGHS-2, 5 \( \mu \)M hematin, and 1 mM phenol in 0.1 M Tris-HCl (pH 8.0) were incubated at 37°C for 1–8 min, and the products were separated and quantified by radio-reverse-phase (RP)-HPLC, as detailed previously (7, 10). The principle underlying this method is described in (10). Agents that displace [1-\(^{14}\)C]AA from E\textsubscript{allo} of huPGHS-2 cause the disappearance of [1-\(^{14}\)C]AA, which, following its displacement from E\textsubscript{allo}, is converted by E\textsubscript{cat} to an oxygenated product.

**Structural analysis of products formed upon oxygenation of 2-AG ether by huPGHS-2**

A reaction was performed using a standard COX assay mixture that included 20 \( \mu \)g of 2-AG ether as the substrate and sufficient huPGHS-2 to consume approximately 80% of the substrate during a 2 min incubation. Immediately afterwards, a volume of 100 mM SnCl\textsubscript{2} in methanol was added to the sample such that the final SnCl\textsubscript{2} concentration was 1 mM. The sample was vortexed and incubated at room temperature, then extracted with ethyl acetate, dried under N\textsubscript{2}, and kept in a sealed tube until analyzed. Control reactions were performed with assay buffer alone, with assay buffer that included heme and phenol, with huPGHS-2 in buffer alone, with huPGHS-2 in buffer that included heme and phenol, with 2-AG ether in buffer alone, with 2-AG ether in buffer containing heme and phenol, and with 2-AG ether plus huPGHS-2 in buffer alone.

Acetylation of the extracted reaction products was performed with a 1:1 mixture of acetic anhydride and pyridine for 20 min at 100°C. The reaction mixture was dried under nitrogen, dissolved in methanol, and then diluted with water to a final methanol concentration of less than 15%. Solid phase extraction and reversed phase chromatography were performed essentially as previously described (16). The LC effluent was directly interfaced into the electrospray ionization source of a triple quadrupole mass spectrometer (Sciex API 5500; PE-Sciex, Thornhill, ON, Canada) where mass spectrometric analyses were performed in the positive ion mode (m/z 250–800) or as MS\textsuperscript{2} product ions using nitrogen as collision gas at a collision voltage of 25 V. TMS ether derivatives were prepared from HPLC-purified metabolites and analyzed by electron ionization by capillary GC/MS, as previously described (17), using a Finnigan DSQ GC/MS system (Thermo Finnigan, Thousand Oaks, CA) with a ZB-1 column (30 m, 0.25 mm inner diameter 0.25 mm film thickness; Phenomenex). The gas chromatograph was programmed from 150 to 270°C at 30°C/min, 270 to 315°C at 10°C/min, and finally held at 315°C for 6 min. The injector was maintained at 230°C, the transfer line was maintained at 290°C, and the ion source at 200°C.

**Statistical analyses**

Student’s t-tests were performed in Microsoft Excel. If the experiments had the same numbers of repetitions, probabilities were calculated with a Student’s paired t-test, with a two-tailed distribution. If the experiments had different numbers of repetitions, probabilities were calculated with a Student’s unequal variance t-test, with a two-tailed distribution.
RESULTS

Products formed from 2-AG ether by huPGHS-2

When 2-AG ether (75 nmoles) was incubated with an excess of huPGHS-2 (240 nmoles) at 37°C in a standard COX assay mixture for 2 min, 112 nmoles of O₂ were consumed. We assumed that the excess enzyme led to complete conversion of the substrate and that either one or two O₂ molecules were incorporated into the 2-AG ether substrate. These assumptions were corroborated by the mass spectrometric results described below. Accordingly we calculated that 1.49 mol O₂ were incorporated per mole of 2-AG ether, indicating that about 70% of the products were bis-oxygenated (i.e., 2-PGH₂-glycerol ether) and 30% were mono-oxygenated [i.e., 2-(hydroxy-eicosatetraenoyl)-glycerol ether(s)].

Mass spectrometric studies using LC-MS and LC-MS/MS were performed to directly characterize the structural products obtained following the action of huPGHS-2 on 2-AG ether (Fig. 1). In order to impart favorable mass spectrometric characteristics, the products extracted from the reaction mixture were first reduced with SnCl₂ and then derivatized by acetylation. RP-HPLC was able to separate two less lipophilic products from the starting 2-AG ether that were not present in control incubations with huPGHS-2 when no phenol or heme was present (Fig. 1A). These are labeled peak A and the more lipophilic peak B. Because these were presumed to be acetylated ether diglycerides, positive ion electrospray ionization as the ammonium adduct ion (NH₄⁺) was employed. The observed [M+NH₄]⁺ adduct ions were m/z 642 (peak A; Fig. 1B) and m/z 524 (peak B; Fig. 1C), while the signal for unreacted starting material was found to produce m/z 466 (peak C; Fig. 1D). Collisional activation of the starting material (peak C) yielded a major product ion at m/z 273 corresponding to cleavage of the arachidonyl chain at the ether bond (Fig. 1D).

The spectrum of least lipophilic reaction product (acetyl derivative, ammonium ion adduct, m/z 642) (Fig. 1B) was consistent with the addition of 3-hydroxyl groups (analyzed as acetyl esters) and reduction of one double bond. This was consistent with a PGF₂-like structure generated from a PGH₂ endoperoxide intermediate formed by the COX activity of huPGHS-2 acting on the arachidonyl ether chain of 2-AG ether. The collisional activation of m/z 642 [M+NH₄]⁺ yielded a very prominent product ion at m/z 445 (Fig. 1B), consistent with three neutral losses of acetic acid (60 Da each) and ammonia (NH₃). The abundant ion at m/z 269 could then be understood as cleavage of the arachidonyl carbon-ether bond with positive charge retention on the 20-carbon alkyl leaving group via the mechanism suggested in supplementary Fig. 1. The corresponding ether-bond fragment ion was observed in the MS/MS spectra of peaks B and C, but each was 2 Da and 4 Da, respectively, higher in measured m/z because the alkyl carbocation generated by collisional activation (supplementary Fig. 1) has five and six rings or double bonds, respectively, compared with the arachidonyl carbocation generated from CID of 2-AG ether, which has only four.

![Fig. 1. Structural analysis of products formed by the oxygenation of 2-AG ether by huPGHS-2.](image-url)
rings or double bonds. Considering the presence of three hydroxyl groups and loss of a double bond, the data are consistent with the presence of a novel ether lipid having a PGF₂ structural element, 2-O-(PGF₂)-glycerol. The scale employed in these experiments was not sufficient for NMR analysis of this metabolite.

The increase in the observed adduct molecular ion mass for peak B was 58 Da (Fig. 1C), consistent with an additional hydroxyl group along the arachidonyl carbon chain that had been converted to an acetate ester. Collisional activation of peak B yielded ions at m/z 447 [M+H-CH₃COOH]⁺, m/z 387 (m/z 477-CH₃COOH), and the most abundant product ion at m/z 271 (Fig. 1C). This latter ion corresponded to the most abundant product ion observed in the MS/MS spectrum of the starting material (that being m/z 275), but 2 Da lower, corresponding to cleavage of the ether bond in 2-AG ether and an additional double bond introduced by the presence of one acetoxy group that had been lost as acetic acid following collisional activation. The position of this hydroxyl group on the 20-carbon chain was determined by electron ionization MS as the TMS derivative (Fig. 2), and the ion at m/z 225 [CH₃(CH₂)₄CH=CH-CH=CH-CH=O+-TMS] was consistent with an introduction of a hydroxyl group at C-11 of the arachidonoyl carbon chain and migration of the Δ¹¹,¹² double bond to Δ¹²,¹³. Thus, this metabolite was determined to be 2-O-(11-hydroxy-eicosatetraenyl)-glycerol.

Overall, our mass spectrometric studies and measurements of O₂ consumption indicate that 2-AG ether is converted by huPGHS-2 to two major products: 2-O-(11-hydroxy-eicosatetraenyl)-glycerol ether (25%) and 2-O-(PGH₂)-glycerol ether (75%). These two products are the glycerol ether homologs of the 2-(11-hydroxy-eicosatetraenyl)-glycerol and 2-PGHe-glycerol products that are formed in similar proportions upon incubation of 2-AG with muPGHS-2 (18).

Comparison of 2-AG and 2-AG ether as huPGHS-2 substrates

We determined the following Vₘₐₓ and Kₘ values for recombinant huPGHS-2 when comparing 2-AG ether, 2-AG, and AA as substrates: Vₘₐₓ of 27 units/mg and Kₘ = 4.6 μM for 2-AG ether (Fig. 3); Vₘₐₓ of 30 units/mg and Kₘ~7 μM for 2-AG (15); and Vₘₐₓ of 43 units/mg and Kₘ~10 μM for AA (15). Thus, the catalytic efficiencies (Vₘₐₓ/Kₘ) with huPGHS-2 are similar when each substrate is tested individually.

To estimate the relative affinities of Eₐₗₜₜₜ and Eₐₗₜₜₜ of 2-AG ether compared to 2-AG, we first examined the effects of

![Fig. 2. EI mass spectrum of the TMS-derivative of peak B obtained by GC/MS. Structure inset with origin of major EI ions indicated from the TMS derivative.](image)

![Fig. 3. Oxygenation of 2-AG ether by huPGHS-2 in the presence and absence of PA. Results are shown as rates of O₂ consumption determined by measuring COX activity using an O₂ electrode as described in the Experimental Procedures. Reactions were initiated by adding an aliquot of purified native huPGHS-2 to an assay chamber containing the indicated concentration of 2-AG ether in the absence or presence of 25 μM PA. Experiments were performed with at least two different preparations of huPGHS-2 with similar results. Results are shown for a representative experiment involving triplicate determinations. The error bars indicate the average ± SD. Asterisks denote significant differences between the specific activity without PA versus with 25 μM PA at the indicated 2-AG ether concentration, as determined by the Student’s t-test (P<0.01).](image)

| FA Addedₜ | Unreacted [1-14C]AA Remainingₜ |
|-----------|-------------------------------|
| Control (no FA added) | 18 ± 0.08                     |
| 5 μM AA | 6.6 ± 1.4ₜ                     |
| 5 μM PA | 6.2 ± 1.2ₜ                     |
| 2.5 μM 2-AG ether | 17 ± 0.22                     |
| 5 μM 2-AG ether | 16 ± 0.33                     |
| 7.5 μM 2-AG ether | 14 ± 0.09                     |
| 15 μM 2-AG ether | 11 ± 0.09ₜ                     |
| 25 μM 2-AG ether | 7.9 ± 1.8ₜ                     |

The [1-14C]AA (1 μM) was incubated with huPGHS-2 (1 μM) at 37°C for 4 min, then unlabeled AA, PA, or 2-AG ether was added, and the incubation continued for another 4 min. Reactions were stopped by adding ethyl acetate/acidic acid (20:1), and an aliquot of the organic phase was subjected to radio-RP-HPLC to separate the radioactive products and unreacted AA, as described in the Experimental Procedures. The results are shown as the percentage of total ¹⁴C label that remained in the RP-HPLC fraction co-eluting with unreacted AA; the data represent averages of replicate samples ± SD. The results are shown for a single experiment that was performed a total of three times with similar results using different enzyme preparations in each case and concentrations of 2-AG ether ranging from 2.5-50 μM.

ₜFA was added 4 min after initiating the reaction.

ₜThe amount of unreacted [1-14C]AA remaining after 8 min (percent of starting radioactivity, average ± SD from two reactions).

ₜValue shown is average ± SD for replicate determinations from two reactions.

ₜSignificantly different from the control value (no FA or other agent added at 4 min) in Student’s t-test (P<0.05).
the nsFA, PA, on the oxygenation of 2-AG ether (Fig. 3). Increasing the ratio of 2-AG ether to PA did not change the ratio of the rates with 2-AG ether alone versus 2-AG ether plus PA. This indicates that 2-AG at any of the concentrations used in the assays fails to compete with 25 μM PA for E_allo. PA binds to E_allo of huPGHS-2 with a K_d ∼ 7.5 μM, but binds only very weakly to E_cat (K_d > 50 μM) (7). PA increased the V_max but did not change the K_m of huPGHS-2 for 2-AG ether. Because it increases the rate of 2-AG ether oxygenation, PA is not competing for E_cat, but rather must act via E_allo; moreover, 2-AG ether did not compete with PA for E_allo at the concentrations tested. This indicates that 2-AG ether binds significantly less tightly to E_allo than PA, and thus, less tightly to E_allo than E_cat. The K_d for PA binding to E_allo is ∼ 7.5 μM, which is below its critical micelle concentration [∼ 25 μM (7, 9)], where PA effectively binds only E_allo. The 2-AG ether did not displace PA from E_allo when the ratio of 2-AG ether/PA was 1.0, indicating that the K_d for 2-AG ether binding is significantly greater than 7.5 μM and, thus, greater than the K_d of 2-AG ether for E_cat. The K_d for 2-AG ether binding to E_cat is the K_m of huPGHS-2 for 2-AG [∼ 5 μM (Fig. 3)]. This situation is unlike what is observed with AA (7) or EPA (10) that bind E_allo 30 times more tightly than E_cat of huPGHS-2.

The results in Table 1 provide further evidence that 2-AG ether binds more tightly to E_cat than E_allo of huPGHS-2. In contrast to what is observed with PA or with 5 μM AA itself, 2-AG ether, at an initial concentration of 5 μM, more than 25 times that of unreacted [1-14C]AA (0.18 μM), failed to displace [1-14C]AA from E_allo of huPGHS-2. Only at concentrations higher than 7.5 μM does 2-AG ether cause any significant displacement of [1-14C]AA from E_allo. These findings provide additional evidence that 2-AG ether fails to bind efficiently to E_allo of PGHS-2. We also compared the interactions of 2-AG and 2-AG ether with nsFAs (Fig. 4). PA was previously found to activate the oxygenation of 2-AG by huPGHS-2, although the magnitude of the effect was much less than that seen with AA (15). As shown in Fig. 4, nsFAs that bind to E_allo cause very similar levels of activation of both 2-AG and 2-AG ether oxygenation by huPGHS-2. These data, viewed in

Fig. 4. Comparison of the effects of nsFAs on the oxygenation of 2-AG and 2-AG ether by huPGHS-2. Measurements of COX activity were performed in a standard O_2 electrode assay with 5 μM 2-AG (A) or 5 μM 2-AG ether (B) as the substrate in the presence or absence of 25 μM PA (16:0), stearic acid (18:0), oleic acid (18:1ω9), or eicosanoic acid (20:1ω9), as described in the Experimental Procedures. The same amounts of enzyme protein were used in all assays, and the value without a nsFA was normalized to 100%. Results are shown for a single experiment involving triplicate determinations. The error bars indicate the average ± SD. When control values are normalized to 100% activity, significant differences from the control value (with no ibuprofen) were seen at all the ibuprofen concentrations tested, as determined by the Student’s t-test (P < 0.05). IC_50 values shown in the two panels were calculated using GraphPad Prism software. For the monophasic mode shown in the two panels in this figure, IC_50 values were calculated by using nonlinear regression to fit the data to the log [ibuprofen] versus normalized response (variable slope) curve.
the context of the kinetic data indicating that 2-AG and 2-AG ether have similar properties as huPGHS-2 substrates, establish that 2-AG ether can be used as a stable surrogate for 2-AG in monitoring interactions between this latter endocannabinoid and PGHS-2.

**Comparison of IBP as an inhibitor of the oxygenation of 2-AG, 2-AG ether, and AA**

Many COX inhibitors are time-dependent inhibitors, but IBP is a rapidly reversible inhibitor of AA oxygenation (19, 20). As first reported by Marnett and coworkers, IBP is a more potent inhibitor of the oxygenation of 2-AG than of AA by muPGHS-2 (13). According to their model, IBP binds one monomer of PGHS-2 to allosterically inhibit 2-AG oxygenation occurring in the partner catalytic monomer, whereas IBP binding to both monomers is required to inhibit AA oxygenation.

We compared the effects of IBP on the oxygenation of 2-AG, 2-AG ether, and AA by huPGHS-2. As shown in Fig. 5, instantaneous inhibition of huPGHS-2-mediated oxygenation of 2-AG and 2-AG ether occurs at similar concentrations of IBP.

We recently described a recombinant huPGHS-2 heterodimer variant, denoted as Y385F/Native huPGHS-2 (15). This variant has 90% of the activity of native huPGHS-2, indicating that 90% of Eallo monomers bear the Tyr-385Phe mutation and 90% of Ecat monomers are native monomers; moreover, once Eallo and Ecat of huPGHS-2 are formed, they do not interconvert (15). This variant can be used as a platform to determine the effect of amino acid substitutions in the COX binding site of Eallo, the subunit with the Tyr385Phe substitution, as compared with the native Ecat subunit (15). **Figure 6** presents the results of studies of Arg-120 substitutions in the Y385F/Native huPGHS-2 platform on the effect of IBP on 2-AG ether oxygenation; supplementary Fig. 2 presents these data, showing actual rates as opposed to the relative COX activities shown in Fig. 6. Arg-120 is the residue that interacts with the carboxylate group on substrates and inhibitors that bind within the COX active site. Inhibition of oxygenation of 2-AG ether by IBP is largely impeded by having Arg-120 substitutions in both subunits (R120A/R120A huPGHS-2), but only modestly attenuated by having an Arg120Ala substitution in Eallo (i.e., Y385F R120A/Native huPGHS-2). This suggests that
IBP functions by binding Eallo, in native huPGHS-2 to cause near complete allosteric inhibition of 2-AG ether oxygenation. However, IBP appears to bind only slightly less well to Ecat to cause complete inhibition when IBP cannot bind to Eallo (i.e., in Y385F R120A/Native huPGHS-2).

As shown in Figs. 5, 6, 50% inhibition of 2-AG ether oxygenation occurs with 5–10 μM IBP with essentially complete inhibition occurring with 30–40 μM IBP. As illustrated in Fig. 5 with native huPGHS-2, the inhibition curves for 2-AG and 2-AG ether involve a single phase. Figure 7 shows the effect of IBP on AA oxygenation by huPGHS-2. Unlike that seen with 2-AG and 2-AG ether, the best fit curve is biphasic for inhibition of AA oxygenation. IBP causes approximately 50% inhibition through a higher affinity IBP binding event, with an IC50 of about 120 μM. The remainder of the inhibition occurs through a second lower affinity IBP binding event, having an IC50 of about 1 mM (i.e., 948 μM). One simple explanation for these data is that the initial and apparent incomplete phase of inhibition of AA oxygenation reflects partial allosteric inhibition upon IBP occupancy of Eallo, while the second phase represents competitive IBP binding to Ecat. The fact that the IC50 for the first phase is 10–20 times higher than the IC50 for 2-AG ether oxygenation is consistent with the idea that IBP needs to displace AA bound to Eallo to cause the first phase of inhibition.

DISCUSSION

PGHSs function in solution as conformational heterodimers composed of Ecat and Eallo monomers. In previous studies, we have identified the Eallo versus Ecat binding specificities of huPGHSs toward FAs from a number of functional classes and of a variety of NSAIDs (7, 8, 10, 15). Here we have determined the binding specificities of 2-AG ether and ibuprofen toward Eallo and Ecat of PGHS-2. Figure 8 compares the specificities of a number of ligands toward Eallo and Ecat of both PGHS-1 and PGHS-2.

The 2-AG is a relatively good substrate for muPGHS-2 and huPGHS-2, but a poor substrate for ovine PGHS-1 (15, 18). Ovine PGHS-1 oxygenates 2-AG at 5% of the rate of AA, whereas 2-AG is oxygenated at 65–75% of the rate of AA by PGHS-2; moreover, the Km values for PGHS-2 with AA and 2-AG are similar (15, 18).

Our present studies indicate that huPGHS-2 has essentially the same kinetic properties and forms a homologous set of products with 2-AG and 2-AG ether. These two substrates have indistinguishable Km and Vmax values, are activated to similar extents by common nsFAs, and are inhibited to similar extents and apparently via similar mechanisms by IBP.

The 2-AG ether exhibits a preference for binding Ecat versus Eallo. These results are consistent with recent studies by Marnett and coworkers that have indicated that 2-AG binds Ecat with about 10 times higher affinity than Eallo (11). The Km for 2-AG ether, which is equivalent to the Km for 2-AG ether binding to Ecat, is 5–10 μM (15). The marked preference of 2-AG and 2-AG ether for Ecat of huPGHS-2 is unlike that observed with FA substrates, such as AA and EPA, that bind Eallo with 20–30 times greater affinity than Ecat (7, 15). Presumably neither the ester nor ether groups of 2-AG or 2-AG ether, respectively, effectively interact with Arg-120 of Eallo.

Because 2-AG ether is more stable than 2-AG, which readily hydrolyzes or rearranges (12, 21), 2-AG ether can be used as a stable surrogate for examining 2-AG as a COX substrate.
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