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Altered Sensitivity of Aspirin-Acetylated Prostaglandin G/H Synthase-2 to Inhibition by Nonsteroidal Anti-Inflammatory Drugs

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SUMMARY

Aspirin (ASA) acetylates Ser516 of prostaglandin G/H synthase-2 (PGHS-2) resulting in a modified enzyme that converts arachidonic acid to 15(*R*)-hydroxy-eicosatetraenoic acid [15(*R*)-HETE]. ASA has pharmacological benefits that may not all be limited to inhibition of prostaglandin synthesis, and this study was initiated to further investigate the properties of ASA-acetylated PGHS-2 and of the mutation of Ser516 to methionine, which mimics ASA acetylation. Both the S516M mutant and ASA-acetylated form of PGHS-2 (ASA-PGHS-2) synthesize 15(*R*)-HETE and have apparent K_m values for arachidonic acid within 10-fold of the apparent K_m value for untreated PGHS-2. The time courses of turnover-dependent inactivation were similar for reactions catalyzed by PGHS-2 and ASA-PGHS-2, whereas the PGHS-2(S516M) showed a decrease in both the initial rate of 15-HETE production and rate of enzyme inactivation. The production of 15-HETE by modified PGHS-2 was sensitive to inhibition by most nonsteroidal anti-inflammatory drugs (NSAIDs), including selective PGHS-2 inhibitors. As observed for the cyclooxygenase activity of PGHS-2, the inhibition of 15-HETE production by indomethacin was time-dependent for both ASA-PGHS-2 and PGHS-2(S516M). However, two potent, structurally related NSAIDs, diclofenac and meclofenamic acid, do not inhibit either ASA-PGHS-2 or the PGHS-2(S516M) mutant. These results demonstrate that the sensitivity to inhibition by NSAIDs of the 15-HETE production by ASA-treated PGHS-2 is different than that of prostaglandin production by PGHS-2 and that Ser516 plays an important role in the interaction with fenamate inhibitors. The results also indicate that the conversion of arachidonic acid to 15-HETE by ASA-PGHS-2 is an efficient process providing a unique mechanism among NSAIDs that will not lead to arachidonic acid accumulation or shunting to other biosynthetic pathways.

ASA was first introduced into medicine as an analgesic and anti-inflammatory agent in 1899 and was named aspirin (1, 2). Approximately 72 years later, the mechanism of action of ASA and newer NSAIDs such as indomethacin was discovered (1, 2). This mechanism involves the inhibition of the enzyme-catalyzed production of prostaglandins, which act as potent mediators of pain, fever, and inflammation (3). The enzyme that catalyzes the first step in the conversion of arachidonic acid to these potent eicosanoid mediators is PGHS (4, 5). There are currently two isoforms of this enzyme, PGHS-1, a constitutively expressed form, and PGHS-2, an inducible isoform (2, 3, 5, 6). Both isoforms catalyze a similar reaction that involves the bis-dioxygenation of arachidonic acid to form prostaglandin G_2 followed by a reduction step to form prostaglandin H_2 (4, 5). Prostaglandin H_2 serves as the precursor for a variety of biologically active prostanoids.

NSAIDs inhibit both PGHS-1 and PGHS-2 isoforms with varying potencies (7–14). PGHS-1 appears to provide prostaglandins for normal physiological conditions, whereas PGHS-2 is induced in various inflammatory conditions (15, 16). The associated toxicity of present NSAIDs, especially gastric ulceration, has been attributed to the inhibition of PGHS-1 (15–17). Recently, selective PGHS-2 inhibitors such as NS-398 [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide], DuP697, [5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl)thiophene] and L-745,337 [5-methanesulfonamide-6-(2,4-difluorothiophenyl)-1-indanone] have been demonstrated to be effective anti-inflammatory, analgesic, and antipyretic agents but are much less irritating to the gastric lining than conventional NSAIDs (16–20). Inhibition of PGHS can occur via competitive, reversible inhibition at the cyclooxygenase-active site or via time-dependent and effectively irreversible inhibition of PGHS without covalent modification (5, 11, 21). ASA is the only known NSAID that covalently modifies a serine residue near the cyclooxygenase-active site of PGHS (22–25). Acetylation of residue 530 of

ABBREVIATIONS: ASA, aspirin (acetylsalicylic acid); PGHS, prostaglandin G/H synthase; PGE_2, prostaglandin E_2; HETE, hydroxy-eicosatetraenoic acid; NSAID, nonsteroidal anti-inflammatory drug; HPLC, high performance liquid chromatography.

PGHS-1 results in an enzyme form that is unable to oxidize arachidonic acid, whereas acetylation of the corresponding serine residue in PGHS-2 (Ser516) generates an enzyme form that performs an incomplete reaction in which arachidonic acid is converted to 15(*R*)-HETE (8, 22–24, 26). The crystal structure of PGHS-1 complexed with ASA shows that ASA acetylation of Ser530 blocks access to the cyclooxygenase-active site of PGHS (25). Recently, a report was published that demonstrates that 15(*R*)-HETE derived from ASA-acetylated PGHS-2 can be converted by lipoxygenases into a lipoxin metabolite that inhibits adhesion of polymorphonuclear leukocytes (27). It has been suggested that 15(*R*)-HETE and its further oxygenation products may have a therapeutic relevance as an anti-inflammatory agent. Although reports have shown that 15-HETE can inhibit neutrophil migration (28), the relevance of a stereoselective 15-HETE molecule and its role in inflammation is not well defined.

Previous reports also have shown that mutagenesis of the serine residue at position 516 of human PGHS-2 mimics the effect of acetylation by ASA and results in a modified enzyme form that produces primarily 15(*R*)-HETE (23, 24, 29). Because the serine residue of PGHS-1 has been demonstrated to impinge on the active site of the enzyme (25, 30) and PGHS-2 is highly homologous to PGHS-1 (31), the acetylation of this residue by ASA may also affect the interaction of PGHS-2 with NSAIDs. In the present study, we have characterized the production of 15(*R*)-HETE by acetylated PGHS-2 and by PGHS-2(S516M) and have compared the inhibition profiles of various PGHS inhibitors on 15(*R*)-HETE production by PGHS-2 containing a modified Ser516 residue compared with PGE_2 production by the unmodified PGHS-2. Interestingly, diclofenac and meclofenamic acid, which are potent inhibitors of PGHS-2, do not inhibit either the acetylated or the mutant form of PGHS-2. The altered sensitivity of ASA-acetylated PGHS-2 to inhibition by NSAIDs may have implications for the use of PGHS inhibitors in combination with ASA and for elucidating the mode of binding of certain NSAIDs with PGHS-2.

Materials and Methods

Expression of PGHS-2 and PGHS-2(S516M). The PGHS-2 recombinant protein was obtained from the baculovirus Sf9 cell expression system and PGHS-2(S516M) protein was obtained using the vaccinia virus expression system in COS-7 cells as previously described (8, 32, 33). Microsomal preparations were prepared from cellular extracts of Sf9 and COS-7 cells by sonication of cell pellets in 100 mM Tris·HCl, pH 7.4, 10 mM EDTA, 2 mM phenylmethonylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml soybean trypsin inhibitor using a microsonic cell disruptor (Cole-Parmer Instrument, Chicago, IL). The cellular extracts were centrifuged at 1,000 $\times g$ at 4° for 10 min and the resulting supernatant was centrifuged at 100,000 $\times g$ for 60 min at 4°. The resulting membrane pellet was resuspended in 100 mM Tris·HCl, pH 7.4, and 10 mM EDTA.

Assay and inhibition of prostaglandin G/H synthase activity. Microsomal preparations of PGHS-2 were assayed for PGE_2 synthesis using a radioimmunoassay (Amersham, Arlington Heights, IL) as described previously (8, 33). Essentially, enzyme assays were performed at protein concentrations of 5 μg/ml in a volume of 210 μl containing 0.1 M Tris·HCl, pH 7.4, 10 mM EDTA, 1 mM reduced glutathione, 0.5 mM phenol, and 1 μM hematin (buffer A). PGHS inhibitors were dissolved in dimethylsulfoxide and preincubated with enzyme in the assay mixture for 15 min at room temperature. The reaction was initiated by the addition of arachidonic acid (5 μl of an ethanol solution to obtain a final concentration of 2 μM arachidonic acid) and terminated after 3 min with the addition of 0.1 M HCl (final concentration) and neutralized with NaOH. The PGE_2 product formed was quantitated using the radioimmunoassay kit. The apparent K_m and V_{max} values of the microsomal preparations were determined from the PGE_2 product formed after a 3-min reaction time as a function of arachidonic acid concentration. The kinetic analysis was performed on the hyperbolic plotting program by J. S. Easterby of the University of Liverpool (Hyperbolic Regression Analysis of Enzyme Kinetic Data). The inhibitors flurbiprofen and indomethacin were obtained from Cayman Chemical (Ann Arbor, MI) and ketoprofen, indomethacin, meclofenamic acid, and diclofenac were obtained from Sigma Chemical (St. Louis, MO); sulindac sulfide, L-745,337, SC-57666 [1-[2-(4-fluorophenyl)cyclopenten-1-yl]-4-(methylsulfonyl)benzene], NS-398, and DuP697 were synthesized at the Medicinal Chemistry Department at the Merck Frosst Centre.

The peroxidase activities of PGHS-2, acetylated PGHS-2, and PGHS-2(S516M) were determined to compare inactivation rates of these three microsomal enzyme preparations. The peroxidase activity was measured spectrophotometrically at 436 nm using guaiacol and H_2O_2 as the cosubstrates as described previously (32, 34).

15(*R*)-HETE production and measurement by HPLC. Microsomal preparations of recombinant PGHS-2 were treated with 100 μM ASA for 30 min in buffer A. The ASA treated PGHS-2 and PGHS-2(S516M) were then incubated for 15 min in the presence of dimethylsulfoxide vehicle or inhibitor. The reaction was initiated with arachidonic acid (10 μM final concentration) and terminated after 3 or 25 min for ASA-acetylated PGHS-2 and PGHS-2(S516M), respectively. The product and a known quantity of PGB_2 internal standard were extracted with an equal volume of methanol and chloroform. The organic material in the chloroform phase was retained and evaporated to dryness under nitrogen. The material was chromatographed using reverse-phase HPLC (nova-pak C18; Waters, Milford, MA) in 75% methanol, 25% H_20, and 0.01% acetic acid at a constant flow rate of 1 ml/min. Elution of the 15-HETE product was followed at a wavelength of 234 nm as described previously (8).

Results

Comparison of PGE_2 synthesis by PGHS-2 and 15(*R*)-HETE synthesis by acetylated PGHS-2 and PGHS-2(S516M). The crystal structure of PGHS-1 has revealed that Ser530, the acetylation site of ASA, is located in the upper part of the cyclooxygenase-active site and in proximity to the unsubstituted phenyl ring of bound flurbiprofen (25, 30). The importance of the corresponding ASA-acetylated serine residue of PGHS-2(Ser516) on arachidonic acid oxidation and the interaction of the enzyme with NSAIDs were evaluated using acetylated PGHS-2 and the mutant PGHS-2(S516M). PGHS-2 and PGHS-2(S516M) were expressed in a baculovirus or vaccinia virus expression system, respectively, to determine the characteristics of PGE_2 and 15(*R*)-HETE production. Previous studies have shown that PGHS-2 in these preparations makes PGE_2 as major product with a low amount of 15-HETE and modification of Ser516 by acetylation or mutation to methionine abolishes PGE_2 synthesis and stimulates 15(*R*)-HETE formation (8, 23, 32). Fig. 1 shows an HPLC tracing of the products of arachidonic acid oxidation by the microsomal preparation of PGHS-2(S516M). 15(*R*)-HETE is detected as the major reaction product, and its synthesis was completely abolished by flurbiprofen, which confirms that it originates from PGHS. The remaining products are

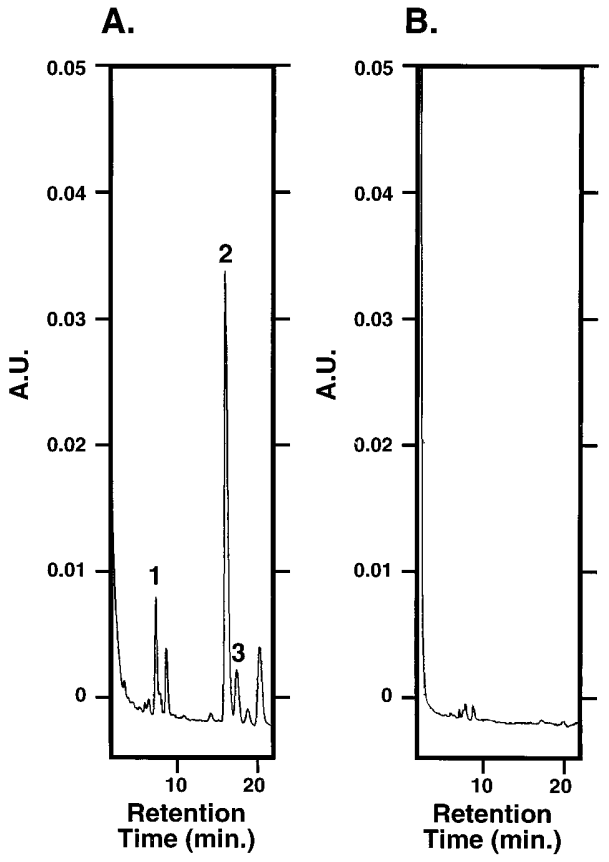


Fig. 1. Oxidation of arachidonic acid to 15(*R*)-HETE by PGHS-2(S516M). Microsomal membrane preparations of PGHS-2(S516M) were incubated with arachidonic acid for 25 min in the absence (A) or presence (B) of 10 μM of flurbiprofen. The reaction products were extracted with chloroform and analyzed for 15(*R*)-HETE production by reverse-phase HPLC. The peaks that have been identified through coelution with authentic standards are labeled: *1*, 12-hydroxy-5,8,10-heptadecatrienoic acid; *2*, 15-HETE; and *3*, 11-HETE.

PGHS-derived products of which 12-hydroxy-5,8,10-heptadecatrienoic acid and 11-HETE have been identified and are inhibited by preincubation with flurbiprofen. Initial characterization of the enzyme preparations was performed by obtaining time courses of PGE_2 and 15(*R*)-HETE production by the various microsomal preparations of PGHS-2 determined at saturating arachidonic acid concentrations. PGE_2 formation by PGHS-2 is increased rapidly during the first minute and reaches a plateau after 5–10 min (Fig. 2A). The time course of the reaction was also determined for the production of 15(*R*)-HETE by the same enzyme preparation after acetylation by ASA. Microsomes containing PGHS-2 were incubated for 30 min with 100 μM ASA before initiation of the reaction by addition of arachidonic acid. Under these conditions, 15(*R*)-HETE is the major reaction product of the ASA-acetylated PGHS-2. Quantitation of the 15(*R*)-HETE produced by reverse-phase HPLC indicated that the time course of 15(*R*)-HETE formation by acetylated PGHS-2 was very similar to the time course of PGE_2 production by the unmodified PGHS-2 and also reached a plateau by 5–10 min (Fig. 2B). The acetylated PGHS-2 produced approximately 10-fold more 15(*R*)-HETE than PGE_2 by the unmodified PGHS-2 by the end of the corresponding reactions.

The PGHS-2(S516M) mutant has been demonstrated to react similarly to the acetylated PGHS-2 and to produce primarily 15(*R*)-HETE when incubated with arachidonic acid in the absence of ASA treatment (23). This PGHS-2 mutant was used in these studies as a comparison with the acetylated form of PGHS-2 to demonstrate independently the effect of the modification of Ser516 in 15(*R*)-HETE production. A time course of 15(*R*)-HETE production following addition of arachidonic acid to the microsomes containing PGHS-2(S516M) shows that this mutated protein has a markedly reduced initial velocity compared with the acetylated PGHS-2 and that product formation was increasing even at the 30-min incubation time point (Fig. 2C).

The peroxidase activity of microsomal preparations of the three enzyme forms mentioned above was determined using H_2O_2 and guaiacol as cosubstrates (34). Acetylation of PGHS-2 by ASA resulted in a decrease of the peroxidase activity of about 40% (642 nmol H_2O_2 reduced/mg of protein/min compared with 1080 nmol H_2O_2 reduced/mg of protein/min for untreated PGHS-2). The specific activity of the preparation of PGHS-2(S516M) was similar to that of PGHS-2 (1037 nmol H_2O_2 reduced/mg of protein/min). Because the protein expression levels may vary between PGHS-2 and the Ser-Met mutant, an immunoblot analysis was performed for each enzyme preparation and is depicted in Fig. 3. The expression of PGHS-2(S516M) is approximately 3-fold lower than PGHS-2 as analyzed by laser densitometric scanning of the immunoblot data. Therefore, the peroxidase activity of PGHS-2(S516M) is not decreased compared with unmodified PGHS-2; the lack of rapid turnover inactivation may be due to a slower turnover rate for this enzyme form. To address this issue, we have decreased the amount of ASA-PGHS-2 microsomes used in a time course assay of product formation and demonstrated that the time course of the reaction becomes similar to that of PGHS-2 (S516M). (Fig. 2, B-D).

Arachidonic acid dependence of PGE_2 and 15(*R*)-HETE production. The dependence on arachidonic acid of 15(*R*)-HETE production by acetylated PGHS-2 and the PGHS-2(S516M) mutant was compared with that of PGE_2 production by PGHS-2. Apparent K_m values for arachidonic acid were evaluated over a broad range of arachidonic acid concentration using a reaction time of 3 min for the production of PGE_2 by PGHS-2 and 15(*R*)-HETE for the ASA-acetylated PGHS-2. Because the reaction catalyzed by the PGHS-2(S516M) mutant was linear over a longer period of time, the 15(*R*)-HETE formation was analyzed after a 25-min reaction. The curves for all three microsomal enzyme preparations of PGHS-2 are shown in Fig. 4. The K_m value for the nonacetylated PGHS-2 was 0.44 μM, a value similar to that reported in similar assays (34). The K_m value for the production of 15(*R*)-HETE by the acetylated PGHS-2 was 4.4 μM, and the K_m value for the PGHS-2(S516M) mutant was 1.6 μM (Table 1). The data in Table 1 also show that the production of 15(*R*)-HETE by both acetylated PGHS-2 and PGHS-2(S516M) is at least 10 times higher than that of PGE_2 by PGHS-2 at the reaction time indicated.

Inhibition of PGHS-2, ASA-PGHS-2, and PGHS-2(S516M). Most of the currently used NSAIDs are potent but rather nonselective inhibitors of PGHS-1 and PGHS-2 (2, 5). Recently, novel classes of selective inhibitors of PGHS-2 have been developed (2, 5, 15, 17–20). We have tested several of these nonselective and selective PGHS inhibitors against the enzymatic activities of PGHS-2, ASA-PGHS-2, and PGHS-

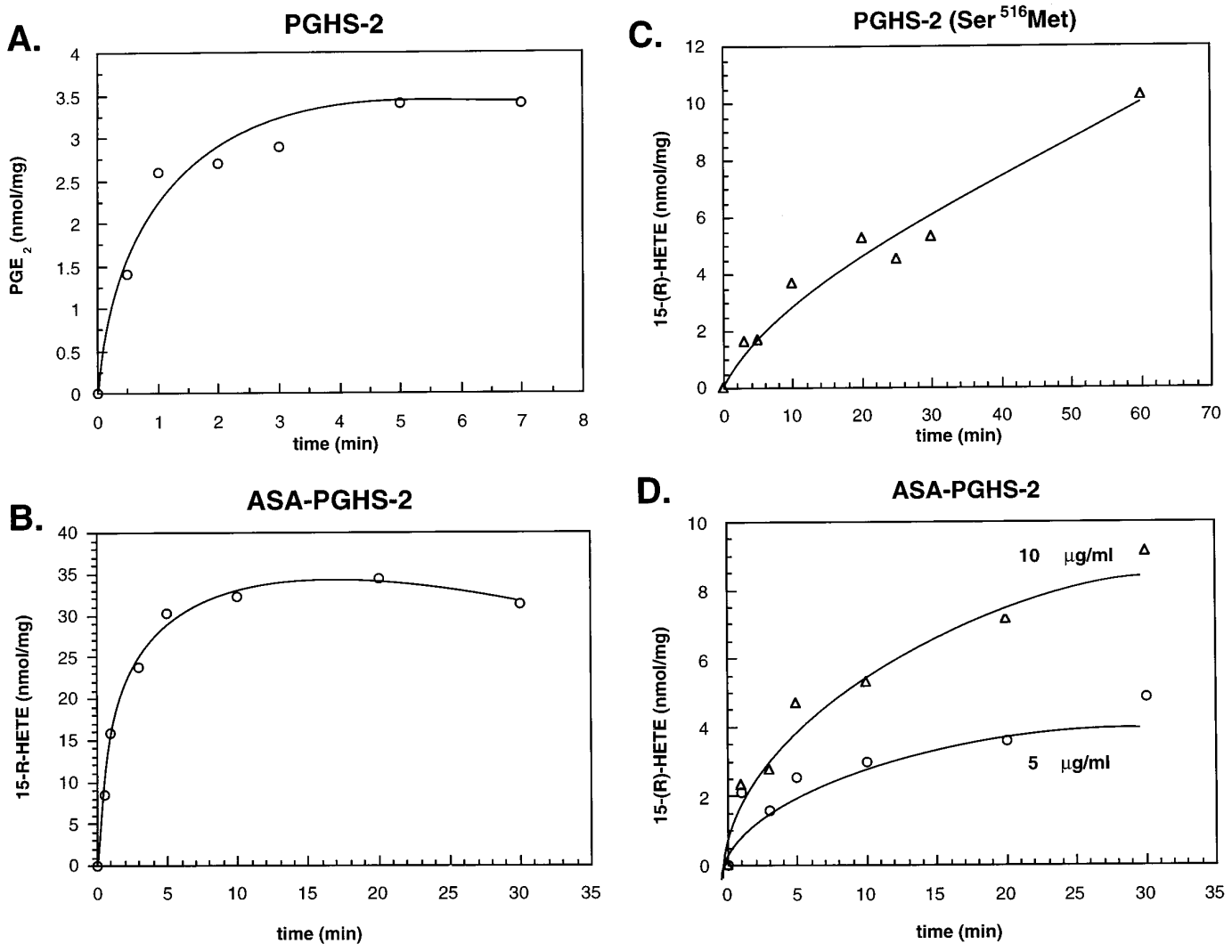


Fig. 2. Time course of product formation by PGHS-2, ASA-PGHS-2, and PGHS-2(S516M). Membrane preparations containing PGHS-2 (A), ASA-treated PGHS-2 (B), and PGHS-2(S516M) (C) were incubated with 2, 10, and 10 μM arachidonic acid, respectively. The reactions were terminated at various time points by acidification. PGE_2 formation for PGHS-2 was detected by radioimmunoassay, whereas microsomal preparations containing PGHS-2 treated with ASA or PGHS-2(S516M) were analyzed for 15-HETE formation by reverse-phase HPLC. A time course of product formation by ASA-PGHS-2 at low enzyme concentrations using a dilution to obtain a similar turnover rate as obtained by PGHS-2(S516M) was also performed (D).

2(S516M). We have depicted the IC_{50} curves of two of these inhibitors (Fig. 5): 1) flurbiprofen, a nonselective time-dependent PGHS inhibitor; and 2) NS-398, a selective time-dependent PGHS-2 inhibitor (21). In addition to their inhibitory effects on PGHS-2, these inhibitors also inhibit the ASA-acetylated PGHS-2 and the PGHS-2(S516M) mutant. Flurbiprofen is about 6- and 20-fold less potent at inhibiting acetylated PGHS-2 and PGHS-2(S516M), respectively, compared with inhibition of the unmodified PGHS-2. NS-398 was an equipotent inhibitor of PGHS-2 and ASA-PGHS-2 and 6-fold less potent as an inhibitor of PGHS-2(S516M) when compared with PGHS-2. A complete summary of the potency of several inhibitors tested against the various PGHS-2 preparations is presented in Table 2. The corresponding structures of several of these inhibitors are depicted in Fig. 6. The most striking difference in inhibition sensitivity was obtained with meclofenamic acid and diclofenac. These compounds are potent PGHS inhibitors with IC_{50} values of approximately 60 nM in our microsomal PGHS-2 assay, but they do not inhibit product formation by the acetylated PGHS-2 or the PGHS-2(S516M) mutant at concentrations up to 240 μM. Diclofenac and meclofenamic acid are from the same fenamate class of PGHS inhibitors (Fig. 6). These results demonstrate that inhibition by certain NSAIDs is sensitive to modification of Ser516 of PGHS-2.

The final experiment was performed to show the mechanism of inhibition of acetylated PGHS-2 and the Ser-Met mutant. NSAIDs such as indomethacin are time-dependent inhibitors of PGHS-2 (11, 21). Microsomal preparations of ASA-PGHS-2 and PGHS-2(S516M) were preincubated with 3 μM indomethacin or dimethylsulfoxide for 0 to 30 min before initiation of the reaction with 20 μM arachidonic acid. The reactions were terminated after 3 or 25 min for ASA-PGHS-2 and PGHS-2(S516M), respectively. The 15-HETE product formation was analyzed by reverse-phase HPLC as described earlier, and the results are shown in Fig. 7. The inhibition of 15-HETE production by ASA-PGHS-2 and PGHS-2(S516M) is via a time-dependent mechanism as has been demon-

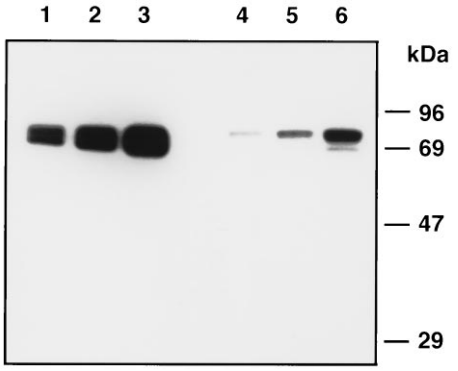


Fig. 3. Immunoblot analysis of PGHS-2 and PGHS-2(S516M). PGHS-2 was expressed in Sf9 insect cells using a baculovirus expression system, and PGHS-2(S516M) was expressed in Cos-7 cells using a vaccinia virus expression system. Microsomal membrane fractions of PGHS-2 and PGHS-2(S516M) were prepared and subjected to sodium dodecyl sulfate/polyacrylamide electrophoresis and processed for immunoblot analysis of PGHS-2 (41). *Lanes 1–3*, 1, 2, and 5 μg of PGHS-2 membrane protein, respectively. *Lanes 4–6*, 1, 2, and 5 μg of PGHS-2(S516M) membrane protein, respectively.

strated previously for PGHS-2. This demonstrates that the ASA acetylation of PGHS-2 or the Ser-Met mutation does not alter the mechanism of inhibition by PGHS inhibitors.

Discussion

PGHS is the enzyme that catalyzes the formation of prostaglandin metabolites from arachidonic acid (5). These prostaglandins are involved in various physiological functions, such as the regulation of renal blood flow, gastric cytoprotection, platelet aggregation, and parturition (3). Prostaglandins are also potent mediators of pain and inflammation, and because PGHS-2 is up-regulated during these pathophysiological conditions, PGHS-2 is an important target for the development of selective PGHS inhibitors (15, 16). ASA, an NSAID, has been demonstrated to covalently acetylate both isoforms of PGHS (23, 24). This covalent modification inhibits its product formation of PGHS-1 but results in an augmentation of 15(*R*)-HETE formation by PGHS-2 (8, 23, 24). Because ASA has many clinical benefits that may not all be related to inhibition of prostaglandins (27, 35, 36), we have compared the effect of inhibition by NSAIDs on the acetylated form of PGHS-2 to that of the unmodified PGHS-2. As a further confirmation that the results are related to the known modification of Ser516 of PGHS-2 by ASA, we also used the PGHS-2(S516M) mutant, which primarily synthesizes 15(*R*)-HETE when incubated with arachidonic acid in the absence of treatment with ASA.

The time course of product formation for PGHS-2 and the acetylated PGHS-2 follows the same pattern with linear product formation for 3 min, reaching a plateau at 5–10 min. Interestingly, the PGHS-2(S516M) is approximately linear for product formation for 30 min. A similar lack of rapid turnover inactivation has been reported for PGHS-1(Arg120Gln) (37). Because these mutants showed reduced reaction rates, their slower rate of inactivation may be accounted for by an overall reduction in turnover rather than by specific effects of the mutation on enzyme stability during the reaction. This conclusion is supported by the observation that acetylated PGHS-2 showed a marked decrease in inactivation at low enzyme concentrations. The ASA-acetylated PGHS-2 retained a significant amount of the peroxidase activity of PGHS-2, whereas PGHS-2(S516M) has no detectable decrease in peroxidase activity compared with the unmodi-

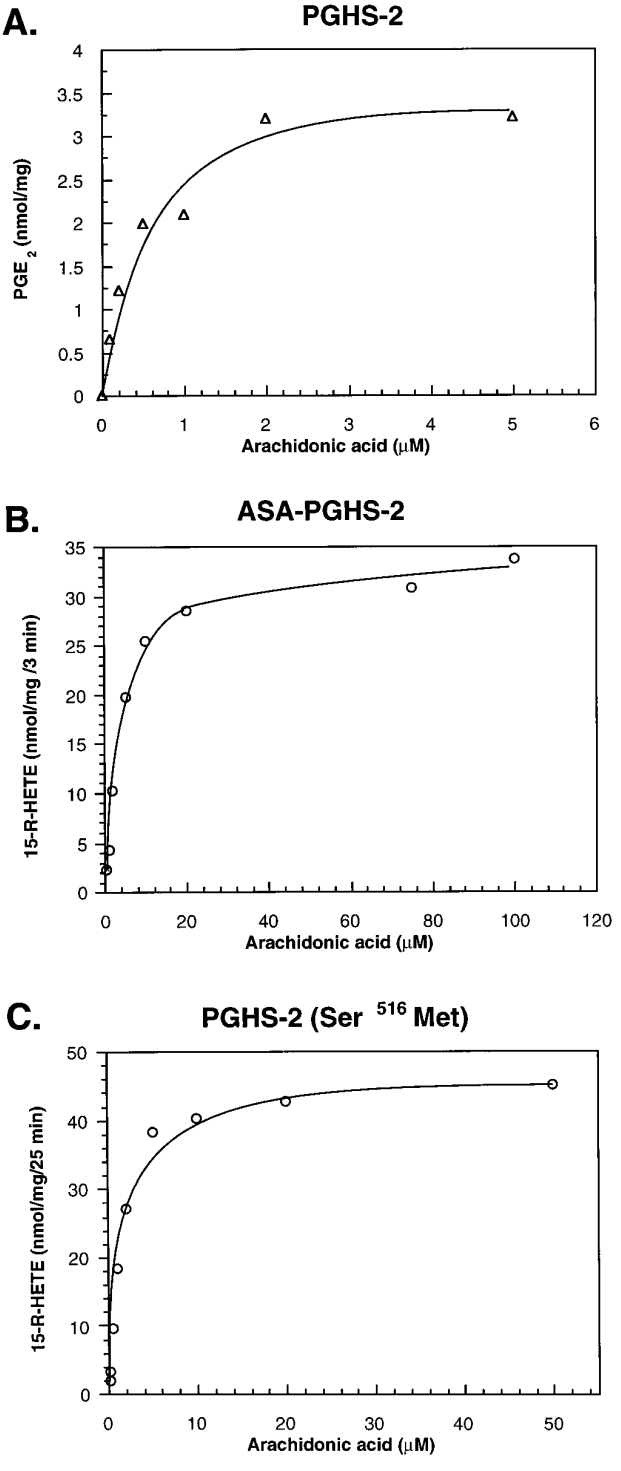


Fig. 4. Arachidonic acid dependence of PGE_2 and 15(*R*)-HETE production by PGHS-2, ASA-PGHS-2, and PGHS-2(S516M). Various concentrations of arachidonic acid were incubated with either PGHS-2 (A), ASA-treated PGHS-2 (B), or PGHS-2(S516M) (C). The reactions were terminated in the corresponding linear portion of product formation obtained from Fig. 2. The product measured (either PGE_2 or 15-HETE) was plotted versus arachidonic acid concentration and the K_m value was obtained using a hyperbolic regression analysis program.

TABLE 1

Summary of the kinetic properties of PGHS-2, ASA-treated PGHS-2, and PGHS-2(S516M)

Apparent K_m values for the microsomal preparations of the various PGHS-2 forms were determined from hyperbolic curve fits of the data from Fig. 5. Accumulation of PGE_2 and 15(*R*)-HETE product was determined at the indicated reaction time. The peroxidase activity was determined from the initial rate of reduction of H_2O_2 with guaiacol as a cosubstrate.

	Reaction product	K_m for arachidonic acid	Product accumulation	Peroxidase act
		μM	*nmol/mg of protein*	*nmol[d]/mg/min*
PGHS-2	PGE_2	0.44 ± 0.32	3.54 ± 0.75[a]	1080
Acetylated PGHS-2	15-(*R*)-HETE	4.4 ± 1.4	34.6 ± 2.8[b]	642
PGHS-2(Ser516Met)	15-(*R*)-HETE	1.6 ± 0.4	47.2 ± 2.6[c]	1037

[a] nmol PGE_2/mg of protein during a 3-min reaction time.
[b] nmol 15-HETE/mg of protein during a 3-min reaction time.
[c] nmol 15-HETE/mg of protein during a 25-min reaction time.
[d] nmol H_2O_2 reduced.

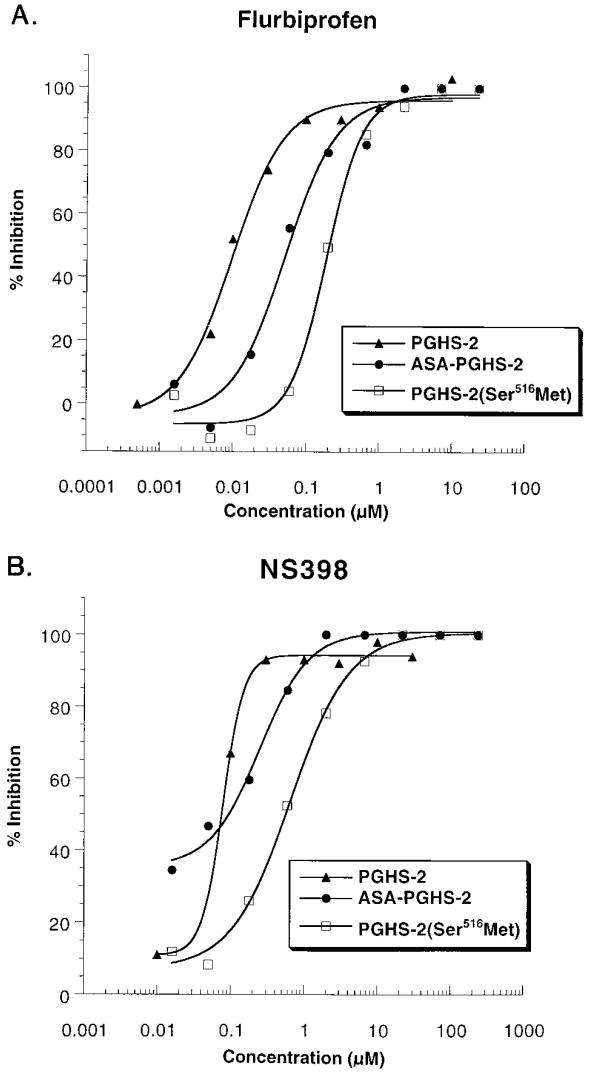


Fig. 5. Inhibition of PGHS-2 by flurbiprofen or NS-398. Microsomal preparations of PGHS-2, ASA-treated PGHS-2, or PGHS-2(S516M) were preincubated for 15 min with either flurbiprofen (A) or NS-398 (B). The reaction was then initiated with arachidonic acid and product formation with and without inhibitor was determined. The results were plotted as percentage of inhibition of PGHS activity versus inhibitor concentration. The IC_{50} values were determined using a 4-parameter logistic fit.

fied PGHS-2. The role of the peroxidase activity in the PGHS reaction is very complex and not completely understood. The peroxidase activity appears to be another site distant from

TABLE 2

Inhibitor sensitivity of PGHS-2, ASA-acetylated PGHS-2, and PGHS-2(S516M)

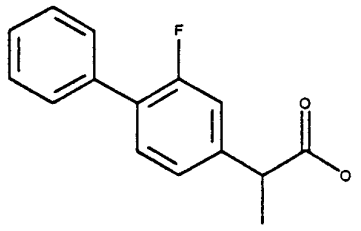
Microsomes containing the indicated PGHS-2 form were preincubated with varying concentrations of inhibitor for 15 min followed by the initiation of the reaction with either 2 or 10 μM arachidonic acid, for PGHS-2 or ASA-PGHS-2 and PGHS-2(S516M), respectively. IC_{50} values for the inhibition of PGE_2 or 15(*R*)-HETE formation were determined by a 4-parameter logistic fit of 8-point titrations of the indicated inhibitor. Each value is an average of at least two determinations. The first four compounds are selective PGHS-2 inhibitors.

Compound	IC_{50}		
	PGHS-2 (PGE_2)	ASA-PGHS-2 (15-(*R*)-HETE)	PGHS-2(S516M) (15-(*R*)-HETE)
		μM	
L-745,337	0.6	1.0	2.7
SC-57666	0.01	0.03	0.06
NS-398	0.08	0.1	0.6
DuP697	0.01	0.005	0.01
Flurbiprofen	0.01	0.06	0.2
Ketoprofen	0.03	0.6	0.5
Sulindac sulfide	0.1	0.12	1.9
Indomethacin	3.0	1.5	1.6
Meclofenamic acid	0.05	>240	>240
Diclofenac	0.07	>240	>240

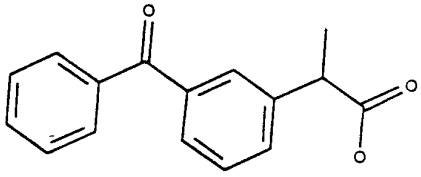
the cyclooxygenase site (38), and this is consistent with the modification of Ser516, which is at the cyclooxygenase-active site, not substantially affecting the peroxidase activity. It has been suggested that the importance of retaining peroxidase activity is to prevent instant inactivation of the enzyme. Because PGHS catalyzes the synthesis of a hydroperoxide, the suggestion is that without peroxidase activity, the enzyme would only catalyze a few turnovers before inactivation due to hydroperoxide accumulation (39).

The apparent K_m values for the mutant and the acetylated PGHS-2 are 4- and 10-fold higher than the nonacetylated PGHS-2, respectively. Previously, the K_m value for PGHS-2(S516M) has been reported by other researchers as 600 μM (24), but we obtain an apparent K_m value that is closer to that of the wild-type PGHS-2. The low rate of enzyme inactivation and of 15(*R*)-HETE formation for the mutant suggests that the Ser-Met mutation of residue 516 of PGHS-2 results in a decreased enzymatic activity compared with acetylation of the Ser516 residue.

Acetylated PGHS-2 and PGHS-2(S516M) produced at least 10-fold higher 15(*R*)-HETE than PGE_2 produced by PGHS-2. This difference is inflated because PGE_2 represents approximately 30% of the oxygenation product of arachidonic acid catalyzed by PGHS-2 (8). However, the increase in 15(*R*)-HETE synthesis of the mutant and acetylated PGHS-2 is still significant and suggests that acetylation by ASA and conver-

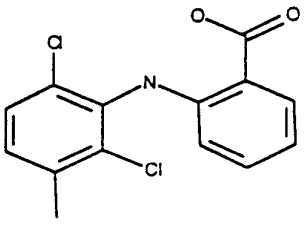


Flurbiprofen

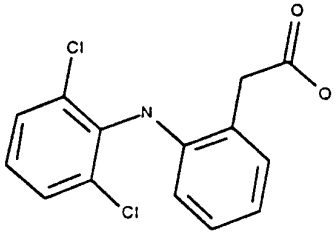


Ketoprofen

Fig. 6. Structures of several NSAIDs.



Meclofenamic Acid



Diclofenac

sion of Ser516 to methionine results in modification of PGHS-2 with an increased capacity for use of the arachidonic acid substrate. This represents a major difference with other NSAIDs in which inhibition of PGHS may result in arachidonic acid accumulation or shunting to other pathways. Shunting of arachidonic acid from PGHS-derived products to leukotriene C_4 production has been demonstrated in mouse peritoneal macrophages treated with the PGHS inhibitor indomethacin (40). The ability of ASA-acetylated PGHS-2 to produce 15(*R*)-HETE may be more important to remove arachidonic acid not used for prostaglandin production than to generate biologically active products.

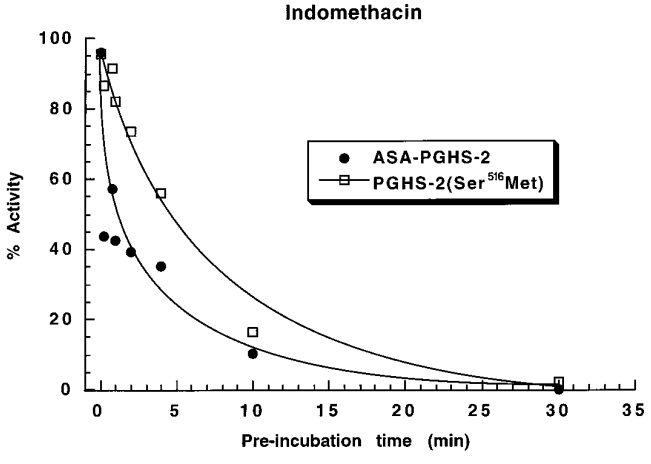


Fig. 7. Time-dependent inhibition of ASA-PGHS-2 and PGHS-2(S516M). Microsomal membrane preparations of ASA-treated PGHS-2 and PGHS-2(S516M) were preincubated with 3 μM indomethacin for 0–30 min before the initiation of the reaction with arachidonic acid. The percentage of activity is plotted versus the preincubation time, and the activity is based on the amount of 15-HETE produced as determined by reverse-phase HPLC.

There has been a recent implication that the products formed from acetylation of PGHS-2 may be converted into bioactive lipid metabolites that inhibit neutrophil adhesion (27). Our present study provides a first report of inhibition of the acetylated PGHS-2 and PGHS-2(S516M) compared with inhibition of PGHS-2 by NSAIDs and selective PGHS-2 inhibitors. Most inhibitors tested demonstrate either equal or slightly decreased potency for inhibition of PGHS-2 and PGHS-2(S516M) compared with inhibition of PGHS-2. Interestingly, the mechanism of time-dependent inhibition by compounds such as indomethacin has been retained by the ASA-acetylated PGHS-2 and PGHS-2(S516M). The most striking result is that both diclofenac and meclofenamic acid have lost all potency for inhibition of the serine-modified forms of PGHS-2. The inhibition by this class of fenamates is therefore extremely sensitive to modification of Ser516. Also, the relative potency of most of the inhibitors follows this decreasing rank order: PGHS-2, acetylated PGHS-2, PGHS-2(S516M). However, differences are not very pronounced. This is consistent with the hypothesis that the increased bulk created by a methionine residue, compared with an acetylated serine, results in a decreased interaction with inhibitors at the active site. The crystal structure of PGHS-1 and recent site-directed mutagenesis studies demonstrate that the carboxylic acid moiety of flurbiprofen and other NSAIDs containing a carboxylic acid group interact with Arg120 (25, 30, 33, 37). In addition, the crystal structure demonstrates that flurbiprofen extends into the active site with the Ser530 of PGHS-1 located in the upper part of the cyclooxygenase-active site (30, 33, 37). Because the crystal

structure of PGHS-2 has not been determined, we can only speculate that acetylation of Ser516 of PGHS-2 may impinge on the active site and effect the inhibition of certain classes of PGHS inhibitors (25). Interestingly, the fenamate (diclofenac and meclofenamic acid) class of inhibitors tested may be binding to the cyclooxygenase-active site in a similar fashion as the profens (flurbiprofen and ketoprofen). Therefore, the unsubstituted phenyl ring (Fig. 6) of flurbiprofen may be in proximity to Ser516 of PGHS-2 and when this ring structure has substituted components such as the chlorine molecules of diclofenac and meclofenamic acid, the acetylated serine of PGHS-2 results in a steric hindrance, which prevents their interaction with the active site of cyclooxygenase.

The important role of NSAIDs in the treatment of inflammatory disorders is unquestionable. The interest in development of PGHS-2-selective inhibitors with a decreased toxicity profile has intrigued several pharmaceutical companies. Understanding the interaction of NSAIDs and selective PGHS-2 inhibitors with various modified versions of the enzyme may provide useful insight into understanding the mechanism of inhibition of these compounds and their specific interactions at the active site.

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