0026-895X/97/050829-10$3.00/0

MOLECULAR PHARMACOLOGY, **52:**829–838 (1997).

The Interaction of Arginine 106 of Human Prostaglandin G/H Synthase-2 with Inhibitors Is Not a Universal Component of Inhibition Mediated by Nonsteroidal Anti-inflammatory Drugs

GILLIAN M. GREIG, DONNA A. FRANCIS, JEAN-PIERRE FALGUEYRET, MARC OUELLET, M. DAVID PERCIVAL, PATRICK ROY, CHRISTOPHER BAYLY, JOSEPH A. MANCINI, and GARY P. O'NEILL

Departments of Biochemistry and Molecular Biology (G.M.G., D.A.F., J.-P.F., M.O., M.D.P, J.A.M., G.P.O) and Medicinal Chemistry (P.R., C.B.), Merck Frosst Centre for Therapeutic Research, Pointe Claire-Dorval, Québec H9R 4P8, Canada

Received May 15, 1997; Accepted July 24, 1997

SUMMARY

The three-dimensional cocrystal structures of ovine prostaglandin G/H synthase-1 (PGHS-1) with *S*-flurbiprofen and murine PGHS-2 with *S*-flurbiprofen and indomethacin reveal that the carboxylate acid groups of these nonsteroidal anti-inflammatory drugs (NSAIDs) form a salt bridge with the guanidinium group of Arg120 in PGHS-1 and Arg106 in PGHS-2. Mutagenesis studies confirmed that the Arg120 residue of PGHS-1 is critical for binding of substrate and inhibitors through ionic interactions of its guanidinium group with the carboxylate moieties of arachidonic acid and certain NSAIDs. We report here that the analogous R106E substitution in human PGHS-2 results in a catalytically active enzyme with a 30-fold higher K_m value for arachidonic acid. Comparison of the inhibition of hPGHS-2(R106E) with wild-type hPGHS-2 by 11 structurally diverse selective and nonselective PGHS inhibitors revealed a 0–1000-fold decrease in inhibitory potency on the mutant enzyme. The loss of inhibitory potency of NSAIDs on hPGHS-2(R106E) could not be correlated with the presence or absence of a carboxylate functional group in the inhibitor, as was demonstrated previously for the PGHS-1(R120E) mutant, or with the selective or nonselective nature of the PGHS inhibitor. The decreases in the inhibitory potencies on hPGHS-2(R106E) by the carboxylate-containing NSAIDs flurbiprofen, indomethacin, meclofenamic acid, and diclofenac on hPGHS-2(R106E) were 965-, 48-, 5.5-, and 4.5-fold, respectively. The nonuniversal requirement for interaction of the carboxylate group of certain NSAIDs with the Arg106 residue in hPGHS-2 is supported by the observation that the methyl ester derivative of indomethacin was a more potent inhibitor than indomethacin on both hPGHS-2 and hPGHS-2(R106E). The greatest loss of potency for inhibition of hPGHS-2(R106E) was observed with the hPGHS-2-selective sulfonamide-containing inhibitors NS-398 and flosulide. The PGHS-2-selective inhibitor DuP697 and a desbromo-sulfonamide analogue of DuP697 displayed equivalent potency on hPGHS-2(R106E) and hPGHS-2. The change in inhibitory potency of NS-398 on hPGHS-2(R106E) was due to a difference in the kinetics of inhibition, with NS-398 displaying time-dependent inhibition of hPGHS-2 but time-independent inhibition of PGHS-2(R106E). The time-dependent inhibition of hPGHS-2 by DuP697 was not affected by the presence of the R106E mutation. We conclude that the Arg106 residue of hPGHS-2 is involved in binding arachidonic acid and certain NSAIDs, but interactions with Arg106 are not a universal requirement for inhibition by either carboxylate-containing NSAIDs or PGHS-2-selective inhibitors.

PGHS-1 and PGHS-2 catalyze the conversion of arachidonic acid to PGH_2, which serves as the precursor for the formation of PGs and thromboxanes (1). The bifunctional PGHS isoenzymes catalyze sequential cyclooxygenase and peroxidase reactions. In the first step, the cyclooxygenase activity oxygenates arachidonic acid, yielding PGG_2, which is then reduced in a second step by the peroxidase activity to PGH_2 (1). The class of drugs known as NSAIDs exerts their anti-inflammatory, antipyretic, and analgesic effects by blocking the production of prostanoids from arachidonic acid through inhibition of PGHS activity (2). Recent studies show that the PGHS isozymes differ markedly in their sensitivities to NSAID inhibition. Essentially all NSAIDs in therapeutic use exhibit nonselective inhibition of both isoforms, whereas

---

ABBREVIATIONS: PGHS, prostaglandin G/H synthase; ibuprofen, α-methyl-4-(2-methylpropyl)benzeneacetic acid; diclofenac, 2-[(2,6-dichlorophenyl)amino]benzeneacetic acid; indomethacin, 1-(*p*-chlorobenzoyl)5-methoxy-2-methylindole-3-acetic acid; flurbiprofen, (±)-2-fluoro-α-methyl(1,1′-biphenyl)4-acetic acid; meclofenamic acid, 2-[(2,6-dichloro-3-methylphenyl)amino]benzoic acid; flosulide, 6-(2,4-difluorophenoxy)-5-methylsulfonylamino-1-indanone; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; CHO[hPGHS-2], Chinese hamster ovary cells stably transfected with human prostaglandin G/H synthase-2; EIA, enzyme-linked immunoassay; DMSO, dimethylsulfoxide; hPGHS-1, human prostaglandin G/H synthase-1; hPGHS-2, human prostaglandin G/H synthase-2; NSAID, nonsteroidal anti-inflammatory drug; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

recently described compounds, such as L-745,337, DFU, SC-58125, DuP697, and NS-398, are potent and selective inhibitors of PGHS-2 (3–12). In several *in vivo* animal models, selective PGHS-2 inhibitors show potent anti-inflammatory, antipyretic, and analgesic properties but display a substantially improved gastrotoxicity profile over nonselective PGHS inhibitors (9, 11–14). These observations support the hypothesis that prostanoids derived from PGH_2 generated by PGHS-2 mediate the onset of the inflammatory response, whereas prostanoids synthesized through the PGHS-1 pathway are involved in normal physiological functions (e.g., gastric acid and mucus secretion in the stomach) (2, 15). Thus, the potential therapeutic advantages of selective PGHS-2 inhibitors have prompted examination of the structural features of the PGHS isozymes that contribute to selective inhibition (16).

Biochemical and biophysical studies have demonstrated that the cyclooxygenase and peroxidase active sites in PGHS isoenzymes are located at distinct sites (1, 17–19). Although human PGHS-1 and -2 share an overall 61% amino acid identity, the residues lining the cyclooxygenase active sites are almost entirely conserved (17–19). The X-ray crystal structures of ovine PGHS-1, murine PGHS-2, and human PGHS-2 reveal that the positioning of carboxylate-containing inhibitors such as *S*-flurbiprofen and indomethacin in the cyclooxygenase active site channel of the PGHS isoforms is similar, such that the carboxylate groups of these NSAIDs interact with the guanidinium group of Arg120 of PGHS-1 and the analogous Arg106 of PGHS-2 (17–20). Other charged residues in the cyclooxygenase active sites include Glu524 in PGHS-1 (Glu510 in PGHS-2) and Arg499 in PGHS-2 (17–19).

Site-specific substitution of Arg120 in PGHS-1 results in dramatic changes to both enzyme activity and sensitivity to NSAID-mediated inhibition (21, 22). We reported recently that replacement of Arg120 with glutamic acid in human PGHS-1 resulted in a mutant enzyme exhibiting a 20-fold reduction in specific activity, a 100-fold increase in the apparent K_m value for arachidonic acid, and a 40–8000-fold loss in sensitivity to inhibition by NSAIDs containing carboxylic acid moieties (21). For example, flurbiprofen and indomethacin did not exhibit any inhibition of hPGHS-1(R120E) (21). A second study by Bhattacharya *et al.* (22) found that R120K, R120Q, and R120E substitutions of Arg120 in ovine PGHS-1 produced mutant enzymes retaining 10%, 5%, and 0%, respectively, of native cyclooxygenase activity and >100%, 60%, and 0%, respectively, of native peroxidase activity. The K_m values for arachidonic acid were also dramatically increased 20–1000-fold for the ovine PGHS-1(R120K) (K_m = 87 μM) and PGHS-1(R120Q) (K_m = 3300 μM) mutants compared with the native ovine PGHS-1 (K_m = 4.0 μM) (22). These biochemical and biophysical studies indicate that in PGHS-1, Arg120 is a key determinant in interaction with arachidonic acid and NSAIDs containing carboxylic acid groups.

The fact that most, but not all, nonselective NSAIDs contain a free carboxylic acid group while all selective PGHS-2 inhibitors reported to date do not contain a carboxylic acid moiety (16) prompted us to examine the contribution of the analogous Arg106 in hPGHS-2 to interactions with selective and nonselective PGHS inhibitors. We constructed and expressed an hPGHS-2(R106E) mutant to compare its enzymatic properties and sensitivity to NSAID-mediated inhibition to native hPGHS-2 and the previously reported R120E mutants of ovine and human PGHS-1 (21, 22).

Experimental Procedures

Materials. Peroxide-free arachidonic acid was purchased from Cayman Chemical (Ann Arbor, MI). Ibuprofen, diclofenac, indomethacin, flurbiprofen, and meclofenamic acid were purchased from Sigma Chemical (St. Louis, MO). DuP697 (5-bromo-2-(4-fluorophenyl)-3-[4-(methylsulfonyl)phenyl]thiophene) (23), NS-398 [*N*-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide] (9), flosulide (24), L-745,296, L-746,483, L-761,066, and L-588,983 (indomethacin methyl ester) were synthesized in the Department of Medicinal Chemistry at Merck Frosst.

Site-directed mutagenesis. An hPGHS-2(R106E) mutant was generated by overlap extension PCR (25, 26) using the wild-type hPGHS-2 coding sequence subcloned into the vector pcDNA3 (7). A 730-bp fragment containing the NH_2-terminal coding sequence of hPGHS-2 was amplified using an oligonucleotide specific to the T7 promoter sequence in the pcDNA3 multiple cloning site (5′-GTAATACGACTCACTATAGGGC-3′) and an oligonucleotide incorporating the desired mutation in the hPGHS-2 sequence, oligonucleotide GMG001 (5′-CAATCAAATGTGATTCGGATGTCAACAC-3′). A second overlapping fragment of 350 bp was amplified using an antisense oligonucleotide complementary to GMG001, designated GMG004 (5′-GTGTTGACATCCGAATCACATTTGATTG-3′), in conjunction with oligonucleotide GO172 (5′-AGATCATCTCTGCCTGAGTATCTT-3′). Underlined nucleotides in GMG001 and GMG004 encode for glutamic acid. For the splicing-by-overlap-extension reaction (25, 26), the 730- and 350-bp products obtained from the first two PCRs were gel purified, combined, and amplified into a single, larger product of 1050 bp using the two flanking oligonucleotides (T7 and GO172). The PCRs were performed using *Taq* DNA polymerase (Boehringer-Mannheim Canada, Laval, Quebec, Canada) for 25 cycles, each consisting of 30 sec at 94°, 1 min at 50°, and 1 min at 72°. The overlap extension product was gel purified, digested at internal restriction enzyme sites for *Blp*I and *Eco*NI (New England Biolabs, Mississauga, Ontario, Canada), and ligated into the corresponding region of the pcDNA3::hPGHS-2 construct (7), followed by transformation into competent *Escherichia coli* DH5-α cells (BRL, Mississauga, Ontario, Canada). Selected positive clones were examined by DNA sequencing, and one clone containing the R106E mutation pcDNA3::hPGHS-2(R106E) was selected for further study.

Cell transfections and tissue culture methods. The hPGHS-2 and hPGHS-2(R106E) cDNAs were subcloned into the multiple cloning site of the eukaryotic expression vector pEE14 (27) to create pEE14::hPGHS-2 and pEE14::hPGHS-2(R106E). Both the wild-type and the mutant constructs, as well as pEE14 vector, were transfected into CHO-K1 cells (American Type Culture Collection, Rockville, MD) using a calcium phosphate-mediated technique, followed by clonal selection of methionine sulfoximine-resistant cell lines as described previously (7). Cell lines were selected and grown using 25 μM methionine sulfoximine in a modified Glasgow's modified Eagle's medium without tryptose phosphate broth or glutamine and supplemented with 8% dialyzed fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, 100 μg/ml gentamicin, 0.5 mM sodium pyruvate, 60 μg/ml L-glutamate, 60 μg/ml L-asparagine, 7 μg/ml concentration each of ribonucleosides adenosine, cytidine, guanosine, and uridine and nonessential amino acids. Cultures were grown as monolayers with 6% CO_2 at 37°.

Immunoblot analysis. Solubilized cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electrophoretic transfer to nitrocellulose membranes as described previously (7, 28). Nonspecific sites were blocked with 5% skim milk powder in Tris-buffered saline/Tween 20 (20 mM Tris, pH 7.5, 0.5 M NaCl, 0.1% Tween 20) for 1 hr at 23°. The primary polyclonal rabbit antiserum to hPGHS-2 (28) was used at a final concentration of 1:5000. The secondary horseradish peroxidase-linked anti-rabbit IgG antibody (Amersham Life Sciences, Oakville, Ontario, Canada) was used at a dilution of 1:3000 in 5% milk/Tris-buffered saline/Tween 20. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

Partial purification of hPGHS-2 and hPGHS-2(R106E). Cells stably transfected with pEE14, pEE14::hPGHS-2, or pEE14::hPGHS-2(R106E) were grown as described above in the presence of 50 μM ibuprofen, a reversible inhibitor of PGHS-2 (7). Cells were harvested by scraping with a rubber spatula, collected by centrifugation at 2,000 × *g*, and resuspended in homogenization buffer (0.1 M Tris, pH 7.4/5 mM EDTA) containing 50 μM ibuprofen and 1 mM diethyldithiocarbamic acid, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride. Cell suspensions were disrupted by sonication, followed by centrifugation at 100,000 × *g* for 2 hr at 4°. The membrane pellets from the 100,000 × *g* centrifugation were solubilized for 1 hr at 4° in homogenization buffer containing 45 mM β-octylglucoside, followed by removal of the insoluble material by centrifugation at 100,000 × *g* for 2 hr. The solubilized PGHS activity in the supernatants was purified by gel filtration on a Superose 6 column (Pharmacia, Dorval, Quebec, Canada) using a column buffer of 20 mM Tris, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 1.2% β-octylglucoside, and 0.25 mM phenol at a flow rate of 0.35 ml/min. PGHS activity in column fractions was measured by determining the level of PGE_2 synthesis from arachidonic acid using a PGE_2 EIA kit (Correlate PGE_2 EIA Kit; Assay Designs, Ann Arbor, MI) (6, 7).

Determination of PGHS activity in intact cells. The activities of hPGHS-2 and hPGHS-2(R106E) and their half-maximal inhibition values (IC_{50}) by inhibitors in intact cells were determined by measuring the level of PGE_2 production using a PGE_2 EIA kit after challenge of the cells with arachidonic acid as described previously (7). Briefly, cells were trypsinized, washed and resuspended in 15 mM HEPES-buffered Hanks' balanced salt solution at a concentration of 1.0×10^6 cells/ml. PGHS inhibitors were dissolved in DMSO, and 3-fold dilutions were prepared for testing. Cells were preincubated with PGHS inhibitors at 37° for either 15 min (IC_{50} determinations) or 0–60 min (time-dependent inhibition studies), followed by a 15-min challenge with 10 μM peroxide-free arachidonic acid. Positive and negative controls were incubated with DMSO vehicle followed by addition of 10 μM peroxide-free arachidonic acid or ethanol vehicle, respectively. PGE_2 synthesis was terminated by the addition of HCl to a final concentration of 0.05 N followed by neutralization by NaOH to a final concentration of 0.05 N. PGE_2 production was quantified by PGE_2 EIA. Inhibition by PGHS inhibitors was calculated as a percentage of total PGE_2 production.

Measurement of K_m and V_{max} values for PGHS-2 and PGHS-2(R106E). Apparent K_m values for arachidonic acid were determined by measuring the production of PGE_2 using 1.5–2.5 μg of partially purified protein in 100-μl reactions in the presence of 0–100 μM arachidonic acid. Reactions were stopped after 45 sec by the addition of 0.05 N HCl and neutralized with 0.05 N NaOH. PGE_2 production was measured using a radioimmunoassay detection kit (Biotrak; Amersham). K_m determinations with purified proteins were made by nonlinear fit of the experimental values to the Michaelis-Menten equation.

Analysis of product synthesis by reverse-phase HPLC. Intact cells stably transfected with hPGHS-2 or hPGHS-2(R106E) were incubated with 5 μM [^{14}C-(U)]arachidonic acid (866 mCi/mmol) for 10 min at 37°. Reactions were stopped by the addition of ethyl acetate/methanol/1 M citric acid (v/v/v, 60:8:2), and products were extracted from the mixture. Reaction products were resolved by reverse-phase HPLC using the following gradient system with solvent A (0.2% HOAc/99.8% H_2O) and solvent B (0.2% HOAc/99.8% CH_3CN). Initial conditions were 70% solvent A and 30% solvent B. At 5 min after injection, the composition of the solvent was changed linearly to 40% solvent A and 60% solvent B over a 30-min period. After a 5-min isocratic period, the composition of the solvent was changed linearly to 100% solvent B over a 15-min period. The flow rate was 1 ml/min. The eluant was monitored using a radioactivity detector and absorbance at 234 nm. Retention times for each observed peak were compared with the retention times of synthetic standards.

Results

Rationale for characterizing wild-type and hPGHS-2(R106E) in stable cell lines. In our previous analysis of an hPGHS-1(R120E) mutant, the enzyme was overexpressed using a vaccinia virus vector and characterized in partially purified microsomal extracts (21). In this system, hPGHS-1(R120E) displayed only 5% of the specific activity of the wild-type enzyme. The analogous ovine PGHS-1(R120E) was enzymatically inactive when assayed in microsomal extracts from transfected or intact cells (22). Because of the low level of catalytic activity we expected for hPGHS-2(R106E), we chose to express this mutant in a cell-based system, several of which have been shown recently to be more sensitive and discriminating than cell-free assays for determining the potency and selectivity of NSAIDs on PGHS isozymes (7, 8, 29–32). For example, the activity of ibuprofen against PGHS-2 in intact cells is 10–300-fold greater than that on broken cells or microsomal PGHS-2 (6–8). The use of a cell-based system to characterize the hPGHS-2(R106E) mutant also avoids the use of detergents that are required for the solubilization, purification, and assay of PGHS-2 (33). The addition of solubilizing agents can lead to marked detergent-dependent changes in the inhibition of PGHS-2 by NSAIDs, which probably result from partitioning of the arachidonic acid and the inhibitor into detergent micelles.[1] In addition, the pharmacological profiles of PGHS inhibitors obtained using several cell-based systems were reviewed recently and shown to correlate with the therapeutic activities of these inhibitors *in vivo* (31, 32). Based on (i) the very low levels of activity of the PGHS-1(R120E) mutants in cell-free assays, (ii) the established ability of cell-based versus cell-free systems to determine NSAID-mediated inhibition of PGHS isozymes, and (iii) our goal of pharmacologically defining the importance of the Arg106 residue of PGHS-2 in NSAID inhibition, we reasoned that a cell-based assay to analyze hPGHS-2(R106E) was appropriate. Thus, stable CHO cell lines were established expressing the mutant and wild-type enzymes using the expression vector pEE14 (7, 27).

Expression of hPGHS-2 and hPGHS-2(R106E). Clonally derived CHO cell lines stably transfected with the expression vector constructs pEE14::hPGHS-2 and pEE14::hPGHS-2 (R106E) were screened for levels of PGHS activity as determined by PGE_2 production after challenge of the cells with 10 μM arachidonic acid for 15 min (7). In comparison to the level of PGE_2 synthesis in the sham-transfected CHO[pEE14] control cell line (0.2 ng $PGE_2/10^6$ cells), both the wild-type CHO[hPGHS-2] (14 ng $PGE_2/10^6$ cells) and mutant CHO[hPGHS-2(R106E)] (10 ng of $PGE_2/10^6$ cells) cell lines exhibited high levels of PGHS activity. Immunoblot analysis using an anti-hPGHS-2 antibody showed that the CHO[hPGHS-2] and CHO[hPGHS-2(R106E)] cell lines, but not the CHO[pEE14] cell line, expressed immunoreactive PGHS-2 proteins with the predicted molecular

---

[1] M. Ouellet and M. D. Percival. Detergent-dependent changes in the inhibition of purified cyclooxygenase-2 by nonsteroidal anti-inflammatory drugs, American Society for Biochemistry and Molecular Biology, New Orleans, LA, June 2–6, 1996, poster no. 1487.

weight of 72,000 that comigrated with the purified sheep PGHS-2 standard (Fig. 1). The relative levels of expression of the wild-type and mutant hPGHS-2 proteins in the cell lines were assessed by scanning densitometry of immunoblots from three independent experiments. By using known amounts of purified sheep PGHS-2 to construct a standard curve (Fig. 1), we determined that the CHO[hPGHS-2] and CHO[hPGHS-2(R106E)] cell lines expressed an average of 2.7 and 15.7 ng, respectively, of hPGHS-2 immunoreactive protein/10^4 cells. Although the CHO[hPGHS-2(R106E)] cell line exhibited 70% of the level of PGE_2 production of the CHO[hPGHS-2] cell line at a concentration of 10 μM arachidonic acid, the densitometric quantification of the immunoblots demonstrated a 6-fold higher level of hPGHS-2(R106E) protein versus hPGHS-2 protein in the cell lines, indicating that hPGHS-2(R106E) exhibits ~12% of the specific activity of hPGHS-2.

To determine whether the reduced specific activity of hPGHS-2(R106E) could be attributed to changes in substrate binding, the K_m values were measured for arachidonic acid with partially purified wild-type and mutant hPGHS-2 enzymes. Average apparent K_m values of 0.85 and 27.6 μM were obtained for hPGHS-2 and hPGHS-2(R106E), respectively (Fig. 2). A range of K_m values of 0.74–5.6 μM have been reported for purified and cellular hPGHS-2 (5, 7, 33). We found previously that the analogous R120E mutation in hPGHS-1 resulted in a 100-fold increase in the K_m value for arachidonic acid (21), whereas Bhattacharyya *et al.* (22) reported that the same mutation in ovine PGHS-1 resulted in a completely inactive enzyme. The 32-fold increase in the K_m value for hPGHS-2(R106E) indicates that Arg106 is involved in fatty acid binding, although its contribution to substrate binding seems to be less than that in PGHS-1 (21, 22). The increase in the K_m value for arachidonic acid could also result from repulsive interactions between the carboxylate groups on both arachidonic acid and the Glu106 of the mutant hPGHS-2. Because modification of the cyclooxygenase active site in PGHS-2 can result in an altered metabolite profile (6, 34, 35), the product profiles of the hPGHS-2 and hPGHS-2(R106E) were compared by reverse-phase HPLC analysis (Fig. 3). The primary products formed in both cell lines were PGE_2, $PGF_{2\alpha}$, and PGD_2, with trace amounts of the reaction by-products 11- and 15-hydroxyeicosatetraenoic acid and 12-hydroxy-5,8,10-heptadecatrienoic acid. Therefore, although the R106E substitution alters substrate binding, in hPGHS-2 this mutation does not seem to affect the product formation profile.

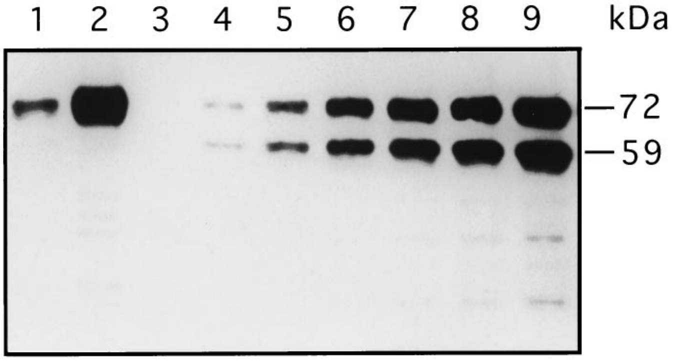

Fig. 1. Immunoblot analysis of CHO cells stably expressing hPGHS-2 and hPGHS-2(R106E) protein. Solubilized protein samples from 1.0 × 10^4 cells stably expressing hPGHS-2 or hPGHS-2(R106E) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and immunoblotted with anti-PGHS-2 antisera, with detection by chemiluminescence as described in Experimental Procedures. *Lane 1*, CHO[hPGHS-2]. *Lane 2*, CHO[hPGHS-2(R106E)]. *Lane 3*, control CHO cells transfected with pEE14. *Lanes 4–9*, 1, 2.5, 5, 10, 15, and 20 ng of ovine PGHS-2 standard, respectively.

NSAID inhibition of hPGHS-2 and hPGHS-2(R106E). In addition to the importance of Arg120 in substrate binding in PGHS-1, this residue is critical for the interaction between PGHS-1 and many carboxylic acid-containing NSAIDs (21, 22). To determine whether Arg106 of hPGHS-2 plays a similar role in binding NSAIDs, we examined the inhibition profiles of hPGHS-2 and hPGHS-2(R106E) by nonselective carboxylic acid containing inhibitors (indomethacin, flurbiprofen, diclofenac, meclofenamic acid, and L-761,066), hPGHS-2-selective inhibitors lacking a carboxylic acid group (NS-398, flosulide, DuP697, and L-746,483), and an hPGHS-1-selective compound (L-745,296) (Fig. 4). Two of the largest decreases, 600- and 1000-fold, in inhibitory potencies on hPGHS-2(R106E) were observed with the methyl sulfonamide-containing compounds NS-398 and flosulide (Fig. 5 and Table 1). Of the five carboxylic acid-containing inhibitors tested, only flurbiprofen displayed a large decrease (965-fold) in inhibitory potency on hPGHS-2(R106E) (Table 1), whereas indomethacin, L-761,066, meclofenamic acid, and diclofenac exhibited much smaller decreases in inhibition of hPGHS-2(R106E) (48-, 21-, 5.5-, and 4.5-fold, respectively) (Fig. 5 and Table 1). The nonacid PGHS-2-selective tricyclic inhibitor DuP697 and a desbromo-sulfonamide analogue of DuP697 (L-746,483) displayed equivalent and highly potent inhibition (≤2.8 nM) of hPGHS-2(R106E) and hPGHS-2 (Fig. 5 and Table 1). Replacement of the sulfonamide substituent of L-746,483 with a cyano group in L-745,296 resulted in a weak inhibitor of both the wild-type and mutant enzyme with IC_{50} values of 8300 ± 2890 and 1960 ± 290 nM, respectively.

These results suggest that the contribution of the Arg106 residue of hPGHS-2 to interactions with structurally diverse selective and nonselective PGHS inhibitors varies. Clearly, the interactions between Arg106 of hPGHS-2 and the acidic groups of indomethacin, diclofenac, and meclofenamic acid NSAIDs are not absolutely required for binding. Two possibilities that may contribute to the effect or effects of the R106E substitution include (i) a loss of the ionic interaction between the acidic group of the inhibitor and the positively charged guanidinium group of Arg106 and/or (ii) an ionic repulsion between the acidic group of an inhibitor with the negatively charged Glu106 in hPGHS-2(R106E). To test the importance of these ionic interactions, L-588,983 (Fig. 4) was synthesized, and its inhibitory potency on hPGHS-2 and hPGHS-2(R106E) was examined. Interestingly, L-588,983 (Fig. 5E and Table 1) was an equipotent inhibitor of both the wild-type (IC_{50} = 19.7 nM) and mutant (IC_{50} = 6.6 nM) enzymes. That the nonacid methyl ester of indomethacin is a potent inhibitor suggests that in both hPGHS-2 and hPGHS-2(R106E), the interaction between the carboxylic acid group of indomethacin and the positively charged Arg106 is not required for inhibition. However, the increased inhibitory potency of L-588,983 (IC_{50} = 6.6 nM) versus indomethacin (IC_{50} = 1899 nM) on hPGHS-2(R106E) (Fig. 5 and Table 1) suggests that an ionic repulsion between the acidic group of indomethacin with the negatively charged Glu106 in

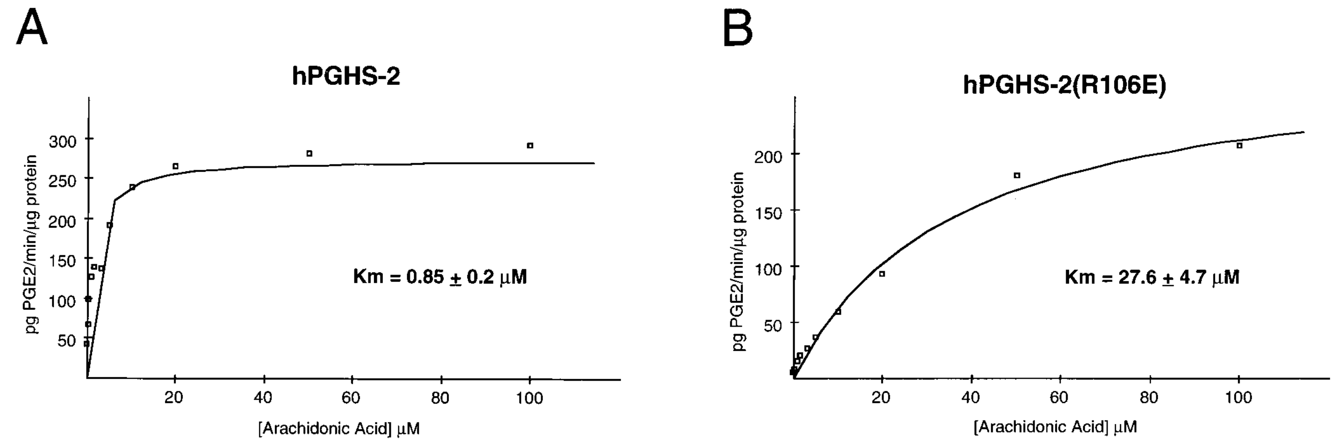

Fig. 2. Determination of apparent K_m values for arachidonic acid for hPGHS-2 and hPGHS-2(R106E). Solubilized, partially purified preparations of (A) hPGHS-2 and (B) hPGHS-2(R106E) were assayed for PGE_2 production using 2.5 μg of protein in a 100-μl volume. Reactions were initiated by the addition of 0–100 μM arachidonic acid, and product formation was quantified as described in Experimental Procedures. The velocity of the reaction, as determined from the change in PGE_2 present, was evaluated by linear fit for the first 45 sec of the reaction. K_m determinations (± standard error) were made by nonlinear fit of the experimental values to the Michaelis-Menten equation. *K_m values,* average values of four and three experiments for hPGHS-2 and hPGHS-2(R106E), respectively.

hPGHS-2(R106E) contributes to the decreased inhibitory potency of indomethacin on hPGHS-2(R106E).

Effect of R106E mutation on time-dependent inhibition by NSAIDs. Compounds of two classes, aryl methyl sulfonamides and aryl methyl sulfonyls, exemplified by NS-398 and DuP697, respectively, have been shown to be potent, time-dependent, selective, and essentially irreversible inhibitors of PGHS-2 while being less potent reversible time-independent inhibitors of PGHS-1 (7, 10, 36). The selective inhibition of PGHS-2 has been suggested to result from this difference in the nature of the time-dependent inhibition of PGHS isozymes (10, 36). We investigated whether the dramatic loss of inhibition of hPGHS-2(R106E) by NS-398, but not DuP697, was due to loss of time-dependent inhibition of the mutant enzyme by NS-398. Methods for characterizing time-dependent inhibition for PGHS isozymes have been reported using intact cells (7, 37) and purified enzymes in the presence of detergents (10, 36, 38). Because the time-dependent inhibition of PGHS isozymes is related to the competitive nature of the inhibitor and the presence of detergents in purified PGHS preparations has been shown to affect the competitive nature of PGHS inhibition,[1] a cell-based method (7) was used to examine the time-dependent inhibition of the hPGHS-2(R106E) mutant. For this experiment, CHO[hPGHS-2] and CHO[hPGHS-2(R106E)] cells were first preincubated for 0–60 min with concentrations of NS-398 yielding 50% inhibition (7.2 nM for CHO[hPGHS-2] cells, 7440 nM for CHO[hPGHS-2(R106E)] cells; see Table 1), and levels of PGHS activity were then measured. NS-398 displayed time-dependent inhibition of hPGHS-2 but time-independent inhibition of hPGHS-2(R106E), with ~50% inhibition of the maximal PGE_2 production observed at all preincubation times (Fig. 6A). In contrast, time-dependent inhibition of PGE_2 synthesis by DuP697 was observed for both CHO[hPGHS-2] and CHO[hPGHS-2(R106E)] (Fig. 6B).

Discussion

The availability of the cDNAs and crystallographic structures for ovine PGHS-1 and human and murine PGHS-2 (17–19) permits an examination of the conserved and non-

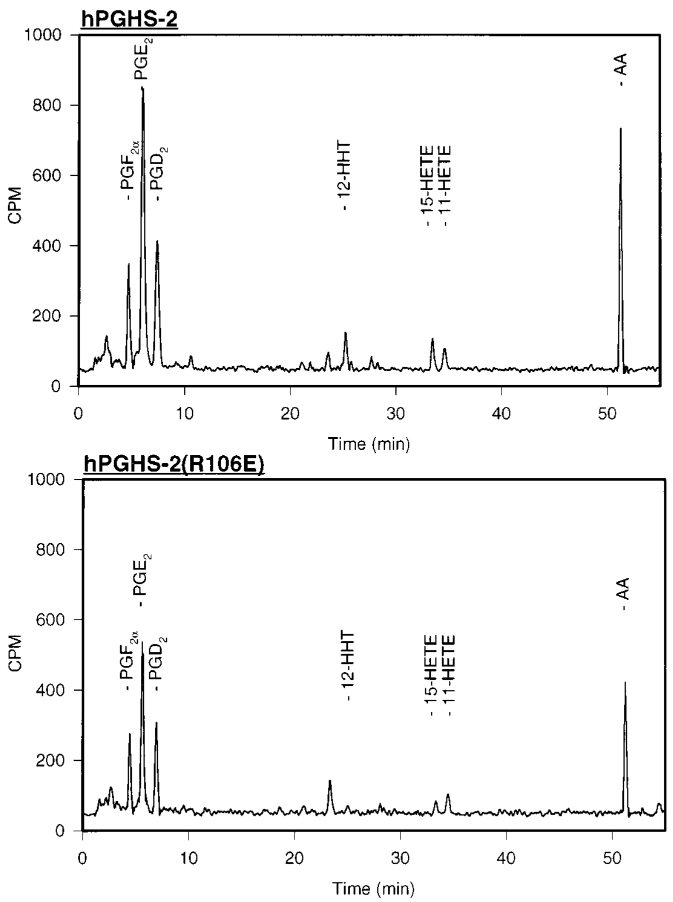

Fig. 3. Analysis by reverse-phase HPLC of product synthesis by intact cells stably expressing hPGHS-2 or hPGHS-2(R106E). Intact cells stably expressing hPGHS-2 or hPGHS-2(R106E) were incubated with 5 μM [^{14}C]arachidonic acid for 10 min at 37°. Reactions were stopped by the addition of ethyl acetate/methanol/1 M citric acid (v/v/v, 60:8:2). Reaction products were resolved by reverse-phase HPLC as described in Experimental Procedures. Peaks labeled as $PGF_{2\alpha}$, PGE_2, PGD_2, 12-hydroxy-5,8,10-heptadecatrienoic acid (*12-HHT*), 15-hydroxyeicosatetraenoic acid (*15-HETE*), and 11-hydroxyeicosatetraenoic acid (*11-HETE*) were identified on the basis of the retention times of synthetic standards.

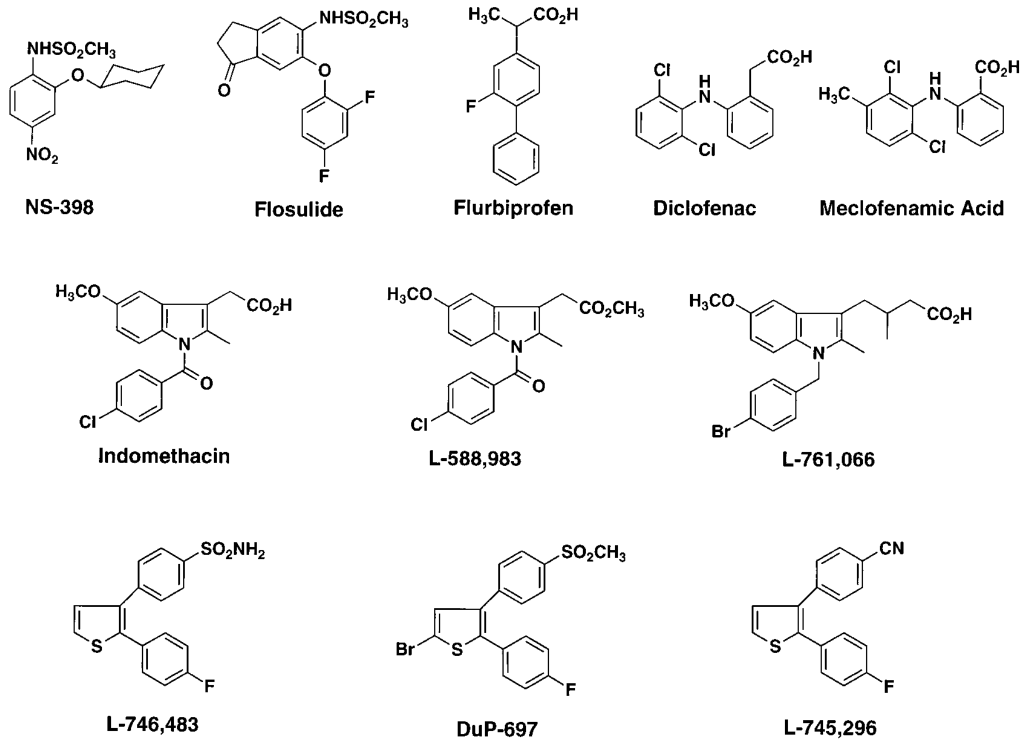

Fig. 4. Chemical structures for PGHS inhibitors tested.

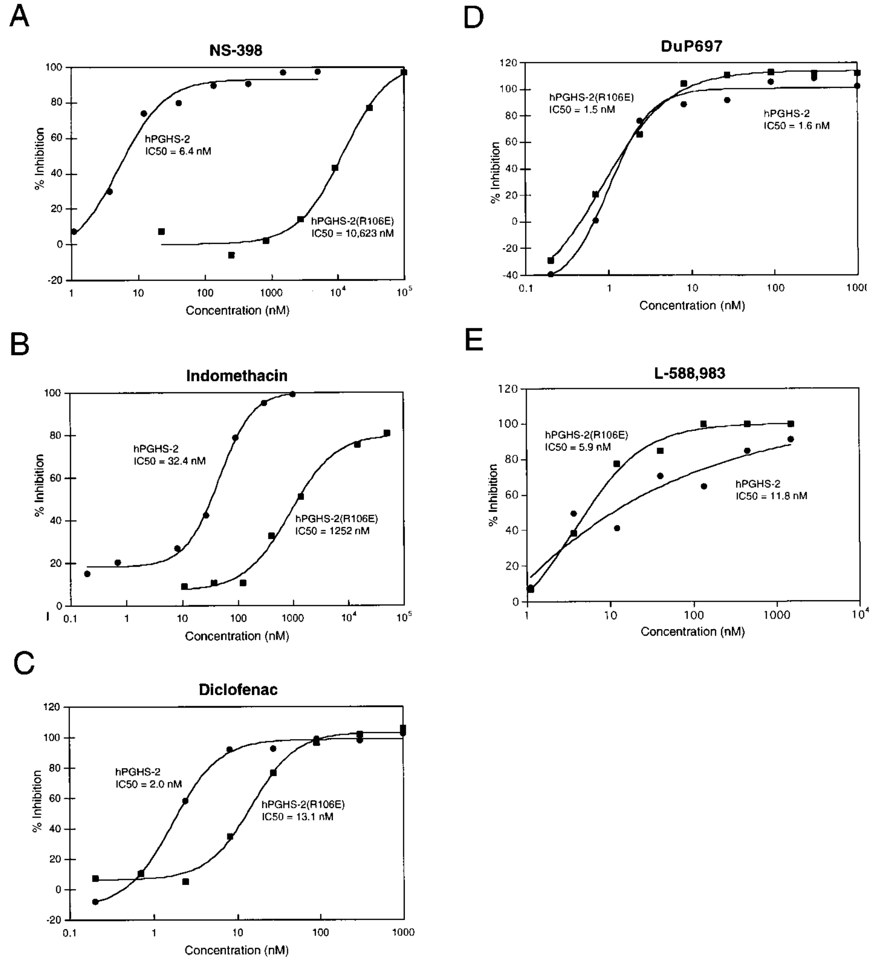

Fig. 5. Dose-response curve for inhibition of PGE_2 production by hPGHS-2 and hPGHS-2(R106E). CHO cells (1.0×10^6) expressing hPGHS-2 (●) or hPGHS-2(R106E) (■) were preincubated at 37° in the presence of eight concentrations of inhibitor for 15 min followed by the addition of 10 μM arachidonic acid for 15 min at 37°. Reactions were terminated by the addition of HCl, and quantification of PGE_2 was determined by specific PGE_2 EIA as described in Experimental Procedures. For each inhibitor, the dose-response curves are representative of 1 of 4–13 separate experiments, as noted in Table 1.

TABLE 1

Inhibition of hPGHS-2 and hPGHS-2(R106E) by acidic and nonacidic NSAIDs

IC_{50} values for various inhibitors were determined in intact CHO cells stably expressing hPGHS-2 and hPGHS-2(R106E) as described in the legend to Fig. 5 and in Materials and Methods. The values represent the average ± standard deviation of four to 13 separate experiments (*n*).

Inhibitor	IC_{50} hPGHS-2	*n*	IC_{50} hPGHS-2(R106E)	*n*	Ratio IC_{50} hPGHS-2(R106E)/hPGHS-2
	nM		*nM*		
NS-398	7.2 ± 0.9	8	7,440 ± 2,000	6	1,030
Flosulide	43.9 ± 4.1	5	>30,000	5	>683
Flurbiprofen	35.9 ± 13.5	5	34,700 ± 8,680	6	965
Diclofenac	3.3 ± 0.6	6	15 ± 1.7	6	4.5
Meclofenamic acid	3 ± 0.3	7	16.4 ± 2	7	5.5
Indomethacin	39.6 ± 5.0	11	1,899 ± 354	13	47.9
L-588,983	19.7 ± 7.6	4	6.6 ± 2.9	4	0.3
L-761,066	352 ± 57	6	7,370 ± 2,730	4	20.9
L-746,483	1.4 ± 0.5	5	2.8 ± 0.3	6	2
DuP697	1.3 ± 0.2	7	1.8 ± 0.3	7	1.4
L-745,296	8,300 ± 2,890	4	1,960 ± 290	7	0.24

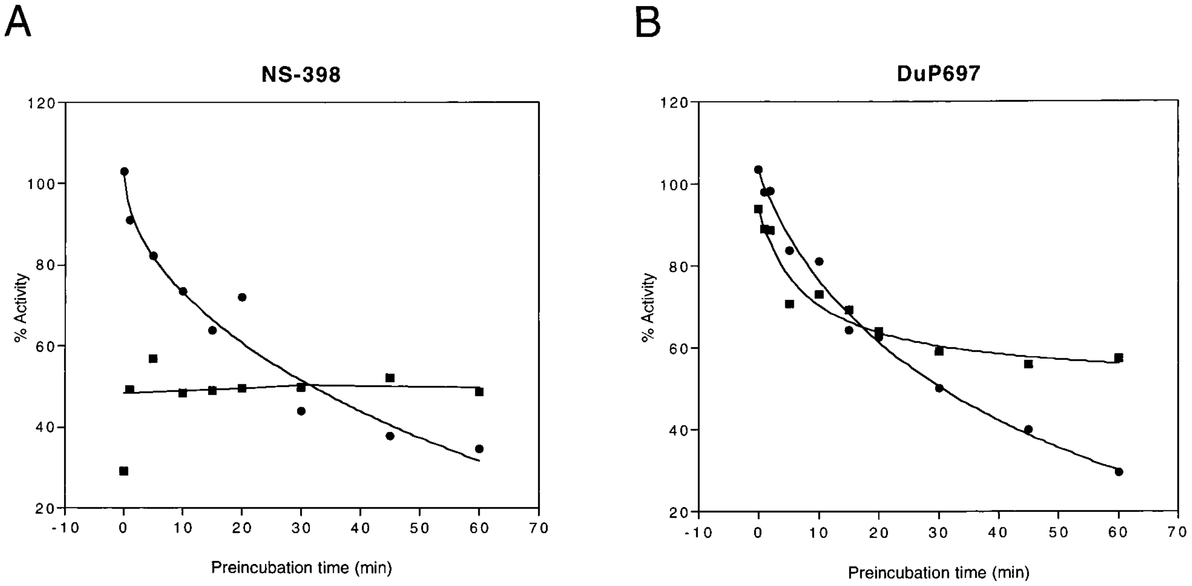

Fig. 6. Effect of inhibitor preincubation time on inhibition of PGE_2 synthesis by hPGHS-2 (●) and hPGHS-2(R106E) (■) in intact cells. Inhibitor concentrations used correspond to the determined IC_{50} values for (A) NS-398 on hPGHS-2 (IC_{50} = 7.2 nM) and hPGHS-2(R106E) (IC_{50} = 7438 nM) and for (B) DuP697 on hPGHS-2 (IC_{50} = 1.3 nM) and hPGHS-2(R106E) (IC_{50} = 1.8 nM). Percent activity is determined from the PGE_2 produced in the presence of inhibitor relative to the PGE_2 produced in the presence of DMSO vehicle.

conserved determinants in the PGHS isozymes that contribute to selective binding of inhibitors. We expected human PGHS-2(R106E) to exhibit an enzymatic and pharmacological profile similar to hPGHS-1(R120E) for several reasons. First, the Arg106 of PGHS-2 and the analogous Arg120 of PGHS-1 residues are completely conserved in all PGHS isozymes from human, sheep, rat, mouse, hamster, guinea pig, and chicken. Second, mutagenesis experiments have shown the critical role of Arg120 of PGHS-1 in interaction with both substrate and carboxylic acid-containing NSAIDs (21, 22). Third, the crystallographic structures of ovine PGHS-1 and murine and human PGHS-2 reveal ionic interactions between Arg120 of PGHS-1 and Arg106 of PGHS-2 with the carboxylic acid groups of arachidonic acid and certain NSAIDs (17–20). Unexpectedly, we found that the R106E substitution in hPGHS-2 has a much less dramatic effect on inhibition mediated by certain NSAIDs than the analogous substitution in PGHS-1 (21, 22). We show that Arg106 of hPGHS-2 is important for interaction with both arachidonic acid and certain PGHS inhibitors, including NS-398, flosulide, and flurbiprofen, but that Arg106 is not essential for binding all carboxylic acid-containing NSAIDs. The Arg106 residue does seem to be critical for substrate binding in that the specific activity of hPGHS-2(R106E) is reduced 8-fold when measured in cells with a 32-fold increase in the K_m value for arachidonic acid as measured using partially purified enzyme. In comparison, the R120E substitution in PGHS-1 yielded a completely inactive enzyme in one report (22) and a 20-fold reduction in specific activity and a 100-fold increase in K_m value for arachidonic acid in another report (21). The increased K_m value for arachidonic acid by the mutant hPGHS-2(R106E) could result from (i) a loss of a positive ionic interaction between the carboxylic acid group of arachidonic acid and the guanidinium group of Arg106 and/or (ii) the introduction of a repulsive interaction between the carboxylic acid groups of glutamate in the hPGHS-2(R106E) mutant and arachidonic acid. We cannot exclude that repulsion between these negatively charged carboxyl groups affects the affinity of hPGHS-2(R106E) for arachidonic acid. In the case of PGHS-1, site-specific substitution of the Arg120 residue with a positively charged lysine still results in a K_m value for arachidonic acid that increases from 4.0 μM for the wild-type to 87 μM for PGHS-1(R106K), whereas the introduction of a neutral glutamine in PGHS-1(R120Q) increases the K_m value for arachidonic acid 1000-fold (22). It is not known how arachidonic acid is bound in the cyclooxygenase active site. In the absence of a crystal structure of PGHS with arachidonic acid in the cyclooxygenase active site, the site-specific substitutions of Arg120/106 in the PGHS isozymes provide evidence of residues that are involved in substrate binding but not required for catalytic activity.

Characterization of hPGHS-1(R120E) revealed that the carboxylate-containing NSAIDs indomethacin and flurbiprofen did not exhibit any inhibitory effects against PGHS-1(R120E), whereas meclofenamic acid and diclofenac were ≤100-fold less potent inhibitors of hPGHS-1(R120E) compared with hPGHS-1 (21). Our analysis reveals that among the carboxylate-containing NSAIDs, flurbiprofen was a weak inhibitor of hPGHS-2(R106E), whereas meclofenamic acid and diclofenac displayed potent inhibition of the mutant hPGHS-2 enzyme, with only a 5–6-fold reduction in potency from 3 nM on the wild-type hPGHS-2 to 16 nM on hPGHS-2(R106E). The relatively strong inhibition of hPGHS-2(R106E) by diclofenac and meclofenamic acid suggests that the contribution to inhibitor binding of an ionic interaction between the carboxylic acid group of diclofenac and meclofenamic acid with the Arg106 of hPGHS-2 is minimal. Although there are currently no cocrystal structures of PGHS-2 with an inhibitor of the diclofenac/meclofenamic acid series, studies on inhibitor-induced changes in the intrinsic fluorescence of hPGHS-2 and the altered sensitivity of aspirin-acetylated hPGHS-2 to inhibition by NSAIDs reveal that the binding mode of diclofenac is distinct from inhibitors such as NS-398, flurbiprofen, DuP697, and indomethacin (35, 39). The binding of diclofenac, but not other NSAIDs tested by Mancini *et al.* (35) and Houtzager *et al.* (39), is blocked by acetylation of Ser516, which is located in the upper portion of the cyclooxygenase active site in hPGHS-2. It is possible that the fenamate series of NSAIDs bind PGHS-2 so that the carboxylate group of the inhibitor is not in close proximity to Arg106.

The cocrystal structure of murine PGHS-2 with indomethacin reveals a salt bridge between the carboxylate group of the inhibitor and the guanidinium group of Arg106 and ≥15 other interactions between indomethacin and the main chain atoms and side chains of 11 amino acids (18). The 48-fold reduction in inhibitory potency of indomethacin on hPGHS-2(R106E) versus hPGHS-2 suggests that the guanidinium/carboxylate interaction is important in indomethacin binding by hPGHS-2. The reduced inhibitory potency of indomethacin on hPGHS-2(R106E) could be due to a ionic repulsion between the substituted Glu106 residue of hPGHS-2(R106E) and the carboxylate of indomethacin. However, the increased inhibitory potency of L-588,983 against both hPGHS-2 and hPGHS-2(R106E) does not support a strong contribution by the guanidinium-carboxylate interaction to overall indomethacin binding. In the case of ovine PGHS-1, Rome and Lands (38) reported that the methylation of carboxylic acid-containing NSAIDs such as indomethacin, flurbiprofen, and meclofenamate did not alter the ability of the inhibitors to competitively inhibit the oxygenase activity but eliminated the time-dependent properties of the inhibitors. Notably, the methyl ester of indomethacin had a K_I value 100-fold lower than that for indomethacin on ovine PGHS-1 (38). As with diclofenac and meclofenamic acid, the lack of an ionic interaction between the carboxylic acid group of indomethacin and Arg106 in hPGHS-2 seems to play a minor role in binding of indomethacin by hPGHS-2.

All highly selective PGHS-2 inhibitors described to date, including NS-398, flosulide, and DuP697, possess a sulfonyl group (16). Weakly acidic inhibitors possessing an aryl methyl sulfonamide, such as NS-398 and flosulide, were among the compounds most affected by the R106E substitution in hPGHS-2, suggesting that Arg106 is required by this class of compounds for effective inhibition. This is in agreement with the three-dimensional cocrystal structure of hPGHS-2 with an acyl sulfonamide derivative of the NSAID zomepirac, in which the nitrogen and oxygen atoms of the sulfonamide group interact with Arg106 in a manner similar to the interaction of the carboxylic acid group of flurbiprofen with Arg120 in PGHS-1 (18). However, the interaction of the sulfonyl groups of other selective PGHS-2 inhibitors with Arg106 in PGHS-2 does not seem to be a conserved feature of binding for this series of inhibitors. The cocrystal structure of murine PGHS-2 with SC-558, a diaryl heterocyclic inhibitor with a sulfonamide substituent attached to one of the aryl rings (19), shows that the phenylsulfonamide group is accommodated by a cavity in the PGHS-2 cyclooxygenase active site with the oxygen atoms of the sulfonamide group of SC-558 hydrogen-bonded to His76 and Arg499 (19). The Arg499 of murine and human PGHS-2 is replaced by a histidine in PGHS-1 isozymes from several species (18, 19). Although Arg120 is the only positively charged residue in the cyclooxygenase active site of PGHS-1 (17), the Arg499 residue of PGHS-2 provides a second positively charged side chain in the cyclooxygenase active site in addition to Arg106 for ionic interactions with substrate and inhibitors. Two other sulfonyl inhibitors, the aryl methyl sulfone DuP697 and the aryl sulfonamide L-746,483, were both potent inhibitors of hPGHS-2 and hPGHS-2(R106E), suggesting that these compounds do not require Arg106 for effective inhibition and may be interacting with Arg499. The importance of the sulfonyl group to selective and potent inhibition of both hPGHS-2 and hPGHS-2(R106E) is highlighted by the greatly reduced potency of L-745,296, a derivative of L-746,483 in which the sulfonamide group is replaced by a cyano group. L-745,296 is a weak inhibitor of both the wild-type and mutant PGHS-2 with IC_{50} values of 8300 ± 2890 and 1960 ± 290 nM, respectively (Table 1). It is difficult to interpret whether the 3–4-fold difference in potency of L-745,296 on hPGHS-2(R106E) compared with hPGHS-2 is significant. Our analysis of the inhibition of hPGHS-2(R106E) suggests that the Arg106 residue in hPGHS-2 is required for binding only to a subset of sulfonyl-containing PGHS-2 inhibitors and is not essential for binding of selective PGHS-2 inhibitors .

Time-dependent inhibitors of PGHS isozymes, which form a tight irreversibly bound complex with the enzyme, are generally more potent inhibitors of PGHS isozymes than time-independent, competitive inhibitors. It is the difference in the nature of the time-dependency of the PGHS isoforms that is believed to give rise to the PGHS isoform-selective inhibition (10, 36). Arg120 of PGHS-1 was found to be important for time-dependent inhibition by carboxylic acid-containing compounds (21). Our studies show that Arg106 in hPGHS-2 is also involved in the development of time-dependent inhibition for inhibitors that interact with Arg106, such as NS-398, but not for compounds that are not affected by the R120E substitution. Thus, DuP697 is a time-dependent inhibitor of both the wild-type and the mutant hPGHS-2, whereas NS-398 exhibited time-dependent inhibition of hPGHS-2 but time-independent inhibition of hPGHS-2(R106E).

In this report, we provide evidence that the Arg106 in the active site of the hPGHS-2 is a critical residue in the binding of arachidonic acid, but its participation in binding structurally diverse NSAIDs varies. Notably, carboxylic acid-containing NSAIDs such as diclofenac do not require interactions with Arg106 of PGHS-2 for potent inhibition. It is apparent that other sites of interaction within the active site of PGHS-2 are important in binding both selective and nonselective NSAIDs of several structural classes

Acknowledgments

We acknowledge C. Black for helpful discussions, members of the Merck-Frosst Medicinal Chemistry Department for synthesis of compounds used in this study, and K. Clark for artwork.

References

1. Smith, W. L., and L. J. Marnett. Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta* **1083:**1–17 (1990).
2. Vane, J. R., and R. M. Botting. New insights into the mode of action of anti-inflammatory drugs. *Inflamm. Res.* **44:**1–10 (1995).
3. Meade, E. A., W. L. Smith, and D. L. DeWitt. Differential inhibition of prostaglandin endoperoxide synthase (cyclooxygenase) isozymes by aspirin and other non-steroidal anti-inflammatory drugs. *J. Biol. Chem.* **268:** 6610–6614 (1993).
4. Gierse, J. K., S. D. Hauser, D. P. Creely, C. Koboldt, S. H. Rangwala, P. C. Isakson, and K. Seibert. Expression and selective inhibition of the constitutive and inducible forms of human cyclo-oxygenase. *Biochem. J.* **305:** 479–484 (1995).
5. Barnett, J., J. Chow, D. Ives, M. Chiou, R. Mackenzie, E. Osen, B. Nguyen, S. Tsing, C. Bach, J. Freire, H. Chan, E. Sigal, and C. Ramesha. Purification, characterization and selective inhibition of human prostaglandin G/H synthase 1 and 2 expressed in the baculovirus system. *Biochim. Biophys. Acta* **1209:**130–139 (1994).
6. O'Neill, G. P., J. A. Mancini, S. Kargman, J. Yergey, M. Y. Kwan, J.-P. Falgueyret, M. Abramovitz, B. P. Kennedy, M. Ouellet, W. Cromlish, S. Culp, J. F. Evans, A. W. Ford-Hutchinson, and P. J. Vickers. Overexpression of prostaglandin G/H synthases-1 and -2 by recombinant vaccinia virus: inhibition by nonsteroidal anti-inflammatory drugs and biosynthesis of 15-hydroxyeicosatetraenoic acid. *Mol. Pharmacol.* **45:**245–254 (1994).
7. Kargman, S., E. Wong, G. M. Greig, J.-P. Falgueyret, W. Cromlish, D. Ethier, J. A. Yergey, D. Riendeau, J. F. Evans, B. Kennedy, P. Tagari, D. A. Francis, and G. P. O'Neill. Mechanism of selective inhibition of human prostaglandin G/H synthase-1 and -2 in intact cells. *Biochem. Pharmacol.* **52:**1113–1125 (1996).
8. Mitchell, J. A., P. Akarasereenont, C. Thiemermann, R. J. Flower, and J. R. Vane. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc. Natl. Acad. Sci. USA* **90:**11693–11697 (1994).
9. Futaki, N., K. Yoshikawa, Y. Hamasaka, I. Arai, S. Higuchi, H. Iizuka, and S. Otomo. NS-398, a novel non-steroidal anti-inflammatory drug with potent analgesic and antipyretic effects, which causes minimal stomach lesions. *Gen. Pharmacol.* **24:**105–110 (1993).
10. Copeland, R. A., J. M. Williams, J. Giannaras, S. Nurnberg, M. Covington, D. Pinto, S. Pick, and J. M. Trzaskos. Mechanism of selective inhibition of the inducible isoform or prostaglandin G/H synthase. *Proc. Natl. Acad. Sci. USA* **91:**11202–11206 (1994).
11. Chan, C.-C., S. Boyce, C. Brideau, A. W. Ford-Hutchinson, R. Gordon, D. Guay, R. Hill, C.-S. Li, J. Mancini, M. Penneton, P. Prasit, R. Rasori, D. Riendeau, P. Roy, P. Tagari, P. Vickers, E. Wong, and I. W. Rodger. Pharmacology of a selective cyclooxygenase-2 inhibitor, L-745,337: a novel nonsteroidal anti-inflammatory agent with an ulcerogenic sparing effect in rat and nonhuman primate stomach. *J. Pharmacol. Exp. Ther.* **274:**1531–1537 (1996).
12. Riendeau, D., M. D. Percival, S. Boyce, C. Brideau, S. Charleson, W. Cromlish, D. Ethier, J. Evans, J.-P. Falgueyret, A. W. Ford-Hutchinson, R. Gordon, G. Greig, M. Gresser, J. Guay, S. Kargman, S. Leger, J. A. Mancini, G. O'Neill, M. Ouellet, I. W. Rodger, M. Therien, Z. Wang, J. K. Webb, E. Wong, L. Xu, R. N. Young, R. Zamboni, P. Prasit, and C.-C. Chan. Biochemical and pharmacological profile of a tetrasubstituted furanone as a highly selective COX-2 inhibitor. *Br. J. Pharmacol.* **121:**105–117 (1997).
13. Masferrer, J. L., B. S. Zweifel, P. T. Manning, S. D. Hauser, K. M. Leahy, W. G. Smith, P. C. Isakson, and K. Seibert. Selective inhibition of inducible cyclooxygenase 2 in vivo is anti-inflammatory and nonulcerogenic. *Proc. Natl. Acad. Sci. USA* **91:**3228–3232 (1994).
14. Seibert, K., Y. Zhang, K. Leahy, S. Hauser, J. Masferrer, W. Perkins, L. Lee, and P. Isakson. Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc. Natl. Acad. Sci. USA* **91:**12013–12017 (1994).
15. Eberhart, C. E., and R. N. Dubois. Eicosanoids and the gastrointestinal tract. *Gastroenterology* **109:**285–301 (1995).
16. Griswold, D. E., and J. L. Adams. Constitutive cyclooxygenase (COX-1) and inducible cyclooxygenase (COX-2): rationale for selective inhibition and progress to date. *Med. Res. Rev.* **16:**181–206 (1996).
17. Picot, D., P. J. Loll, and R. M. Garavito. The X-ray crystal structure of the membrane protein prostaglandin H_2 synthase-1. *Nature (Lond.)* **367:**243–249 (1994).
18. Luong, C., A. Miller, J. Barnett, J. Chow, C. Ramesha, and M. Browner. Flexibility of the NSAID binding site in the structure of human cyclooxygenase-2. *Nature Struct. Biol.* **11:**927–933 (1996).
19. Kurumbail, R. G., A. M. Stevens, J. K. Gierse, J. J. McDonald, R. A. Stegeman, J. Y. Pak, D. Gildehaus, J. M. Miyashiro, T. D. Penning, K. Seibert, P. C. Isakson, and W. C. Stallings. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature (Lond.)* **384:**644–648 (1996).
20. Loll, P. J., D. Picot, and R. M. Garavito. The structural basis of aspirin activity inferred from the crystal structure of inactivated H_2 synthase. *Nature Struct. Biol.* **2:**637–643 (1995).
21. Mancini, J. A., D. Riendeau, J.-P. Falgueyret, P. J. Vickers, and G. P. O'Neill. Arginine 120 of prostaglandin G/H synthase-1 is required for the inhibition by nonsteroidal anti-inflammatory drugs containing a carboxylic acid moiety. *J. Biol. Chem.* **270:**29372–29377 (1995).
22. Bhattacharyya, D. K., M. Lecomte, C. J. Rieke, R. M. Garavito, and W. L. Smith. Involvement of arginine 120, glutamate 524, and tyrosine 355 in the binding of arachidonate and 2-phenylpropionic acid inhibitors to the cyclooxygenase active site of ovine prostaglandin endoperoxide H synthase-1. *J. Biol. Chem.* **271:**2179–21844 (1996).
23. Gans, K. R., W. Galbraith, R. J. Roman, S. B. Haber, J. S. Kerr, W. K. Schmidt, C. Smith, W. E. Hewes, and N. R. Ackerman. Anti-inflammatory and safety profile of DuP 697, a novel orally effective prostaglandin synthesis inhibitor. *J. Pharmacol. Exp. Ther.* **254:**180–187 (1990).
24. Wiesenberger-Boettcher, I., A. Schweizer, J. R. Green, K. Mueller, F. Maerki, and J. Pfeilschifter. The pharmacological profile of CGP28238, a novel highly potent anti-inflammatory compound. *Drugs Exp. Clin. Res.* **15:**501–509 (1989).
25. Tomic, M., I. Sunjevaric, E. S. Savtchenko, and M. Blumenberg. A rapid and simple method for introducing specific mutations into any position of DNA leaving all other positions unaltered. *Nucleic Acids Res.* **18:**1656 (1990).
26. Horton, R. M., Z. Cai, S. N. Ho, and L. R. Pease. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* **8:**528–535 (1990).
27. Bebbington, C. R., and M. R. Rolfe. Amplification using glutamine synthetase, in *Current Protocols in Molecular Biology* (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and J. Struhl, eds.). John Wiley, New York, 16.4.7–16.14.13, (1994).
28. Kargman, S., G. P. O'Neill, P. J. Vickers, J. F. Evans, J. A. Mancini, and S. Jothy. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. *Cancer Res.* **55:**2556–2559 (1995).
29. Laneuville, O., D. K. Breuer, D. L. DeWitt, T. Hla, C. Funk, and W. L. Smith. Differential inhibition of human prostaglandin endoperoxide H synthases-1 and -2 by nonsteroidal anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.* **271:**927–934 (1995).
30. Chulada, P. C., and R. Langenbach. Differential inhibition of murine prostaglandin synthase-1 and -2 by nonsteroidal anti-inflammatory drugs using exogenous and endogenous sources of arachidonic acid. *J. Pharmacol. Exper. Ther.* **280:**606–613 (1997).
31. Frolich, J. C. A classification of NSAIDs according to the relative inhibition of cyclooxygenase isoenzymes. *Trends Pharm. Sci.* **18:**30–34 (1997).
32. Vane, J. R., and R. M. Botting. Mechanism of action of anti-inflammatory drugs. *Scand. J. Rheumatol.* **25:**9–21 (1996).
33. Percival, M. D., M. Ouellet, C. Vincent, J. Yergey, B. P. Kennedy, and G. P. O'Neill. Purification and characterization of recombinant human cyclooxygenase-2. *Arch. Biochem. Biophys.* **315:**111–118 (1994).
34. Mancini, J. A., G. P. O'Neill, C. Bayly, and P. Vickers. Mutation of serine-516 in human prostaglandin G/H synthase-2 to methionine or aspirin acetylation of this residue stimulates 15-*R*-HETE synthesis. *FEBS Lett.* **342:**33–37 (1994).
35. Mancini, J. A., P. J. Vickers, G. P. O'Neill, C. Boily, J.-P. Falgueyret, and D. Riendeau. Altered sensitivity of aspirin-acetylated prostaglandin G/H synthase-2 to inhibition by nonsteroidal anti-inflammatory drugs. *Mol. Pharmacol.* **51:**52–60 (1997).
36. Ouellet, M., and M. D. Percival. Effect of inhibitor time-dependency on selectivity towards cyclooxygenase isoforms. *Biochem. J.* **306:**247–251 (1995).

37. Wong, E., C. DeLuca, C. Boily, S. Charleson, W. Cromlish, D. Denis, S. Kargman, B. P. Kennedy, M. Ouellet, K. Skorey, G. P. O'Neill, P. J. Vickers, and D. Riendeau. Characterization of autocrine inducible prostaglandin H synthase-2 (PGHS-2) in human osteosarcoma cells. *Inflamm. Res.* **46:**51–59 (1997).
38. Rome, L. H., and W. E. M. Lands. Structural requirements for time-dependent inhibition of prostaglandin biosynthesis by anti-inflammatory drugs. *Proc. Natl. Acad. Sci. USA* **72:**4863–5865 (1975).
39. Houtzager, V., M. Ouellet, J.-P. Falgueyret, L. A. Passmore, C. Bayly, and M. D. Percival. Inhibitor-induced changes in the intrinsic fluorescence of human cyclooxygenase-2. *Biochemistry* **35:**10974–10984 (1996).

Send reprint requests to: G. P. O'Neill, Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Québec H9R 4P8, Canada. E-mail: gary_oneill1@merck.com