The therapeutic action of nonsteroidal anti-inflammatory drugs (NSAIDs) is exerted through the inhibition of prostaglandin G/H synthase (PGHS), which is expressed as two isoenzymes, termed PGHS-1 and PGHS-2. From the crystal structure of sheep PGHS-1, it has been proposed that the carboxylic acid group of flurbiprofen is located in a favorable position for interacting with the arginine 120 residue of PGHS-1 (Picot, D., Loli, P. J., and Garavito, R. M. (1994) Nature 367, 243–249). Mutation of this Arg120 residue to Glu was performed and expressed in COS-7 cells using a vaccinia virus expression system. Comparison of microsomal enzyme preparations show that the mutation results in a 20-fold reduction in the specific activity of PGHS-1 and in a 100-fold increase in the apparent Km for arachidonic acid. Indomethacin, flurbiprofen, and ketoprofen, inhibitors of PGHS activity containing a free carboxylic acid group, do not exhibit any inhibitory effects against the activity of PGHS-1(Arg120 → Glu). Diclofenac and meclofenamic acid, other NSAIDs containing a free carboxylic acid group, were 50–100-fold less potent inhibitors of the activity of the mutant as compared with the wild type PGHS. In contrast, the nonacid PGHS inhibitors, 5-bromo-2-(4-fluorophenyl)-3-(4-methylsulfonyl)thiophene (DuP697) and a desbromo-sulfonamide analogue of DuP697 (L-746,483), were both more potent inhibitors of PGHS-1(Arg120 → Glu) than of the wild type PGHS-1. Inhibition of PGHS-1(Arg120 → Glu) was time-dependent for diclofenac and time-independent for DuP697, as observed for the wild type enzyme, indicating that the mutation does not alter the basic mechanism of inhibition. Aspirin is an acid NSAID that inhibits PGHS-1 through a unique covalent acetylation of the enzyme and also showed a reduced rate of inactivation of the mutated enzyme. These data provide biochemical evidence of the importance of the Arg120 residue in PGHS-1 for interaction with arachidonic acid and NSAIDs containing a free carboxylic acid moiety.

Prostaglandins, which are derived from arachidonic acid, act as potent mediators of pain, fever, and inflammation (1). The enzyme that catalyzes the initial step in their formation is prostaglandin G/H synthase (PGHS) (2). There are two isoforms of human PGHS, a constitutively expressed form termed PGHS-1 and an inducible isoform, termed PGHS-2, which can be induced in cells and tissues by growth factors and cytokines and also in several models of inflammation (3–8). The PGHS-catalyzed reaction consists of the bis-dioxygenation of arachidonic acid to form prostaglandin G2 and the reduction of this metabolite to prostaglandin H2, which then serves as the precursor for a variety of biologically active prostanoids (1, 2, 9).

Nonsteroidal anti-inflammatory drugs (NSAIDs), which were originally shown to inhibit PGHS-1 (10), have also been demonstrated to inhibit PGHS-2 (11–14). At least three types of NSAID-mediated inhibition of PGHS isoforms have been reported. In the first case, aspirin has been demonstrated to irreversibly inhibit prostaglandin production of PGHS-1 and stimulate (1S,5R)-hydroxyeicosatetraenoic acid production by PGHS-2 through the acetylation of a specific serine residue in each PGHS isoform (12, 15, 16). In a second type of NSAID-mediated inhibition, drugs such as indomethacin and flurbiprofen act as time-dependent, irreversible inhibitors of both isoforms without resultant covalent modification (17–19). In a third type of PGHS-mediated inhibition, compounds such as N-(2-cyclohexyloxy-4-nitrophenyl)methanesulfonamide (NS-398) and 3-bromo-2-(4-fluorophenyl)-3-(4-thiophene) (lithio)phenone (DuP697) act as time-independent, reversible inhibitors of PGHS-1 but as time-dependent, irreversible inhibitors of PGHS-2 (17, 18).

NSAIDs are the most widely utilized drugs for the treatment of inflammatory disorders such as arthritis. The major problem in the use of these compounds is their toxicity profile, which includes ulcerogenicity and renal dysfunction and has been attributed mainly to inhibition of the PGHS-1 isoform. Understanding the interaction of NSAIDs with PGHS can provide insight into the development of NSAIDs with enhanced therapeutic potential and a decreased capacity for toxicity. The recent determination of the x-ray crystal structure of sheep PGHS-1 has led to the development of a model for the topography of the NSAID binding site in PGHS-1 (20–22). Docking flurbiprofen in the long hydrophobic channel of the proposed active site of PGHS shows the carboxylic acid group of flurbiprofen located in a favorable position for interacting with the guanidinium group of Arg120. The only other charged residue in the active site cavity and hydrophobic channel is a Glu224.
which is proposed to form a salt bridge with Arg

Various studies on PGHS have utilized site-directed mutagenesis to demonstrate a Tyr residue essential for PGHS catalysis (23), essential histidines involved in heme binding of PGHS (24), residues glycosylated on PGHS (25), and the residue on PGHS acetylated by aspirin (15, 16). This study reports on the mutagenesis of Arg to glutamate in human PGHS-1 in order to delineate the interaction between NSAIDs and PGHS. Our observations show that PGHS-1(Arg Glu) has a decreased affinity for arachidonic acid and is no longer sensitive to inhibition by several NSAIDs containing a carboxylic acid moiety.

**MATERIALS AND METHODS**

Mutagenesis of PGHS-1 to PGHS-1(Arg Glu)—General recombinant DNA techniques were performed as described elsewhere (12, 15, 26). A 700-base pair BamHI-Aco DNA fragment, encoding the first 220 codons of human PGHS-1, was excised from plasmid pTM1-PGHS-1, and subcloned into the multiple cloning site of pBluescript (Stratagene, La Jolla, CA), yielding pBS-Bam-Acc-PGHS-1. Mutagenesis of Arg to PGHS-1 to Glu was performed using an oligonucleotide-directed mutagenesis kit (Amer sham Corp.), single-stranded DNA generated from pBS-Bam-Acc-PGHS-1, and a 33-mer oligonucleotide (5'-CAACACGCGCTTCCATCCGATGCCCTTAGCC-3'). The amino acid change of arginine residue 120 to glutamate is encoded by the underlined three nucleotide mismatch of the synthesized oligonucleotide.

Confirmation of the desired mutation was by dyeoxy DNA sequencing using a T7 DNA polymerase sequencing kit (Pharmacia Biotech Inc.). The mutated 700-base pair BamHI-Aco DNA fragment was then subcloned back into pTM1-PGHS-1, resulting in pTM1-PGHS-1(Arg Glu). Homologous recombination between the transfer vector pTM1-PGHS-1(Arg Glu) and wild type vaccinia virus (VV) was performed following standard protocols elsewhere (12, 27) and resulted in the recombinant VV:PGHS-1(Arg Glu). All procedures for the infection of COS-7 cells with VV:PGHS-1(Arg Glu) and the helper virus VV:TF7-3 and the preparation of the recombinant protein fractions from infected cells have been described elsewhere (12). Essentially, monolayer cultures of confluent COS-7 cells were co-infected with either VV:PGHS-1 or VV:PGHS-1(Arg Glu) and the helper virus VV:TF7-3, each at a multiplicity of infection of 1. At 24 h postinfection, cells were harvested by scraping, washed twice with phosphate-buffered saline, resuspended in lysis buffer (100 mM Tris, pH 7.4, 10 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml soybean trypsin inhibitor), and then disrupted using a microsonic cell disruptor (Coles Parmer). Samples were centrifuged for 10 min at 1,000 × g at 4°C, and the resulting supernatant was centrifuged for 60 min at 100,000 × g at 4°C. The 100,000 × g microsome fraction was resuspended in 100 mM Tris, pH 7.4, 10 mM EDTA to yield a protein concentration of 1–8 mg/ml. Protein concentrations were determined using the Pierce Coomassie protein assay reagent as described by the manufacturer’s instructions.

**Immunoblot Analysis**—Protein samples obtained from microsomal preparations were solubilized in 0.5 volume of electrophoresis buffer (20 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 4% glycerol, 0.24 M β-mercaptoethanol, and 0.05% bromphenol blue), boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis on precast 10% Tris-glycine acrylamide gels (Novex) and electrophoretically transferred to nitrocellulose membranes. Immunoblot detection was performed using a PGHS-1 polyclonal antiserum (29) and [125I]-protein A as previously described (28). Total microsomal protein at 2 and 5 μg from PGHS-1 expressing cells is shown in lanes 1 and 2, respectively. Total microsomal protein at 2, 5, and 10 μg from hPGHS-1(Arg Glu) expressing cells is shown in lanes 3, 4, and 5, respectively. The immunoblot was exposed to Kodak XAR-2 film at −80°C for 4 h. The migration position of molecular mass standards is depicted.

(DuPont NEN). Apparent Km values were found from PGHS-1 and PGHS-1(Arg Glu) expressed from the product of PGE production by the microsomal preparations at a short reaction time of 30 s as a function of arachidonic acid concentration. The Km analysis was performed using a program by J. S. Easterby (Hyperbolic Regression Analysis of Enzyme Kinetic Data) and is presented as double reciprocal plot. The time dependence of inhibition was determined using the assays for PGE production as described, and the inhibitor was preincubated for 0–30 min with PGHS-1 or PGHS-1(Arg Glu) before arachidonic acid addition. Inhibition was plotted as the percentage of PGHS activity remaining at the designated time points in the presence of inhibitor as compared with the control. The inhibitors indomethacin and flurbiprofen were obtained from Cayman Chemical Co., diclofenac was from Sigma, and DuP697 and L-746,483 were synthesized by the Department of Medicinal Chemistry (Merck Frosst, Canada).

**RESULTS**

Expression of PGHS-1 and PGHS-1(Arg Glu) in COS-7 Cells—PGHS-1(Arg Glu) was mutagenized to a glutamate residue to evaluate the importance of this arginine residue in the inhibition of activity of PGHS by acid NSAIDs and nonacid PGHS inhibitors. PGHS-1 and PGHS-1(Arg Glu) were expressed in COS-7 cells by co-infection with the helper virus VV:TF7-3 and either of the recombinants, VV: PGHS-1 or VV:PGHS-1(Arg Glu). At 24 h postinfection, the level of expression of these PGHS forms was analyzed by preparation of 100,000 × g membrane pellets from the infected cells and immunoblot analysis using an anti-PGHS-1 antiserum (Fig. 1). The level of recombinant protein expression of PGHS-1 and PGHS-1(Arg Glu) was equivalent as determined by scanning laser densitometry of the immunoblots (data not shown). The similar levels of recombinant PGHS-1 and PGHS-1(Arg Glu) protein in the microsomes permit the measurement of enzymatic activity and the evaluation of inhibitor potency at the same protein concentration without a difference in partitioning of substrate or inhibitors by the microsomal membranes. Similar microsomal preparations of PGHS have been described in the literature for analysis of various mutants of PGHS (15, 16, 23, 25).

Effect of Mutation of Arg Glu on Arachidonic Acid Oxidation by PGHS—Microsomal preparations from VV:PGHS-1-infected COS-7 cells were previously demonstrated to produce primarily PGE when incubated with arachidonic acid (12). The specific
activity of the mutant PGHS-1(Arg120→Glu) was 20-fold lower than that observed for the wild type PGHS-1. Interestingly, the peroxidase activity of PGHS-1(Arg120→Glu) was reduced to a similar extent as the cyclooxygenase activity of this mutant as measured with a peroxidase assay using hydrogen peroxide as a substrate and guaiacol as a reducing agent (30). Although the PGE2 production of the mutant was low, this amount of synthesis was 40-fold higher than that of mock infected COS cells, and product formation was dependent on exogenous arachidonic acid, thus allowing the further characterization of its activity. The time course of PGE2 product formation over a range of arachidonic acid concentrations was performed for both PGHS-1 and PGHS-1(Arg120→Glu) and showed similar hyperbolic curves with product formation plotted as a function of time. The time course for the mutant contained no detectable lag phase as compared with the wild type, even though the cyclooxygenase and peroxidase activity of the mutant PGHS-1 was decreased. Also, 0.1 or 1 μM 13-hydroperoxyoctadienoic acid did not stimulate arachidonic acid-dependent PGE2 production by either the mutant or wild type enzyme preparation. The production of PGE2 by microsomal preparations of PGHS-1 and the Arg120→Glu mutant was evaluated as a function of arachidonic acid concentration at a short reaction time of 30 s, and Lineweaver-Burk plots for PGHS-1 and PGHS-1(Arg120→Glu) are shown in Fig. 2. The apparent Km for arachidonic acid of PGHS-1(Arg120→Glu) was 60 μM, about 100-fold higher than that of hPGHS-1 (Km = 0.5 μM). The same Km values were obtained using a 3-min enzymatic reaction (data not shown). This large shift in the apparent Km suggests that the Arg120 is involved in binding arachidonic acid but is not absolutely required for catalytic activity. Microsomal assays performed at pH 5.0 resulted in a loss of only 20% of the activity for hPGHS-1(Arg120→Glu), whereas the activity of hPGHS-1 was decreased 20-fold to a level similar to that of the mutant form (data not shown). Although several interpretations can be envisioned for these results, they are consistent with the ionization of the carboxylic acid of arachidonic acid being more important for the binding of substrate to the wild type as opposed to the mutant enzyme.

Inhibition of hPGHS-1 and hPGHS-1(Arg120→Glu) by NSAIDs—If NSAIDs with an acidic carboxylic acid moiety, such as indomethacin, flurbiprofen, and diclofenac, interact with the Arg120 of PGHS-1 through an ionic interaction, then these NSAIDs should show a reduced inhibitory potency against hPGHS-1(Arg120→Glu). Conversely, the potency of PGHS inhibitors that do not contain a carboxylic acid, such as DuP697 (17), should be less affected by the mutation of the arginine residue.

PGHS inhibitors such as indomethacin, flurbiprofen, and diclofenac inhibit this enzyme by an essentially irreversible time-dependent mechanism (17, 18). The inhibitory potencies of these NSAIDs were determined using a preincubation time of inhibitor with enzyme to allow the time-dependent inhibition to develop prior to initiation of the reaction with 20 μM arachidonic acid. Flurbiprofen, indomethacin, and ketoprofen are potent inhibitors of PGHS-1 with IC50 values of 3.2, 90, and 60 nM, respectively (Fig. 3 and Table I). In contrast, no significant inhibition of PGHS-1(Arg120→Glu) at concentrations up to 24 μM was observed with indomethacin, flurbiprofen, or ketoprofen. The fenamic acid NSAIDs, diclofenac and meclofenamic acid, retained some inhibitory effects against PGHS-1(Arg120→Glu), with IC50 values of 1.5 and 5.0 μM for the Arg120→Glu mutant, respectively. Although, these latter NSAIDs inhibited PGHS-1(Arg120→Glu), the potency was decreased 40–100-fold as compared with the inhibition of wild type PGHS-1. The acid NSAID ibuprofen was also tested and found to inhibit PGHS-1 with an IC50 of 100 μM, whereas no detectable inhibition of PGHS-1(Arg120→Glu) was observed at 240 μM ibuprofen. Clearly, all of the acid NSAIDs tested are significantly more potent for inhibition of the wild type PGHS-1 as compared with the mutant PGHS-1(Arg120→Glu).

The DuP697 compound inhibits PGHS-1 by a competitive reversible inhibitory mechanism (17, 18). DuP697 displayed significant potency for inhibition of both PGHS-1 and PGHS-1(Arg120→Glu) with IC50 values of 1.2 and 0.1 μM, respectively (Fig. 4A). Also, a desbromo-sulphonamide analogue of DuP697 (L-746,483) (31) was found to inhibit both PGHS-1 (IC50 = 10 μM) and PGHS-1(Arg120→Glu) (IC50 = 0.84 μM). The increased potency of inhibition against the mutant by DuP697 and L-746,483 appears to be related to the higher Km of the mutant for arachidonic acid. These compounds are competitive with arachidonic acid, and because the inhibition was performed at a substrate concentration (20 μM arachidonic acid) approaching the Km of the PGHS-1(Arg120→Glu) mutant (Km = 60 μM) but higher than the Km for PGHS-1 (Km = 0.5 μM), the IC50 for inhibition of activity of the mutant is expected to be lower than that of the wild type. This was confirmed by performing assays with the wild type PGHS-1 at lower arachidonic acid concentration (0.6 μM) approaching the Km of the microsomal preparation of PGHS-1 (Km = 0.5 μM). At this substrate concentration approaching the Km of PGHS-1, DuP697 results in an IC50 of 40 nM, a value similar to that observed for inhibition of the mutant (data not shown). In sharp contrast to acid NSAIDs, these results demonstrate that the nonacid PGHS inhibitors, DuP697 and L-746,483, retain their potency for
inhibition of the mutant PGHS-1(Arg\textsuperscript{120} → Glu).

Experiments were performed to confirm the type of inhibition by diclofenac and DuP697 for PGHS-1(Arg\textsuperscript{120} → Glu) by incubating microsomal preparations for 0–30 min with the inhibitors prior to initiation of the reaction with arachidonic acid (Fig. 5). The results from these experiments demonstrate

\begin{table}
\centering
\caption{Inhibition of PGHS-1 and PGHS-1 (Arg\textsuperscript{120} → Glu) by Various NSAIDs and recently developed inhibitors of PGHS were tested for the inhibition of PGE\textsubscript{2} production by microsomal preparations of PGHS-1 and PGHS-1 (Arg\textsuperscript{120} → Glu). The IC\textsubscript{50} values were obtained from experiments in which the inhibitor was preincubated with these enzyme preparations for 15 min before a 3-min incubation with 20 \mu M arachidonic acid.}
\begin{tabular}{lcc}
\hline
\textbf{Compound} & \textbf{PGHS-1 IC\textsubscript{50}} & \textbf{PGHS-1 (Arg\textsuperscript{120} → Glu) IC\textsubscript{50}} \\
\hline
Indomethacin & 0.09 & >24 \\
Flurbiprofen & 0.003 & >24 \\
Diclofenac & 0.035 & 1.5 \\
Ketoprofen & 0.06 & >24 \\
Medofenamic & 0.05 & 5.0 \\
Ibuprofen & 100 & >240 \\
DuP697 & 1.2 & 0.1 \\
L-746,483 & 10 & 0.84 \\
\hline
\end{tabular}
\end{table}

that DuP697 at the given inhibitor concentrations inhibits both PGHS-1 and PGHS-1(Arg\textsuperscript{120} → Glu) in a time-independent manner. In sharp contrast, diclofenac inhibits both the wild type and mutant enzyme in a time-dependent mechanism.

Aspirin is an acid NSAID that is a time-dependent inhibitor of PGHS-1 but whose mechanism of action is through acetyla-
tion of PGHS. Microsomal preparations of PGHS-1 and PGHS-
1(Arg120 → Glu) were preincubated with 1 mM aspirin for 0–90
min before the addition of arachidonic acid and measurement
of enzymatic activity (Fig. 6). Aspirin inhibited PGHS-1 in a
time-dependent mechanism with a complete inhibition at 10
min independent of the arachidonic acid concentration used for
the experiment (0.5 or 20 μM). Interestingly, aspirin at all time
points tested only inhibits the mutant PGHS-1(Arg120 → Glu)
by 40% after a 90-min preincubation. This is further evidence
that an acid NSAID that inhibits PGHS through a novel cova-
lent modification requires an initial interaction with the Arg120
residue.

DISCUSSION

The present results demonstrate that the Arg120 residue of
hPGHS-1 is essential for the potent inhibitory effects of the
acid inhibitors flurbiprofen, indomethacin, diclofenac, ketopro-
fen, and meclofenamic acid. Also, the less potent PGHS inhib-
itors, ibuprofen and aspirin, are acid NSAIDs that also have a
diminished potency for inhibition of PGHS-1(Arg120Glu) as com-
pared with PGHS-1. Interestingly, Arg120 is not essential for
interaction with the nonacid DuP697 and L-746,483 com-
ounds. The decreased or lack of inhibition of PGHS-1(Arg120
→ Glu) by acid NSAIDs supports the model proposed by
Garavito and his colleagues in which the Arg120 residue of
PGHS-1 interacts with the carboxylic acid group of flurbiprofen
(20). Based on the x-ray crystal structure of sheep PGHS-1, there
are only two charged residues that are found within the
hydrophobic channel leading up to the cyclo-oxygenase active
site, Arg120 and Glu524. In the absence of arachidonic acid or an
NSAID, Arg120 and Glu524 may form a salt bridge. The impor-
tance of this arginine residue for PGHS activity is demon-
strated by both a large increase in the Km of the mutant for
arachidonic acid and the decrease in both the cyclo-oxygenase
and the peroxidase activities of the enzyme. The conversion of
the positive charged arginine to a negative charged glutamate
residue abolishes the possible interaction between the carbox-
ylic acid group of acid NSAIDs and arachidonic acid with the
guanidinium group of Arg120 of PGHS. This ionic interaction
may be essential for the formation of a tight binding inhibitor
complex leading to the time-dependent inhibition of PGHS by
inhibitors such as indomethacin and flurbiprofen. The presence
of the common arginine residue (PGHS-1 and PGHS-2) for the
interaction of the carboxylic acid group from both arachidonic
acid and certain NSAIDs with PGHS is consistent with the
competitive binding of inhibitor preceding the formation of the
irreversible enzyme-inhibitor complex (19).

The inhibition by DuP697 and L-746,483, which are nonacid
PGHS inhibitors, is not affected by the mutation, implying that
the interaction of DuP697 and PGHS-1 involves amino acids
other than Arg120 whose spatial arrangement is not affected by
the substitution of Glu for Arg. In addition, the time-inde-
dependent inhibition of PGHS-1 by DuP697 is maintained in the
mutant PGHS-1 (Arg120 → Glu), and the time-dependent inhi-
bition by diclofenac is similar for the mutant as compared with
the wild type, albeit at a higher concentration of inhibitor. The
mechanism of inhibition of the Arg120 → Glu mutant by
DuP697 and diclofenac shows that some of the essential fea-
tures for the binding of inhibitors at the active site are pre-
served and confirms the selectivity of the interaction between
Arg120 and NSAIDs possessing a free carboxylic acid group.
Aspirin is a unique acid NSAID that inhibits PGHS-1 through
a time-dependent covalent acylation. The slower time-de-
dependent inhibition of the mutant PGHS-1 (Arg120 → Glu)
by aspirin demonstrates the importance of the interaction of
aspirin with Arg120 for its ability to inhibit PGHS.

It has been suggested that PGHS-1 provides prostaglandins
for use under normal physiological conditions, whereas
PGHS-2 is involved in inflammatory responses (7, 8). NSAIDs
in current therapeutic use inhibit both isoforms of PGHS with
no or little selectivity and have associated toxicities (i.e. ulcer-
Most of the conventional NSAIDs contain a carboxylic acid group. Surprisingly all of the selective inhibitors of PGHS-2 reported to date lack a carboxylic acid group but contain a sulfonyl group. Further understanding of the mode of binding of these NSAIDs with both PGHS-1 and PGHS-2 should provide insight into developing better NSAIDs with a decreased potential for toxicity.

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Arginine 120 of Prostaglandin G/H Synthase-1 Is Required for the Inhibition by Nonsteroidal Anti-inflammatory Drugs Containing a Carboxylic Acid Moiety

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