Acetylation of Human Prostaglandin Endoperoxide Synthase-2 (Cyclooxygenase-2) by Aspirin*

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Aspirin (acetylsalicylate) treatment of human (h) prostaglandin endoperoxide H synthase (PGHS-1) expressed in cos-1 cells caused a time-dependent inactivation of oxygenase activity. Aspirin treatment of hPGHS-2 produced an enzyme which retained oxygenase activity but formed exclusively 15-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) instead of PGH₂. The 15-HETE was exclusively of the 15R configuration. The Kₐ values for arachidonate of native and aspirin-treated hPGHS-2 were about the same, suggesting that arachidonate binds to both aspirin-treated and native hPGHS-2 in a similar manner. If, as expected, the formation of 15R-HETE proceeds through abstraction of the 13α-proton from arachidonate, O₂ insertion must occur from the same side as the hydrogen abstraction; with all other lipoygenases and cyclooxygenases, O₂ addition is antarafacial. When microsomal hPGHS-2 was incubated with [acetyle-¹⁴C]aspirin, the enzyme was acetylated. An SS16A mutant of hPGHS-2, which retains enzyme activity, was not acetylated. This indicates that Ser-516 is the site of aspirin acetylation of hPGHS-2; this residue is homologous to the “active site” serine of PGHS-1. An SS16N mutant of hPGHS-2 was catalytically active; in contrast, an SS16Q mutant lacked cyclooxygenase but retained peroxidase activity. Because in the case of PGHS-1 a smaller asparagine substitution is sufficient to eliminate cyclooxygenase activity, we conclude that the active site of PGHS-2 is slightly larger than that of PGHS-1. An SS16M mutant of hPGHS-2 was obtained which resembled aspirin-acetylated hPGHS-2 in that this mutant made 15R-HETE as its major product; however, unlike the aspirin-acetylated hPGHS-2, the Kₐ value of the SS16M mutant for arachidonate was 100 times that of native hPGHS-2.

There are two isozymes of prostaglandin endoperoxide H synthase, PGHS-1 and PGHS-2, that are able to catalyze the conversion of arachidonic acid to prostaglandin endoperoxide H₂ (PGH₂). Both enzymes exhibit both cyclooxygenase and peroxidase activities (1-3). PGHS-1 was initially purified and its cDNA cloned from ovine vascular glands (1, 2, 4-7). The sequences of murine (8), rat (9), and human (10) PGHS-1 are also now known. Recent studies from several laboratories have reported the cloning of cDNAs for the second isozyme, PGHS-2, from chicken (11), mouse (12), rat (9, 13), and human (14, 15) sources.

PGHS-1 and -2 have quite different patterns of expression. PGHS-1 is constitutively expressed in most tissues and in many cultured cells including NIH 3T3 cells (16), rat mesangial cells (17), and kidney (18). PGHS-1 is probably involved in the production of prostaglandins involved in cellular “housekeeping” functions (19, 20). In contrast, PGHS-2 is rapidly, but transiently, induced in a variety of cell types following treatment with mitogens, growth factors, cytokines, and tumor promoters (15, 21-27). Prostaglandins produced through the action of PGHS-2 may be specifically involved in cellular differentiation and proliferation (20).

The cyclooxygenase activity of PGHS-1 had been thought to be the pharmacological target of aspirin and related nonsteroidal anti-inflammatory drugs (NSAIDs) (28, 29). However, PGHS-2 is induced under conditions of inflammation (13, 25) and recent evidence suggests that this second isozyme may be the target of NSAIDs acting in their anti-inflammatory capacities (20, 30, 31). Accordingly, it is important to understand how various NSAIDs interact with PGHS-2. Previous studies have shown that aspirin and related NSAIDs inhibit the cyclooxygenase but not the peroxidase activities of both PGHS-1 and -2 (3, 32-34). However, the two isozymes interact differently with different NSAIDs (3, 34). The situation with aspirin is particularly intriguing. Aspirin causes a time-dependent inhibition of murine PGHS-2 converting this enzyme to a form which catalyzes the production of 15-hydroxy-eicosatetraenoic acid (15-HETE) instead of PGH₂ from arachidonate (34, 35). In this study we have determined the stereochemistry of the 15-HETE which is produced. We have also characterized the biochemical alteration induced by treatment of hPGHS-2 with aspirin.

EXPERIMENTAL PROCEDURES

**Materials**—Dulbecco’s modified Eagle’s medium was from Life Technologies Inc. Fetal calf serum and calf serum were obtained from HyClone. Chloroquine, bovine hemoglobin, hematin, guaiacol, acetylsalicylic acid, and penicillin G were from Sigma. Arachidonic acid, prostaglandin D₂, E₂, and F₂α, and 15-HETE were purchased from Cayman Chemical Co. [¹⁴C]Arachidonic acid (40-60 mCi/mmol), [α-³²P]ATP (1000-1500 Ci/mmol), and EN³HANCE autoradiography enhancer were purchased from DuPont NEN; [acetyle-¹⁴C]acetylsalicylic acid (15 mCi/mmol, 98% pure) was from Research Products International Corp. ¹⁴C-Methylated protein molecular weight markers (Mᵣ, 14,300-200,000) and ECL Western blotting reagents were from Amersham Corp. BA85 (0.4% v/v) nitrocellulose was from Schleicher & Schuell. Acrylamide, bis-acrylamide, SDS, streptomycin, restriction endonucleases, T4 DNA ligase, and T4 DNA polymerase were purchased from Boehringer Mannheim. Sequenase was from U. S. Biochemical Corp. Horseradish peroxidase-labeled goat anti-rabbit IgG was from

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1. The abbreviations used are: PGHS-1 or -2, prostaglandin endoperoxide H synthase isoform 1 or -2; aspirin, acetylsalicylic acid; 15R-HETE, 15R-hydroxy-5,8,11,13-eicosatetraenoic acid; NSAID, nonsteroidal anti-inflammatory drug; PGH₂, PGG₂, PGE₂, PGD₂, and PGF₂α, prostaglandin H₂, G₂, E₂, D₂, and F₂α; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; h, human.

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Bio-Rad. DEAE-dextran was purchased from Pharmacia Biotech Inc. CaCl₂ was from Var-Lac-Oid Chemical Co. The oligonucleotides used as primers for preparing the mutants of human PGHS-1 and -2 and for DNA sequencing were prepared by the Michigan State University Macromolecular Sequencing Facility. The mammalian expression vector pOSML (3) was derived from pMT2 (36) as described previously (3). pcDNA vectors containing sequences for human PGHS-1 and -2 were subcloned into the SalI site of pOSML (3). The correct orientation of the insert was determined by restriction digestion with PstI. Plasmids used for preparing the mutants of human PGHS-2 and for DNA sequencing were isolated by CsCl gradient ultracentrifugation. The mammalian expression vector pcDNA vectors containing sequences for human PGHS-1 and -2 were purified from the plasmid DNA preparations by CsCl gradient ultracentrifugation. The synthetic oligonucleotide 5'-CGGCGAGCCACGCTTTATGC-3' (14) was used as a sequencing primer to identify all recombinants.

Preparation of PGHS-2 Mutants—The cDNAs containing the coding regions for each of the human PGHS synthase isozymes (1.8 kilobase) with unique SalI restriction sites in both the 5' and 3' untranslated sequences were as described previously (3). Mutants of human PGHS-2 (5S16A, 5S16N, 5S16Q, and 5S16 M) were prepared starting with M13 mp19-human PGHS-2, which contains a 1.8-kilobase SaI1 fragment encoding the native human PGHS-2, according to the method of Kunkel et al. (38) using a Bio-Rad kit essentially as described previously (59, 60). Table I shows the oligonucleotides used for the preparation of each of these mutants. Phage saponies were sequenced using the dideoxy method (41) to identify mutants. The 1.8-kilobase insert from the replicative form of M13 mp19-human PGHS-2 containing the desired mutation was isolated after digestion with SalI restriction enzyme and subcloned into the SalI site of pOSML (3). The correct orientation of the insert was determined by restriction digestion with PstI. Plasmids used for transfections were purified by CaCl₂ gradient ultracentrifugation.

Transient Expression of Human PGHS-1 and PGHS-2—Cos-1 cells (ATTC CR-1860) were grown to near confluence in Dulbecco's modified Eagle's medium containing 8% calf serum and 2% fetal calf serum, streptomycin (0.1 g/liter), and penicillin G (0.1 g/liter) in a water-saturated 5% CO₂ atmosphere. About 16 h before transfections, the cells were subcultured 1:2. Cos-1 cells were transfected as described previously (8) using the DEAE dextran/chloroquine method. Cells were sham-transfected or transfected with 15 μg of pSPTV-PGHS-1, pSOML-hPGHS-1, or pOSML-hPGHS-2 per 100-mm culture dish (3×10⁶ cells). Forty hours post-transfection, cells were harvested using a rubber policeman, resuspended in 0.1 M Tris-HCl, pH 7.4, to a final protein concentration of about 5 mg/ml (8).

Acetylation of PGHS Isozymes by Acetyl-14C]-Aspirin—Microsomal preparations of various PGHS isozymes (250 μl) were incubated with 1 μmol of aspirin (0.1 μCi of 14C]-aspirin) for 1-5 min with Tris-HCl buffer, pH 7.2, 5.6 mm guaiacol, 50-250 μg of microsomal protein, 1 μmol hematin (added in dimethyl sulfoxide, 1.5% final concentration), and 0.4 mm H₂O₂ in a total volume of 0.9 ml. The reactions were initiated by the addition of H₂O₂ and the progress of the oxidation of guaiacol to 3,3'-dimethoxy diphenyl-4,4'-quinone was monitored at 440 nm (45).

Western Transfer Blotting—Solubilized microsomal membranes were resolved by one-dimensional SDS-PAGE and transferred electrophoretically to BA85 nitrocellulose filters (0.45 μm) essentially as described previously (40, 46). For detection of human PGHS-2 by enhanced chemiluminescence, filters were incubated for 1-2 h with a 1:2000 dilution of a nonimmune rabbit anti-human PGHS-2 serum (47). The filters were washed and incubated with a 1:2000 dilution of goat anti-rabbit IgG horseradish peroxidase. The filters were again washed and incubated for 1-5 min with Amersham ECL Western blotting detection reagents. The filters were immediately blot-dried and exposed to XAR-5 film.

The reaction mixture contained 3 ml of 0.1 M Tris-HCl buffer, pH 7.2, 5.6 mm guaiacol, 50-250 μg of microsomal protein, 1 μmol hematin (added in dimethyl sulfoxide, 1.5% final concentration), and 0.4 mm H₂O₂ in a total volume of 0.9 ml. The reactions were initiated by the addition of H₂O₂ and the progress of the oxidation of guaiacol to 3,3'-dimethoxy diphenyl-4,4'-quinone was monitored at 440 nm (45).

Assay of Cyclooxygenase Activity—The cyclooxygenase activities were assayed on microsomal preparations made the same day and were measured by monitoring the initial rate of O₂ uptake at 37 °C (200 μm O₂) using an O₂ electrode as described previously (44). A typical assay mixture contained 3 ml of 0.1 M Tris-HCl buffer, pH 7.0, 1.5% final concentration), and 0.4 mm H₂O₂ (a total volume of 0.9 ml). The reactions were initiated by the addition of H₂O₂ and the progress of the reaction was monitored (43) at 440 nm (45).

Bio-Rad. DEAE-dextran was purchased from Pharmacia Biotech Inc. CaCl₂ was from Var-Lac-Oid Chemical Co. The oligonucleotides used as primers for preparing the mutants of human PGHS-1 and -2 and for DNA sequencing were prepared by the Michigan State University Macromolecular Sequencing Facility. The mammalian expression vector pOSML (3) was derived from pMT2 (36) as described previously (3). pcDNA vectors containing sequences for human PGHS-1 and -2 were purified by CsCl gradient ultracentrifugation. The synthetic oligonucleotide 5'-CGGCGAGCCACGCTTTATGC-3' (14) was used as a sequencing primer to identify all recombinants.

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Acetylation of PGHS Isozymes by Acetyl-14C]-Aspirin—Microsomal preparations of various PGHS isozymes (250 μl) were incubated with 1 μmol of aspirin (0.1 μCi of 14C]-aspirin) dissolved in ethanol (1% final concentration) and 80-μl aliquots were removed after 0, 1, or 2 h incubations at 37 °C. Reactions were terminated by the addition of 2 ml of ice-cold saline, protein was collected by centrifugation, and washed twice with cold saline. After evaporation of residual acetone under a stream of N₂ gas, the protein pellet was solubilized in 150 ml of phosphatase loading buffer (0.2 M Tris-HCl, pH 6.8, containing 2.3% SDS, 0.5% mercaptoethanol, and 10% glycerol). Aliquots (10-20 μl) were submitted to SDS-PAGE on 10% polyacrylamide gels containing 0.1% SDS, with a 5% stacking gel and the discontinuous buffer system of Laemmli (42). Radiolabeled bands were detected after fluorography of the gel using EN3HANCE® according to the specifications of the manufacturer. Autoradiographs of dried gels were exposed to Kodak XAR-5 film (preflushed in order to reach an optical density of 0.15 above the background (43)) at -80 °C for periods up to 40 days.
Fig. 2. Radio-TLC of the products made from \(^{14}\text{C}\)arachidonate by human PGHS-1 and -2 after incubation with aspirin. Aspirin (500 μM) was added (+) or not (−) directly to the culture medium of cos-1 cells following transfection with an expression vector containing either human PGHS-1 or -2 or following transfection without any DNA (sham). After incubation with aspirin, cells were harvested and washed before incubation with \(^{14}\text{C}\)arachidonate (60 μM) for 15 min at 37 °C, as described under “Experimental Procedures.” Radioactive products in the supernatant were then extracted, separated by thin layer chromatography, and visualized by autoradiography, as described under “Experimental Procedures.” The positions of migration of several standards are indicated on the right of the figure. This figure is representative of two experiments with similar results.

Fig. 3. Chiral HPLC of [methyl-1-\(^{14}\text{C}\)]15-HETE formed from arachidonic acid by aspirin-treated human PGH synthase-2. [1-\(^{14}\text{C}\)]15-HETE was isolated from an incubate of [1-\(^{14}\text{C}\)]arachidonate with aspirin-treated cos-1 cells expressing hPGHS-2, methylated, and subjected to chiral HPLC on a Chiralcel OD column (250 x 4.6 mm) eluted with hexane/isopropyl alcohol (100:3, v/v) at a flow rate of 0.35 ml/min. Details are presented in the text. The mobility of methyl-15-(R/S)-HETE standards is indicated in the figure.

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PHY and autoradiography, respectively. Proteins in the cell supernatant were precipitated by adding 1.5 ml of ice-cold acetone. After centrifugation (2000 x g, 10 min) to remove precipitated proteins, the resulting supernatant was acidified with 0.2 ml of 0.2 M HCl and the aqueous phase extracted with 2.5 ml of CHCl. The organic phase was evaporated under nitrogen and the residue dissolved in 50 μl of CHCl before spotting on a Silica Gel 60 thin layer chromatography plate. The lipid products were chromatographed twice for 1 h in benzene/dioxane/formic acid/acetic acid (82:14:1:1, v/v). The thin layer chromatography plate was then air-dried and subjected to autoradiography for 40 h using Kodak XAR-5 film.

Chiral HPLC of Methyl-15-HETE—[1-\(^{14}\text{C}\)]15-HETE was isolated from cos-1 cells expressing either: (a) aspirin-treated hPGHS-2 or (b) the S516M mutant of hPGHS-2 following incubation with [1-\(^{14}\text{C}\)]arachidonate, as described previously (34). Radioactive 15-HETE samples were treated with an excess of ethereal diazomethane at 0 °C for 10 min to prepare the methyl esters. The samples were purified by straight phase HPLC on a Partisil silica column (250 x 4.6 mm, 10 μm, Alltech Associates, Inc.) eluting with hexane containing 1% isopropyl alcohol (v/v) at a flow rate of 1.0 ml/min. The eluate was collected at the time corresponding to the retention time of methyl-15-HETE, evaporated under a stream of N₂, and redissolved in 50 μl of hexane containing 3% isopropyl alcohol. The purified samples were subjected to Chiral-phase HPLC essentially as described by Braith and Hawkins (48). Separations were performed using a Chiralcel OD column (250 x 4.6 mm; J. T. Baker, Inc.) eluted with hexane/isopropyl alcohol (100:3, v/v) at a flow rate of either 0.25 or 0.35 ml/min. The mobility of racemic methyl-15-(RS)-HETE standards was monitored at 235 nm with a Varian model 2050 UV detector, and radioactivity was monitored using a β-RAM radiochromatographic detector (IN/US Systems, Inc.).

RESULTS

Differential Effects of Aspirin on Prostaglandin Endoperoxide Formation by Human PGH Synthases-1 and -2—Human PGHS-1 and PGHS-2 were expressed transiently in cos-1 cells and microsomal membranes isolated from the transfected cells were assayed for their sensitivity to inhibition by aspirin using both an O₂ electrode assay and direct measurement of arachidonate-derived products. Aspirin (1 mM) completely in-
Fig. 4. Acetylation of human PGHS-1 and -2 by [14C]aspirin. Microsomal membrane preparations from cos-1 cells transfected without any DNA (sham) or from cells expressing either human PGHS-1 or -2 or ovine PGHS-1 were prepared as described under "Experimental Procedures" and incubated with [acetyl-14C]aspirin (1 μM) for 0, 1, or 2 h. At the end of the incubation, protein was precipitated, washed two times by ice-cold acetone and subjected to SDS-PAGE as described under "Experimental Procedures." This gel was then fluorographed and exposed to a preflashed x-ray film for 36 days at -80°C as described under "Experimental Procedures." The position of molecular weight standards on the gel is indicated (Mr x 10^3). This gel is representative of two experiments with similar results.

Fig. 5. Acetylation of native and S516A mutant of human PGHS-2 by [14C]aspirin. The experiment was performed essentially as described in the legend to Fig. 3, except that the cells were transfected without any DNA (sham) or with an expression vector containing either native PGHS-2 or the S516A mutant of PGHS-2 (S516A). The exposure time for the autoradiography was 39 days.
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TABLE II

| Human PGHS-2 expressed in cos-1 cells | Cyclooxygenase | Peroxidase |
|-------------------------------------|----------------|------------|
|                                     | % of control   | μM         |
| Native                              | 100            | 100        |
| Acetylated native                   | 75\(^a\)        | ND\(^b\)   |
| S516A                               | 62             | 98         |
| S516N                               | 98             | 114        |
| S516Q                               | 0              | 82         |
| S516M                               | 22             | 100        |

\(^a\) Data from Laneuville et al. (3).
\(^b\) Data from Fig. 1.
\(^c\) ND, not determined.

Cyclooxygenase and peroxidase activities of native human PGH synthase-2 and PGH synthase-2 mutants

Cyclooxygenase and peroxidase activities were assayed using microsomes prepared from cos-1 cells transfected with pOSM1, constructs containing the coding regions for native or mutant hPGHS-2, or from cos-1 cells transfected with pOSML-hPGHS-2 and treated with 1 mM aspirin at 37 °C before assaying the activity. Methodological details are presented under "Experimental Procedures." Activities are presented as percentages of native human PGHS-2 cyclooxygenase or peroxidase activities and represent the mean of triplicate determinations in one transfection. Results are representative of at least two separate transfections which displayed qualitatively similar results. Native cyclooxygenase activity in this experiment was 49.0 ± 1.1 nmol (mean ± S.D., n = 3) O₂/min/mg microsomal protein; native peroxidase activity was 34.3 nmol of H₂O₂/min/mg of microsomal protein.

with hPGHS-1. First, to determine if hPGHS-1 and -2 are acetylated by aspirin, microsomal preparations of cos-1 cells expressing either ovine or human PGHS-1 or human PGHS-2 were incubated with [acetyl-1-\(^14\)C]aspirin (1 mM), and the radiolabeled proteins were separated by SDS-PAGE and analyzed by fluorography (Fig. 4). With microsomes from cells transfected with ovine PGHS-1 (as compared to sham-transfected cells), one unique radiolabeled protein with an apparent molecular mass of 70 kDa was present as expected for acetylation of the ovine PGHS-1 (4). Similar results were obtained with microsomes from cells transfected with hPGHS-1; a single radiolabeled protein band was present which comigrated with the one observed with ovine PGHS-1. With microsomes from cells transfected with hPGHS-2, three radiolabeled protein bands with apparent molecular masses of 70, 72, and 74 kDa were observed. Western transfer blotting of hPGHS-2 also yielded a triplet of immunoreactive proteins of the same mobilities; pre-treatment of the sample with endoglycosidase H prior to SDS-PAGE yielded a single immunoreactive protein with Mᵦ = 65,000 corresponding to the expected mobility of deglycosylated hPGHS-2. Our results indicate that hPGHS-2 is acetylated by aspirin.

To determine if the site of acetylation of hPGHS-2 by aspirin involved the serine residue homologous to that acetylated in PGHS-1 we substituted an alanine residue for Ser-516 in hPGHS-2 by site-directed mutagenesis and then compared the acetylation of native hPGHS-2 and the S516A mutant of hPGHS-2 by [acetyl-1-\(^14\)C]aspirin (Fig. 5). With membranes prepared from cos-1 cells transfected with native hPGHS-2, we observed the expected three proteins with estimated molecular masses of 70, 72, and 74 kDa as compared to sham-transfected cells. In contrast, the aspirin labeling pattern observed with the S516A mutant of hPGHS-2 was indistinguishable from the one observed with sham-transfected cells. This result establishes that the S516A mutant cannot be acetylated by aspirin and suggests that Ser-516 is the single site of acetylation of hPGHS-2 by aspirin. We next measured the activity of the S516A hPGHS-2 to determine if its insensitivity toward aspirin acetylation was simply the result of a dramatic structural

FIG. 6. Radio-TLC of the products made from [\(^14\)C]arachidionate by native human PGHS-2 or the S516A mutant of human PGHS-2. This figure shows a radio-thin layer chromatogram (radio-TLC) of [\(^14\)C]arachidonic acid metabolites made by cos-1 cells transfected with no DNA (sham) or expression vectors containing either native PGHS-2 (native) or the S516A mutant of PGHS-2. Before incubation with arachidonate, the cells were treated with (+) or without (−) aspirin (ASA) (500 μM), as described under "Experimental Procedures." The position of migration of several standards is indicated on the right side of this figure.

hindered the cyclooxygenase activity of hPGHS-1 following a 1-h incubation at 37 °C (Fig. 1, panel A); in the absence of aspirin the cyclooxygenase activity of hPGHS-1 was stable. When hPGHS-2 was treated with aspirin (1 mM), the initial rate of oxygenation of arachidonate dropped by about 50% after a 1-h incubation; however, a simple control incubation of hPGHS-2 at 37 °C for 10 min also caused the oxygenase activity to fall by 30%. In some experiments, the inhibition of hPGHS-2 was more pronounced, but there was always detectable oxygenase activity after aspirin treatment. The profiles of arachidonate-derived products made by intact cos-1 cells transfected with one of the two PGHS isoforms and treated with or without aspirin (500 μM) for 40 min at 37 °C is shown in Fig. 2. Sham-transfected cos-1 cells did not transform arachidonate to products. Cos-1 cells transfected with either hPGHS-1 or hPGHS-2 synthesized essentially the same products; the major radiolabeled bands comigrated with standard PGD₂, PGE₂, and PGF₂₀ (Fig. 2). After treatment of the cells expressing hPGHS-1 with aspirin, the synthesis of prostaglandins was almost completely abolished. Aspirin treatment of cells expressing hPGHS-2 resulted in almost complete inhibition of prostaglandin synthesis, but there was a substantial increase in the synthesis of a product which comigrated with 15-HETE. Analysis of the stereochemistry of the 15-HETE by chiral HPLC column indicated that ≈97% of the 15-HETE was in the R configuration (Fig. 3).

Acetylation of Human PGH Synthases-1 and -2 by Aspirin—Previous studies on purified PGHS-1 obtained from sheep vesicular glands have demonstrated that aspirin inhibits the cyclooxygenase activity of this enzyme via irreversible acetylation of a unique serine residue, the "active site" serine located at position 530 (8). There is an homologous serine residue (Ser-516) in hPGHS-2 in a region which shares about 70% identity...
Acetylation of Human PGHS-2 by Aspirin

**Fig. 7.** Radio-TLC of the products made from [14C]arachidonate by native human PGHS and the S516N mutant of this enzyme. The experiment was performed as described in the legend Fig. 6, except that cos-1 cells were transfected with the S516N mutant of human PGHS-2.

change. Fig. 6 shows an autoradiogram of the products synthesized from [14C]arachidonate by cos-1 cells transfected with native hPGHS-1 and with the S516A mutant. The synthesis of prostaglandins by cells expressing native hPGHS-2 is almost completely abolished by aspirin and 15R-HETE is produced as the major product. Cells expressing the S516A mutant of hPGHS-2 do synthesize about the same amount and type of products as native hPGHS-2. However, aspirin had no effect on product formation by the S516A mutant of hPGHS-2. When assayed using an O2 electrode the S516A mutant was found to exhibit cyclooxygenase activity comparable to that of the native enzyme. The K_m for arachidonate for the S516A mutant was determined to be 2.9 μM, a value comparable to that obtained with native hPGHS-2 (5.4 μM) (3). Finally, the peroxidase activities of hPGHS-2 and S516A hPGHS-2 were found to be quite similar to one another. We conclude from these results that hPGHS-2 is acetylated by aspirin at Ser-516.

We made three additional substitutions of Ser-516 in hPGHS-2 replacing the serine with asparagine, glutamine, and methionine residues. The purpose of these experiments was to determine the effect of the size of the side chain at position 516 of hPGHS-2 on cyclooxygenase and peroxidase activities (Table II). The S516N mutant, which contains a side chain roughly isosteric with an acetylated serine, retains activity and the products made from arachidonate by the cells expressing this mutant are the same as for native hPGHS-2 (Fig. 7). The K_m of this mutant for arachidonate was determined to be 3.6 μM. The S516Q mutant which has a side chain one methylene group larger than the S516N mutant lacked cyclooxygenase activity but retained peroxidase activity (see Table II). Intact cos-1 cells expressing the S516Q mutant failed to synthesize prostaglandin or hydroxy acid products from [1-14C]arachidonate (see Fig. 8).

An S516M mutant which also has a side chain significantly larger than an acetylated serine was active but exhibited only 22% of the oxygenase activity of the native enzyme (see Table II). The K_m value of this mutant for arachidonate is ~600 μM, which is roughly 100 times that observed for the native enzyme. An unexpected result was that the S516M mutant enzyme, when expressed in cos-1 cells, synthesized 15-HETE (Fig. 9) which, like that formed by the aspirin-acetylated hPGHS-2, was of the 15R configuration (data not shown). There were some differences in the nature and the relative abundance of the products made by acetylated hPGHS-2 versus the S516M mutant of PGHS-2. The relative amount of 15R-HETE produced by the acetylated hPGHS-2 was greater than that observed for the S516M mutant, and PGF_2 alpha was not produced in the case of the S516M mutant. All the mutants we made at position 516 retained peroxidase activity (Table II). Western blot analysis with rabbit monospecific IgGs directed against human PGHS-2 revealed three bands of 70, 72, and 74 kDa which comigrated with the ones observed for the native PGHS-2 (data not shown), indicating that mutations at position 516 did not significantly alter the structure of the enzyme as compared to the native enzyme.

**DISCUSSION**

In the present study, we have demonstrated that aspirin acetylates hPGHS-2 expressed in cos-1 cells. Three radiolabeled proteins not observed in sham-transfected cells were observed when microsomal membranes from cos-1 cells expressing the S516Q mutant failed to synthesize prostaglandin or hydroxy acid products from [1-14C]arachidonate (see Fig. 8).

An S516M mutant which also has a side chain significantly larger than an acetylated serine was active but exhibited only 22% of the oxygenase activity of the native enzyme (see Table II). The K_m value of this mutant for arachidonate is ~600 μM, which is roughly 100 times that observed for the native enzyme. An unexpected result was that the S516M mutant enzyme, when expressed in cos-1 cells, synthesized 15-HETE (Fig. 9) which, like that formed by the aspirin-acetylated hPGHS-2, was of the 15R configuration (data not shown). There were some differences in the nature and the relative abundance of the products made by acetylated hPGHS-2 versus the S516M mutant of PGHS-2. The relative amount of 15R-HETE produced by the acetylated hPGHS-2 was greater than that observed for the S516M mutant, and PGF_2 alpha was not produced in the case of the S516M mutant. All the mutants we made at position 516 retained peroxidase activity (Table II). Western blot analysis with rabbit monospecific IgGs directed against human PGHS-2 revealed three bands of 70, 72, and 74 kDa which comigrated with the ones observed for the native PGHS-2 (data not shown), indicating that mutations at position 516 did not significantly alter the structure of the enzyme as compared to the native enzyme.

In the present study, we have demonstrated that aspirin acetylates hPGHS-2 expressed in cos-1 cells. Three radiolabeled proteins not observed in sham-transfected cells were observed when microsomal membranes from cos-1 cells ex-
pressing hPGHS-2 were treated with [acetyl-\textsuperscript{14}C]aspirin; moreover, the radiolabeled proteins had the same mobility as immunoreactive hPGHS-2 expressed in cos-1 cells.\textsuperscript{2} Aspirin was unable to acetylate the S516A mutant of hPGHS-2. S516A hPGHS-2 exhibits cyclooxygenase (and peroxidase) activity and catalytically active forms of PGHS-1 are known to be acetylated by aspirin. Accordingly, studies with the S516A mutant of hPGHS-2 support the concept that native hPGHS-2 is acetylated at a single site by aspirin-Ser-516. Moreover, the Ser-516 of hPGHS-2 is homologous to the ovine PGHS-1 active site Ser-530 which is known to be acetylated by aspirin (8, 49). In contrast to the results with ovine and human PGHS-1 (Fig. 3), acetylation of human PGHS-2 by aspirin produces three labeled proteins at 74, 72, and 70 kDa instead of one band at 70 kDa. These three bands are also detected by Western blot analysis with monospecific rabbit IgGs directed against hPGHS-2 and represent forms of hPGHS-2 which are variously glycosylated.\textsuperscript{3} The fact that each of these bands is reactive with aspirin suggests that each of these three forms of hPGHS-2 retains cyclooxygenase activity.

Previous studies with murine PGHS-2 had shown that this isozyme formed 15-HETE when the enzyme was treated with aspirin. Related studies on a novel form of ovine PGHS, most likely ovine PGHS-2, had also established that aspirin treatment increases 15-HETE formation and that the 15-HETE which was produced had a stereochernical composition of 3:1 of R:S (35). Our studies indicate that all of the 15-HETE formed by aspirin-treated hPGHS-2 is in the R configuration. 15-HETE formed via lipooxygenases is of the S configuration (50). Thus, our results indicate that one can distinguish between 15-HETE products formed via lipooxygenase versus those formed via aspirin-treated PGHS-2 by determining the stereochernistry. In fact, we speculate that this is probably what occurred in the system examined by Holtzman and co-workers (35) which probably contained a mixture of 15-lipooxygenase and ovine PGHS-2.

The mechanism by which aspirin-acetylated hPGHS-2 forms 15\textsuperscript{R}-HETE is not clear. With cyclooxygenase and lipooxygenase \( \text{O}_3 \) insertion reactions studied to date, \( \text{O}_3 \) insertion occurs antarafacial to hydrogen abstraction (50–55). If antarafacial \( \text{O}_3 \) insertion occurs with aspirin-acetylated hPGHS-2, the 13pro\( \alpha \) hydrogen would need to be removed. Previous studies with cyclooxygenase have indicated that it is the 13pro\( \alpha \) hydrogen which is abstracted (51). Because the \( K_m \) for arachidonate for native and aspirin-treated hPGHS-2 are essentially the same, we suspect that arachidonate binding occurs similarly with both forms of the enzyme; if so, removal of the 13pro\( \alpha \) hydrogen would occur. Given this scenario for hydrogen abstraction and the fact that the \( \text{O}_3 \) is inserted in the \( R \) configuration, our results indicate that acetylation must alter the orientation of incoming \( \text{O}_3 \) so that insertion occurs from the same side (synfacial) of the carbon chain as hydrogen abstraction. Moreover, aspirin acetylation of PGHS-2 also appears to prevent the oxygennation of arachidonate at C-11.

Studies with ovine PGHS-1 have indicated that Ser-530 is uniquely acetylated by aspirin (8). An alanine substitution at that position had no effect on the cyclooxygenase activity of the enzyme, but increasing the size of the side chain at position 530 interfered with arachidonate binding. For example, the S530N and S530L mutants of ovine PGHS-1 lacked cyclooxygenase but
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Fig. 9. Radio-TLC of the products made from [14C]arachidonate by native human PGHS-2 and the S516M mutant of this enzyme. The experiment was performed as described in the legend for Fig. 6, except that cos-1 cells were transfected with the SS16M mutant of human PGHS-2.

retained peroxidase activity (8). Our mutational analysis of the Ser-516 residue of hPGHS-2, which is homologous to Ser-530 of ovine PGHS-1, indicates that increasing the size of the side chain at this position can also eliminate cyclooxygenase activity. However, larger groups are required in the case of hPGHS-2 as compared to ovine PGHS-1. For example, the S516N mutant of hPGHS-2 is active whereas the SS30N mutant of ovine PGHS-1 is not. Elimination of cyclooxygenase activity of hPGHS-2 requires that the larger glutamine group be used to replace Ser-516. The present results obtained with Ser-516 mutants of hPGHS-2 when compared with those obtained earlier with ovine PGHS-1 suggest that the cyclooxygenase active site pocket of PGHS-2 is larger than that of PGHS-1. This view is also consistent with studies of competitive inhibition of human and murine PGHS-1 and PGHS-2 by various NSAIDs other than aspirin (3, 34).

The expected effect of ingestion of an anti-inflammatory dose of aspirin would be the inhibition of prostaglandin synthesis and production of 15R-HETE. A possible anti-inflammatory effect of 15R-HETE needs to be further analyzed, although it should be noted that other anti-inflammatory drugs do not cause murine PGHS-2 to form 15R-HETE (34). It is also worth noting that R-HETEs are generally more potent chemotactic agents than their S counterparts (56, 57).

In conclusion, we have shown that human PGHS-2 is acetylated by aspirin on a single serine residue, Ser-516, the residue homologous to the active site serine (Ser-530) of ovine PGHS-1. The acetylation causes the enzyme to make 15R-HETE. Mutational analysis of Ser-516 suggests that the active site pocket of PGHS-2 is larger than that of PGHS-1. The Met-516 mutant is like the acetylated wild-type enzyme in that both make 15R-HETE.

REFERENCES
1. Miyamoto, T., Ogino, N., Yamamoto, S., and Hayashi, O. (1976) J. Biol. Chem. 251, 2629–2636
2. van der Ouderaa, F. J., Buytenhek, M., Nugteren, D. H., and van Dorp, D. A. (1977) Biochem. Biophys. Acts 487, 315–331
3. Laneuville, O., Breuer, D. K., DeWitt, D. L., Hla, T., Funk, C. D., and Smith, W. L. (1994) J. Pharm. Exp. Therap., in press
4. Hemler, M., Lands, W. E. M., and Smith, W. L. (1976) J. Biol. Chem. 251, 5575–5579
5. DeWitt, D. L., and Smith, W. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1412–1416
6. Merlie, J. P., Fagan, D., Mudd, J., and Needleman, P. (1988) J. Biol. Chem. 263, 3550–3553
7. Yokoyama, C., Takai, T., and Tanabe, T. (1988) FEBS Lett. 231, 347–351
8. DeWitt, D. L., El-Harith, E. A., Kraemer, S. A., Andrews, M. J., Yao, E. F., Armstrong, R. L., and Smith, W. L. (1990) J. Biol. Chem. 265, 5192–5198
9. Feng, L., Sun, W., Xia, Y., Tang, W. W., Channugan, P., Soyoola, E., Wilson, C. B., and Hwang, D. (1993) Arch. Biochem. Biophys. 307, 361–368
10. Funk, C. D., Funk, L. B., Kennedy, M. E., Pong, A. S., and FitzGerald, G. A. (1994) PASEJ 5, 2304–2312
11. Xie, W., Chipman, J. G., Robertson, D. L., Erikson, R. L., and Simmons, D. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 2692–2696
12. Kujubu, D. A., Fletcher, B. S., Varun, B. C., Lim, R. W., and Herschman, H. R. (1991) J. Biol. Chem. 266, 12866–12872
13. Kennedy, B. P., Chan, C. C., Culp, S. A., and Cromlish, W. A. (1993) Biochem. Biophys. Res. Commun. 197, 494–500
14. Hla, T., and Neilson, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7384–7388
15. Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. (1993) J. Biol. Chem. 268, 9049–9054
16. Lin, A. H., Bienkowski, M. J., and Gorman, R. R. (1989) J. Biol. Chem. 264, 17379–17383
17. Simonson, M. S., Wolfe, J. A., Konieczkowski, M., Sodor, J. R., and Dunn, M. J. (1991) Mol. Endocrinol. 5, 441–451
18. Wong, W. Y. L., and Richards, J. S. (1991) Mol. Endocrinol. 5, 1269–1279
19. Smith, W. L. (1989) Biochem. J. 259, 315–329
20. Smith, W. L., and DeWitt, D. L. (1994) Curr. Opin. Invest. Drugs 3, 1–11
21. Kujubu, D. A., Reddy, S. T., Fletcher, B. S., and Herschman, H. R. (1993) J. Biol. Chem. 268, 5425–5430
22. Fletcher, B. S., Kujubu, D. A., Perrin, D. M., and Herschman, H. R. (1992) J. Biol. Chem. 267, 4338–4344
23. Kujubu, D. A., and Herschman, H. R. (1992) J. Biol. Chem. 267, 7991–7994
24. DeWitt, D. L., and Meade, E. A. (1993) Arch. Biochem. Biophys. 306, 94–102
25. O'Sullivan, G. M., Huggeo, E. M., Meade, E. A., DeWitt, D. L., and McCall, C. E. (1992) Biochem. Biophys. Res. Commun. 187, 1123–1127
