The Catalytic Outcomes of the Constitutive and the Mitogen Inducible Isoforms of Prostaglandin H_2 Synthase Are Markedly Affected by Glutathione and Glutathione Peroxidase(s)†

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ABSTRACT: Reduced glutathione (GSH), at physiological concentrations, was found to markedly alter the profile of arachidonate metabolism by prostaglandin H_2 synthase. In 1 mM GSH, the constitutive (COX-1) and the mitogen inducible (COX-2) isoforms metabolized arachidonate to 12-hydroxyheptadecatrienoic acid (12-HHT) (88% and 78% of total products, respectively). Prostanoid formation was consequently reduced to only 12% (COX-1) and 19% (COX-2) of the total metabolites. The GSH-dependent production of 12-HHT was regio- and enantioselective for the 12(*S*)-isomer. We propose that 12(*S*)-HHT is formed by a GSH-dependent enzymatic cleavage of the PGH_2 8,9 and 11,12 carbon−carbon bonds based on the following: (a) nonsignificant GSH-dependent formation of 12(*S*)-HHT during chemical decomposition of synthetic PGH_2, (b) the structural similarities between the asymmetric carbons at C(12) in 12-HHT and C(15) in PGH_2, (c) the GSH concentration-dependent product/precursor relationship between 12-HHT and prostanoid production, and (d) aspirin inhibition of 12-HHT formation by both enzymes. Arachidonic acid oxidation by COX-1, and not by COX-2, was inhibited by the combined presence of GSH and liver cytosol. In contrast, metabolism by neither isoform was inhibited when the cytosol was obtained from selenium-depleted animals. This is consistent with a unique, selenium dependent, cytosolic GSH peroxidase that inhibits specifically prostanoid and 12(*S*)-HHT formation by COX-1. These results suggest an additional role for GSH and GSH peroxidase(s) in regulating prostanoid biosynthesis. Differences between the isoforms in their sensitivities to GSH peroxidase may reflect differences in their requirements for an "initiator hydroperoxide".

Prostaglandin H_2 synthase catalyzes the oxidative metabolism of AA[1] to PGH_2, the precursor for the physiologically important prostaglandins thromboxanes and prostacyclins (Smith et al., 1981; Marnett, 1992; Smith, 1992). Recent studies have also documented the existence of additional PGH_2 synthase isoforms (COX-2) expressed and/or induced during cell activation by cytokines, growth factors, tumor promoters, or mediators of inflammation (Kubuju et al., 1991; Xie et al., 1991; Marnett, 1992; O'Banion et al., 1992; Fletcher et al., 1994). At present, the constitutive form of prostaglandin H_2 synthase (COX-1) appears catalytically indistinguishable from COX-2. However, upregulated expression suggests that COX-2 may be the isoform responsible for prostanoid biosynthesis during inflammation and tumor progression (Kubuju et al., 1991; Xie et al., 1991; Marnett, 1992; O'Banion et al., 1992; Fletcher et al., 1994). The potential involvement of COX-2 in the pathophysiology of inflammation and cancer has focused interest in its molecular, biochemical, and functional characterization (Marnett, 1992; Mitchell et al., 1994). While differential regulation and/or tissue specific localization provides the rationale for the existence of multiple prostaglandin synthase isoforms, documentation of catalytic differences between these proteins is critical not only for functional assignments, but also to our understanding of their physiological roles and to the design of specific tools for biochemical, pharmacological and, eventually, clinical intervention.

The mechanism of PGH_2 formation by COX-1 has been extensively studied (Smith et al., 1981; Kulmacz et al., 1994). Catalytic turnover is initiated by the abstraction of the arachidonate C(13)-*pro-S* hydrogen atom and followed by the coupling of the resulting carbon centered radicals to dioxygen. The intermediate thus formed, 15(*S*)-hydroperoxy endoperoxide, is finally reduced to the corresponding alcohol, releasing PGH_2 and water as products (Smith et al., 1981; Kulmacz et al., 1994). While this reaction is formally analogous to a free radical mediated autoxidation, the enzyme

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[1] Abbreviations: AA, arachidonic acid; prostaglandin synthase, prostaglandin H_2 synthase; COX-1 and COX-2, the constitutive and mitogen inducible forms of prostaglandin H_2 synthase, respectively; GSH-Px, glutathione peroxidase(s); GSH and GSSG, the reduced and oxidized forms of glutathione, respectively; DTT, 1,4-dithiothreitol; RP, reversed phase; HPLC, high pressure liquid chromatography; GC, gas−liquid chromatography; MS, mass spectral; NICI, electron capture negative ion chemical ionization; PG, prostaglandin; HHT, hydroxyheptadecatrienoic acid; HETE, hydroxyeicosatetraenoic acid; PFB, pentafluorobenzyl; TMS, trimethylsilyl.

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provides a remarkable kinetic effect as well as regio- and stereochemical control. Both the cyclooxygenase and peroxidase activities associated with PGH_2 biosynthesis are catalyzed by a single heme containing polypeptide (Smith et al., 1981). Several lines of evidence have demonstrated that, prior to hydrogen abstraction, COX-1 must be activated by an initiator hydroperoxide (Smith et al., 1981; Kulmacz et al., 1994). Activation involves a heme-dependent two-electron reduction of the initiator hydroperoxide, forming a hypervalent heme iron−oxygen complex, isoelectronic with horseradish peroxidase compound I (Smith et al., 1981). A protein tyrosyl radical, generated during the enzyme's heme−initiator hydroperoxide redox interaction, was proposed in the final step leading to the H atom abstraction from the substrate molecule (Smith et al., 1981; Dietz et al., 1988). These and other studies indicated the presence in cells and tissues of an "endogenous peroxide tone" necessary for *in vivo* prostanoid formation (Kulmacz & Lands, 1983; Kulmacz et al., 1994). While not yet studied in detail, it has been assumed that arachidonate oxidation by COX-2 also requires the activation of the enzyme by an initiator hydroperoxide and that catalysis proceeds by a reaction mechanism essentially similar to that of COX-1.

Selenium-dependent and selenium-independent glutathione peroxidase (GSH-Px) are cytosolic enzymes that catalyze the GSH-dependent, two-electron reduction of a variety of fatty acid hydroperoxides to the corresponding alcohols (Rotruck et al., 1973; Takahashi et al., 1987; Maiorino et al., 1990; Thomas et al., 1990; Fong-Fong et al., 1992; Burk & Hill, 1993). Glutathione peroxidases are important components of the body's defense mechanisms against reactive oxygen species that have been implicated in the pathophysiology of tumor formation and inflammation (Cerutti, 1985; Halliwell, 1987; Cerutti & Trump, 1991; Mirault et al., 1991). As is the case with COX-2, preliminary studies have indicated increased activity and/or levels of GSH-Px in several inflammatory processes (Parnham et al., 1987; Grisham et al., 1990). In early studies, it was shown that COX-1 activity was completely abolished after GSH-dependent GSH-Px catalyzed reduction of the endogenous initiator hydroperoxides, present in most AA preparations (Kulmacz & Lands, 1983; Kulmacz et al., 1994).

During studies of the effects of GSH on prostanoid production by ram seminal vesicle COX-1 and sheep placenta COX-2, it was observed that the catalytic output of these isoforms was markedly and distinctly affected by GSH and cytosolic GSH-Px(s). We report here the following: (a) in the presence of physiological concentrations of GSH, AA metabolism by purified forms of COX-1 and COX-2 generates 12(*S*)-HHT as the predominant product, and under these conditions, only small quantities of prostanoids are recovered, and (b) while AA oxidation by COX-1 is suppressed in the presence of GSH and cytosolic GSH-Px(s), under similar conditions, the mitogen inducible isoform or COX-2 remains catalytically active. These results raise significant questions regarding (a) the generality of endogenous hydroperoxide initiation during prostanoid biosynthesis and (b) the existence of a common and/or general mechanism for prostanoid biosynthesis. Additionally, the demonstration of potential differences in the mechanism(s) of PGH_2 production by COX-1 and COX-2 isoforms may be of significance for current efforts to design isoform specific inhibitors of prostanoid formation. Finally, the results also indicate that under conditions of oxidative stress, for example, during inflammation, prostaglandin production by COX-2 can remain unaffected by changes in the activities or concentrations of GSH-dependent peroxide metabolizing enzymes.

MATERIALS AND METHODS

Cytosolic Fractions. Anesthetized (Nembutal, 5 mg/kg, intraperitoneally) adult Sprague-Dawley male rats (300−340 g body weight) were sacrificed and their livers immediately removed, freed of connective tissue, weighed, minced in 0.15 M KCl, suspended in 10 mM Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose (10% w/v), and homogenized utilizing a glass−Teflon homogenizer. After centrifugation at 3500*g* (15 min) once and twice at 12000*g* (20 min each), the supernatants were centrifuged at 150000*g* for 90 min. The 150000*g* supernatants were stored at 4 °C for <24 h and utilized as the cytosolic fractions. Selenium-deficient animals, a gift of Dr. Raymond F. Burk, Department of Medicine, Vanderbilt University, were fed either selenium-depleted or selenium-supplemented (as Na_2SeO_4) diets for 4 weeks prior to sacrifice. The details of diet preparation and animal treatment are described in a reference (Yang et al., 1989).

Purified Enzymes. Commercially available samples of prostaglandin H_2 synthase purified from either ram seminal vesicles (constitutive isoform, COX-1) or sheep placenta (mitogen inducible isoform, COX-2) were utilized throughout these studies. COX-1 (38 000 units/mg of protein) was obtained from Oxford Biomedical (Oxford, MI) and found to be ≥90% pure by SDS−polyacrylamide electrophoresis. COX-2 (6400 units/mg of protein) was obtained from Cayman Biochemicals (Ann Arbor, MI) and found to be ≥70% pure by SDS−polyacrylamide electrophoresis. Both enzymes were aliquoted into small volumes and kept at −80 °C until use. Ram seminal vesicle microsomes (10 mg of protein/mL), from Oxford Biomedical, were aliquoted into small volumes and maintained at −80 °C until use. Glutathione peroxidase (GSH-Px), purified from bovine erythrocytes, was obtained from Sigma Chemical Co. (St. Louis, MO). The enzyme is packed as a lyophilized powder containing approximately 2.5% w/w DTT. Upon arrival, GSH-Px was dissolved in 0.1 M Tris-HCl buffer (pH 7.8) containing 0.1 mM GSH (200 units/mL) and dialyzed at 4 °C overnight against 1000 volumes of the same buffer. After dialysis, the peroxidase activity of the GSH-Px was measured utilizing the spectrophotometric glutathione reductase coupled assay and NADPH and H_2O_2 exactly as described (Lawrence et al., 1974). After determination of its peroxidase activity, the dialyzed enzyme was aliquoted into small volumes and kept at −80 °C until use. Glutathione reductase (0.14 unit/mg of protein), type II from wheat germ, was purchased from Sigma Chemical Co. The enzyme was suspended in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.1 mM EDTA and 1 mM NaN_3 and maintained at −80 °C.

Enzymatic Incubations. For measurements of prostaglandin synthase activity we utilized a modification of literature procedures (Kulmacz & Lands, 1983; Kulmacz et al., 1994). Briefly, immediately prior to use, COX-1 and COX-2 were suspended in 0.1 M Tris-HCl buffer (pH 7.8) at a concentration of 1−2 and 5−10 μg of protein/mL, for COX-1 and COX-2, respectively. After 1 min at room temperature, the following reagents in 0.1 M Tris-HCl buffer (pH 7.8) were

sequentially added: 30 mM phenol, 50 μM hematin, and when needed, 50 mM GSH, to obtain final concentrations of 0.5, 0.002, and 1 mM for phenol, hematin, and GSH, respectively. The mixture was preincubated for 2 min at 30 °C and then the reactions were initiated by addition of an equal volume of 100 μM [1-^{14}C]arachidonic acid (10−50 μCi/μmol) in Tris-HCl buffer (pH 7.8) containing 50 μg/mL dilauroylphosphatidylcholine. Prior to utilization, the [1-^{14}C]AA was purified by SiO_2 exactly as described (Capdevila et al., 1990). After mixing for 0.5−4 min at 30 °C, the reactions were stopped by addition of ethyl acetate containing 0.05% HOAc (5 volumes) and 0.15 M NaCl (2 volumes). Samples were mixed vigorously and centrifuged to achieve phase separation. The organic phases were collected and the water phases extracted two additional times with ethyl acetate. The pooled organic phases were evaporated under a N_2 stream and dissolved in a small volume of ethanol containing 0.001% butylated hydroxytoluene, and the reaction products were resolved by RP-HPLC as described below. In initial studies, the effects of substrate and enzyme concentrations and of incubation time on the rates of product production were studied. On the basis of these results, we chose to use AA at concentrations of $\geq$50 μM and COX-1 and COX-2 at concentrations of $\leq$2 or $\leq$10 μg of protein/mL, and for most cases, the incubation times were maintained below 2 min. Dilauroylphosphatidylcholine was utilized to improve AA solubility at concentrations $\geq$50 μM. In control experiments it was determined that the addition of this phospholipid (tested between 20 and 80 μg/mL) did not alter the nature and/or the rates of product formation by the prostaglandin synthases. Stock solutions of phenol (30 mM) and GSH (50 mM) were made in 0.1 M Tris buffer (pH 7.8), kept at 4 °C, and discarded after 4 h. Stock hematin solutions were prepared by dissolving hematin (Sigma Chemical Co.) in 0.1 M Tris-HCl (pH 9.0). The pH of the solution was then titrated to 7.8 with HCl. Tris-HCl buffer (pH 7.8) was utilized to adjust the concentration of hematin to 50 μM. Hematin solutions were maintained at 4 °C and discarded after 4 days. It was noted that when hematin solutions were kept for more than 5 days at 4 °C or 2 weeks at −20 °C, they failed to sustain full COX-1 catalytic activity, particularly when GSH was included as a cofactor. When GSH-Px (0.5−20 units/mL, final concentrations) or cytosolic fractions (0.5 mg of protein/mL, final concentration) were utilized, either they were added prior to initiation with AA or, alternatively, the reactions were started by the addition of AA solutions in Tris-HCl buffer (pH 7.8) preincubated with GSH and GSH-Px or cytosol for 1−10 min prior to use. For these experiments, dilauroylphosphatidylcholine was omitted from the reaction mixtures.

Product Resolution. The reaction products extracted from incubates containing prostaglandin synthase isoforms were resolved by RP-HPLC on a 5-μm Dynamax Microsorb C_{18} column (4.6 × 250 mm, Rainin Instruments Co., Woburn, MA) utilizing mixtures of solvent A (0.1% HOAc/99.9% H_2O) and solvent B (0.1% HOAc/99.9% CH_3CN). Initial conditions were 70% A/30% B. Five minutes after injection, the solvent composition was changed linearly to 40% A/60% B over 30 min. After a 5 min isocratic period, the composition of the solvent mixture was changed linearly to 100% B over 15 min and maintained at 100% B for an additional 5 min. The flow rate was 1 mL/min. Product quantification was done by on-line liquid scintillation utilizing a Radiomatic Flo-one β-detector (Radiomatic Instruments, Tampa, FL). For structural characterization, radioactive fractions eluting from the HPLC column with retention times corresponding to those of authentic $PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2, PGB_2, 12-HHT, 11-HETE, and 15-HETE were collected, further purified, derivatized, and characterized as described below. The HPLC elution profiles of nonradiolabeled synthetic eicosanoids were determined by on-line UV absorption at 210 ($PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2, and PGB_2) or 234 nm (12-HHT, 11- and 15-HETE).

Structural Characterization. Fractions with RP-HPLC retention times corresponding to those of synthetic prostanoids were collected as a group, and after derivatization to either the methyl or PFB ester, TMS ether, and/or methyloxime derivatives, they were resolved and analyzed by GC/MS exactly as described (Morrow et al., 1990; Wendelborn et al., 1990). Fractions with RP-HPLC retention times corresponding to synthetic 12-HHT (36.8 min) were collected from the column and, after solvent evaporation, further purified by normal phase HPLC on a μPorasil column (4.6 × 300 mm, Waters Associates, Milford, MA) utilizing a linear solvent gradient from 2-propanol/HOAc/hexane (0.5:0.1:99.4 v/v) to 2-propanol/HOAc/hexane (1.5:0.1:98.4 v/v) over 30 min at 3 mL/min. Fractions with a retention time corresponding to that of authentic 12-HHT (17.9 min) were collected and, after solvent evaporation, derivatized to the corresponding PFB ester TMS ether derivative and analyzed by GC/MS. Fractions with reversed phase HPLC retention times corresponding to authentic 15- and 11-HETE (44.3 and 45.5 min, respectively) were collected and, after solvent evaporation, individually purified by normal phase HPLC on a μPorasil column utilizing the solvent conditions described above. Fractions with normal phase HPLC retention time corresponding to those of synthetic 15- and 11-HETE (7.9 and 12.1 min, respectively) were collected, converted to the corresponding PFB ester, TMS ether derivatives, and characterized by GC/MS.

The GC/MS characterization of prostanoid derivatives was done using a 15-m DB1701 capillary column (J and W Scientific, Folsom, CA) with He and CH_4 as carrier and reagent gases, respectively. Full mass spectra were obtained employing either electron impact ionization or NICI. The conditions of analysis and of GC resolution are those of Morrow et al. (1990) and Wendelborn et al. (1990). The GC/NICI/MS characterization of PFB-TMS derivatives of 12-HHT and 11- and 15-HETE was done utilizing a 30-m SPB-20 fused silica capillary column (0.32 mm i.d., 0.25 μm coating thickness, Supelco Inc., Bellefonte, PA) with He as carrier gas. After 1 min at 100 °C, the oven temperature was raised to 300 °C at 20 °C/min. Mass fragmentographic analysis was done under NICI conditions utilizing CH_4 as reagent gas. NICI/MS was performed using a Nermag R10-10C instrument. Electron impact ionization MS was done on a Finnigan Incos B50 mass spectrometer.

Stereochemical Analysis. 12-HHT, isolated from enzymatic incubations, was hydrogenated and derivatized to the corresponding phenacyl ester as described below. Prior to chiral resolution, phenacyl 12-hydroxyheptadecanoate was purified by RP-HPLC on a 5-μm Dynamax Microsorb C_{18} column (4.6 × 250 mm) utilizing mixtures of solvent A (0.1% HOAc/99.9% H_2O) and solvent B (0.1% HOAc/99.9% CH_3CN) at a flow rate of 1 mL/min. Initial conditions were 20% A/80% B. Ten minutes after injection, the solvent

composition was changed linearly to 100% B over 20 min. Isocratic elution at 100% B was then continued for an additional 10 min. The phenacyl ester of hydrogenated 12-HHT (retention time: 23.3 min) was collected and, after solvent evaporation, submitted to chiral phase HPLC on a Chiralcel OD column (4.6 × 250 mm, J. T. Baker Chemical Co., Phillisburg, NJ). The optical antipodes of the purified phenacyl 12-hydroxyheptadecanoate were separated, with base line resolution, using a solvent mixture of 2-propanol/hexane (3:97 v/v) isocratically at 1.5 mL/min. The *R* and *S* enantiomers eluted from the column with retention times of 42 and 53 min, respectively.

The PFB esters of 11- and 15-HETE were synthesized as described below. Prior to chiral resolution, 11- and 15-HETE-PFB were purified by RP-HPLC on a 5-μm Dynamax Microsorb C_{18} column (4.6 x 250 mm) utilizing a linear solvent gradient of H_2O/CH_3CN/HOAc (49.9:50.0:0.1 v/v) to CH_3CN/HOAc (99.9:0.1 v/v) over 40 min at 1 mL/min. The 11- and 15-HETE-PFB, eluting from the HPLC column at 35.5 and 37.5 min, respectively, were collected and, after solvent evaporation, submitted to chiral phase HPLC. The optical antipodes of 11-HETE-PFB were separated, with near base line resolution (≤10% of maximal peak height), on a Chiralcel OC column (4.6 × 250 mm) using an isocratic mixture of 2-propanol/hexane (0.7:99.3 v/v) at 1 mL/min. The 11(*R*)- and 11(*S*)-HETE-PFB enantiomers eluted with retention times of 37.8 and 41.2 min, respectively. The optical antipodes of 15-HETE-PFB were resolved, with near base line resolution (≤10% of maximal peak height), on a Chiralcel OC column (4.6 × 250 mm) using an isocratic mixture of 2-propanol/hexane (0.8:99.2 v/v) at 1.2 mL/min. The 15(*S*)- and 15(*R*)-HETE-PFB enantiomers eluted with retention times of 24.8 and 27.6 min, respectively.

Chemical Procedures. Samples of authentic 11(*R*)-, 11-(*S*)-, and 15(*S*)-HETE and of 12(*S*)-HHT were obtained from either Oxford Biomedical and/or Cayman Chemical Co. 12-(*S*)-Hydroxyheptadecanoic acid was obtained by catalytic hydrogenation of 12(*S*)-HHT. Racemic 11- and 15-HETE were synthesized from AA by reaction with 1,1-dimethylethyl hydroperoxide. Briefly, approximately 1 mg of neat AA was dissolved in 1 mL of CH_3OH containing 0.5 μM hematin and 50 mM hydroperoxide. After 14−20 h mixing at room temperature, the reaction was stopped by adding 100 μL of a 1 mM solution of triphenylphosphine in CH_2Cl_2, and after 10 min, the mixture was evaporated to dryness under a N_2 stream. The oily residue was dissolved in 5 mL of ethyl ether containing 0.01% HOAc and then washed twice with 2 mL of 0.2 M KCl. The organic phase was evaporated, and the resulting 11- and 15-HETE were purified first by RP-HPLC and then by normal phase HPLC exactly as described (Capdevila et al., 1986).

Methyl (±)-12-hydroxyheptadecanoate was prepared as follows: to a −78 °C solution of dimethyl sulfoxide (150 μL, 2.08 mmol) in 10 mL of anhydrous CH_2Cl_2 was added oxalyl chloride (100 μL, 1.15 mmol) dropwise. After 15 min, a solution of methyl 12-hydroxydodecanoate (Aldrich Chemical Co., Milwaukee, WI) (100 mg, 0.46 mmol) in 2 mL of dry CH_2Cl_2 was added over 10 min. After 1 h, dry triethylamine (640 μL) was added, and the reaction mixture was warmed to room temperature over 1h. The reactants were diluted with 10 mL of H_2O and extracted with CH_2Cl_2 (3 × 5 mL), and the combined organic extracts were dried over Na_2SO_4. Evaporation of the solvent afforded the corresponding aldehyde. ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.26−1.48 (m, 12H), 1.53−1.75 (m, 4H), 2.31 (t, 2H, $J \sim$ 7.5 Hz), 2.43 (dt, 2H, $J \sim$ 1.8 and 7.4 Hz), 3.67 (s, 3H), 9.77 (t, 1H, $J \sim$ 1.8 Hz). To a 0 °C ethereal solution (5 mL) of the above aldehyde (40 mg) was added an ethereal solution of pentylmagnesium bromide (0.7 M, 120 μL). After 30 min, the reaction was quenched with 0.1 N HCl (10 mL) and extracted with Et_2O (3 × 3 mL). The combined organic extracts were dried and evaporated, and the residue was purified by SiO_2 column chromatography to give methyl (±)-12-hydroxyheptadecanoate (32 mg, 57%). ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.87 (t, 3H, $J \sim$ 6.9 Hz), 1.20−1.50 (m, 24 H), 1.56−1.66 (m, 2H), 2.29 (t, 2H, $J \sim$ 6.2 Hz), 3.55−3.65 (m, 1H), 3.67 (s, 3H); TLC (SiO_2), EtOAc/hexane (1:9), $R_f \sim$ 0.1. Saponification of methyl (±)-12-hydroxyheptadecanoate afforded (±)-12-hydroxyheptadecanoic acid. Briefly, to a 0 °C solution of methyl 12-hydroxyheptadecanoate (17 mg) in THF/H_2O (3 mL, 5:1) was added a 1 N solution of LiOH (290 μL). The mixture was stirred for 16 h at room temperature and then quenched with 0.1 N HCl. After adjusting the pH to 4.0, the reaction mixture was extracted with ethyl acetate (3 × 3 mL), the combined organic extracts were dried over Na_2SO_4, and all volatiles were removed *in vacuo* to give 12-hydroxyheptadecanoic acid (15.8 mg, 100%).

Catalytic hydrogenations were done in the presence of PtO_2 as in Capdevila et al. (1984). Methylations were done using CH_2N_2 in ether as described (Capdevila et al., 1984). TMS ether derivatives were prepared by reaction with bis-(trimethylsilyl)trifluoroacetamide in pyridine as described (Capdevila et al., 1992). PFB esters were prepared by reaction with pentafluorobenzyl bromine in dimethylformamide and *N,N'*-diisopropylethylamine exactly as in Capdevila et al. (1992). The phenacyl esters of racemic 12- and 12-(*S*)-hydroxyheptadecanoic acid were prepared by reaction with Phenacyl-8 (Pierce, Rockford, IL) as follows: the fatty acid (≤0.5 mg) in 0.2 mL of CH_3CN was mixed with 0.1 mL of Phenacyl-8 and 0.02 mL of diisopropylethylamine and incubated at 50 °C. After 1 h, 1 mL of water was added, the mixture was extracted three times with hexane, and the combined organic phases were evaporated under N_2.

RESULTS AND DISCUSSION

Enzymology of AA Oxidation by the Isoforms of Prostaglandin Synthase. The recent documentation of the existence of several prostaglandin synthase isoforms has attracted a considerable amount of interest due to their unique regulatory properties as well as the recognized role of prostanoids in cell and body physiology and/or disease (Smith et al., 1981; Marnett, 1992; Smith, 1992). Protein polymorphism is accompanied, in many cases, by both regulatory and functional polymorphism (Gonzalez, 1989). It is evident that the delineation of the biochemical properties of the individual prostaglandin synthase isoforms is of importance for the assignment of their functional properties and significance. For the studies reported here, we utilized experimental conditions that guaranteed enzyme−substrate saturation during analysis, i.e., low enzyme concentrations, high AA concentrations, and short incubation times. Under these conditions, the low detection sensitivity of polarographic techniques for the measurement of oxygen concentrations made their use impractical. Consequently, an expanded HPLC method, capable of separating a wide range of polar

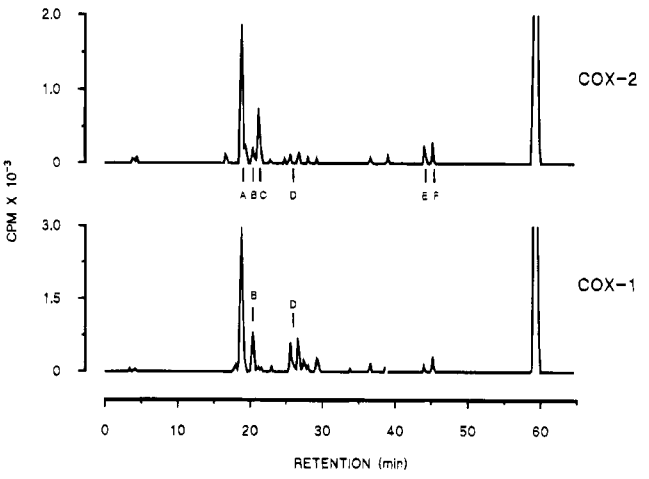

FIGURE 1: Reversed phase HPLC resolution of the arachidonic acid metabolites generated by incubates containing purified samples of the constitutive (COX-1) and mitogen inducible (COX-2) isoforms of prostaglandin synthase. Purified COX-1 (1.5 μg of protein/mL) or COX-2 (8 μg of protein/mL) was incubated with [1-^{14}C]-arachidonic acid (50 μM, final concentration) in 0.1 M Tris-HCl (pH 7.8) containing 0.5 mM phenol and 2 μM hematin. After 1 min mixing at 30 °C, the reaction products were extracted into acidified ethyl acetate and resolved by reversed phase HPLC as described under Materials and Methods. Shown are the HPLC profiles of the metabolites generated by incubates containing 0.2 and 1.0 μg of COX-1 (bottom) and COX-2 (top), respectively. The HPLC retention times for synthetic PGE_2 (A), PGD_2 (B), a mixture of PGA_2 and PGB_2 (D), 15-HETE (E), and 11-HETE (F), as well as fraction X (C), are indicated.

Table 1: Catalytic Properties of the Constitutive (COX-1) and Mitogen Inducible (COX-2) Isoforms of Prostaglandin Synthase[a]

	COX-1		COX-2	
metabolite	reaction rate	% distribution	reaction rate	% distribution
$PGF_{2\alpha}$	≤1		≤1	≤1
PGD_2	154 ± 25	16	11 ± 1.0	9
PGA_2/PGB_2	128 ± 12	13	16 ± 2.0	13
15-HETE	≤1		8 ± 0.4	7
11-HETE	≤10	≤1	6 ± 0.2	5
X	≤10	≤1	16 ± 3.0	13
total	978 ± 90	100	122 ± 8	100

[a] Reaction rates are given as amounts of products recovered after incubating COX-1 (0.5−1.5 μg of protein/mL) or COX-2 (6−9 μg of protein/mL) for 1 min and at 30 °C. Values are averages calculated from at least 10 different experiments ± SEM.

AA metabolites, was developed for the resolution of the products of AA oxidation by the prostaglandin synthase isoforms. A comparison of the reaction products generated when AA was incubated with COX-1 and COX-2 showed that both isoforms actively catalyzed PGH_2 formation, as revealed by the recovery of its isomerization and/or reduction products PGE_2 (51% and 31% of the total for COX-1 and COX-2, respectively) and PGD_2 (16% and 9% for COX-1 and COX-2, respectively) as well as small and variable amounts of PGA_2 and PGB_2 (13% each for COX-1 and COX-2) (Figure 1 and Table 1). These structural assignments were corroborated by GC/MS analysis of each individual fraction. For this purpose, HPLC fractions with retention times corresponding to synthetic $PGF_{2\alpha}$, PGE_2, PGD_2, PGA_2, and PGB_2 were collected batchwise and, after solvent evaporation, derivatized and analyzed by GC/MS as described in the Materials and Methods section. The mass fragmentographic properties, under EI or NICI conditions, of the samples were compared to that of authentic standards analyzed under identical conditions. Since the purified COX-1 protein is devoid of additional prostanoid metabolizing activities, we concluded that these metabolites were formed from PGH_2 by nonenzymatic processes. In addition to these prostanoids, COX-2 generated variable quantities of an unidentified metabolite (13% of total) (fraction X, Figure 1, Table 1) with HPLC properties different from those of synthetic $PGF_{2\alpha}$, $PGF_{1\alpha}$, PGE_2, PGD_2, PGA_2, or PGB_2. Since the molecular weight of metabolite(s) X is similar to that of PGE_2 and PGD_2 but its GC retention time is different, we propose this fraction contains a structural isomer(s) of PGD_2. The facile isomerization of PGD_2 in aqueous solution has been reported (Wendelborn et al., 1988). Furthermore, COX-2 also functioned consistently as an arachidonate 11- and 15-lipoxygenase, generating significant quantities of 11- and 15-HETE (7% and 5% of total products, respectively) (Table 1). Chiral analysis demonstrated that HETE formation by COX-2 was stereoselective and that the 11(*R*)- and 15(*S*)-HETE enantiomers were generated with 86% and 81% optical purities, respectively. The COX-1 isoform also generated radioactive products with HPLC properties corresponding to that of synthetic 11-HETE; however, low and highly variable yields (≤1% of the total metabolites) precluded further structural characterization. Finally, approximately 18% (COX-1) and 21% (COX-2) of the radioactivity recovered from the HPLC columns eluted in poorly resolved fractions and remained, therefore, unidentified. As previously noted (Smith et al., 1981; Marshal et al., 1987), we were unable to determine initial rates of AA metabolism by either COX-1 or COX-2. Within 30 s of the addition of substrate, the rates of product formation decreased, presumably due to a suicidal inactivation of the enzymes (Smith et al., 1981). The rates of metabolism shown in Table 1 were obtained after 1 min incubation at 30 °C and, consequently, are only given as a reference for comparative purposes.

GSH-Dependent Metabolism of AA to 12(S)-HHT by the Isoforms of Prostaglandin Synthase. Several studies have indicated the existence of a GSH-dependent PGE_2 isomerase (reviewed in Smith et al., 1981). The AA and 15-HPETE supported oxidation of GSH to a thiyl radical by ram seminal vesicle microsomes has been demonstrated (Eling et al., 1986). However, the enzymology of these reactions remained undefined since purified COX-1, obtained from ram seminal vesicles, failed to catalyze AA-dependent oxidation of GSH to thiyl radicals (Eling et al., 1986). On the basis of these results, it was proposed that GSH could serve as an endogenous reducing cofactor for the prostaglandin hydroperoxidase (Eling et al., 1986). A comparison of the HPLC chromatograms in Figures 1 and 2 shows that GSH, when added to incubation mixtures containing either COX-1 or COX-2, had a profound effect in the profile of metabolites generated by the purified isoforms. Thus, in the presence of the GSH (1 mM, final concentration), prostanoid and HETE production was nearly abolished and replaced by the enzyme-catalyzed formation of a radioactive product(s) eluting from the HPLC column at approximately 36.8 min (Figure 2). This product was identified as 12-HHT based on the following criteria: (1) coelution with a synthetic sample of 12(*S*)-HHT in reversed (t_R = 36.8 min) and normal phase HPLC (t_R = 17.9 min), (2) the presence of a conjugated diene functionality as revealed by an absorption maxima at 235 nm (in CH_3OH), (3) coelution with a synthetic

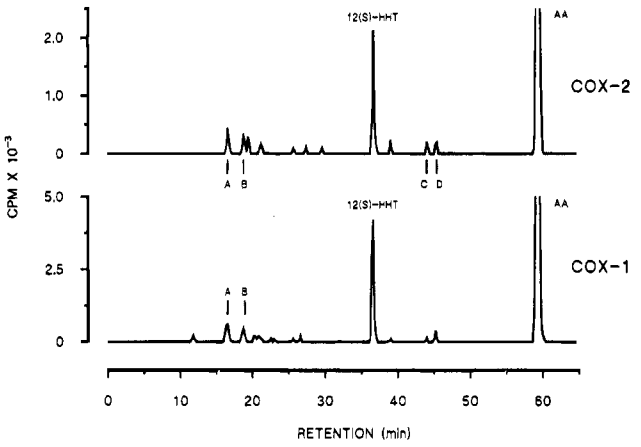

FIGURE 2: Effect of GSH on the catalytic output of the constitutive (COX-1) or mitogen inducible isoforms (COX-2) of prostaglandin synthase. Samples of purified COX-1 (1.5 μg of protein/mL) or COX-2 (8 μg of protein/mL) were incubated with [1-^{14}C]arachidonic acid (50 μM, final concentration) in 0.1 M Tris-HCl (pH 7.8) containing 0.5 mM phenol, 2 μM hematin, and 1 mM GSH. After 1 min at 30 °C, the reaction products were extracted, resolved, and analyzed as described in Figure 1 and under Materials and Methods. Shown are the profiles of metabolites generated by incubates containing 0.2 and 1.0 μg of COX-1 (bottom) and COX-2 (top), respectively. The HPLC retention times for synthetic $PGF_{2\alpha}$ (A), PGE_2 (B), 15-HETE (C), and 11-HETE (D) are indicated.

sample of the pentafluorobenzyl ester of 12(*S*)-HHT in reversed phase HPLC (t_R = 45 min), (4) a molecular weight of 352 as shown by NICI/GC/mass fragmentographic analysis of its pentafluorobenzyl ester, trimethylsilyl ether derivative [major fragment ions at *m/z* 351 and 261, corresponding to the loss of PFB (80% abundance) and PFB and HOTMS (base peak), respectively], and (5) the presence of three olefins as revealed by NICI/GC/MS of its hydrogenated PFB ester, TMS ether derivative [major ion fragments at *m/z* 357 and 268, corresponding to the loss of PFB (base peak) and PFB and HOTMS (40% abundance), respectively)]. Finally, chiral analysis showed that 12-HHT had the same absolute configuration as the corresponding C(15)-alcohol in PGH_2 (Smith et al., 1981), with both prostaglandin synthase isoforms generating 12(*S*)-HHT as the predominant enantiomer (93% and 81% optical purities for COX-1 and COX-2, respectively).

In summary, these results demonstrate that the catalytic outcome of purified COX-1 and COX-2 can be markedly altered by GSH. Thus, in the presence of physiological concentrations of GSH (1 mM GSH), the metabolism of AA by COX-1 and COX-2 results in the predominant formation of 12(*S*)-HHT instead of prostanoids (88% and 78% of the products generated from AA by COX-1 and COX-2, respectively) (Table 2). It is important to note that while at these concentrations GSH induced major changes in the catalytic outcome of COX-1 and COX-2, it only minimally affected the overall rates of AA oxidation. As shown in Table 2, the GSH-dependent generation of 12(*S*)-HHT by COX-1 and COX-2 proceeded at rates which were approximately 98−83% of the rates of AA metabolism in the absence of GSH.

As determined with COX-1, 12-HHT formation had a nearly absolute requirement for the reduced glutathione thiol functionality. Neither GSSG nor GSH *S*-methyl ether supported this reaction. On the other hand, in the presence of 1 mM cysteine, COX-1 metabolism decreased to 65% of

Table 2: Effect of GSH on the Metabolism of AA by the Constitutive (COX-1) and the Mitogen Inducible (COX-2) Isoforms of Prostaglandin Synthase[a]

	COX-1		COX-2	
metabolite	reaction rate	% distribution	reaction rate	% distribution
$PGF_{2\alpha}$	95 ± 10	10	12 ± 1	12
PGE_2	24 ± 4	2	7 ± 1	7
12-HHT	846 ± 80	88	80 ± 5	78
unknown	≤1	≤1	≤4	≤4
total	966 ± 27	100	103 ±6	100
% of control	98		83	

[a] Reaction rates are given as the amounts of products recovered after incubating COX-1 (0.5−1.5 μg of protein/mL) or COX-2 (6−9 μg of protein/mL) for 1 min at 30 °C and in the presence of 1 mM GSH. Values are averages calculated from at least 10 different experiments ± SEM. Control values were obtained in the absence of GSH.

Table 3: Effect of Thiol Reagents on the Metabolism of AA by the Constitutive Form of Prostaglandin Synthase[a]

thiol reagent	% GSH rate	% prostanoids	% 12-HHT
GSH-CH_3	99	100	0
DTT	10	≤1	100
cysteine	65	11	89
cysteamine			
0.05 mM	47	20	80
0.50 mM	15	≤1	100
1.00 mM	≤1	≤1	≤1
penicillamine			
0.25 mM	51	54	46
1.00 mM	28	65	35

[a] Reaction mixtures containing COX-1 (1.5 μg of protein/mL) were preincubated with the indicated thiols for 1 min at 30 °C prior to initiation by the addition of AA. Reaction rates were obtained by measuring the amount of products recovered after a 1 min incubation at 30 °C. Values shown are the following: (a) % of the rate obtained in the presence of 1 mM GSH (991 ± 59 nmol of product/(mg of protein:min), n = 3) and (b) the % of products recovered as either prostanoids or 12-HHT. Values are averages calculated from at least 3 different experiments with SEM ≤15% of the averages. Final concentrations for GSH *S*-methyl ether, cysteine, and DDT were 1, 1, and 0.05 mM, respectively.

that obtained in 1 mM GSH, yet 12-HHT accounted for 89% of the AA metabolites (Table 3). Cysteamine (2-aminoethanethiol) and penicillamine (β,β-dimethylcysteine) supported 12-HHT formation to variable degrees (Table 3). At 50 μM, cysteamine reduced the extent of AA oxidation by COX-1 to 47% of that observed in the presence of 1 mM GSH, with 12-HHT accounting for 80% of the reaction products. Higher concentrations of cysteamine resulted in a marked inhibition of AA metabolism by COX-1 (Table 3) and COX-2 (not shown). The results with penicillamine were similar. Although to a more limited extent, penicillamine also inhibited the overall metabolism of AA (Table 3). Finally, at 50 μM, DTT was a powerful inhibitor of AA oxidation by COX-1 (Table 3) and COX-2 (not shown).

GSH-Dependent 12(S)-HHT Formation and the Cyclooxygenase Activity of Prostaglandin Synthase. Incubations of COX-1 or COX-2 with AA in the presence of increasing concentrations of GSH (from 0 to 5 mM) demonstrated that GSH concentration-dependent decreases in prostanoid recovery were accompanied by concomitant increases in 12-(*S*)-HHT formation, suggestive of a product/precursor relationship between the intermediate PGH_2 and 12(*S*)-HHT (Figure 3A,B). As shown in Figure 3A,B, the relationship

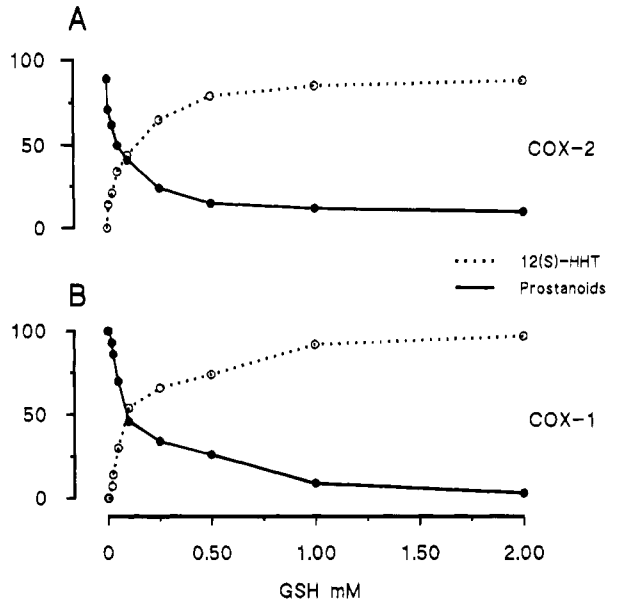

FIGURE 3: Effect of increasing concentrations of GSH on the catalysis of prostanoid and 12(*S*)-HHT production by the constitutive (COX-1) and mitogen inducible (COX-2) isoforms of prostaglandin synthase. Samples of purified COX-1 (A) and COX-2 (B) were incubated with arachidonic acid in the presence of increasing concentrations of GSH, exactly as described in Figures 1 and 2. After extraction, the reaction products were resolved by reversed phase HPLC and quantified as described in the Materials and Methods section. Values shown are averages calculated from at least four different experiments with SEM ≤10% of the mean.

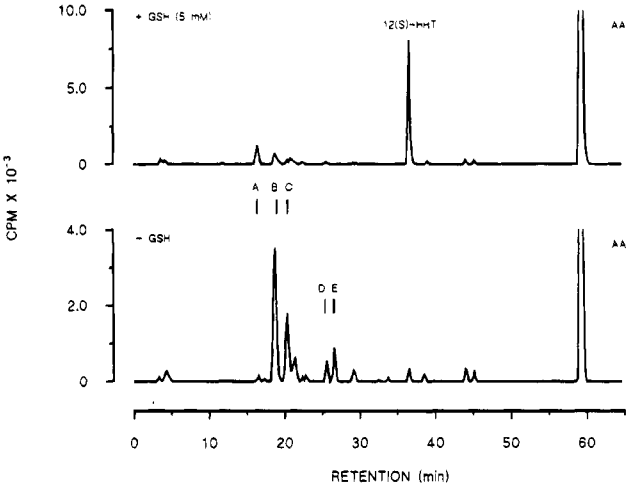

FIGURE 4: Effect of GSH on the catalytic output of the constitutive prostaglandin synthase isoform present in ram seminal vesicle microsomal fractions. Ram seminal vesicle microsomal fractions suspended in 0.1 M Tris-HCl (pH 7.8) (50 μg of microsomal protein/mL) containing 0.5 mM phenol and 2 μM hematin were incubated with [1-^{14}C]arachidonic acid (50 μM, final concentration) in the presence (top) or absence (bottom) of GSH (5 mM, final concentration). After 1 min of mixing at 30 °C, the reaction products were extracted, resolved, and quantified as described in Figure 1 and in the Materials and Methods section. Shown are the radiochromatograms derived from incubates containing 5 μg of microsomal protein. The HPLC retention times for synthetic $PGF_{2\alpha}$ (A), PGE_2 (B), PGD_2 (C), PGA_2 (D), and PGB_2 (E) are shown.

between the extent of HHT formation (measured after 1 min incubation) and GSH concentrations (Figure 3A,B) follows a saturation type of kinetics, typical of enzyme mediated processes. While, as mentioned above, we were unable to determine initial reaction rates, the apparent half-maximal rates of HHT formation were reached between 75 and 150 μM GSH. 12(*S*)-HHT was recovered as nearly the only reaction product at GSH concentrations between 1 and 2 mM (Figure 3A,B). Finally, although less prominently, the effects of GSH were also evident when ram seminal vesicle microsomes were used as the source of COX-1. In the presence of GSH (1 mM), 12-HHT accounted for nearly half of the products generated by the microsomal COX-1 (60 ± 10% of total products; $n = 3$). However, at 5 mM GSH, the microsomal enzyme also generated 12(*S*)-HHT as its major reaction product (Figure 4).

Aspirin is a potent and irreversible inhibitor of prostanoid formation (Smith et al., 1981; Marnett, 1992; Shimokawa & Smith, 1992; Smith, 1992). Studies with COX-1 demonstrated that enzyme inhibition was associated with an aspirin-dependent acetylation of a serine residue positioned near the enzyme active site (Shimokawa & Smith, 1992). The amino acid sequences surrounding the active site and the aspirin target serine residue are similar for COX-1 and COX-2 (Meade et al., 1993). Furthermore, the aspirin-dependent acetylation of a serine residue (Ser-516) in COX-2 was reported recently (Lecomte et al., 1994). A differential response to aspirin inhibition by COX-1 and COX-2 was initially suggested by Holtzman and collaborators (Holtzman et al., 1992) using cultured epithelial cells. In these cells, aspirin treatment inhibited cellular prostanoid formation and resulted in a dose-dependent increase in 15(*R*)-HETE formation (Holtzman et al., 1992). More recently, a murine and a human recombinant COX-2 were expressed in COS-1 cells and shown to catalyze the aspirin-dependent metabolism of AA to 15-HETE and 15(*R*)-HETE, respectively (Meade et al., 1993; Lecomte et al., 1994)). As reported (Vane & Botting, 1988), aspirin pretreatment (1 mM) resulted in a ≥94% inhibition of prostanoid production by COX-1. On the other hand, aspirin-treated sheep placenta COX-2 was catalytically active and metabolized AA to 15-HETE (≥91% of total products), instead of prostanoids (Meade et al., 1993) (Figure 5). Thus, aspirin treatment blocked the cyclooxygenase activity of COX-2 and converted this enzyme into a 15-lipoxygenase-like enzyme (Meade et al., 1993). Under our experimental conditions, no significant 15-hydroperoxyeicosatetraenoic acid intermediate was observed, indicating that the enzyme's 15-hydroperoxidase activity was either unaffected by aspirin or that 15-HETE formation proceeds via alternate mechanisms. Chiral analysis demonstrated that aspirin-treated COX-2 metabolized AA with high enantioselectivity, generating 15(*R*)-HETE with an optical purity of 91% (Figure 5). Importantly, the product of control, non-aspirin-treated, COX-2 is 15(*S*)-HETE, the optical antipode of 15(*R*)-HETE. Although the catalytic center or centers responsible for the 15-lipoxygenase-like activities of control and aspirin-treated COX-2 exhibit similar regioselectivities for oxygen insertion, they show opposite enantiofacial selectivities. These results indicate that either COX-2 contains more than one active site responsible for AA oxidation at C(15), with the 15(*R*)-lipoxygenase-like center becoming predominant only after aspirin inhibition of cyclooxygenase and 15(*S*)-lipoxygenase activities, or that, alternatively, aspirin treatment results in marked changes in the active site molecular coordinates responsible for the stereoselective oxygenation of AA at C(15).

The aspirin induced modification of the catalytic activity of COX-2 was utilized to further document the proposed precursor/product relationship between PGH_2 and 12-HHT.

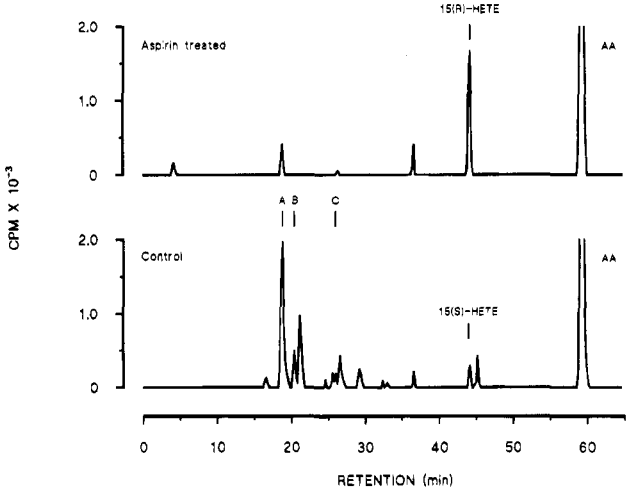

FIGURE 5: Effect of aspirin treatment on the catalytic activity of the mitogen inducible isoform of prostaglandin synthase. Samples of purified COX-2 (8 μg of protein/mL) in 0.1 M Tris-HCl (pH 7.8) were incubated in the presence or absence of aspirin (5 mM, final concentration). After 5 min at room temperature, aspirin-free and aspirin-treated COX-2 were incubated with [1-^{14}C]arachidonic acid (50 μM, final concentration), phenol (500 μM), and hematin (2 μM) for 1 min at 30 °C. The reaction products were extracted, resolved, and quantified as described in Figure 1 and in the Materials and Methods section. The radiochromatograms shown were derived from incubates containing 1 μg of aspirin-free (bottom) or aspirin-treated (top) COX-2. The HPLC retention times for synthetic PGE_2 (A), PGD_2 (B), and PGA_2/PGB_2 (C) are indicated in the figure.

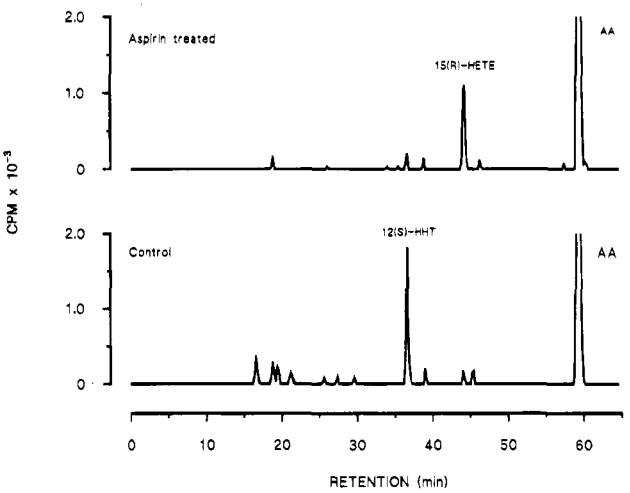

FIGURE 6: Effect of GSH on the catalytic activity of aspirin-treated purified sheep placenta prostaglandin synthase. Samples of purified COX-2 (8 μg of protein/mL) were preincubated in the presence or absence of aspirin as described for Figure 5. After 5 min at room temperature, the enzymes were incubated for 1 min at 30 °C with [1-^{14}C]arachidonic acid (50 μM, final concentration) in Tris-HCl buffer (pH 7.8) containing 1 mM GSH, 0.5 mM phenol, and 2 μM hematin. Reaction products were extracted, resolved, and quantified as described in Figure 1 and in the Materials and Methods section. Shown are the radiochromatograms derived from incubates containing 1 μg of aspirin-free (bottom) or aspirin-treated (top) COX-2.

Control and aspirin-treated COX-2 were incubated with AA in the presence of GSH (1 mM), and after 1 min at 30 °C, the reaction products were extracted into ethyl acetate and analyzed by HPLC. A comparison of the chromatograms in Figures 5 and 6 demonstrates that 15-HETE formation by aspirin-treated COX-2 was unaffected by the presence of GSH (1−2 mM, final concentration). Thus, both control and aspirin-treated COX-2 generated 15(*R*)-HETE in the presence or absence of GSH (Figures 5 and 6). In summary, although catalytically active, aspirin-treated COX-2 does not generate significant amounts of PGH_2, and consequently, 12-(*S*)-HHT was not a product when the aspirin-treated COX-2 was incubated in the presence of GSH (Figure 6). Finally, the role of PGH_2 as the precursor for the chemical, GSH-dependent formation of 12-HHT was studied by incubating samples of purified PGH_2 with 1 mM GSH in the presence or absence of hematin (2 μM) and/or phenol (0.5 mM). After 1 min at 30 °C and in 0.1 M Tris-HCl (pH 7.8), organic soluble products were extracted into ethyl acetate and resolved by reversed phase HPLC. Under these experimental conditions, we were unable to detect significant GSH-dependent 12-HHT formation from PGH_2. On the other hand, PGE_2 accounted for most of the PGH_2 products generated nonenzymatically in the presence or absence of GSH. The evidence so far discussed indicates that 12(*S*)-HHT is formed by an enzymatic process that requires GSH-dependent cleavage of PGH_2.

The structural similarities between the asymmetric carbons at C(12) in 12-HHT and at C(15) in PGH_2, the apparent product/precursor relationship between these eicosanoids, and the requirement for reduced thiols indicate that the 12(*S*)-HHT may be derived from reductive cleavage of the 8,9 and 11,12 carbon−carbon bonds in PGH_2, with the elimination of malondialdehyde and the formation of GSSG. Thromboxane synthase catalyzes the GSH-independent metabolism of PGH_2 to a nearly equimolar mixture of TXA_2 and 12(*S*)-HHT (Smith et al., 1981; Ullrich et al., 1981; Pace-Asciak & Smith, 1983). While the mechanism of 12(*S*)-HHT formation by thromboxane synthase is unknown, the enzyme generates 1 mol of malondialdehyde/mol of 12(*S*)-HHT formed from PGH_2 (Smith et al., 1981; Ullrich et al., 1981; Pace-Asciak & Smith, 1983). GSH is known to support the slow chemical reduction of hydroperoxides to the corresponding alcohols. However, the present study indicates that, in addition to oxygen−oxygen bond reductive cleavage, GSH supports a presumably enzymatic carbon−carbon bond scission similar to that observed during TXA_2 biosynthesis (Ullrich et al., 1981; Pace-Asciak & Smith, 1983). As with thromboxane synthase, the role of GSH and the mechanism of 12-HHT formation from PGH_2 remain to be determined.

Effects of Cytosolic GSH Peroxidases on AA Metabolism by the Isoforms of Prostaglandin Synthase. In the absence of other known redox transitions, a mechanism based on the COX-1 heme-dependent peroxidase activity was proposed to account for the initial enzymatic abstraction of a H atom from the substrate (Smith et al., 1981; Kulmacz & Lands, 1983; Kulmacz et al., 1994). Central to this proposal was the concept of an endogenous peroxide, present in most substrate preparations, serving as the initiator of an intramolecular interaction between the heme peroxidase and cyclooxygenase activities of COX-1 (Kulmacz et al., 1994). Thus, peroxide heme high oxidation states, common to several heme peroxidases, provided the required redox potential for the initial H abstraction, a prerequisite for initiation of subsequent cyclooxygenase activity and the formation of PGG_2. The product of the cyclooxygenase cycle, PGG_2, served then as peroxide donor for sustained catalytic turnover (Kulmacz et al., 1994). Implicit in this proposal was the concept that catalytic activity could be modulated by the experimental manipulation of the initiator hydroperoxide concentrations (Smith et al., 1981; Kulmacz

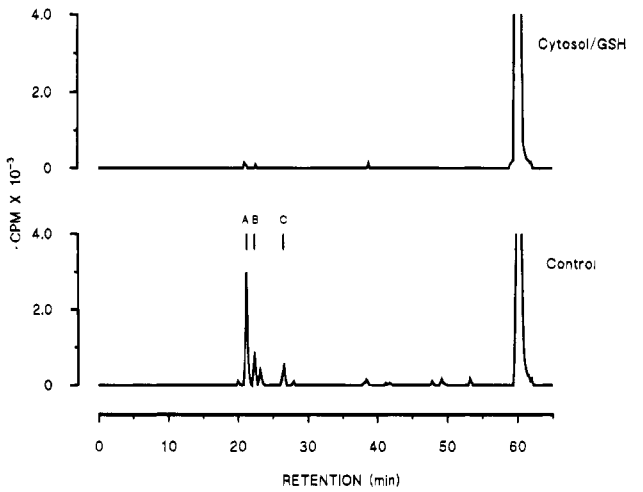

FIGURE 7: Effect of GSH and rat liver cytosol on the catalytic output of the constitutive isoform of prostaglandin synthase. Samples of purified COX-1 (1.5 μg of protein/mL) were incubated for 1 min at 30 °C with [1-^{14}C]arachidonic acid (50 μM, final concentration) in the presence or absence of rat liver cytosol (0.5 mg of protein/mL) and GSH (1 mM, final concentration). The conditions utilized for incubation, product extraction, resolution, and quantification are as described in Figure 1 and in the Materials and Methods section. Shown are the radiochromatograms derived from reaction mixtures containing 0.2 μg of COX-1 and incubated in the absence (bottom) or the presence of liver cytosol and GSH (top). The retention times for synthetic PGE_2 (A), PGD_2 (B,) and PGA_2/PGB_2 (C) are indicated.

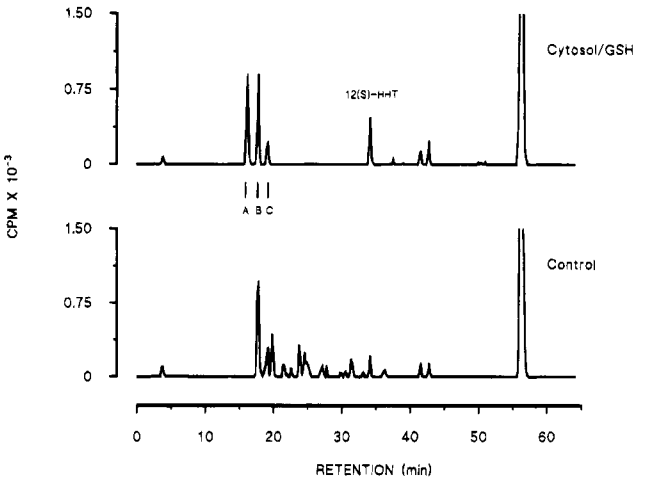

FIGURE 8: Effect of GSH and rat liver cytosol on the catalytic output of the mitogen inducible isoform of prostaglandin synthase. Samples of purified COX-2 (8 μg of protein/mL) were incubated for 1 min at 30 °C with [1-^{14}C]arachidonic acid (50 μM, final concentration) in the presence or absence of rat liver cytosol (0.5 mg of protein/mL) and GSH (1 mM, final concentration). The conditions utilized for incubation, product extraction, resolution, and quantification are as described in Figure 1 and in the Materials and Methods section. Shown are the radiochromatograms derived from reaction mixtures containing 0.8 μg of COX-2 and incubated in the absence (bottom) or the presence of liver cytosol and GSH (top). The HPLC retention times for synthetic $PGF_{2\alpha}$ (A), PGE_2 (B), and PGD_2 (C) are indicated.

& Lands, 1983; Kulmacz et al., 1994). Thus, addition of catalytic amounts of hydroperoxides resulted in significant increases in the rates of prostanoid formation by COX-1 while, on the other hand, manipulations that reduced the concentrations of adventitious peroxides substrates resulted in a markedly reduced COX-1 activity (Kulmacz & Lands, 1983; Kulmacz et al., 1994). Earlier work demonstrated that preincubation of AA with GSH and selenium-dependent GSH-Px, prior to metabolism by COX-1, significantly reduced or, in some cases, abolished AA metabolism and prostanoid formation (Kulmacz et al., 1994). These observations provided important support to the idea that an initiator hydroperoxide, reduced to the corresponding alcohol in the presence of GSH/GSH-Px, was essential for COX-1 catalytic activity.

Selenium-dependent GSH-Pxs are cytosolic enzymes that actively catalyze the GSH-dependent reduction of organic hydroperoxides, including fatty acid hydroperoxides (Rotruck et al., 1973; Takahashi et al., 1987; Maiorino et al., 1990; Thomas et al., 1990; Fong-Fong et al., 1992; Burk & Hill, 1993). While selenium-dependent GSH-Pxs have recognized protective roles during pathological conditions resulting in lipid peroxidation, little is known with regard to their ability to regulate cellular prostanoid formation (Buckley et al., 1991; Mirault et al., 1991). It is of interest that, as for COX-2, glutathione peroxidases are modulated during inflammation and tumor development (Parnham et al., 1987; Grisham et al., 1990; Segura-Aguilar et al., 1990; Mirault et al., 1991; Rana et al., 1994). To ascertain the functional significance of the GSH effects described above, samples of COX-1 and COX-2 were incubated with AA and GSH in the presence or absence of a rat liver cytosol preparation, an abundant source of GSH-dependent peroxidases (Burk & Hill, 1993). As demonstrated by the chromatograms in Figures 7 and 8, the prostaglandin synthase isoforms showed a markedly

Table 4: Effects of Rat Liver Cytosol and GSH on the Metabolism of AA by the Mitogen Inducible Isoform of Prostaglandin Synthase (COX-2)[a]

metabolite	reaction rate	% distribution
$PGF_{2\alpha}$	31 ± 3	27
PGE_2	34 ± 5	30
PGD_2	21 ± 6	18
12-HHT	17 ± 2	15
15-HETE	5 ± 1	4
11-HETE	6 ± 1	5
total	117 ± 20	100

[a] Reaction rates are given as the amount of products recovered after incubating COX-2 (6−9 μg of protein/mL) in the presence of rat liver cytosol (0.5 mg of protein/mL) and GSH (1 mM) for 1 min at 30 °C. Values are averages calculated from at least 5 different experiments ± SEM.

different behavior when incubated in the presence of liver cytosol and GSH. Hence, while the activity of the COX-1 was inhibited completely by the addition of cytosol and GSH, under identical experimental conditions, the rates of AA metabolism by the COX-2 isoform were not significantly changed. As shown in Figure 8 and Tables 1 and 4, there was an increased recovery of $PGF_{2\alpha}$ at the expense of PGE_2, PGA_2, and PGB_2. Variable amounts of 12-HHT were also recovered (Table 4, Figure 8). The presence of cytosolic enzymes that catalyze, on the one hand, the GSH-dependent formation of PGE_2 from PGH_2 and, on the other hand, the reduction of PGH_2 to $PGF_{2\alpha}$ has been described (reviewed in Smith et al., 1981). These effects of the cytosolic fraction were GSH-dependent and were not altered by preincubating the AA with cytosol and GSH, prior to its addition to the reaction mixtures containing prostaglandin synthase isoforms. These results indicated major differences in the response of the prostaglandin synthase isoforms to the addition of a subcellular fraction rich in GSH-dependent peroxidase activ-

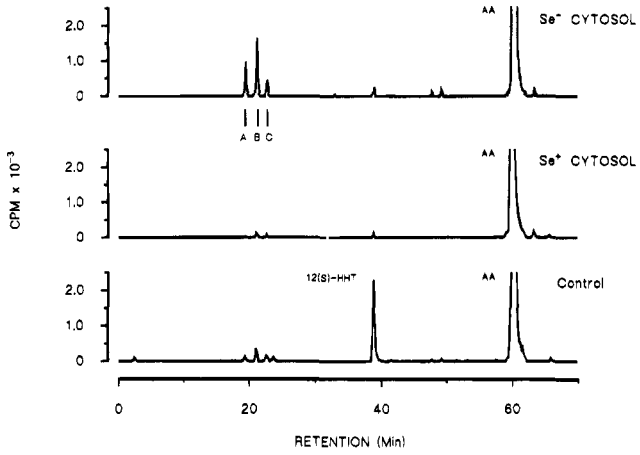

FIGURE 9: Effect of dietary selenium on the GSH-dependent modulation of the activity of the constitutive isoform of prostaglandin synthase by rat liver cytosol fractions. Rat cytosolic fractions were isolated from the livers of animals fed a standard laboratory rat chow or a selenium-deficient chow. Samples of purified COX-1 (1.5 μg of protein/mL) were incubated for 1 min at 30 °C with [1-^{14}C]arachidonic acid (50 μM, final concentration) in the presence GSH only, or in the presence of GSH and liver cytosol isolated from control or selenium deficient animals. Cytosolic fractions and GSH were added to a final concentration of 0.5 mg of protein/mL and 1 mM, respectively. The conditions utilized for incubation, product extraction, resolution, and quantification are as described in Figure 1 and in the Materials and Methods section. Shown are the radiochromatograms derived from reaction mixtures containing 0.2 μg of COX-1 and incubated in the presence of GSH (bottom), GSH and liver cytosol from control rats (center), or GSH and cytosol from a selenium-deficient animal (top). The HPLC retention times for synthetic $PGF_{2\alpha}$ (A), PGE_2 (B), and PGD_2 (C) are indicated.

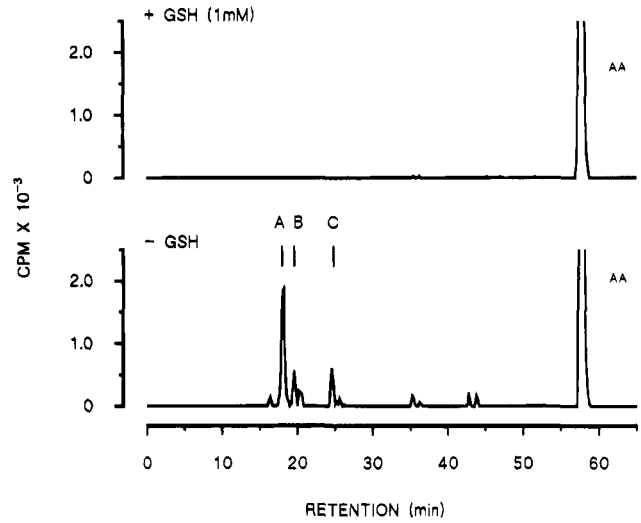

FIGURE 10: Effect of purified erythrocyte glutathione peroxidase and GSH on the catalytic activity of the constitutive isoform of prostaglandin synthase. Samples of purified COX-1 (1.5 μg of protein/mL) were incubated for 1 min at 30 °C with [1-^{14}C]-arachidonic acid (50 μM, final concentration), purified erythrocyte GSH peroxidase (8 units/mL, final concentration), and in the presence or absence of GSH (1 mM, final concentration). The conditions utilized for incubation, product extraction, resolution, and quantification are as described in Figure 1 and in the Materials and Methods section. Shown are the radiochromatograms derived from reaction mixtures containing 0.2 μg of COX-1 and incubated in the absence (bottom) or the presence of GSH (top). The HPLC retention times for synthetic PGE_2 (A), PGD_2 (B), and PGA_2 (C) are indicated.

ity. The nature of the cytosolic GSH-dependent peroxidase(s) responsible for the selective inhibition of COX-1 was further investigated by isolating liver cytosol from rats fed selenium-deficient diets. After 2 weeks on selenium-deficient diets, the activity of rat liver selenium-dependent GSH peroxidases is reduced to nearly undetectable levels (Yang et al., 1989). As previously mentioned, cytosol isolated from rats fed normal, selenium containing diets was a powerful, GSH-dependent inhibitor of COX-1 AA metabolism (Figure 9). However, cytosolic fractions obtained from animals fed selenium-deficient diets had no significant effects on the overall rates of COX-1 AA metabolism (Figure 9). Importantly, a comparison of the chromatograms in Figures 1 and 9 indicated that, like COX-2 (Table 4, Figure 8), when COX-1 was incubated in the presence of GSH and the cytosol from selenium-deficient rats, the recovery of $PGF_{2\alpha}$ was substantially increased. As mentioned, liver cytosol contains several, selenium independent proteins that catalyze the GSH- or NADPH-dependent metabolism of PGH_2 to PGE_2 and $PGF_{2\alpha}$ (Smith et al., 1981).

The sharply distinct behavior of the constitutive and inducible isoforms of prostaglandin synthase in the presence of GSH and liver cytosol raises important questions regarding a possible general role for initiator hydroperoxides in the catalysis of prostanoid formation by these isoforms. The selective inhibition of COX-1 by the combined presence of GSH and liver cytosol suggested that the selenium-dependent GSH-Px(s) reduction of initiator hydroperoxides (Kulmacz et al., 1994) only affected COX-1 and that, therefore, the mechanism by which the COX-2 isoform abstracts H atoms from the substrate and thus initiates and sustains catalytic turnover may be different (Kulmacz et al., 1994). Significantly, the activity of COX-2 was not modified even after preincubation of the AA with GSH and cytosol prior to its addition to the incubates containing COX-2. To further define the enzyme(s) responsible for this selective inhibition of COX-1 activity, samples of COX-1 and COX-2 were incubated with AA in the presence of GSH (1 mM, final concentration) and variable concentrations of a commercially available, selenium-dependent, purified erythrocyte glutathione peroxidase. This enzyme preparation was previously shown to inhibit AA oxidation by COX-1 in a GSH-dependent manner (Kulmacz et al., 1994). As with liver cytosol, the constitutive and inducible isoforms of prostaglandin synthase can be distinguished by their capacity to metabolize AA in the presence of GSH-Px and GSH. The metabolism of AA by the constitutive isoform of prostaglandin synthase was completely inhibited by the combined presence of GSH (1 mM, final concentration) and purified GSH-Px (8 units/mL, final concentration) (Kulmacz et al., 1994) (Figure 10). Under similar conditions, the activity of the mitogen inducible form was only slightly altered (Figure 11). Importantly, the effect of GSH-Px on AA metabolism by COX-1 was GSH-dependent and not affected by preincubating the fatty acid with GSH and GSH-Px. On the other hand, COX-2 remained catalytically active in the presence of GSH-Px (8 units/mL, final concentration), regardless of the GSH concentration (0−2 mM) or the time of preincubation of the substrate with GSH-Px and GSH (between 0 and 1 h). The effects of purified GSH-Px on AA metabolism by the prostaglandin synthase isoforms were GSH-Px concentration-dependent (Figure 12). Thus, while both isoforms could be inhibited in the presence of GSH and of increasing concentrations of purified GSH-Px, when com-

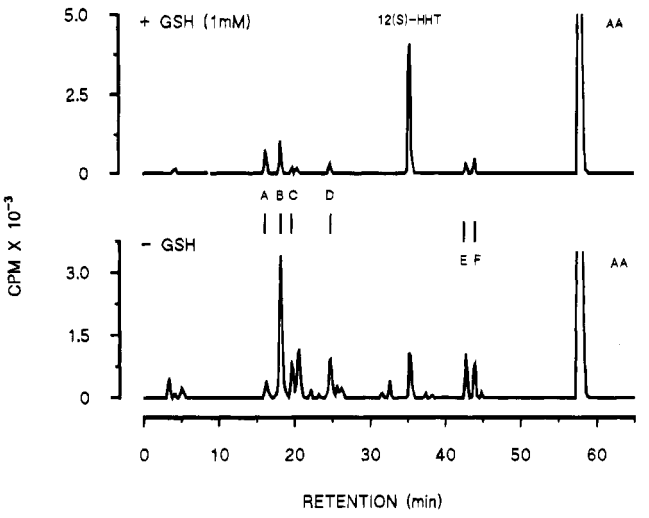

FIGURE 11: Effect of purified erythrocyte glutathione peroxidase and GSH on the catalytic activity of the mitogen inducible isoform of prostaglandin synthase. Samples of purified COX-2 (8 μg of protein/mL) were incubated for 1 min at 30 °C with [1-^{14}C]-arachidonic acid (50 μM, final concentration), purified erythrocyte GSH peroxidase (8 units/mL, final concentration), and in the presence or absence of GSH (1 mM, final concentration). The conditions utilized for incubation, product extraction, resolution, and quantification are as described in Figure 1 and in the Materials and Methods section. Shown are the radiochromatograms derived from reaction mixtures containing 1.0 μg of COX-2 and incubated in the absence (bottom) or the presence of GSH (top). The HPLC retention times for synthetic $PGF_{2\alpha}$ (A), PGE_2 (B), PGD_2 (C), and PGA_2 (D) are indicated.

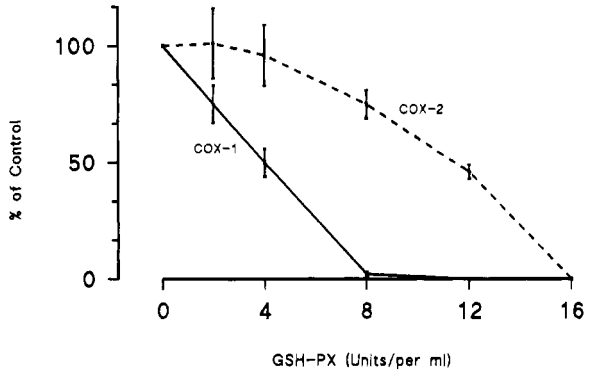

FIGURE 12: Effect of increasing concentrations of erythrocyte glutathione peroxidase on the activities of the constitutive and mitogen inducible isoforms of prostaglandin synthase. Samples of [1-^{14}C]arachidonic acid (100 μM, final concentration) in 0.1 M Tris-HCl buffer (pH 7.8) were incubated with increasing concentrations of GSH-Px (0−16 units/mL) and GSH (2 mM final concentration). After 5 min at 30 °C, they were mixed with equal volumes of a suspension of COX-1 (3 units/mL) or of COX-2 (18 units/mL) in 0.1 M Tris-HCl buffer (pH 7.8) containing 0.5 mM phenol and 2 μM hematin, and the incubations were continued for an extra 1 min. The reaction products were extracted, resolved, and quantified as described in Figure 1 and in the Materials and Methods section. Values shown are averages calculated from three different experiments ±SEM.

pared to COX-1, the enzymatic activity of COX-2 was markedly less sensitive (Figure 12). As discussed above and shown in Figures 7 and 8, the addition of GSH and liver cytosol (tested between 0.25 and 1.5 mg of cytosolic protein/mL of incubation mixture) blocked only the metabolism of AA by COX-1. In addition to potential isoform differences in their requirements for endogenous initiator hydroperoxide(s), these results could also indicate the presence of additional liver GSH-dependent peroxidase(s). The affinity of these enzymes for the initiator hydroperoxides would allow for competition by COX-1 and not by COX-2. Finally, commercial preparations of purified erythrocyte GSH-Px contain approximately 2.5% (w/w) DTT (Sigma Chemical Co.). In initial experiments, it was noted that, at high GSH-Px concentrations, both isoforms of prostaglandin synthase were inhibited by GSH-Px, even in the absence of added GSH. Dialysis against 0.1 M Tris-HCl (pH 7.5) containing 0.1 mM GSH substantially lowered DTT levels and greatly reduced this problem.

Endotoxin-Dependent Transcriptional Regulation of the Mitogen Inducible Isoform of Prostaglandin Synthase (COX-2) and GSH Peroxidase. It has been proposed that the contribution of the different prostaglandin synthase isoforms to cellular AA metabolism may be controlled by the physiological or pathophysiological conditions of individual organs (Smith, 1989; Mitchell et al., 1994). Thus, while COX-1 may be responsible for most of the prostanoid biosynthesis occurring under normal, physiological conditions, COX-2, the inducible isoform, may be responsible for prostanoid biosynthesis during inflammation, cancer, or other pathophysiological conditions (Smith, 1989; Marnett, 1992; DuBois et al., 1994a; Mitchell et al., 1994). This hypothesis is attractive not only because it provides an additional rationale for the existence of prostaglandin synthase isoforms but, more importantly, it suggests the potential for a selective clinical intervention that precludes changes in the production of those prostanoids necessary for normal body function (Mitchell et al., 1994). It is known that conditions of oxidative stress, such as those associated with inflammatory processes and certain stages of tumor formation, induce the biosynthesis of protective enzymes, including GSH-dependent peroxidase(s) (Parnham et al., 1987; Grisham et al., 1990; Segura-Aguilar et al., 1990; Rana et al., 1994). The differential sensitivities of COX-1 and COX-2 to the action of GSH-dependent glutathione peroxidase(s) suggest that, under these oxidative stress conditions, COX-2 may be responsible for most of the prostanoid biosynthesis in the affected cell and/or organ. Preliminary studies, utilizing an immortalized cell line derived from mouse macrophages, indicated that treatment of these cells with a single dose of endotoxin resulted in the coordinate upregulation of mRNA transcripts coding for COX-2 and GSH-Px. As shown in Figure 13, nucleic acid blot hybridization showed the apparent simultaneous appearance of COX-2 and GSH-Px transcripts, approximately 4 h after the endotoxin treatment. The relative abundance of the COX-2 mRNA continued to increase 24 h after treatment while that coding for GSH-Px was already in decline (Figure 13). This, as well as published data (Marnett, 1992; Mitchell et al., 1994), suggests that these enzymes may be part of a coordinated cellular response to injury and that, under these pathological conditions, prostanoid production by COX-2, and not by COX-1, may be an important component of the protective mechanisms to tissue injury and disease. Recent studies have demonstrated the oxygen-dependent regulation of a selenium-dependent GSH-Px (Cowan et al., 1993). It will be of interest to determine the role that oxygen and/or reduced oxygen species may play in the regulation of COX-2 levels.

The differential response of COX-1 and COX-2 to the GSH-dependent peroxidase activities associated with liver cytosol in general, and with the selenium-dependent GSH-Px in particular, has important implications for the com-

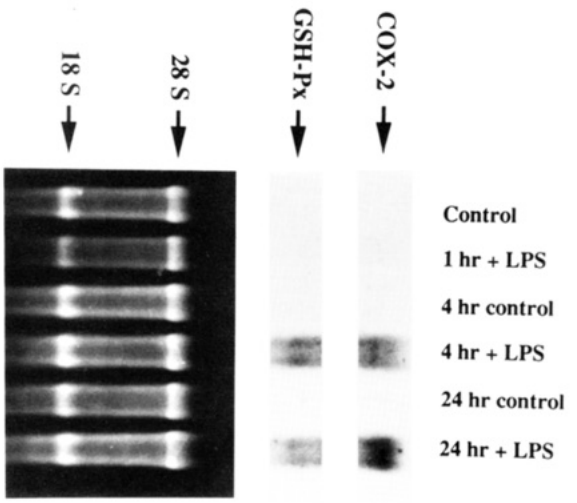

FIGURE 13: Effect of lipopolysaccharide treatment on the level of mRNA transcripts coding for the mitogen inducible isoform of prostaglandin synthase and glutathione peroxidase. Total RNA, isolated from RAW 264.7 cells 1, 4, and 24 h after a single treatment with lipopolysaccharide (LPS, 10 μg/mL, Sigma Chemical Co.) and from untreated controls, was fractionated by agarose electrophoresis, transferred to nitrocellulose filters, and probed using the following [^{32}P]-labeled cDNAs: (a) a cDNA, cloned from rat intestinal epithelial cells (DuBois et al., 1994b), containing the full coding sequence for COX-2 and (b) a 300 bp fragment containing part of the open reading frame 5′ end of the cDNA coding for mouse liver GSH-Px (a gift from Dr. N. Imura, Kitasato University, Japan) (Toyoda et al., 1989). Approximately 20 μg of total RNA was loaded into each lane. The RNA extraction procedure and the conditions utilized for hybridization, electrophoresis, and labeling are those of the references: DuBois et al. (1994b and 1990). Shown in the figure are 4.5 and 1.0 kb mRNAs that hybridized to the COX-2 and GSH-Px probes, respectively. Prior to transfer, the agarose−ethidium bromide gel was exposed to UV and photographed to verify RNA loading.

monality of the mechanisms by which these two prostaglandin synthase isoforms catalyze AA metabolism and prostanoid formation. The lack of an effect by cytosolic GSH-dependent peroxidases suggests that, in contrast to COX-1, substrate H atom abstraction by the COX-2 isoform may not involve the same type of interaction between activator hydroperoxide(s) and the enzyme's peroxidase heme group or the formation of COX-2 peroxidase hypervalent heme−oxygen complexes. If indeed the constitutive and the mitogen inducible prostaglandin synthase isoforms metabolize AA by substantially different reaction mechanisms and/or through different rate limiting intermediates, then new strategies for a meaningful and rational approach to the design of selective inhibitors for biochemical and/or clinical intervention will be become available. As sufficient quantities of recombinant, catalytically active COX-2 become available, metabolic, spectral, and site-specific protein modification studies will help to establish the commonality of the phenomena described here and their mechanistic implications.

In conclusion, the experimental evidence presented here demonstrates that GSH and selenium-dependent cytosolic GSH-Px(s) have profound and distinct effects on the catalytic outcome of the constitutive and mitogen inducible forms of prostaglandin synthase. These results suggest that, under pathological conditions associated with oxidant stress, the mitogen inducible form of prostaglandin synthase may be responsible for a significant portion of cellular prostanoid biosynthesis. Additionally, the evidence also indicates the potential for marked differences in the mechanism(s) of prostanoid production by these prostaglandin synthase isoforms. These results should be of help during the design of meaningful approaches to the selective manipulation of the individual prostaglandin synthase isoforms.

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