Comparison of Hydroperoxide Initiator Requirements for the Cyclooxygenase Activities of Prostaglandin H Synthase-1 and -2*

(Received for publication, May 5, 1995, and in revised form, June 30, 1995)

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Two isoforms of prostaglandin H synthase have been described: isoform-1 (PGHS-1), which is ascribed a role in basal or housekeeping prostaglandin synthesis; and isoform-2 (PGHS-2), which has been found to be strongly inducible in many tissues and has been associated with inflammatory processes. Recent observations have indicated that cyclooxygenase catalysis by the two isoforms can be differentially regulated when both are present simultaneously (Reddy, S. T., and Herschman, H. R. (1994) J. Biol. Chem. 269, 15473–15480). The requirement of the cyclooxygenase for hydroperoxide initiator has been proposed as an important limit on cellular prostaglandin synthesis (Marshall, P. J., Kulmacz, R. J., and Lands, W. E. M. (1987) J. Biol. Chem. 262, 3510–3517). To compare the levels of hydroperoxide required for cyclooxygenase initiation in the two PGHS isoforms, we have examined the ability of a hydroperoxide scavenger, glutathione peroxidase, to suppress the cyclooxygenase activity of purified preparations of human PGHS-2, ovine PGHS-2, and ovine PGHS-1. Half-maximal prostaglandin synthetic activity was found to require a much lower hydroperoxide level with human PGHS-2 (2.3 nM) and ovine PGHS-2 (2.2 nM) than with ovine PGHS-1 (21 nM). Similar results were obtained when cyclooxygenase activity was monitored by chromatographic analyses of radiolabeled arachidonate metabolites or with oxygen electrode measurements. Mixing four parts of ovine PGHS-1 with one part of human PGHS-2 did not markedly change the sensitivity of the overall cyclooxygenase activity to inhibition by glutathione peroxidase, indicating that the PGHS-1 activity was not easily initiated by PGHS-2 activity in the same cell. Effective catalysis by PGHS-2 can thus proceed at hydroperoxide levels too low to sustain appreciable catalysis by PGHS-1. This difference in catalytic characteristics provides a biochemical mechanism for differential control of prostaglandin synthesis by the two PGHS isoforms, even when both are present in the same intracellular compartment.

Prostaglandin H synthase (PGHS) catalyzes a key irreversible step in the biosynthesis of prostaglandins, the oxygenation and rearrangement of arachidonic acid to form prostaglandin G2 (Samuelsson et al., 1978). Two isoforms of PGHS have been described. The first, PGHS-1, was purified from ovine and bovine seminal vesicles (Hemler et al., 1976; Miyamoto et al., 1976; van der Ouderaa et al., 1977). The second isoform, PGHS-2, was discovered more recently (Xie et al., 1991; Kujubu et al., 1991; O’Banion et al., 1991; Sirois and Richards, 1992) and has been purified from heterogeneous expression systems (Barnett et al., 1994; Perl et al., 1994). The two human isoforms have about 60% sequence identity overall, with much higher sequence conservation in catalytically important regions (Hla and Nielsen, 1992). Currently, it is thought that PGHS-2, which is strongly induced by various mitogens, has a role in inflammation, whereas PGHS-1, whose cellular levels vary over a smaller range, is a housekeeping enzyme (Mitchell et al., 1993; Masferrer et al., 1994).

It has been found recently that the two PGHS isoforms can be regulated separately even when both enzymes are present in the same cell (Reddy and Herschman, 1994; Murakami et al., 1994). Differential control of the isoforms was particularly striking in fibroblast and macrophage systems, where prostaglandin synthesis from endogenous substrate was due almost entirely to PGHS-2 catalysis, even though high levels of latent PGHS-1 were present (Reddy and Herschman, 1994).

The cyclooxygenase activity of PGHS-1 has long been known to require initiation by hydroperoxide (Smith and Lands, 1972), and it has been proposed that cellular prostaglandin synthesis is controlled in part by intracellular hydroperoxide levels (Marshall et al., 1987). Hydroperoxide initiation represents a potential route for differential control of cyclooxygenase catalysis by the two PGHS isoforms. To investigate this, the hydroperoxide requirements of cyclooxygenase activity in purified preparations of human PGHS-2 and ovine PGHS-2 were compared with that of ovine PGHS-1. The results indicate that cyclooxygenase initiation in PGHS-2 requires considerably lower levels of hydroperoxide than required by PGHS-1. This would allow prostaglandin synthesis by PGHS-2 to proceed at cellular hydroperoxide levels too low to support cyclooxygenase catalysis by PGHS-1 in the same cell.

MATERIALS AND METHODS

Hematin, Tween 20, reduced glutathione, erythrocyte glutathione peroxidase, glutathione reductase, and NADPH were obtained from Sigma. Glutathione peroxidase activity was assayed spectrophotometrically (Lawrence et al., 1974). One unit of glutathione peroxidase represents an initial velocity of 1 nmol of GSH oxidized/min. Unlabeled arachidonate was obtained from NuChek Prep (Elysian, MN) and [1-14C]arachidonate from Amersham Corp. Working stocks of arachidonate (0.50 mM in 50 mM Tris, pH 8.5) were pretreated with a small amount of GSP to remove residual hydroperoxides as described previously (Kulmacz et al., 1994b).

PGHS-1 was purified to homogeneity from ovine seminal vesicles (Kulmacz and Lands, 1987) and reconstituted with a 3-fold molar excess of hematin. In some cases the partially purified enzyme from the first gel-filtration chromatography step was used, after addition of excess...
hemin. oPGHS-2 (purified to about 70% electrophoretic homogeneity from placenta) was purchased from Cayman Chemical and reconstituted with excess hematin. Immunoblotting of the oPGHS-2 with rabbit polyclonal antibody raised against the C-terminal fragment indicated that each antibody fragment stained a broad band with a molecular weight of about 72 kDa (data not shown), confirming that the ovine placental enzyme is isoform 2.

hPGHS-2 was expressed in Sf9 (American Type Culture Collection) or High-5 (InVitrogen) cells using a Baculovirus vector containing the cDNA for human PGHS-2 under control of the polyhedrin promoter. Insect cell culture and Baculovirus manipulations followed procedures recommended by the suppliers. The coding region of the hPGHS-2 cDNA was released by NcoI digestion from a pDONR Vector (generously provided by Dr. Timothy Hla, American Red Cross) and ligated into the Nol site of the pVL1393 twin vector (Pharminen). The 1.9-kilobase pair insert includes about 30 base pairs of 5′ and 60 base pairs of 3′ untranslated sequence. A clone was done with the hPGHS-2 insert in the correct orientation was confirmed by restriction enzyme digest. This clone was mixed with linearized DNA from Baculovirus containing a lethal deletion (Baculogold, Pharmingen) and used to transfect Sf9 insect cells. The recombinant virus was amplified in fresh Sf9 cells; membranes from these cells had considerable cyclooxygenase activity and immunoreactive protein of the expected molecular weight, confirming expression of hPGHS-2. Large scale expression of recombinant hPGHS-2 was done in either Sf9 or High-5 cells in suspension culture. Insect cells were harvested 3–4 days after infection, homogenized by brief sonication, and a membrane fraction isolated by centrifugation at 100,000 × g for 1 h. The recombinant hPGHS-2 was solubilized with 1% Tween 20 and chromatographed on an AcA34 gel-filtration column essentially as described for oPGHS-1 (Kulmacz and Lands, 1987). The specific activity (measured in 0.1M potassium phosphate, pH 7.2, with 1 mM phenol and 100 μM arachidonate) of the purified hPGHS-2 used in these studies averaged 9 units/mg of protein.

Protein levels were assayed as described by Peterson (1983). Proteins were analyzed by electrophoresis under denaturing conditions following the procedure of Laemmli (1970). Immunoblotting was done as described earlier (Kulmacz and Wu, 1989) with rabbit polyclonal antibody raised against residues 579–598 of hPGHS-2 (kindly provided by Dr. Paul Marshall, Ciba-Geigy); this antibody did not stain oPGHS-1 under the same conditions.

Cyclooxygenase activity was measured at 30°C with an oxygen electrode (Kulmacz and Lands, 1987). The standard reaction mixture contained 3 ml of 0.1 m potassium phosphate, pH 7.2, 1 mM phenol, 0.5 mM GSH, and 50 μM arachidonate. Reaction was started by addition of enzyme. One unit of cyclooxygenase activity represents a peak velocity of 1 nmol of oxygen/min.

The hydroperoxide activator requirements of oPGHS-1, oPGHS-2, and hPGHS-2 were assessed by titration with a hydroperoxide scavenger, GSP (Kulmacz et al., 1994b). Briefly, a fixed amount of PGHS was injected into a reaction mixture containing a variable amount of GSP. A plot of the fraction of activated cyclooxygenase determined from the oxygen consumption kinetics.

Theoretical predictions of the sensitivities of the cyclooxygenase activities of oPGHS-1, hPGHS-2, and mixtures of the two enzymes to inhibition by GSP were generated with an expanded version of an earlier kinetic model (Kulmacz and Lands, 1983). In the expanded model, written in Microsoft QuíckBasic, hydroperoxide (PGG2) generation and decomposition by PGHS-1, PGHS-2, and GSP was governed by the following equations.

\[
\Delta PGG_2 + \text{GSP} \rightarrow \text{PGG}_2 + \text{GSP}
\]

The parameter values were: \(K_{p1}, 21 \text{ mM} ; K_{p2}, 2.3 \text{ mM} ; K_{p3}, 1.25 \mu M ; K_{p4}, 2.5 \mu M ; K_{p5}, 15 \mu M\). The total cyclooxygenase activity was set at 0.38 μM O2/s and divided between PGHS-1 and PGHS-2 activity as desired. The total PGHS peroxidase activity (assuming saturating substrate levels) was 1.37 μM ROOH/s and divided between PGHS-1 and PGHS-2 in the same proportion as the cyclooxygenase activity. The GSP activity was set at levels desired. The initial hydroperoxide concentration was 1 mM and the hydroperoxide produced and consumed by the five activities was calculated for a 0.001-s increment using Equations 3–5. The reiterative program then adjusted the hydroperoxide level to reflect the net change before repeating the calculations for the next time increment. Self-inactivation was accounted for by decreasing PGHS-1 (or PGHS-2) cyclooxygenase and peroxidase activities in proportion to cyclooxygenase catalysis by that isoform in the previous interval, so that each PGHS-1 (or PGHS-2) molecule synthesized 1200 PGG2 mol-1/s; it was injected into a reaction mixture containing a variable amount of PGHS-1 (or PGHS-2) peroxidase activity.

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The current kinetic model was found to predict a slightly different relationship between the GSP/Cox ratio needed for complete suppression of cyclooxygenase activity and the \(K_p\) value. For example, with the current model a GSP/Cox ratio of 75 corresponded to a \(K_p\) value of 21 nm; with the old model (Kulmacz and Lands, 1985) the same GSP/Cox ratio implied a \(K_p\) value of 16 nm. This small difference was due to the smoother reaction course predicted with the shorter time increments used in the current model. A standard curve was generated with the current model for 10 separate \(K_p\) values between 1.5 and 30 nm using the procedure described previously (Kulmacz and Lands, 1985). The points on this standard curve were fitted to an equation of the following form.

\[
\frac{\text{GSP/Cox ratio (at suppression)}}{\text{GSP/Cox ratio (at suppression)}} = \frac{1}{K_p} \left(1 + \frac{1}{K_p}\right)
\]

(Eq. 6)

The value of the constant, \(C_p\), was found to be 1556 ± 4 nm. This standard curve was used in the present study to calculate \(K_p\) values from the observed GSP/Cox ratios.

Reactions with \([14C]\text{arachidonate contained 0.6 ml of the standard reaction mixture in a glass tube thermostatted at 30°C. The oxygenase was added and the mixture vortexed briefly to start the reaction. After 1 min of further incubation, the reaction was stopped, and lipid metabolites were extracted by addition of 3 volumes of ice-cold diethyl ether/methanol/1% citric acid (30:41) (Miymoto et al., 1976). To minimize decomposition of prostaglandin endoperoxide, the lipid extracts were dried over anhydrous sodium sulfate and stored at −20°C until analysis by thin layer chromatography at 0°C (Tsai et al., 1992). Radioactive bands were located by autoradiography and quantitated by liquid scintillation counting. The concentration of metabolite in each band was calculated by multiplying the fraction of total radioactivity in that band by the initial arachidonate concentration. PGG2 and PGH2 standards were prepared by reacting oPGHS-1 with arachidonate in the absence of cosubstrate or with 1 mM phenol present (Hecker et al., 1987). Other standard eicosanoids were obtained from Cayman Chemical Co. (Ann Arbor, MI).

RESULTS AND DISCUSSION

Characterization of hPGHS-2—Recombinant hPGHS-2 expressed in Baculovirus-infected cells was solubilized with Tween 20 and partially purified by gel-filtration chromatography as described under "Materials and Methods." Electrophoretic analysis revealed the major protein component to be a rather broad band with a molecular weight of about 74 kDa (lane 1 in Fig. 1). Densitometry of the stained gel indicated that the 74-kDa band accounted for about one-third of the total protein in lane 1. Immunoblotting with antibody directed at the hPGHS-2 C-terminal peptide insert stained the same 74-kDa band (lane 2 in Fig. 1), confirming that the major protein

![Fig. 1. Electrophoretic analysis of recombinant hPGHS-2.](image)
Hydroperoxide Initiator Requirements of PGHS-1 and -2

The GSP sensitivity of the cyclooxygenase activity of paracrine PGHS, measured by oxygen consumption, declined in direct proportion to the relative amount of added GSP (Fig. 2). Extrapolation of the fitted line to the x axis indicated complete suppression at a GSP/Cox ratio of about 75. This is quite comparable with the value of 65 determined earlier for oPGHS-1 (Kulmacz and Lands, 1985). The oPGHS-1 cyclooxygenase activity was not decreased by GSP in control reactions lacking GSH, demonstrating that the inhibition was due to peroxidase catalysis by GSP rather than some direct effect of GSP on oPGHS-1. With oPGHS-2, considerable cyclooxygenase activity was observed at rather high GSP/Cox ratios, with about 25% of the control activity remaining when the ratio was 380. The cyclooxygenase activity of purified hPGHS-2 was also relatively resistant to inhibition by GSP. For hPGHS-2, the activity declined linearly as the GSP level was increased, with complete suppression indicated at a GSP/Cox ratio of about 670, almost 9-fold higher than observed for oPGHS-1. As with oPGHS-1, GSP did not inhibit the hPGHS-2 cyclooxygenase activity in the absence of GSH (Fig. 2), showing that peroxidase catalysis by GSP was responsible for the inhibitory effect. For both oPGHS-1 and hPGHS-2, addition of 1 mM N-ethylmaleimide, which alkylates GSH and thus vitiates GSP activity, after the initial burst of cyclooxygenase activity was complete resulted in a secondary burst of activity which increased in intensity as the GSP concentration was increased (data not shown). This confirms that the GSP activity converted a portion of the cyclooxygenase activity to a latent form that could be recovered if the GSP was inactivated.

The GSP sensitivity of the cyclooxygenase activity of partially purified oPGHS-1, at the same gel-filtration stage as the...
expected if the basic catalytic mechanism was similar in the two isoforms. As the GSP/Cox ratio was increased, the amounts of individual metabolites decreased in parallel for both isoforms (Fig. 3). This suggests a common hydroperoxide activator requirement, and a common catalytic origin, for all metabolites produced by each isoform.

The amount of total eicosanoid metabolites produced by oPGHS-1 declined sharply as the GSP/Cox ratio increased, with an extrapolated suppression ratio of about 80 (Fig. 4). Total metabolite production by hPGHS-2 was less sensitive to inhibition by the hydroperoxide scavenger, with an extrapolated suppression ratio of about 700. The GSP/Cox ratios producing suppression of arachidonate metabolism by oPGHS-1 and hPGHS-2 (Fig. 4) are in good agreement with those obtained from oxygen consumption measurements (Fig. 2), providing an important confirmation of the reliability of the more detailed and convenient kinetic measurements made with the oxygen electrode. While this manuscript was in preparation, Capdevila et al. (1995) reported GSP inhibition results with oPGHS-2 similar to those found in the present study with hPGHS-2 (Fig. 4).

Effect of hPGHS-2 Addition on the Sensitivity of oPGHS-1 to Inhibition by GSP—If the two isoforms are present in the same compartment, PGHS-2 cyclooxygenase catalysis, initiated at a low hydroperoxide level, might conceivably trigger initiation of PGHS-1 cyclooxygenase catalysis. Such a situation would allow cyclooxygenase catalysis by PGHS-1 at essentially the same hydroperoxide levels as those needed for PGHS-2 catalysis. To examine the propensity for PGHS-1 to be “primed” in this fashion by PGHS-2, cyclooxygenase catalysis by oPGHS-1 alone was compared with catalysis by a mixture of oPGHS-1 and hPGHS-2 at various GSP levels (Fig. 5). Cyclooxygenase catalysis by oPGHS-1 alone was strongly inhibited by the added hydroperoxide scavenger, reproducing the results in Fig. 2. When hPGHS-2 was present along with oPGHS-1, the decline in cyclooxygenase activity with increases in the GSP level was essentially the same as that observed with oPGHS-1 alone (Fig. 5). The observed decline agreed reasonably well with the behavior predicted by a simple model for the mixture of the two isoforms. A portion of the overall cyclooxygenase activity persisted at higher GSP levels, much of it presumably hPGHS-2 catalysis. Cyclooxygenase catalysis in the oPGHS-1/hPGHS-2 mixture was markedly more sensitive to inhibition by GSP than was the activity of hPGHS-2 alone (Figs. 2 and 5). This clearly demonstrates that the mere presence of PGHS-2 in the same compartment does not release PGHS-1 cyclooxygenase activity because of PGHS-1 cyclooxygenase inhibition by added GSP, reproducing the results in Fig. 2. Details are described under “Materials and Methods.”

Implications of Intrinsic Isoform Differences in Cyclooxygenase Inhibition Efficiency—The autocatalytic acceleration observed during the cyclooxygenase reaction (Hemler et al., 1978) can be understood in terms of a branched chain mechanism where the hydroperoxide (PGG₂) produced by one molecule of activated cyclooxygenase subsequently initiates cyclooxygenase catalysis in multiple molecules of latent enzyme (Wei et al., 1995). Because of the powerful feedback amplification produced, only low hydroperoxide concentrations are needed to sustain cyclooxygenase catalysis in a bulk reaction. The exact level of hydroperoxide initiator required is likely to depend on several factors. These would include the rate constants for formation of peroxidase Compound I, for cyclooxygenase initiation, and for cyclooxygenase propagation, and the concentrations of fatty acid and peroxidase cosubstrate. Because the substrate and cosubstrate concentrations were held constant in the present studies, the observed differences between PGHS-1 and -2 hydroperoxide requirements reside in differences in one or more intrinsic rate constants. In this connection, a slower rate of cyclooxygenase propagation has been shown to account for the higher hydroperoxide initiator required when oPGHS-1 reacts with eicosapentaenoate instead of arachidonate (Kulmacz et al., 1994b), and a slower rate for the initial perox-
idase reaction can account for the increase in initiator requirement when manganese protoporphyrin IX is substituted for heme in dPGHS-1 (Odenwallner et al., 1992; Kulmacz et al., 1994a). A detailed comparison of peroxidase and cyclooxygenase reaction kinetics of the two isoforms should identify the mechanistic basis for the lower hydroperoxide initiator requirement in hPGHS-2.

The sensitivities of the cyclooxygenase activities of the PGHS isoforms to suppression by GSP in vitro are likely to reflect the responses of the activities to hydroperoxide scavengers in vivo. GSP itself is a major peroxidase activity in many cells (Flohe, 1978) and other hydroperoxide scavengers have been shown to suppress cyclooxygenase activity, including the peroxidase activity of aspirin-treated dPGHS-1 (Kulmacz et al., 1985). It is worth noting that the GSP/Cox ratios found to suppress the cyclooxygenase activities of the purified PGHS isoforms fall in the range of those observed in several cell and tissue homogenates (Marshall et al., 1987). The general cellular hydroperoxide scavenging “potential” furnished by GSP is thus roughly analogous to the range of those observed in several cell and tissue homogenates.

Because of its lower requirement for initiator hydroperoxide, modulation of the hydroperoxide level could provide differential catalytic control of the isoforms no matter what their intracellular location(s). In any case, the large intrinsic difference between the isoforms in cyclooxygenase initiation efficiency provides a biochemical mechanism which may help explain the differential regulation of PGHS-1 and PGHS-2 catalysis in cells where the two enzymes serve very different physiological roles.

Acknowledgments—We thank Chris Walker and Chunhong Wei for help with enzyme purification, Dr. Timothy Hla for providing the human PGHS-2 cDNA, Dr. Paul Marshall for providing antibody against the hPGHS-2 C-terminal peptide, Dr. Pei-Feng Chen for advice on the Baculo virus expression system, and Dr. Ah-Lin Tsai for a critical reading of the manuscript.

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