Prostaglandin Synthase-Mediated Metabolism of Carcinogens and a Potential Role for Peroxyl Radicals As Reactive Intermediates

by Lawrence J. Marnett*

Prostaglandin-H synthase is unique among enzymes of the plant and animal kingdom in its ability to biosynthesize and metabolize hydroperoxides. Its cyclooxygenase activity oxygenates polyunsaturated fatty acids to hydroperoxy endoperoxides, and its peroxidase activity reduces the hydroperoxy group to hydroxy groups. Higher oxidation states of the peroxidase oxidize reducing substrates to electron-deficient derivatives that react with macromolecular nucleophiles. In the case of aromatic amines, the electron-deficient derivatives are mutagenic to bacterial and mammalian cells. β-Dicarbonyl compounds and retinoic acid are oxidized to carbon-centered radicals that react with O₂ to form peroxyl free radicals. Peroxyl radicals are the most stable oxy radicals and are able to diffuse some distance from the site of their generation. Peroxyl radicals are also formed during lipid peroxidation and in the reaction of polyunsaturated fatty acid hydroperoxides with metal complexes and metalloproteins. Peroxyl radicals epoxidize isolated double bonds of compounds such as 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (BP-7,8-diol); 3,4-dihydroxy-3,4-dihydrobenzo(a)anthracene; and aflatoxin B₁. The epoxide products represent the ultimate carcinogenic forms of the respective compounds. Techniques for quantitating the extent of peroxidase dependent or peroxyl radical-dependent metabolism in vivo make use of differences in the structure or stereochemistry of reactive intermediates formed by peroxidases relative to cytochromes P-450. Differences in the relative amounts of hydrolysis products and DNA adducts derived from anti- and syn-dihydropirole-oxides following application of BP-7,8-diol to mouse skin in vivo indicate peroxyl radicals play a significant role in metabolism of BP-7,8-diol in uninduced animals.

Introduction

Prostaglandin-H (PGH) synthase catalyzes the oxygenation of arachidonic acid to the hydroperoxy endoperoxide, PGG₂, and the reduction of PGG₂ to the hydroxy endoperoxide, PGH₂ [Eq. (1)] (1–3). PGH synthase is unique among all plant and animal peroxidases because it has the ability to biosynthesize the hydroperoxide substrate for its peroxidase activity. There has been a great deal of interest in the possible involvement of PGH synthase in the oxidative metabolism of drugs and carcinogens (4–7). The tissue distribution and mechanisms of oxidation of PGH synthase are distinct from those exhibited by the principal drug-metabolizing enzyme in animal tissue, cytochrome P-450. This raises the possibility that PGH synthase serves as an alternative metabolic activation enzyme to the P-450 isoenzymes. There appears little chance that PGH synthase plays a major role in systemic drug metabolism; but it may be involved in tissue-specific pathology by certain toxins or carcinogens. For example, PGH synthase-dependent oxidation is implicated in bladder carcinogenesis by aromatic amines and nitrofurans and in teratogenicity by trimethadone (7,8).

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\text{Arachidonic Acid} \xrightarrow{2 \text{O}_2} \text{PGG}_2 \xrightarrow{\text{Peroxidase}} \text{PGH}_2
\]

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Properties of PGH Synthase

Other mammalian peroxidases carry out similar oxidations to PGH synthase. Why, then, is there special interest in this particular peroxidase? Figure 1 illustrates some of the influences on PGH synthase that provide a partial answer to this question. The profile of polyunsaturated fatty acids in phospholipids and triglycerides is sensitive to the makeup of the fat eaten by particular individuals. Thus, there are dietary influences on the substrates that PGH synthase oxidizes. The cyclooxygenase activity of PGH synthase only oxidizes free fatty acids, the concentrations of which are very low in most cells (9,10). Release of substrate is the limiting step in prostaglandin biosynthesis and is triggered by the interaction of membrane-active agents with the cell surface (11). This makes PGH synthase and the oxidations it catalyzes exquisitely sensitive to events at the plasma membrane. The oxygenation of arachidonic acid by PGH synthase is potently inhibited by a variety of nonsteroidal anti-inflammatory drugs. In fact, it is generally believed that this inhibition explains the mechanism of anti-inflammatory activity of such drugs. Aspirin is noteworthy among the well-known anti-inflammatory agents because it inhibits PGH synthase by selectively acetylating the hydroxyl group of Ser506 (12).

Recent work establishes that the levels of PGH synthase in cells are sensitive to stimulation at the transcriptional and translational level by a variety of growth factors and tumor promoters, including epidermal growth factor (EGF), transforming growth factor β, interleukin-1 (IL-1), tetradecanoyl phorbol acetate, and tumor necrosis factor (13–17). Enhanced expression of the PGH synthase gene by EGF and IL-1 appear to be inhibited by corticosteroids (18,19), which may explain the potent anti-inflammatory activity of EGF and IL-1. An alternative mechanism for rapid modulation of PGH synthase protein levels is supported by the report that the half-life of the protein in rat muscle is less than 10 min (20). This half-life implies rapid turnover by proteolysis, but the proteases responsible and the generality of their action in other tissues has not been established. The summation of these observations identifies PGH synthase as an enzyme that triggers intracellular oxidations in response to events at the cell surface while demonstrating sensitivity to a variety of physiological and pharmacological modulators.

The initial product of PGH synthase action is the hydroperoxy endoperoxide PGG2. Its hydroperoxide group triggers the formation of potent oxidizing agents, either metal-oxo derivatives of the heme prosthetic group or oxygen radicals derived from the hydroperoxide (21,22). The endoperoxide group is metabolized to prostaglandins and thromboxane, which are potent autocrine and paracrine mediators of cell responses. PGG2 is also converted to malondialdehyde, which is a mutagen and carcinogen (23–25). Although the emphasis of this paper is on mechanisms of oxidation by PGH synthase, which is usually associated with tumor initiation, PGH synthase may also play an important role in proliferative events such as those associated with tumor promotion and progression (26).

PGH synthase is widely distributed in mammalian tissue but is particularly abundant in accessory sex glands, lung, and vasculature (27). PGH synthase is a membrane protein located in the endoplasmic reticulum, the nuclear membrane, and occasionally in the plasma membrane (28,29). The enzyme purified from ovine or bovine seminal vesicles is a homodimer of 70 kDa subunits (30–38). It contains 3.5% carbohydrate of the high mannose type and variable amounts of ferric protoporphyrin IX (Fe3+-PPIX) (39). The heme group is essential for both cyclooxygenase and peroxidase activities but is readily lost during solubilization and chromatography. Both activities are reconstituted following addition of Fe3+-PPIX, whereas reconstitution with Mn3+-PPIX produces an enzyme with high cyclooxygenase but low peroxidase activity (40). There is some disagreement about the stoichiometry of heme binding, but most estimates place it between one and two hemes per homodimer (41,42). The amino acid sequence of PGH synthase is available from its cDNA sequence (43–45). The open reading frame codes for 600 amino acids, of which the first 24 represent a signal sequence that is absent in the mature protein. Computer analysis reveals no striking parallels to other functionally related proteins. Speculation has been offered for the location of membrane binding and heme binding regions of the polypeptide (43,46).
Hydroperoxide-Dependent Oxidations

Cooxidation during arachidonic acid oxygenation results from interactions of PGH$_2$ or other hydroperoxy fatty acids with the heme prosthetic group of PGH synthase or other metals and metalloproteins in the cells. The two general pathways of hydroperoxide reduction by metal complexes are shown in Equations (2) and (3). One-electron reduction of the hydroperoxide produces an alkoxyl free radical and a ferryl-oxo complex that is one redox equivalent above Fe$^{3+}$-PPIX [Eq. (2)]. Two-electron reduction of the hydroperoxide produces an alkoxide ion and a ferryl-oxo complex that is two redox equivalents above Fe$^{3+}$-PPIX [Eq. (3)]. Both ferryl-oxo complexes and the alkoxyl free radical are potent oxidizing agents. Studies with a diagnostic hydroperoxide probe, 10-hydroperoxy-octadeca-8,12-dienoic acid, establish that the heme prosthetic group of PGH synthase reduces hydroperoxides by two electrons [Eq. (3)] (46). The two-electron oxidized heme has been detected by rapid scan spectrophotometry and appears electronically very similar to the higher oxidation state known as compound I of horseradish peroxidase (47,48). The prosthetic group of compound I contains a ferryl-oxo complex (Fe$^{4+}$-O) and a porphyrin cation radical (49).

\[ \text{ROOH} + M^{n+} \rightarrow RO^- + M^{n+1} \]  \hspace{1cm} (2)

\[ \text{ROOH} + M^{n+} \rightarrow ROH + M^{n+2} \]  \hspace{1cm} (3)

PGH synthase catalyzes a number of oxidations that appear similar to those of horseradish peroxidase, but a comprehensive comparison of the substrate specificity of the two enzymes has not been performed. All the available mechanistic information indicates PGH synthase follows a classic peroxidase catalytic cycle (Fig. 2). Reduction of the hydroperoxide oxidizes the heme to compound I. This intermediate must be reduced by two electrons to regenerate resting enzyme. Heme reduction occurs by either stepwise electron transfer or what appears to be concerted oxygen transfer. Oxygen transfer is only observed with alkyl aryl sulfides (50,51).

Certain amines and phenols are excellent substrates for electron transfer oxidation by peroxidases. Amines produce cation radicals and phenols produce phenoxyl radicals (52). Certain ß-dicarbonyl compounds and polynes are oxidized to carbon-centered radicals by electron transfer or hydrogen atom abstraction (53–55). The fate of the one-electron oxidized derivatives of the reductants determines the eventual products of the reaction; for example, amine cation radicals disproportionate to iminium cations and the parent amine [Eq. (4)] (56). The iminium compounds undergo subsequent reactions such as hydrolysis. Phenoxyl radicals couple to form biphens that can be further oxidized to quinonoid compounds [Eq. (5)] (52). Diamines and polyphenolic compounds (benzidine, catechol) are particularly good substrates and are oxidized to quinoneimines and quinones, respectively (6,57,58). Some of the one-electron oxidized derivatives of the reducing substrates or their metabolites (e.g., amine cation radicals, quinoneimines, and quinones) react with nucleophilic sites on proteins and nucleic acids. This reaction has been suggested to account for the toxicity/mutagenicity or transforming activity of aromatic amines, nitrofurans, and diethylstilbestrol (7,59–61).

Peroxyl Free Radicals

In contrast to the reactions of nitrogen- and oxygen-centered radicals, carbon-centered radicals couple to molecular oxygen to form peroxyl radicals (62). This is a very important reaction because peroxyl radicals are long-lived and capable of epoxidation of isolated double

![Figure 2. Peroxidase catalytic cycle.](image-url)
bonds. A particularly interesting example of this epoxidation is provided by retinoic acid. Retinoic acid is a potent inhibitor of tumor promotion in mouse skin (63–65) and is a moderately good reducing substrate for the peroxidase of PGH synthase (54). Retinoic acid is oxidized to a carbon-centered radical that forms a peroxyl radical [Eq. (6)]. This peroxyl radical then epoxidizes another molecule of retinoic acid, producing equal amounts of 4-hydroxy retinoic acid and 5,6-epoxy retinoic acid [Eq. (7)] (54). The reactivity of retinoic acid toward peroxyl radicals is quite high among a series of polyenes we have tested. This makes retinoic acid an excellent radical scavenger and antioxidant (66). Whether this property contributes to its chemopreventive activity is unknown.

\[
\begin{align*}
\text{H} & \quad \text{COH} \\
\text{H} & \quad \text{COH}
\end{align*}
\]

Peroxyl radicals are recognized as the principal mediators of lipid peroxidation, but they have been essentially overlooked as mediators of hydroperoxide-dependent oxidations until recently (69,70). In contrast to alkoxyl radicals and metal-oxo complexes, peroxyl radicals are relatively stable and somewhat selective in their reactions. The half-lives of peroxyl radicals range from 0.1 to 10 sec in the biological milieu (71). This half-life means that peroxyl radicals are able to diffuse to cellular locations remote from the site of their generation to effect oxidations. An important reaction of peroxyl radicals is their ability to epoxidize olefins [Eq. (9)] (72). It is not certain what endogenous cellular compounds, if any, are epoxidized, but there is no doubt xenobiotics and their metabolites are epoxidized by peroxyl radicals (70).

\[
\begin{align*}
\text{R} & \quad \text{O} \\
\text{R} & \quad \text{O}
\end{align*}
\]

In addition to the anticarcinogen retinoic acid, peroxyl radicals epoxidize the procarcinogens 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene (BP-7,8-diol); 3,4-dihydroxy-3,4-dihydrobenzo(a)anthracene; and aflatoxin B1 (73–77). The epoxide products of each of these reactions represent the ultimate carcinogenic forms of the parent molecules [benzo(a)pyrene and benzo(a)anthracene for the first two compounds]. Peroxyl radical-dependent epoxidation of BP-7,8-diol has been detected during such diverse processes as PGH synthase-dependent cooxidation, NADPH-dependent lipid peroxidation, cumene hydroperoxide-dependent lipid peroxidation, hematin-catalyzed decomposition of polyunsaturated fatty acid hydroperoxides, peroxidase-dependent oxidation of \( \beta \)-dicarbonyl compounds, and peroxidase-dependent oxidation of bisulfite (74,76,78–81). The common thread through these reactions is the generation of peroxyl free radicals.

Techniques for Quantitating Hydroperoxide-Dependent Oxidations

Quantitating the extent to which hydroperoxide-dependent xenobiotic oxidations occur in intact cells or in vivo requires specific methodology for each compound of interest. In some cases, one can exploit the different mechanisms of oxidation of xenobiotics by peroxidases compared to cytochrome P-450 to design experiments to detect peroxidase products. For example, aromatic
Monoamines are oxidized by peroxidases to radical cations that form different adducts to DNA than do the nitrenium ions that formed in cytochrome P-450-dependent reactions (82). Peroxidase-dependent adducts have been detected in DNA from the urinary bladders of dogs fed β-naphthylamine (83). Unfortunately, the identities of the adducts have not been elucidated, and their formation appears quite variable. Thus, the generality of the formation of the adducts is not certain.

Stereochemistry has been used to differentiate epoxidation of polycyclic hydrocarbon dihydrodiols by peroxyl radicals and cytochromes P-450 (84). Figure 3 summarizes the stereochemical considerations that make this approach feasible. The (−)-enantiomer of BP-7,8-diol is epoxidized to the (−)-enantiomer of the anti-dihydrodiolepoxide by both peroxyl radicals and cytochromes P-450. In contrast, the (+)-enantiomer of BP-7,8-diol is epoxidized to the (+)-enantiomer of the anti-dihydrodiolepoxide by peroxyl radicals, but to the (+)-enantiomer of the syn-dihydrodiolepoxide by cytochromes P-450 (84). Dihydrodiolepoxides hydrolyze to tetrahydrotetraols that are readily separable by HPLC, which enables one to quantify the amounts of the dihydrodiolepoxides produced and to identify the oxidant responsible for their formation (85). Alternatively, one can isolate DNA modified by BP-7,8-diol oxidation products, hydrolyze it to deoxynucleoside adducts, and quantitative the levels of adducts produced by reaction with the various dihydrodiolepoxide enantiomers and diastereomers. By starting with commercially available (+)-BP-7,8-diol, one can directly determine the percentage of epoxides produced by peroxyl radicals or cytochrome P-450.

The stereochemical approach has been employed to detect peroxyl radical-dependent epoxidation of BP-7,8-diol in rat liver microsomes, mouse skin homogenates, cultured fibroblasts, cultured hamster trachea, and freshly isolated mouse epidermal cells (79,86–89). Figure 4 displays HPLC profiles of the products of (+)-BP-7,8-diol oxidation by epidermal homogenates from control female CD-1 mice and mice pretreated with the cytochrome P-450 inducer β-naphthoflavone (86). Peroxyl radical products predominate in the uninduced animals, whereas cytochrome P-450 products are the principal metabolites in β-naphthoflavone-pretreated animals. This suggests that peroxyl radicals are important contributors to BP-7,8-diol oxidation in the skin of normal animals, whereas cytochrome P-450 has primacy in induced animals. Since BP-7,8-diol is a tumor initiator in mouse skin but is not a cytochrome P-450 inducer, it seems peroxyl radicals play a significant role in the metabolic activation of BP-7,8-diol to an ultimate carcinogenic form in at least one tissue commonly used to assay tumor initiators (86,89). These conclusions are sup-

**Figure 3.** Comparison of the stereochemistry of epoxidation of BP-7,8-diol by cytochrome P-450 and peroxyl radicals.

**Figure 4.** Reverse-phase HPLC profiles of the tetraol hydrolysis products formed after (+)-BP-7,8-diol epoxidation by mouse skin homogenates. (TA) trans-anti-tetraol, the major hydrolysis product of the (−)-anti-dihydrodiolepoxide; (CS) cis-syn-tetraol, the major hydrolysis product of the (+)-syn-dihydrodiolepoxide; and (TS) trans-syn-tetraol, the minor hydrolysis product of the (+)-syn-dihydrodiolepoxide. Reprinted with permission (89).
ported by studies of BP-7,8-diol metabolism to tetraols or DNA adducts in mouse skin in vivo (86,90).

A common approach to detecting metabolic pathways is the use of specific inhibitors. Numerous compounds inhibit the cyclooxygenase activity of PGH synthase (91). These compounds do not block the peroxidase activity of the enzyme, but by preventing PGG synthesis, they inhibit PGH synthase-dependent cooxidation. Agents such as aspirin and indomethacin are quite useful in determining the percentage oxidation of a particular compound dependent on PGG2 biosynthesis. However, PGH synthase inhibitors do not block hydroperoxide synthesis by lipoygenases or by lipid peroxidation. This fact makes PGH synthase inhibitors of limited utility for diagnosing the overall contribution of hydroperoxide-dependent oxidation to the metabolism of a given compound, particularly in intact cells or in vivo. For example, indomethacin does not inhibit oxidation of (+)-BP-7,8-diol to (-)-anti-dihydrolleipoxyde by freshly isolated mouse epidermal cells (89). Other biochemical studies establish that the peroxides responsible for BP-7,8-diol oxidation in epidermis arise from lipid peroxidation (89).

Even if one does see inhibition of oxidation of a compound by an inhibitor of PGH synthase, one must interpret the observation with caution. In intact cells, toxicity or alteration of cell cycle kinetics can be depress the extent of metabolism and/or DNA adduction without actually inhibiting the oxidative reaction. Conclusions about the role of PGH synthase cooxidation in xenobiotic metabolism based on inhibitor studies should be supported by parallel studies of the effects of the inhibitors on the metabolites and macromolecular adducts derived from the test compounds.

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