Mechanism of Hydroperoxide Reduction by Mangano-Prostaglandin Endoperoxide Synthase

Lisa M. Landino and Lawrence J. Marnett*

A. B. Hancock, Jr., Memorial Laboratory for Cancer Research, Center in Molecular Toxicology, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received October 25, 1995[⊗]

ABSTRACT: Reaction of manganese-reconstituted prostaglandin endoperoxide synthase (Mn-PGHS) with 15-hydroperoxyeicosatetraenoic acid (15-HPETE) generates two products in nearly equal amounts: 15-hydroxyeicosatetraenoic acid (15-HETE) and 15-ketoeicosatetraenoic acid (15-KETE) [Kulmacz et al. (1994) *Biochemistry 33*, 5428−5439]. Their proposed mechanism to explain 15-KETE formation, namely oxidation of 15-HETE by the peroxidase activity of Mn-PGHS, was tested and found not to occur. Instead, 15-KETE may arise by one-electron reduction of 15-HPETE followed by oxidation of an intermediate alkoxyl radical. The mechanism of hydroperoxide reduction by Mn-PGHS was investigated using 10-hydroperoxyoctadeca-8,12-dienoic acid (10-OOH-18:2), a diagnostic probe of hydroperoxide reduction pathways. Reaction of Mn-PGHS with 10-OOH-18:2 generated the two-electron reduced product, 10-hydroxyoctadeca-8,12-dienoic acid (10-OH-18:2), as well as the one-electron reduction products, 10-oxooctadeca-8,12-dienoic acid (10-oxo-18:2) and 10-oxodec-8-enoic acid (10-oxo-10:1) in relative yields of 82, 10, and 7%, respectively. The identity of the one-electron reduction products was confirmed by electrospray ionization mass spectrometry. The detection of 10-oxo-10:1 provides strong evidence for the production of an alkoxyl radical during 10-OOH-18:2 reduction by Mn-PGHS. Like 15-HPETE, reaction of Mn-PGHS with 13-hydroperoxyoctadeca-8,12-dienoic acid (13-OOH-18:2) generated two products in equal amounts: 13-hydroxyoctadeca-8,12-dienoic acid (13-OH-18:2) and the keto fatty acid 13-oxooctadeca-8,12-dienoic acid (13-oxo-18:2). Comparison of the three hydroperoxides demonstrates that 15-HPETE is a much better substrate for Mn-PGHS than 10-OOH-18:2 or 13-OOH-18:2 with 10-fold greater turnovers. The results show that Mn-PGHS catalyzes both one- and two-electron hydroperoxide reduction and that the pathway of alkoxyl radical decomposition is influenced by the protein component of Mn-PGHS and the structure of the alkoxyl radical intermediate.

Prostaglandin endoperoxide (G/H) synthase (PGHS)[1] is a hemeprotein that catalyzes the first two steps in the prostaglandin and thromboxane biosynthetic pathway. The cyclooxygenase activity of PGHS catalyzes the bis-oxygenation of arachidonic acid to form the hydroperoxy endoperoxide, PGG_2, which is then reduced to the hydroxy endoperoxide, PGH_2, by the enzyme's peroxidase activity (Hamberg et al., 1974; Nugteren & Hazelhof, 1973). Both the cyclooxygenase and peroxidase activities of PGHS require the participation of the heme prosthetic group.

The catalytic cycle of the PGHS peroxidase is typical of heme-containing peroxidases and displays highest activity toward fatty acid hydroperoxides. Reaction with hydroperoxide leads to the rapid formation of a spectroscopically detectable enzyme intermediate known as compound **I** (eq 1) (Lambeir et al., 1985; Anni & Yonetani, 1992). Release of the two-electron reduced product, the fatty acid alcohol, concomitant with formation of compound **I** indicates that the hydroperoxide oxygen−oxygen bond is cleaved heterolytically (Markey et al., 1987). The reaction of fatty acid hydroperoxides with Fe-PGHS gives >95% heterolytic cleavage and generates a detectable compound **I** intermediate (Labeque & Marnett, 1987; Lambeir et al., 1985). The spectral properties of compound **I** of Fe-PGHS are consistent with a Fe^{IV}═O porphyrin cation radical. One-electron reduction of compound **I** by an electron donor leads to formation of compound **II**, a Fe^{IV}═O species with the porphyrin fully covalent (eq 2). One-electron reduction of compound **II** regenerates the resting Fe(III) state of the enzyme (eq 3).

$$Fe^{3+}PPIX + ROOH \rightarrow O{=}Fe^{4+}PPIX^{\bullet+} + ROH \quad (1)$$

$$O{=}Fe^{4+}PPIX^{\bullet+} + e \rightarrow O{=}Fe^{4+}PPIX \quad (2)$$

$$O{=}Fe^{4+}PPIX + e \rightarrow Fe^{3+}PPIX + H_2O \quad (3)$$

The protein component of PGHS facilitates formation of the peroxidase higher oxidation states and also defines the mechanism of hydroperoxide cleavage by the heme prosthetic group. Whereas FePGHS catalyzes exclusively two-electron reduction of a hydroperoxide, FePPIX in the absence of

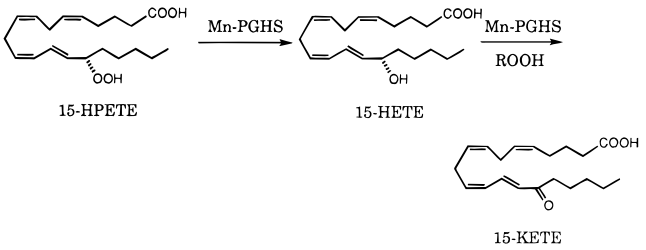
* To whom correspondence should be addressed. Telephone: 615-343-7329. Fax: 615-343-7534. E-mail: marnettlj@macpost.vanderbilt.edu.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1996.

[1] Abbreviations: PGHS, prostaglandin endoperoxide synthase (EC 1.14.99.1); FePPIX, iron(III) protoporphyrin IX; MnPPIX, manganese(III) protoporphyrin IX; Mn-PGHS, manganese-reconstituted prostaglandin H synthase; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 15-KETE, 15-ketoeicosatetraenoic acid; 10-OOH-18:2, 10-hydroperoxyoctadeca-8,12-dienoic acid; 10-OH-18:2, 10-hydroxyoctadeca-8,12-dienoic acid; 10-oxo-18:2, 10-oxooctadeca-8,12-dienoic acid; 10-oxo-10:1, 10-oxodec-8-enoic acid; 13-OOH-18:2, 13-hydroperoxy-8,12-octadecadienoic acid; 13-OH-18:2, 13-hydroxy-8,12-octadecadienoic acid; 13-oxo-18:2, 13-oxooctadeca-8,12-dienoic acid; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine; BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography.

0006-2960/96/0435-2637$12.00/0

Scheme 1



protein catalyzes primarily one-electron hydroperoxide reduction (eq 4) (Dix & Marnett, 1985; Labeque & Marnett, 1987; Wilcox & Marnett, 1993).

$$Fe^{3+}PPIX + ROOH \rightarrow O{=}Fe^{4+}PPIX + RO^{\bullet} \quad (4)$$

Manganese-substituted PGHS (Mn-PGHS) can be formed by reconstitution of apo-PGHS with manganese(III) protoporphyrin IX (MnPPIX). Mn-PGHS has been a valuable mechanistic tool in the investigation of the relationship between the cyclooxygenase and peroxidase activities of PGHS. Whereas the cyclooxygenase activity of Mn-PGHS is similar to that of Fe-PGHS, the peroxidase activity of Mn-PGHS is only 0.8% of Fe-PGHS activity (Odenwaller et al. 1992, Streider et al., 1992). The reaction of hydroperoxides with Mn-PGHS generates a spectroscopically detectable higher oxidation state of the enzyme identified as a Mn(IV) species (Odenwaller et al., 1992; Streider et al., 1992). A very short-lived Mn(V) species that is a precursor to the Mn(IV) intermediate is inferred because two-electron reduction of 5-phenyl-4-pentenyl 1-hydroperoxide (PPHP) to 5-phenyl-4-pentenyl-1-alcohol (PPA) is observed (Odenwaller et al., 1992).

Kulmacz et al. recently reported that the reaction of 15-hydroperoxyeicosatetraenoic acid (15-HPETE) with Mn-PGHS, in the presence of a reducing substrate, generates two products in approximately equal amounts: 15-hydroxyeicosatetraenoic acid (15-HETE) and a less polar product, 15-ketoeicosatetraenoic acid (15-KETE) (Scheme 1) (Kulmacz et al., 1994). They proposed that 15-KETE is formed by oxidation of 15-HETE by the higher oxidation states of Mn-PGHS. A precedent exists for alcohol oxidation by metal−oxo derivatives of heme proteins in that catalase oxidizes primary alcohols to aldehydes during reduction of primary hydroperoxides (Schonbaum & Chance, 1976). However, alcohol oxidation is not normally observed with peroxidases which have a different proximal heme ligand compared to catalase (histidine vs tyrosine).

The suggestion of this unusual mechanism for hydroperoxide metabolism by Mn-PGHS prompted us to conduct several direct tests of its occurrence. None of the results of these experiments was consistent with 15-HETE oxidation by higher oxidation states of Mn-PGHS. Therefore, we performed additional experiments designed to evaluate the mechanism of hydroperoxide reduction by Mn-PGHS. The results of these experiments indicated that Mn-PGHS catalyzes a significant percentage of one-electron reduction of fatty acid hydroperoxides to fatty acid alkoxyl radicals. We propose that the alkoxyl radicals are oxidized to ketones by the higher oxidation state of Mn-PGHS which is detected during catalytic turnover. This accounts for the generation of ketones during the metabolism of fatty acid hydroperoxides by Mn-PGHS.

MATERIALS AND METHODS

Materials. Unlabeled arachidonic acid (20:4) and linoleic acid (18:2) were from Nu-Chek Prep, Inc. (Elysian, MN). [1-^{14}C]Arachidonic acid (57 mCi/mmol) and [1-^{14}C]linoleic acid (53 mCi/mmol) were from New England Nuclear (Wilmington, DE). Soybean lipoxygenase (type I-B), glutathione peroxidase (from bovine erythrocytes), reduced glutathione, and hematin (FePPIX) were from Sigma. Guaiacol, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine (TMPD), and BHT were from Aldrich. Manganese(III) protoporphyrin IX chloride (MnPPIX) was from Porphyrin Products (Logan, UT).

PGHS was purified from ram seminal vesicles as described previously and stored at −80 °C (Marnett et al., 1984). Apo-PGHS was prepared as described and was 99.5−100% apo as judged by cyclooxygenase activities in the presence and absence of FePPIX (Odenwaller et al., 1990). The cyclooxygenase activity was determined by measuring oxygen consumption at 37 °C with a Gilson model 5/6 oxygraph equipped with a Clark electrode and a thermostatted cuvette (Gilson Medical Electronics, Inc., Middleton, WI). The specific activity of apo-PGHS was 20−25 μmol of AA min^{-1} mg^{-1}. All hydroperoxide reduction assays were performed with apo-PGHS reconstituted with either FePPIX or MnPPIX.

Preparation of [1-^{14}C]-15-HPETE. [1-^{14}C]-15-HPETE was prepared enzymatically from [1-^{14}C]arachidonic acid (20 μmol, 20 μCi) in 40 mL of 0.1 M borate buffer (pH 9.0) using soybean lipoxygenase (0.6 mg) (Funk et al., 1976). [1-^{14}C]-15-HPETE was purified either by preparative HPLC on a Zorbax Rx-Sil column (5 μm, 9.4 × 250 mm) or by silicic acid chromatography. 15-HPETE was eluted isocratically from the preparative HPLC column in hexane/2-propanol/glacial acetic acid (984/15/1) at a flow rate of 2.0 mL/min. The eluent was monitored at 235 nm, and the 15-HPETE was collected manually. For the silicic acid chromatography purification, arachidonic acid was eluted with 95/5 hexane/ethyl acetate and 15-HPETE was eluted with 85/15 hexane/ethyl acetate. In both cases, the solvent was evaporated and the 15-HPETE was stored at −20 °C in methanol. The concentration of [1-^{14}C]-15-HPETE was determined from the UV absorption at 235 nm using a molar absorptivity of 23 000 M^{-1} cm^{-1}(Funk et al., 1976). The [1-^{14}C]-15-HPETE was >99% pure by HPLC with radioactivity detection.

Preparation of [1-^{14}C]-15-HETE. [1-^{14}C]-15-HETE was prepared enzymatically from [1-^{14}C]arachidonic acid using soybean lipoxygenase, glutathione peroxidase, and reduced glutathione. Soybean lipoxygenase (0.5 mg) was added to [1-^{14}C]arachidonic acid (10 μmol, 12 μCi) in 20 mL of 0.1 M borate buffer (pH 9.0), and the reaction mixture was stirred for 8 min at room temperature. At that point, 20 units of glutathione peroxidase and reduced glutathione (final concentration of 5 mM) were added directly to the reaction mixture. After 5 min, the reaction was quenched by addition of 2 mL of 1 N HCl, and the products were extracted with ether. The combined ether layers were dried with anhydrous $MgSO_4$, and the solvent was evaporated. Under these conditions, complete conversion of [1-^{14}C]arachidonic acid to [1-^{14}C]-15-HETE was achieved, and the 15-HETE was >99% pure by HPLC with radioactivity detection. The material was used without further purification. The con-

centration of [1-^{14}C]-15-HETE was determined from the UV absorption at 235 nm using a molar absorptivity of 23 000 M^{-1} cm^{-1} (Funk et al., 1976).

Synthesis of [1-^{14}C]Linoleate Hydroperoxides. [1-^{14}C]-Linoleate hydroperoxides were synthesized from linoleic acid by photosensitized oxygenation as described (Labeque & Marnett, 1987, 1988) with minor modifications. A solution of linoleic acid (45 mg, 45 μCi) in methanol (40 mL) containing methylene blue (3 mg) and BHT (5 mg) was irradiated with a 150 W flood lamp. The solution was also bubbled with oxygen or air during irradiation. The solution was irradiated for a total of 34 h, and 70% of the linoleic acid was converted to products. The unreacted linoleic acid was separated from the hydroperoxides by silicic acid chromatography. Linoleic acid was eluted with 95/5 hexane/ethyl acetate, and the hydroperoxides were eluted with 85/15 hexane/ethyl acetate. The solvent was evaporated, and the hydroperoxides were stored in methanol at −20 °C.

Purification of [1-^{14}C]-10-OOH-18:2 and [1-^{14}C]-13-OOH-18:2. The positional hydroperoxide isomers generated by photooxygenation of linoleic acid were separated by HPLC using a Zorbax Rx-Sil semipreparative column (5 μm, 9.4 × 250 mm) with UV detection at 235 nm. Hydroperoxides were eluted isocratically in hexane/2-propanol/glacial acetic acid (984/15/1) at a flow rate of 2.0 mL/min. 13-OOH-18:2 eluted first (retention time = 25 min), followed by the 12-, 10-, and 9-isomers. Both 13-OOH-18:2 and 9-OOH-18:2 absorb strongly at 235 nm, but the 12- and 10-isomers do not. The solvent was evaporated, and the individual isomers were resuspended in methanol and stored at −20 °C. The isomers were >98% pure by HPLC with radioactivity detection.

Hydroperoxide Reduction Assays. Apo-PGHS was reconstituted with 1 or 0.5 equiv of the metal porphyrin, FePPIX or MnPPIX, in 1 mL of 0.1 M Tris-HCl (pH 8.0) for 2 min. The reducing substrate, guaiacol or TMPD (100−500 μM), was then added. Reaction was initiated with the addition of radiolabeled hydroperoxide in methanol to a final concentration of 50 or 100 μM.

Apo-PGHS (100 nM) was used for assays with [1-^{14}C]-15-HPETE, and 500 nM apo-PGHS was used with [1-^{14}C]-10-OOH-18:2 and [1-^{14}C]-13-OOH-18:2. Following incubation at room temperature for varying periods of time, the reaction was quenched with 1 N HCl and the lipid products were extracted with 2 volumes of ether. The ether layers were dried with anhydrous $MgSO_4$, and the solvent was evaporated under nitrogen. The dried lipid products were resuspended in hexane and subjected to HPLC analysis using a Zorbax Rx-Sil analytical column (4.6 × 250 mm). Products were eluted isocratically in hexane/2-propanol/glacial acetic acid (987/12/1) at a flow rate of 1 mL/min. The radiolabeled products were quantitated using a radioactivity flow detector (IN/US Systems, Inc., Tampa, FL).

The hydroperoxides (50 μM) were reacted with the free porphyrins (0.5 μM) in 0.1 M $NaPO_4$ (pH 7.8) containing 0.1% Tween 20 and 200 μM guaiacol. Reactions were quenched after 5 min with 1 N HCl, and the lipid products were analyzed as described above.

RESULTS

Hydroperoxide reduction by Mn-PGHS was assayed with [1-^{14}C]-15-HPETE and a reducing substrate such as guaiacol or TMPD. The ^{14}C-labeled metabolites were separated by straight phase HPLC and quantitated using an in-line radioactivity flow detector (Figure 1). The top frame displays the elution profile of UV-absorbing compounds, whereas the bottom frame displays the elution profile of radioactive compounds. The reaction of Mn-PGHS with [1-^{14}C]-15-HPETE consistently generated two products: 15-HETE and 15-KETE (Figure 1). Nearly equal amounts of the two products were formed under varying conditions of incubation time and enzyme concentration as reported by Kulmacz et al. (1994). Reaction of [1-^{14}C]-15-HPETE with the same apoenzyme preparation reconstituted with FePPIX generated >95% 15-HETE (Figure 2).

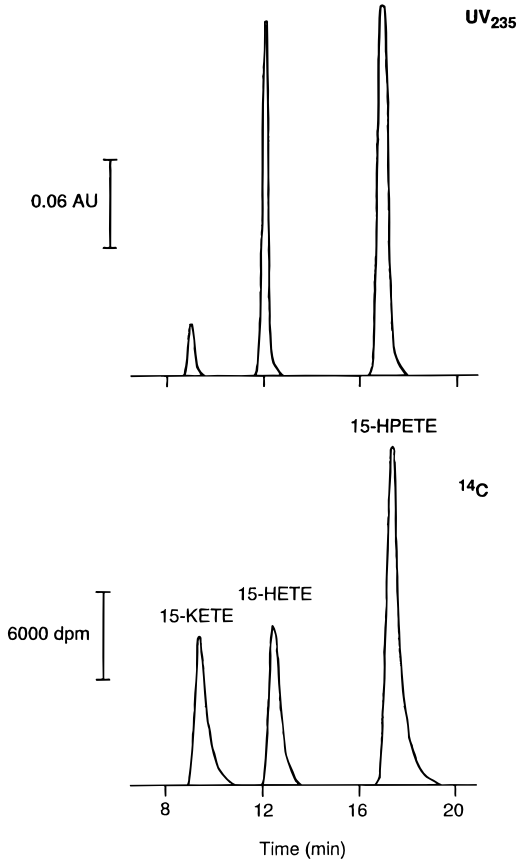


FIGURE 1: Radioactivity and UV_{235} profiles of products of reaction of Mn-PGHS with [1-^{14}C]-15-HPETE. Mn-PGHS (100 nM apo-PGHS reconstituted with 50 nM MnPPIX) was assayed with 100 μM [1-^{14}C]-15-HPETE and 200 μM guaiacol in 0.1 M Tris (pH 8.0) for 2 min. Elution was performed with hexane/2-propanol/acetic acid (987/12/1) at a flow rate of 1.0 mL/min using a Zorbax Rx-Sil column (5 μm silica, 4.6 × 250 mm).

If Mn-PGHS utilizes 15-HETE as a peroxidase-reducing substrate, incubation of 15-HETE with Mn-PGHS and a hydroperoxide should produce 15-KETE. Therefore, we incubated Mn-PGHS with equimolar amounts of [1-^{14}C]-15-HETE and unlabeled 15-HPETE (50 μM) in the presence of 200 μM guaiacol. A chromatogram of the product profile is shown in Figure 3. Three peaks were observed in the UV trace: the reaction products, 15-KETE and 15-HETE, and unreacted 15-HPETE. The products were formed from the reaction of unlabeled 15-HPETE with Mn-PGHS. The UV profile at 235 nm shows a much smaller 15-KETE peak relative to 15-HETE because its UV maximum is 280 nm. In addition, the 15-HETE peak observed in the UV trace is comprised of both the unlabeled 15-HETE generated as a reaction product and [1-^{14}C]-15-HETE added as a potential

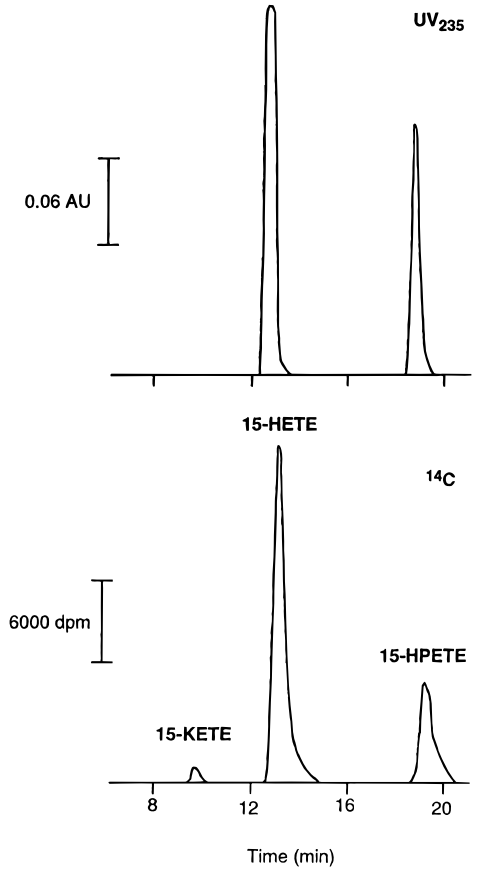


FIGURE 2: Radioactivity and UV_{235} profiles of products of reaction of Fe-PGHS with [1-^{14}C]-15-HPETE. Fe-PGHS (100 nM apo-PGHS reconstituted with 50 nM FePPIX) was assayed with 100 μM [1-^{14}C]-15-HPETE and 200 μM guaiacol in 0.1 M Tris (pH 8.0) for 2 min. Elution was performed with hexane/2-propanol/acetic acid (987/12/1) at a flow rate of 1.0 mL/min using a Zorbax Rx-Sil column (5 μm silica, 4.6 $\times$ 250 mm).

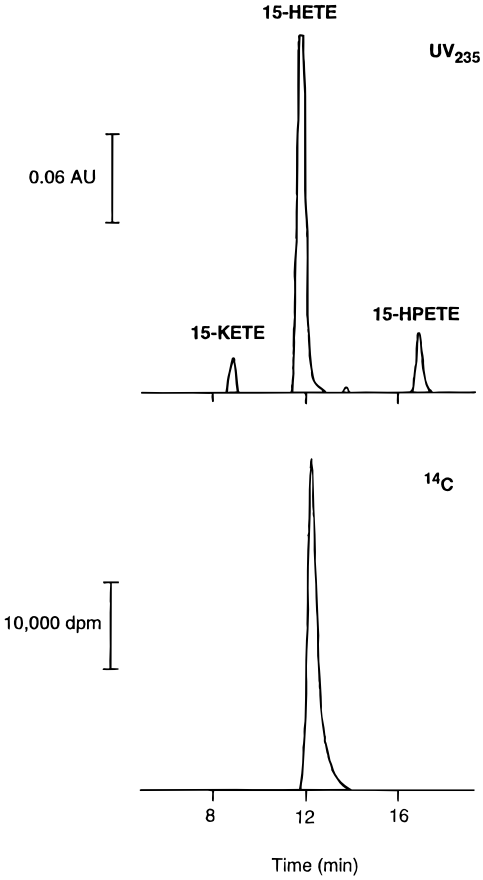


FIGURE 3: Radioactivity and UV_{235} profiles of metabolism of [1-^{14}C]-15-HETE in the presence of unlabeled 15-HPETE by Mn-PGHS. Mn-PGHS (100 nM apo-PGHS reconstituted with 50 nM MnPPIX) was assayed with 50 μM 15-HPETE, 50 μM [1-^{14}C]-15-HETE, and 200 μM guaiacol in 0.1 M Tris (pH 8.0) for 2 min. Elution was performed with hexane/2-propanol/acetic acid (987/12/1) at a flow rate of 1.0 mL/min using a Zorbax Rx-Sil column (5 μm silica, 4.6 $\times$ 250 mm).

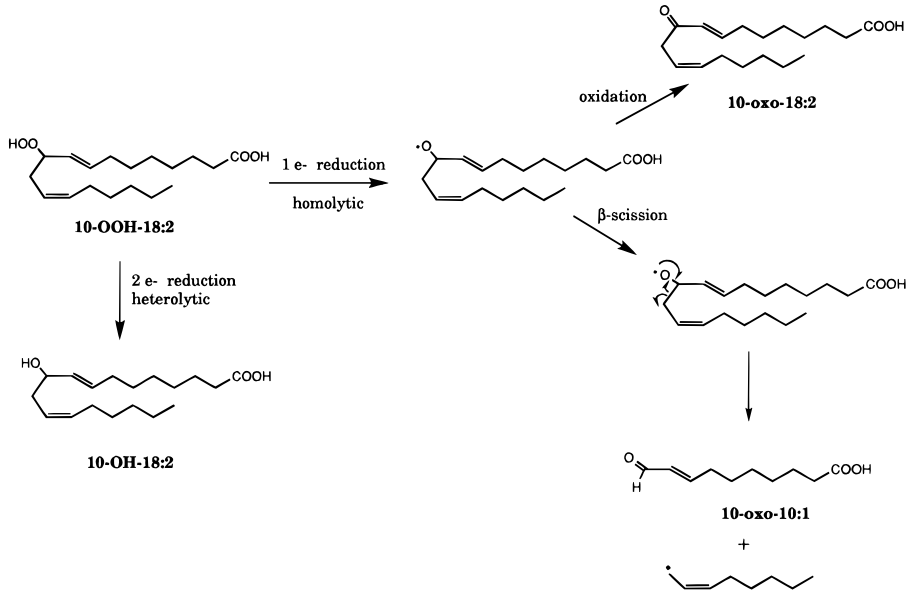
substrate. Although the UV profile demonstrates that metabolism of unlabeled 15-HPETE occurred, the radioactivity profile shows that no metabolism of [1-^{14}C]-15-HETE to 15-KETE occurred.

It is possible that exogenously added 15-HETE does not reach the peroxidase active site but that 15-HETE generated at the active site by 15-HPETE reduction remains at the active site and is oxidized by a second round of peroxidase turnover. If so, Mn-PGHS should convert 15-HPETE to 15-HETE, then to 15-KETE in the absence of a reducing substrate. To test this, Mn-PGHS was assayed with [1-^{14}C]-15-HPETE in the absence of a reducing substrate. Little or no turnover of [1-^{14}C]-15-HPETE was observed ($<$5% products). The amount of turnover was comparable to the amount of nonenzymatic 15-HPETE degradation that occurred during incubations without enzyme. This lack of [1-^{14}C]-15-HPETE metabolism in the absence of a reducing substrate is not consistent with a mechanism of 15-KETE generation in which 15-HETE is an intermediate.

The assays performed with Mn-PGHS and 15-HPETE indicate that 15-KETE does not arise from oxidation of 15-HETE by a higher oxidation state of the peroxidase. An alternative hypothesis is that 15-KETE arises directly from 15-HPETE without the intermediacy of 15-HETE. A mechanism for which substantial precedent exists is one in which 15-HPETE is reduced to a fatty acid alkoxyl radical (eq 4) which is then oxidized to 15-KETE (Dix & Marnett, 1985; Labeque & Marnett, 1987; Wilcox & Marnett, 1993). To test this, Mn-PGHS was reacted with [1-^{14}C]-10-hydroperoxyoctadeca-8,12-dienoic acid (10-OOH-18:2), a diagnostic probe for the formation of alkoxyl radicals (Labeque & Marnett, 1987, 1988, 1989) (Scheme 2). Heterolytic hydroperoxide cleavage generates only the alcohol, 10-hydroxyoctadeca-8,12-dienoic acid (10-OH-18:2). If homolytic hydroperoxide cleavage occurs, the resulting alkoxyl radical can be further oxidized to the corresponding ketone, 10-oxooctadeca-8,12-dienoic acid (10-oxo-18:2), or it can undergo β-scission to form 10-oxodec-8-enoic acid (10-oxo-10:1).

Assays with [1-^{14}C]-10-OOH-18:2 as the substrate required higher enzyme concentrations (500 vs 100 nM for 15-HPETE) in order to generate significant yields of products. A representative chromatogram of the products obtained following reaction of 50 μM [1-^{14}C]-10-OOH-18:2 with 500 nM Mn-PGHS is shown in Figure 4. A peak was observed at 25 min in the radioactivity profile that corresponded to the two-electron reduction product, 10-OH-18:2. Two other peaks were observed at 13 and 30 min that coeluted with standards of the oxidation product, 10-oxo-18:2, and the β-scission product, 10-oxo-10:1. The hydroperoxide, 10-OOH-18:2, and the alcohol, 10-OH-18:2, do not absorb at 235 nm, but the one-electron reduction products have conjugated chromophores and are detected in the UV profile.

Scheme 2



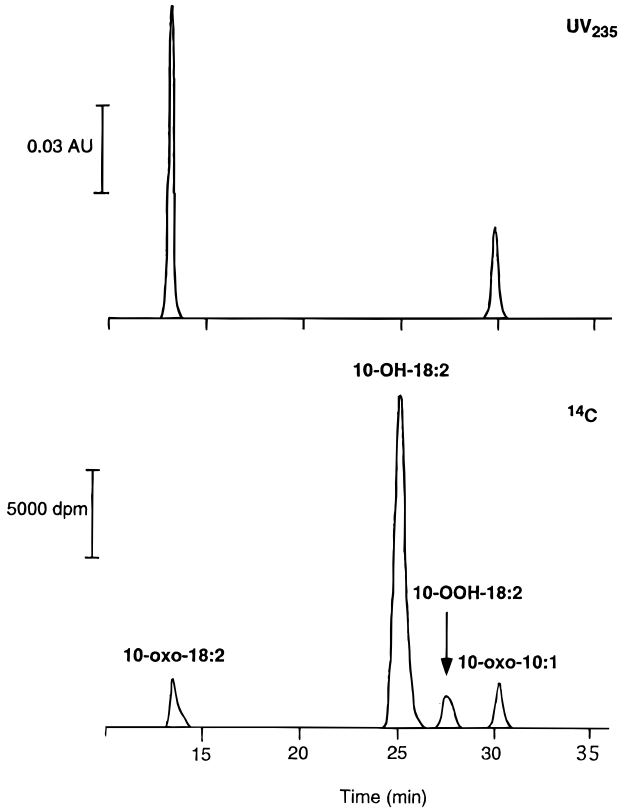


FIGURE 4: Radioactivity and UV_{235} profiles of products of reaction of Mn-PGHS with [1-^{14}C]-10-OOH-18:2. Mn-PGHS (500 nM apo-PGHS reconstituted with 500 nM MnPPIX) was assayed with 50 μM [1-^{14}C]-10-OOH-18:2 and 200 μM TMPD in 0.1 M Tris (pH 8.0) for 3 min. Elution was performed with hexane/2-propanol/acetic acid (984/15/1) at a flow rate of 1.0 mL/min using a Zorbax Rx-Sil column (5 μm silica, 4.6 × 250 mm). The identity of the one-electron reduction products was confirmed by electrospray ionization mass spectrometry.

Standards of the one-electron reduction products were generated by reaction of 10-OOH-18:2 with FePPIX in 0.1 M $NaPO_4$ (pH 7.8) containing 0.1% Tween 20. The standards as well as the MnPGHS reaction products were collected following HPLC separation and analyzed by electrospray ionization mass spectrometry. Molecular ions (M − 1) at *m/z* 293 and 183 were observed for authentic and enzymatically produced 10-oxo-18:2 and 10-oxo-10:1, respectively.

A number of control reactions were performed to confirm that the one-electron reduction products were not formed by the reaction of free MnPPIX with 10-OOH-18:2. Apo-PGHS was reconstituted with varying amounts of MnPPIX and assayed with 50 μM [1-^{14}C]-10-OOH-18:2. Varying the ratio of MnPPIX to apo-PGHS from 0.5/1 to 2/1 did not alter the percentage of one-electron products generated by Mn-PGHS. The reaction of Mn-PGHS with [1-^{14}C]-10-OOH-18:2 consistently generated the one-electron reduction products as 20−25% of the total products. In addition, heat-inactivated Mn-PGHS was incubated with [1-^{14}C]-10-OOH-18:2, and only trace amounts of products were detected. Table 1 summarizes the product distributions obtained from the reaction of Mn-PGHS with [1-^{14}C]-10-OOH-18:2. For Mn-PGHS, the reducing substrate, guaiacol or TMPD, influenced the amount of 10-oxo-10:1 formed, although the amount of 10-oxo-18:2 was greater than the amount of 10-oxo-10:1 for both reducing substrates.

The extent of one-electron hydroperoxide reduction catalyzed by Mn-PGHS was significantly different for 15-HPETE (50%) and 10-OOH-18:2 (20−25%). Therefore, another fatty acid hydroperoxide, 13-OOH-18:2, was incubated with Mn-PGHS. The maximal number of enzyme turnovers (moles of product/mole of Mn-PGHS) and the number of two-electron reduction events (mole of ROH/mole of Mn-PGHS) were determined for the three hydroperoxides (Table 2). PGHS undergoes rapid inactivation in the presence of hydroperoxides (less than 15 s) which hinders rate determination; therefore, the maximal number of turnovers was used as a measure of enzyme activity. 15-HPETE, derived from arachidonic acid, is a much better substrate for the peroxidase activity of Mn-PGHS than the linoleate hydroperoxides with 10-fold greater turnovers than 10-OOH-18:2. Comparison of the 10- and 13-hydroperoxy linoleates shows differences in the number of enzyme turnovers as well as the number of two-electron reduction events. Mn-PGHS catalyzed 2-fold greater turnovers with 13-OOH-18:2 than with 10-OOH-18:2. In addition, the number of two-electron reduction events

Table 1: Metabolism of 10-Hydroperoxy-8,12-octadecadienoic Acid by Mn-PGHSa

catalyst	maximal turnover (mol of product/mol of catalyst)	10-oxo-18:2 (nmol)	10-oxo-10:1 (nmol)	10-OH-18:2 (nmol)
Mn-PGHS + guaiacol	76.2 ± 5	4.6 ± 0.6	1.4 ± 0.1	32.2 ± 2.7
Mn-PGHS + TMPD	87.3 ± 5	4.5 ± 0.5	3.2 ± 0.2	35.7 ± 3.1
Mn-PGHS (boiled)	4.1 ± 1	0.3 ± 0.1	0.4 ± 0.1	ND[b]

[a] For Mn-PGHS reactions, 500 nM apo-PGHS was reconstituted with 500 nM MnPPIX in 0.1 M Tris (pH 8.0) containing either 200 μM guaiacol or TMPD. Reaction time was 2 min. All reactions were initiated with the addition of [1-^{14}C]-10-OOH-18:2 (50 μM) in methanol. For the boiled control, Mn-PGHS was heat-inactivated for 5 min at 65 °C prior to addition of [1-^{14}C]-10-OOH-18:2. [b] ND is not detected.

Table 2: Metabolism of Fatty Acid Hydroperoxides by Mn-PGHS[a]

substrate	maximal turnover (mol of product/mol of Mn-PGHS)	two-electron reduction (mol of ROH/mol of Mn-PGHS)
15-HPETE	1010 ± 80	566
10-OOH-18:2	89 ± 7	69
13-OOH-18:2	219 ± 27	125

[a] Hydroperoxide reduction assays contained 100 μM 1-^{14}C-labeled substrate and 200 μM guaiacol in 0.1 M Tris (pH 8.0). Concentrations of Mn-PGHS (apo-PGHS reconstituted 1/1 with MnPPIX) from 100 to 500 nM were used, and reactions were terminated after 2 min. Reaction products were separated by HPLC and quantitated using a radio-flow detector.

for the isomers was substantially different. Whereas 10-OOH-18:2 consistently generated 75−80% of the two-electron reduction product, 13-OOH-18:2 generated 50% two-electron reduction. Like 15-HPETE, 13-OOH-18:2 generated the corresponding alcohol and the keto derivative as the exclusive Mn-PGHS products.

DISCUSSION

The reaction of 15-HPETE with Mn-PGHS consistently generates the two-electron reduction product, 15-HETE, as well as an additional product with an absorbance maximum at 280 nm which has been identified as 15-KETE (Kulmacz et al., 1994). The origin of the keto fatty acid 15-KETE was of interest because this product is not formed by Fe-PGHS. We first tested the possibility that the initial product of 15-HPETE reduction, i.e., 15-HETE, is oxidized by Mn-PGHS to 15-KETE. When Mn-PGHS was incubated with [1-^{14}C]-15-HPETE in the absence of a reducing substrate, no metabolism of 15-HPETE occurred. In addition, when [1-^{14}C]-15-HETE was incubated with unlabeled 15-HPETE during Mn-PGHS turnover, no metabolism of [1-^{14}C]-15-HETE was observed and unlabeled 15-KETE was formed (Figure 3). These observations are inconsistent with a mechanism for 15-KETE generation in which 15-HETE is an intermediate (Kulmacz et al., 1994).

Previous studies in our laboratory have demonstrated that oxidation of intermediate alkoxyl radicals derived from one-electron reduction of fatty acid hydroperoxides generates the corresponding keto compounds (Dix & Marnett, 1985; Labeque & Marnett, 1987; Wilcox & Marnett, 1993). These studies, coupled with the control experiments described above, suggest an alternative mechanism in which 15-KETE is a primary metabolite derived from one-electron reduction of 15-HPETE and oxidation of the intermediate alkoxyl radical. The detection of the β-scission product, 10-oxo-10:1, following reaction of 10-OOH-18:2 with Mn-PGHS, supports an initial one-electron reduction step. This product can only arise from an intermediate alkoxyl radical and provides the most compelling evidence that Mn-PGHS catalyzes significant levels of one-electron reduction.

If Mn-PGHS reduces 15-HPETE by one electron to form an alkoxyl radical, a one-electron oxidized Mn-PGHS intermediate would be formed (eq 5). This intermediate would be analogous to compound **II**, a Fe^{IV}=O species with a fully covalent porphyrin (eq 4). In fact, previous studies by Odenwaller et al. (1992) and Streider et al. (1992) have detected and characterized this intermediate.

$$Mn^{3+}PPIX + ROOH \rightarrow Mn^{IV}{=}O + RO^{\bullet} \qquad (5)$$

The detection of 15-KETE and 15-HETE as products of Mn-PGHS action indicates that Mn-PGHS is able to catalyze both one- and two-electron reduction of fatty acid hydroperoxides. Precedent exists for competing one- and two-electron reduction pathways by other hemeproteins, such as cytochromes P_{450}. For example, the ferric form of cytochrome P_{450} catalyzes one- and two-electron reduction of peroxyacids such as peroxyphenylacetic acid (McCarthy & White, 1983) and a peroxyquinol, 2,6-di-*tert*-butyl-4-hydroperoxy-4-methyl-2,5-cyclohexadienone (Thompson & Wand, 1985; Wand & Thompson, 1986). In addition, our laboratory has demonstrated that addition of excess imidazole can alter the mechanism of hydroperoxide reduction by chloro(*m*-tetraphenylporphorinato)iron(III) (Fe-TPP) (Labeque & Marnett, 1989). Imidazole coordinates to the metal and alters the mechanism from exclusively one-electron to predominantly two-electron reduction.

The ability of Mn-PGHS to generate either compound **I** or compound **II**-like intermediates is dependent on the reactivity of the metal porphyrin. MnPPIX-substituted peroxidases are clearly less reactive than FePPIX-substituted peroxidases; however, like Mn-PGHS, Mn-reconstituted horseradish peroxidase and cytochrome *c* peroxidase generate higher oxidation states and retain partial enzyme activity (Yonetani & Asakura, 1968; Hori et al., 1987). Characterization of oxidized manganoporphyrin complexes by spectroscopic methods reveals an unusually weak Mn^{IV}=O bond attributed to a high-spin d^3 electronic state unique to manganese (Czernuszewicz et al., 1988; Nick et al., 1991). A consequence of this weak bond is decreased reactivity of Mn^{IV}=O relative to the corresponding iron−oxo complex. This makes it less likely that the higher oxidation state of Mn-PGHS would oxidize 15-HETE, a reaction not catalyzed by the metal−oxo derivative of Fe-PGHS.

Although the manganoporphyrin slows the rate of formation of higher oxidation states of PGHS, the protein component of PGHS influences the fate of the alkoxyl radical generated by one-electron hydroperoxide reduction. Extensive work from several laboratories has shown that the functional groups adjacent to the alkoxyl radical determine the pathway of decomposition (Gardner & Crawford, 1981;

Labeque & Marnett, 1987, 1988). For example, β-scission of an alkoxyl radical derived from 10-OOH-18:2 generates a relatively stable allylic radical (Scheme 2). However, β-scission of alkoxyl radicals derived from 15-HPETE or 13-OOH-18:2 is not observed because unstable primary alkyl radicals would be formed. Alternatively, alkoxyl radicals derived from 15-HPETE or 13-OOH-18:2 are oxidized by the Mn^{IV}═O complex to the corresponding ketones with concomitant reduction of Mn^{IV}═O to Mn(III). An additional pathway of decomposition for alkoxyl radicals derived from 15-HPETE or 13-OOH-18:2 is cyclization to an epoxyallylic radical with the ultimate formation of epoxyalcohols (Dix & Marnett, 1985; Wilcox & Marnett, 1993). The lack of cyclization products in the present study implies that the alkoxyl radical generated at the active site of Mn-PGHS is held rigidly in a conformation that prevents cyclization. In addition, the low yield of the β-scission product, 10-oxo-10:1, relative to the oxidation product, 10-oxo-18:2 (Table 1), following reaction of 10-OOH-18:2 with Mn-PGHS may be due to constraints imposed by the protein.

In summary, the present experiments establish that the formation of keto acids by Mn-PGHS-catalyzed metabolism of fatty acid hydroperoxides does not occur by oxidation of putative hydroxy fatty acid intermediates. Rather, the keto acids appear to arise by oxidation of alkoxyl radical intermediates generated by one-electron reduction of the hydroperoxide. Thus, the chemistry of hydroperoxide metabolism by Mn-PGHS corresponds to chemistry that is well-established for other hemeproteins and metal complexes.

REFERENCES

Anni, H., & Yonetani, T. (1992) in *Metal Ions in Biological Systems Vol. 28* (Sigel, H., & Sigel, A., eds.) pp 219−241, Marcel Dekker, Inc., New York.

Czernuszewicz, R. S., Su, Y. O., Stern, M. K., Macor, K. A., Kim, D., Groves, J. T., & Spiro, T. G. (1988) *J. Am. Chem. Soc. 110*, 4158−4165.

Dix, T. A., & Marnett, L. J. (1985) *J. Biol. Chem. 260*, 5351−5357.

Funk, M. O., Isaac, R., & Porter, N. A. (1976) *Lipids 11*, 113−117.

Gardner, H. W., & Crawford, C. G. (1981) *Biochim. Biophys. Acta 665*, 126−133.

Hamberg, M., Svensson, J., Wakabayashi, T., & Samuelsson, B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 345−349.

Hori, H., Ikeda-Saito, M., & Yonetani, T. (1987) *Biochim. Biophys. Acta 912*, 74−81.

Kulmacz, R. J., Palmer, G., Wei, C., & Tsai, A.-L. (1994) *Biochemistry 33*, 5428−5439.

Labeque, R., & Marnett, L. J. (1987) *J. Am. Chem. Soc. 109*, 2828−2829.

Labeque, R., & Marnett, L. J. (1988) *Biochemistry 27*, 7060−7070.

Labeque, R., & Marnett, L. J. (1989) *J. Am. Chem. Soc. 111*, 6621−6627.

Lambeir, A.-M., Markey, C. M., Dunford, H. B., & Marnett, L. J. (1985) *J. Biol. Chem. 260*, 14894−14896.

Markey, C. M., Alward, A., Weller, P. E., & Marnett, L. J. (1987) *J. Biol. Chem. 262*, 6266−6279.

Marnett, L. J., & Maddipati, K. R. (1991) in *Peroxidases in Chemistry and Biology Vol. I* (Everse, J., Everse, K. E., & Grisham, M. E., Eds.) pp 293−334, CRC Press, Boca Raton, FL.

Marnett, L. J., Siedlik, P. H., Ochs, R. C., Pagels, W. D., Das, M., Honn, K. V., Warnock, R. H., Tainer, B. E., & Eling, T. E. (1984) *Mol. Pharmacol. 26*, 328−335.

Marnett, L. J., Chen, Y.-N. P., Maddipati, K. R., Plé, P., & Labeque, R. (1988) *J. Biol. Chem. 263*, 16532−16535.

McCarthy, M. B., & White, R. E. (1983) *J. Biol. Chem. 258*, 11610−11616.

Nick, R. J., Ray, G. B., Fish, K. M., Spiro, T. G., & Groves, J. T. (1991) *J. Am. Chem. Soc. 113*, 1838−1840.

Nugteren, D. H., & Hazelhof, E. (1973) *Biochim. Biophys. Acta 326*, 448−461.

Odenwaller, R., Chen, Y.-N. P., & Marnett, L. J. (1990) *Methods Enzymol. 187*, 479−485.

Odenwaller, R., Maddipati, K. R., & Marnett, L. J. (1992) *J. Biol. Chem. 267*, 13863−13869.

Schonbaum, G. R., & Chance, B. (1976) in *The Enzymes, Vol. 13* (Boyer, P., Ed.) pp 363−408, Academic Press, New York.

Streider, S., Schaible, K., Scherer, H.-J., Dietz, R., & Ruf, H. H. (1992) *J. Biol. Chem. 267*, 13870−13878.

Thompson, J. A., & Wand, M. D. (1985) *J. Biol. Chem. 260*, 10637−10644.

Wand, M. D., & Thompson, J. A. (1986) *J. Biol. Chem. 261*, 14049−14056.

Wilcox, A. L., & Marnett, L. J. (1993) *Chem. Res. Toxicol. 6*, 413−416.

Yonetani, T., & Asakura, T. (1968) *J. Biol. Chem. 243*, 3996−3998.

BI952546H