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Prostaglandin Hydroperoxidase-Dependent Oxidation of Phenylbutazone: Relationship to Inhibition of Prostaglandin Cyclooxygenase

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SUMMARY

Prostaglandin H synthase (PHS) hydroperoxidase-mediated metabolism of phenylbutazone and the relationship of this metabolism to the inhibition of PHS cyclooxygenase by phenylbutazone was investigated. Phenylbutazone was metabolized to several intermediates and metabolites. A phenylbutazone carbon-centered radical (a^N = 14.6 G) formed by PHS hydroperoxidase was trapped by 2-methyl-2-nitrosopropane and detected by ESR in incubations with ram seminal vesicle microsomes. 4-Hydroperoxy- and 4-hydroxyphenylbutazone were isolated from incubations of phenylbutazone with either ram seminal vesicle microsomes or horseradish peroxidase. Phenylbutazone (100 μM–2 mM) inhibited PHS cyclooxygenase in incubations of PHS apoenzyme reconstituted with hematin. Phenylbutazone (5–250 μM) did not inhibit PHS cyclooxygenase in incubations of PHS apoenzyme reconstituted with manganese protoporphyrin IX, which lacks hydroperoxidase activity. Thus, metabolism of phenylbutazone by PHS hydroperoxidase is required for it to inhibit PHS cyclooxygenase. 4-Hydroperoxy- and 4-hydroxyphenylbutazone were ineffective inhibitors of PHS cyclooxygenase. Other hydroperoxides that easily rearrange to peroxyl radicals were potent inhibitors of PHS cyclooxygenase, suggesting that the phenylbutazone peroxyl radical may be the inhibitor. 4-Hydroperoxyphenylbutazone was not reduced to 4-hydroxyphenylbutazone by PHS hydroperoxidase. We propose that 4-hydroxyphenylbutazone formation occurs by a nonenzymatic reaction of two phenylbutazone peroxyl radicals and their subsequent rearrangement to alkoxy radicals, which abstract hydrogen atoms. Our data indicate the importance of PHS hydroperoxidase in the inactivation of PHS cyclooxygenase by peroxides.

Phenylbutazone is a nonsteroidal anti-inflammatory drug. Drugs of this class inhibit PG synthesis in cell-free homogenates (1) and PG release from human platelets (2) and perfused dog spleen (3). These drugs inhibit PG formation by inhibiting the cyclooxygenase activity of PHS, the enzyme that catalyzes formation of PGs. Although the mechanism of the inhibition of PG formation by aspirin is known (4), the mechanism for many of these drugs, including phenylbutazone, is still unknown.

PHS catalyzes two distinct enzymatic reactions that copurify (5–7). The first reaction is a bis-dioxygenation of arachidonic acid, catalyzed by the cyclooxygenase activity of PHS, forming the hydroperoxy endoperoxide PGG_2 (8). The second reaction is the reduction of the hydroperoxide group of PGG_2 by PHS hydroperoxidase to form the hydroxy endoperoxide, PGH_2 (6). During PHS-catalyzed reduction of PGG_2, many xenobiotics including phenylbutazone are cooxidized by PHS hydroperoxidase (9–12). The only oxidized product of phenylbutazone that has been isolated and identified to date from incubations with PHS is 4-hydroxyphenylbutazone (10, 13). Portoghese *et al.* (14) have identified 4-hydroxyoxyphenbutazone in incubations containing PHS and oxyphenbutazone. The metabolic pathway from phenylbutazone to 4-hydroxyphenylbutazone has been proposed (10, 13, 15) to occur via the formation of a carbon-centered radical in the diketone moiety (position C-4). This radical reacts with molecular oxygen to form a peroxyl radical. Abstraction of a hydrogen atom results in formation of 4-hydroperoxyphenylbutazone, which is thought to be reduced by PHS hydroperoxidase to 4-hydroxyphenylbutazone. Evidence for the mechanism is incomplete but studies using $^{18}O_2$ indicate the hydroxyl oxygen is derived from atmospheric oxygen as predicted by the mechanism (10). Evidence suggesting that the phenylbutazone peroxyl radical is formed was reported by Reed

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ABBREVIATIONS: PG, prostaglandin; PHS, prostaglandin H synthase; BP-7,8-diol, 7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene; DTPA, diethylenetriaminepentaacetic acid; BSA, bovine serum albumin; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 15-HPETE, 15-hydroperoxyeicosatetraenoic acid; Tween 20, polyethylene (20) sorbitan monolaurate; RSV, ram seminal vesicles; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography; *t*-NB, 2-methyl-2-nitrosopropane; HRP, horseradish peroxidase.

et al. (16). They observed a phenylbutazone-dependent epoxidation of BP-7,8-diol in incubations with PHS initiated with peroxides. The epoxidation of alkenes (e.g., 9,10 position of BP-7,8-diol) by peroxyl radicals is well known (17–19). Direct evidence of formation of the phenylbutazone carbon-centered radical, the peroxyl radical, and 4-hydroperoxyphenylbutazone by PHS hydroperoxidase have not been reported.

Recently, Reed *et al.* (20) reported that phenylbutazone inactivated PHS and prostacyclin synthase upon addition of peroxides to the incubations. This suggested that the inhibition of these enzymes by phenylbutazone was dependent upon the hydroperoxidase-mediated oxidation of the parent compound. The stable end product of phenylbutazone oxidation is most likely not the inhibitory species, inasmuch as 4-hydroxyoxyphenbutazone did not inhibit PHS (14). Potential inhibitors formed during hydroperoxidase-mediated oxidation of phenylbutazone are the intermediate phenylbutazone peroxyl radical and 4-hydroperoxyphenylbutazone. The latter metabolite is a proposed inhibitor in view of the known sensitivity of PHS (21, 22) and prostacyclin synthase (23–25) to peroxides.

In this paper, we have characterized PHS hydroperoxidase-dependent oxidation of phenylbutazone, by detecting the proposed carbon-centered radical by ESR and isolating and characterizing 4-hydroperoxyphenylbutazone. The relationship between PHS hydroperoxidase oxidation of phenylbutazone and the inhibition of PHS cyclooxygenase was also investigated.

Experimental Procedures

Materials. Phenylbutazone, DTPA, BSA, indomethacin, HRP Type VI, 4-dimethylaminoantipyrine (aminopyrine), hematin, *t*-butyl hydroperoxide, tryptophan, and *t*-NB were purchased from Sigma Chemical Co. (St. Louis, MO). Tris was purchased from Bethesda Research Laboratories, Inc. (Rockville, MD). Hydrogen peroxide (30%) was purchased from Fisher Chemical Co. (Fair Lawn, NJ). Arachidonic acid was purchased from Nu-Chek Prep (Elysian, MN). Atomlight, [^{3}H]arachidonic acid (100 mCi/μmol) and the ^{3}H-labeled arachidonic acid metabolite standards were obtained from New England Nuclear (Boston, MA). Manganese protoporphyrin IX was purchased from Porphyrin Products, Inc. (Logan, UT). PHS apoenzyme (specific activity, 0.5 μmol of O_2 consumed/min without heme) and 12-HPETE were purchased from Biomol Research Laboratories, Inc. (Philadelphia, PA). Tween 20 was purchased from Matheson Coleman and Bell Manufacturing Chemists (Norwood, OH). All other chemicals used were of the highest grade available. RSV were purchased from Dr. Lawrence Marnett, Wayne State University (Detroit, MI) and stored at −70° until used. The RSV were used to prepare microsomal and Tween 20-solubilized PHS as described by Marnett and Wilcox (26). Aliquots of the microsomes were frozen at −70° until used. The protein concentration of the microsomes was determined by the method of Lowry *et al.* (27), with BSA used as the standard.

Measurement of cyclooxygenase activity. Studies measuring the cyclooxygenase activity of PHS were carried out in 66 mM Tris with 1 mM DTPA, pH 7.8 at 37° in a final volume of 1.5 ml. Cyclooxygenase activity was assessed by measuring the incorporation of oxygen into arachidonic acid using a Clark oxygen electrode and oxygen monitor (Yellow Springs Instruments Co., Yellow Springs, OH). In incubations with RSV microsomes, buffer and microsomal protein (0.25–0.5 mg/ml) were preincubated 2 min at 37° followed by addition of arachidonic acid (100 μM). In incubations with PHS apoenzyme, either hematin (1 μM) or manganese protoporphyrin IX (1 μM) were reconstituted with the apoenzyme. Hematin and manganese protoporphyrin IX were initially dissolved in 0.1 N sodium hydroxide and then diluted with distilled water before addition to the incubation mixture. Buffer, apoenzyme (10 μg/ml), and hematin and aminopyrine (1 mM) or manganese protoporphyrin IX were preincubated 2 min at 37° for reconstitution, followed by addition of arachidonic acid (200 μM). The cyclooxygenase activity of the purified PHS and RSV microsomes varied from preparation to preparation. For the peroxide inhibition studies, RSV microsomes and buffer were preincubated for 1 min at 37°. The peroxide was then added and preincubated with enzyme for an additional 1 min. Arachidonic acid was added to initiate the cyclooxygenase reaction.

Measurement of hydroperoxidase activity. Hydroperoxidase activity of PHS was determined by quantitating formation of the chromophoric aminopyrine cation radical at 566 nm (28) using a Hewlett Packard 8450A Diode Spectrophotometer (Palo Alto, CA) equipped with a Model 89100 stirring temperature controller. These incubations were carried out in a final volume of 2 ml of 66 mM Tris with 1 mM DTPA, pH 7.8. Incubations consisted of aminopyrine (1 mM) and either solubilized RSV microsomes (0.25 mg/ml) or PHS apoenzyme (5–10 μg/ml) reconstituted with either hematin (1 μM) or manganese protoporphyrin IX (1 μM) in the manner described above. Enzyme and other additions were preincubated at 37° for 2 min. The reaction was initiated with the addition of 200 μM hydrogen peroxide. In some experiments, 200 μM 4-hydroperoxyphenylbutazone was added to initiate the reaction. The concentration of 4-hydroperoxyphenylbutazone was quantitated by ultraviolet absorbance at 237 nm and based on the extinction coefficient of phenylbutazone (log ϵ = 4.19).

Preparation of compounds. 15-HPETE was prepared by the method of Funk *et al.* (29). 4-Hydroxyphenylbutazone was synthesized by the method of Woodruff and Polya (30) with some modifications. Preparative TLC (silica gel GF, 1000 μm; Analtech, Inc., Newark, DE) was used to isolate 4-hydroxyphenylbutazone from the crude reaction mixture using a solvent system of dichloromethane/ethyl acetate (19:1). The respective area (R_F = 0.15) was scraped from the plate and the silica gel was extracted twice with dichloromethane. The organic fractions were pooled and taken to dryness under vacuum. The HPLC system described by Ichihara *et al.* (31) was used to analyze the compound. This isocratic solvent system consists of acetonitrile/H_2O (3:2) flowing at a rate of 1.5 ml/min (Model 6000A pump, Waters Associates, Milford, MA) through a 3.9 mm × 30 cm C_{18} μBondapak column (Waters). Column effluent was monitored with a Lambda Max 491 LC spectrophotometer (Waters) at a wavelength of 237 nm. One peak was observed with a retention time of 4.6 min. Mass spectral analysis (electron impact, 30 eV) of the compound showed an identical fragmentation pattern to that reported (31) for 4-hydroxyphenylbutazone with a molecular ion peak at *m/z* 324.

The method of Ichihara *et al.* (31) with some modifications was used to synthesize 4-hydroperoxyphenylbutazone. This hydroperoxide has been definitively characterized by HPLC, ultraviolet absorption, nuclear magnetic resonance, and mass spectroscopy (31). This compound was prepared enzymatically with HRP Type VI. Tris buffer (66 mM) containing DTPA (1 mM), pH 6.5, was oxygenated for 30 min at 22°. Phenylbutazone (50 mg) and HRP (20 mg) were added to 200 ml of the oxygenated buffer. Hydrogen peroxide (20 mM) was added to initiate the reaction, which lasted for 30 min. The mixture was oxygenated throughout the incubation. At the end of the incubation, the mixture was extracted twice with 200 ml of ice-cold ethyl acetate. The organic fractions were pooled and washed with an equal volume of ice-cold water. The washed organic fraction was kept on ice and dried over anhydrous $MgSO_4$, filtered, and reduced in volume. The organic extract was passed through a silica gel column (28–200 mesh, grade 12) prepared in dichloromethane. The material was eluted from the column with a solution of dichloromethane/ethyl acetate (19:1). The collected eluate was dried under vacuum and reconstituted in a minimal volume of dichloromethane. The hydroperoxide was isolated by the TLC method described earlier (R_F = 0.25). This compound had a retention time of 5.1 min in the isocratic HPLC system described previously.

Isolation and identification of RSV microsomal metabolites of phenylbutazone. An incubation (10 ml of 66 mM Tris, 1 mM DTPA, pH 6.5) consisting of RSV microsomes (0.25 mg/ml) and

phenylbutazone (200 μM) was initiated with hydrogen peroxide (100 μM). The incubation was done at 22° for 10 min. The buffer was oxygenated for 10 min before the addition of hydrogen peroxide and throughout the incubation. The reaction mixture was then extracted twice with 10 ml of ice-cold ethyl acetate. The organic fractions were pooled, washed with 20 ml of ice cold water, and dried over anhydrous $MgSO_4$. The organic extract was filtered, reduced in volume to dryness, and reconstituted with acetonitrile/H_2O (3:2). The material was analyzed by the isocratic HPLC system described previously.

Oxidation of phenylbutazone to a carbon-centered radical. Incubation mixtures (22°) consisted of RSV microsomes (0.5 mg/ml), the spin trap *t*-NB (10 mM), and phenylbutazone (250 μM) in 3 ml of 66 mM Tris with 1 mM DTPA, pH 7.8. The reactions were initiated with either arachidonic acid (100 μM), hydrogen peroxide (100 μM), or 15-HPETE (25 μM). In some incubations the cyclooxygenase inhibitor indomethacin (200 μM) was preincubated with the microsomes 1 min before addition of phenylbutazone and arachidonic acid or hydrogen peroxide. ESR spectra of the incubations were recorded with a Varian E-109 ESR spectrometer equipped with a TM_{110} microwave cavity and an aqueous flat cell. Incubations were initiated and then loaded into the flat cell by aspiration with a rapid sampling device (32).

Analysis of arachidonic acid metabolites. RSV microsomes (0.25 mg/ml) were preincubated for 2 min at 37° in a volume of 1.5 ml of 66 mM Tris buffer with 1 mM DTPA, pH 7.8, followed by addition of [^{3}H]arachidonic acid (100 μM). In some incubations, 15-HPETE (2 μM) or *t*-butyl hydroperoxide (50 μM) were added 1 min before addition of arachidonic acid. PHS apoenzyme (7 μg/ml) was reconstituted with manganese protoporphyrin IX and preincubated for 2 min at 37°. [^{3}H] Arachidonic acid (200 μM) was then added to the incubate. In some incubations phenylbutazone (500 μM) was preincubated with purified PHS for 1 min before the addition of arachidonic acid. Two minutes after addition of arachidonic acid, the incubate was pipetted into 8 ml of cold ethanol. This mixture was centrifuged to remove the protein, evaporated under vacuum, and reconstituted in methanol/H_2O (50:50). Aliquots of the reconstituted incubate were analyzed for arachidonic acid metabolites on a 4.6 mm × 250 mm Altex C_{18} 5 μm column (Beckman, Berkeley, CA) with two Waters Model 6000A pumps, a Model 721 System controller, and a WISP 710B automatic injector (Waters) by the method described by Henke *et al.* (33). Radioactivity of the eluant was monitored with a Radiometer Flow Detector (Radiomatic Instruments and Chemical Co., Tampa, FL). Atomlight was used as the scintillation fluid. Authentic arachidonic acid metabolites were also injected onto the column and their retention times were compared with those of the metabolites isolated from the incubate.

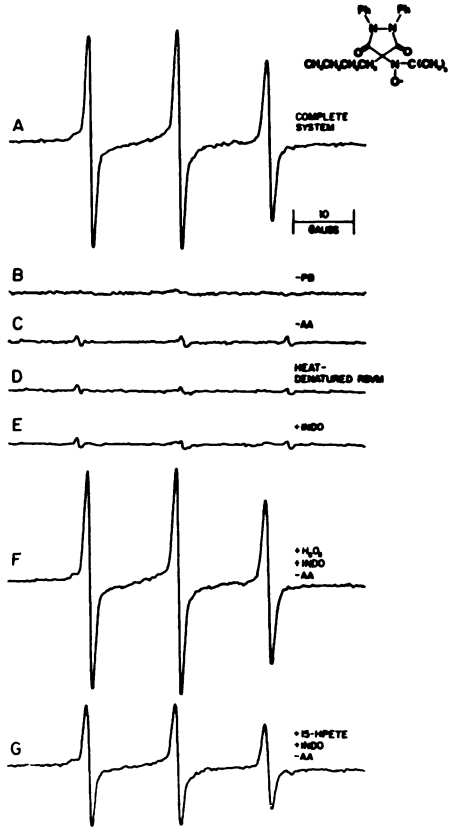


Fig. 1. ESR spectra of the phenylbutazone carbon-centered radical formed in incubations with ram seminal vesicle microsomes ((*RSVM*). A, Complete system including 250 μM phenylbutazone (*PB*), 0.5 mg/ml RSVM, 100 μM arachidonic acid (*AA*), and 10 mM *t*-NB. B, Complete system minus phenylbutazone. C, Complete system minus arachiodonic acid. D, Complete system with heat-denatured RSVM. E, Complete system PLUS 200 μM indomethacin (INDO). F, Complete system with 200 μM indomethacin and initiated with 100 μM hydrogen peroxide instead of arachidonic acid. G, Complete system with 200 μM indomethacin and initiated with 25 μM 15-HPETE instead of arachidonic acid.

Results

Formation of a phenylbutazone carbon-centered radical. The ESR spectra (Fig. 1) of incubations consisting of RSV microsomes and phenylbutazone and initiated with either arachidonic acid, hydrogen peroxide, or 15-HPETE indicated the formation of a phenylbutazone carbon-centered radical (a^N = 14.6 G) that was trapped by the spin trap *t*-NB. This carbon-centered radical was not observed in incubations lacking phenylbutazone (*line B*) or arachidonic acid (*line C*), with heat-denatured RSV microsomes (*line D*), or initiated with arachidonic acid in the presence of the cyclooxygenase inhibitor indomethacin (*line E*). Fig. 1, *lines F* and *G* illustrate that the carbon-centered radical was formed by PHS hydroperoxidase. These reactions were initiated with hydrogen peroxide and 15-HPETE, respectively, in the presence of indomethacin.

Oxygen incorporation into phenylbutazone was measured in incubations initiated with hydrogen peroxide containing indomethacin-inhibited RSV microsomes in the presence and absence of *t*-NB (Fig. 2). The spin trap *t*-NB reacted with the phenylbutazone carbon-centered radical and inhibited the incorporation of molecular oxygen into phenylbutazone. The rate of incorporation of oxygen into phenylbutazone was inhibited by 23% and the total amount of oxygen consumed was inhibited by 30% in the presence of *t*-NB.

Analysis of metabolites of phenylbutazone. In incubations with HRP and PHS, two peaks (nos. 2 and 3) were observed on HPLC with retention times similar to those reported by Ichihara *et al.* (31) (Fig. 3). Peak 2 cochromatographed with authentic 4-hydroxyphenylbutazone. From the HRP incubations, material corresponding to the second peak was isolated by TLC. This material was subjected to several tests in order to confirm that it was the previously reported 4-hydroperoxyphenylbutazone. This compound and 15-HPETE reacted positively with a chemical spray solution (200 mg of NH_4SCN in 15 ml of acetone mixed with 400 mg of $FeSO_4$ in 10 ml of H_2O) that turns orange upon contact with peroxides. 4-Hydroxyphenylbutazone was unreactive in this test. Hematin catalyzes the decomposition of peroxides to alkoxy radicals (34–37), which oxidize aminopyrine (1 mM) to a chromophoric cation radical. The formation of the chromophore was monitored in the same manner described for the hydroperoxidase assay. The compound isolated as 4-hydroperoxyphenylbutazone (50 μM) was positive in this assay, whereas 4-hydroxy-

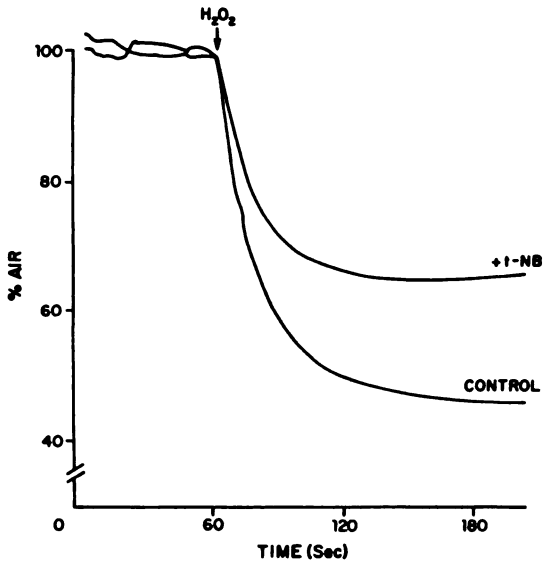


Fig. 2. Inhibition of incorporation of molecular oxygen into the phenylbutazone carbon-centered radical by the spin trap *t*-NB. The amount of oxygen (air) in solution was measured with an oxygen electrode. RSV microsomes (0.25 mg/ml) were incubated (37°) with 100 μM indomethacin and 250 μM phenylbutazone, either in the absence or presence of 1 mM *t*-NB. The reaction was initiated with 100 μM hydrogen peroxide.

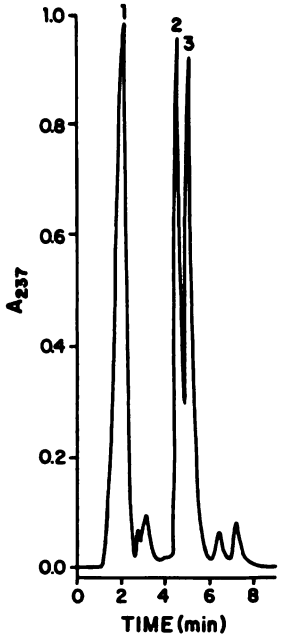


Fig. 3. HPLC chromatogram of the ethyl acetate extract of an incubation of RSV microsomes containing PHS with phenylbutazone. The labeled peaks and retention time are: 1) phenylbutazone, 2.1 min; 2) 4-hydroxyphenylbutazone, 4.6 min; and 3) 4-hydroperoxyphenylbutazone, 5.1 min.

phenylbutazone and phenylbutazone were unreactive. 4-Hydroperoxyphenylbutazone (200 μM) was reduced by dithiothreitol (1 mM, 15 min at 37° in 66 mM Tris, 1 mM DTPA, pH 7.8) to 4-hydroxyphenylbutazone as shown after HPLC analysis. These observations indicated that 4-hydroperoxyphenylbutazone was synthesized by HRP. This material was added to the organic extract from the PHS incubation and analyzed by HPLC. Peak 3 increased upon addition of the standard 4-hydroperoxyphenylbutazone. This indicated that 4-hydroperoxyphenylbutazone was also an intermediate in PHS hydroperoxidase-mediated metabolism of phenylbutazone.

PHS apoenzyme studies. To investigate the role of PHS hydroperoxidase in the inhibition of PHS cyclooxygenase by phenylbutazone, purified PHS apoenzyme was reconstituted with either hematin or manganese protoporphyrin IX, and cyclooxygenase activity was measured. When PHS apoenzyme is reconstituted with hematin, the enzyme possesses both cyclooxygenase and hydroperoxidase activity. PHS apoenzyme possesses only cyclooxygenase activity when reconstituted with manganese protoporphyrin IX (38). To ensure that the apoenzyme did not contain residual hydroperoxidase activity (due to the presence of endogenous heme), the hydroperoxidase activity of the enzyme was measured after reconstitution with hematin and manganese protoporphyrin IX by quantitating formation of the aminopyrine cation radical (28). A peak aminopyrine cation radical concentration of approximately 65 μM was quantitated with hematin-reconstituted preparations whereas the aminopyrine cation radical concentration formed with the manganese protoporphyrin IX-reconstituted enzyme was negligible or below the limits of detection by this assay (data not shown). Both reconstituted enzymes contained cyclooxygenase activity (see Figs. 4 and 6) as measured by oxygen incorporation into arachidonic acid.

The addition of 5–250 μM phenylbutazone to the manganese protoporphyrin IX-reconstituted enzyme did not significantly alter the rate or extent of oxygen incorporation into arachidonic acid (Fig. 4). At 500 μM phenylbutazone, a significant stimulation of oxygen consumption was observed. HPLC analysis of incubation mixtures containing [^{3}H]arachidonic acid indicated that oxygen consumption was the result of increased arachi-

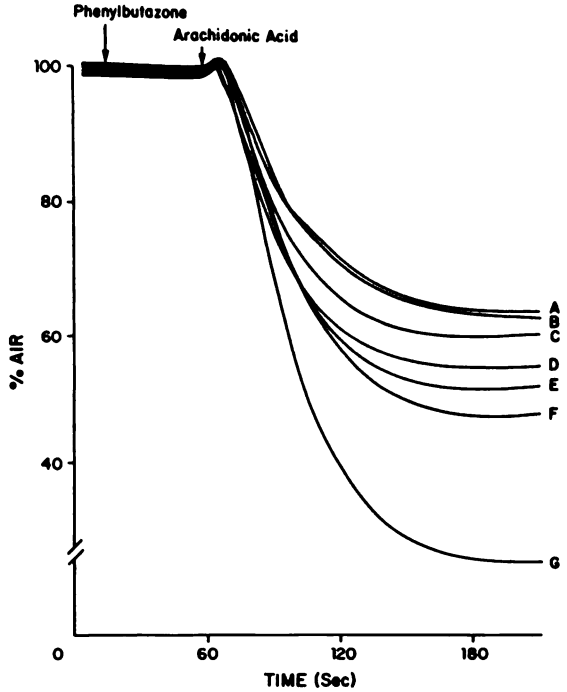


Fig. 4. Effect of phenylbutazone on the cyclooxygenase activity of 10 μg/ml PHS apoenzyme reconstituted with 1 μM manganese protoporphyrin IX. The labeled lines and phenylbutazone concentration are as follows: A, 50 μM; B, 10 μM; C, 5 μM; D, O μM; E, 100 μM; F, 250 μM; and G, 500 μM. The cyclooxygenase activity was assessed by measuring the oxygen (air) in solution (37°) with an oxygen electrode. The reaction was initiated by the addition of 200 μM arachidonic acid.

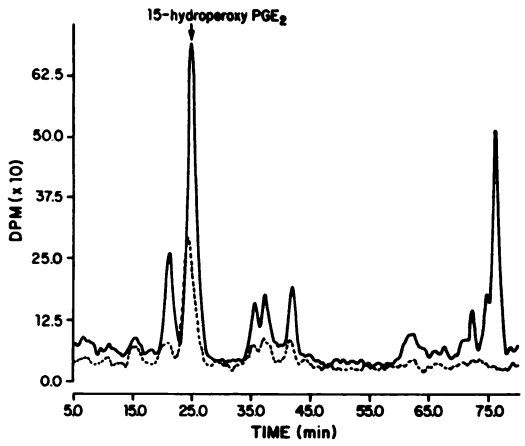


Fig. 5. HPLC chromatogram of an incubation mixture of 7 μg/ml PHS apoenzyme reconstituted with 1 μM manganese protoporphyrin IX and initiated with 200 μM [^{3}H]arachidonic acid in the absence (– – –) and presence (——) of 500 μM phenylbutazone.

TABLE 1

Oxygen consumption and PG formation by PHS apoenzyme reconstituted with manganese protoporphyrin IX in the presence and absence of 500 μM phenylbutazone

	– Phenylbutazone	+ Phenylbutazone
Oxygen consumed (nmol)[a]	101.6 ± 27.0[b]	244.0 ± 47.3
PGs formed (nmol)	56.1 ± 12.2	101.0 ± 9.6

[a] Oxygen consumption was measured with a Clark oxygen electrode after addition of 200 μM [^{3}H]arachidonic acid in the presence of PHS apoenzyme (7 μg/ml) and 1 μM manganese protoporphyrin IX. After 2 min the incubation was stopped by the addition of 8 ml of ethanol. The mixture was centrifuged and the supernatant was analyzed for arachidonic acid metabolites as described in Experimental Procedures.

[b] Mean ± standard deviation (three experiments).

donic acid metabolism to PGs (Fig. 5). The major arachidonic acid metabolite was 15-hydroperoxy-PGE$_2$, both in the presence and absence of phenylbutazone, as expected for an enzyme preparation devoid of hydroperoxidase activity (39). The amount of PGs formed and oxygen consumed increased approximately 2-fold in the presence of phenylbutazone (Table 1). The mechanism responsible for the stimulation is not known.

PHS apoenzyme reconstituted with hematin requires a reducing cofactor for cyclooxygenase activity and tryptophan, aminopyrine, and phenol are commonly used for this purpose (38). The use of 1 and 2 mM phenylbutazone as a reducing cofactor for hematin-reconstituted PHS resulted in negligible arachidonic acid oxidation (Fig. 6). Compared with incubations that contained 1 mM aminopyrine as the reducing cofactor, approximately 100% inhibition of PHS cyclooxygenase activity was observed in the presence of phenylbutazone. Similar results were observed with phenylbutazone concentrations of 100, 250, and 500 μM. Phenylbutazone may be such a poor reducing cofactor that it cannot support arachidonic acid metabolism. To eliminate this possibilitiy, tryptophan (5 mM) was used because it has an efficiency as a reducing cofactor similar to phenylbutazone (12). As shown in Fig. 6, oxygen was consumed upon addition of arachidonic acid in the incubation with hematin-reconstituted PHS apoenzyme in the presence of tryptophan. The data indicate that phenylbutazone inhibition of PHS cyclooxygenase is dependent on the hydroperoxidase activity of PHS.

Inactivation of PHS cyclooxygenase by peroxides. In

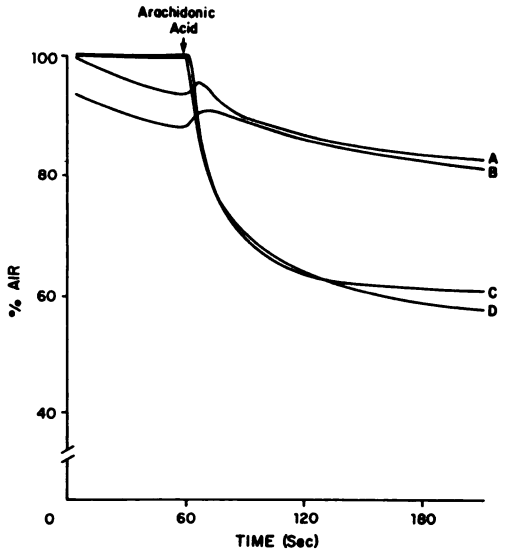


Fig. 6. Effect of the hydroperoxidase cofactors 1–2 mM phenylbutazone (A, B), 1 mM aminopyrine (C), and 5 mM tryptophan (D) on the cyclooxygenase activity of PHS apoenzyme reconstituted with 1 μM hematin. The cyclooxygenase activity was assessed by measuring the oxygen (air) in solution (37°) with an oxygen electrode. The reaction was initiated by the addition of 200 μM arachidonic acid.

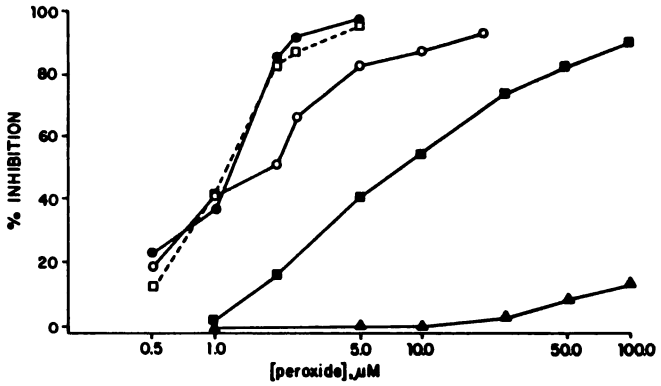


Fig. 7. Inhibition of PHS cyclooxygenase activity in RSV microsomes (0.5 mg/ml) by 15-HPETE (●), 12-HPETE (□), H_2O_2 (○), *t*-butyl hydroperoxide (■), and 4-hydroperoxyphenylbutazone (▲). The peroxides were incubated (37°) with the microsomes 1 min before addition of 100 μM arachidonic acid.

the absence of a reducing cofactor, PHS is rapidly inactivated by peroxides (12). The effect of 4-hydroperoxyphenylbutazone on PHS was compared with other peroxides using RSV microsomes as the enzyme source. The microsomal preparation was essentially devoid of endogenous reducing cofactors inasmuch as HPLC analysis of [^{3}H]arachidonic acid metabolites indicated the major, if not exclusive, formation of hydroperoxy metabolites (data not shown). RSV microsomes were incubated with increasing concentrations of 12-HPETE, 15-HPETE, H_2O_2, *t*-butyl hydroperoxide, and 4-hydroperoxyphenylbutazone. As shown in Fig. 7, the lipid hydroperoxy fatty acids 12-HPETE and 15-HPETE were the most potent inhibitors of PHS cyclooxygenase activity. An I_{50} of approximately 1.2 μM was calculated for both 12-HPETE and 15-HPETE. Similar results with purified PHS and 12-HPETE and 15-HPETE were reported by Markey *et al.* (12). Hydrogen peroxide was also very potent, with an I_{50} of approximately 4 μM. Less potent was *t*-butyl hydroperoxide, with an I_{50} of approximately 10 μM. 15-

HPETE (2 μM) and *t*-butyl hydroperoxide (50 μM) also inhibited formation of PGs by 60 and 54%, respectively, in incubations with RSV microsomes initiated with [^{3}H]arachidonic acid. However, 4-hydroperoxyphenylbutazone was an ineffective inhibitor of PHS cyclooxygenase at the concentration range used. At 100 μM 4-hydroperoxyphenylbutazone, only 10% inhibition was observed. 4-Hydroxyphenylbutazone was completely ineffective as an inhibitor of PHS cyclooxygenase activity (data not shown).

4-Hydroperoxyphenylbutazone-dependent metabolism. The effectiveness of 4-hydroperoxyphenylbutazone as a substrate for PHS hydroperoxidase was examined using the formation of the aminopyrine cation radical as an index of hydroperoxidase metabolism. The addition of 15-HPETE and hydrogen peroxide to incubations containing solubilized RSV microsomes produced a rapid formation of the aminopyrine cation radical (28). *t*-Butyl hydroperoxide was less effective in supporting aminopyrine metabolism. In contrast, 200 μM 4-hydroperoxyphenylbutazone was ineffective in supporting aminopyrine oxidation (Fig. 8). HPLC analysis of ethyl acetate extracts of incubations initiated with 4-hydroperoxyphenylbutazone in the presence and absence of solubilized RSV microsomes (Fig. 9) showed that 4-hydroperoxyphenylbutazone was not significantly reduced to 4-hydroxyphenylbutazone by PHS hydroperoxidase. Thus 4-hydroperoxyphenylbutazone appears to be a poor substrate for PHS hydroperoxidase.

Discussion

We have characterized the intermediates in the PHS hydroperoxidase oxidation of phenylbutazone to 4-hydroxyphenylbutazone and investigated the relationship between phenylbutazone oxidation and inhibition of PHS cyclooxygenase. The hydroperoxidase-dependent metabolism (Scheme 1) begins with a one-electron oxidation of phenylbutazone to a carbon-centered radical. This radical was detected by ESR with the spin trap *t*-NB. The same radical, formed by chemical oxidation of phenylbutazone, was detected by ESR (40).

The carbon-centered radical reacts with molecular oxygen to form a phenylbutazone peroxyl radical. This reaction was inhibited by *t*-NB. Attempts to detect the peroxyl radical by ESR were unsuccessful. Indirect observations suggest that it was an intermediate during phenylbutazone hydroperoxidase-mediated oxidation. Reed *et al.* (16) have reported the enhanced epoxidation of BP-7,8-diol in the presence of phenylbutazone in RSV microsomal incubations.

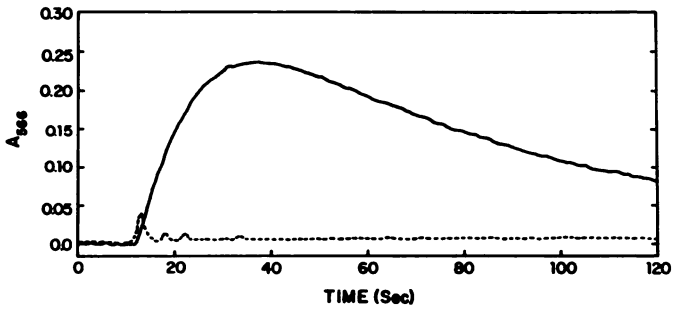


Fig. 8. Formation of the aminopyrine cation free radical by PHS hydroperoxidase initiated with either 200 μM hydrogen peroxide (——) or 200 μM 4-hydroperoxyphenylbutazone (– – –). The peroxides were added to incubation mixtures (37°) consisting of 1 mM aminopyrine and 0.25 mg/ml solubilized RSV microsomes after a 2-min preincubation period.

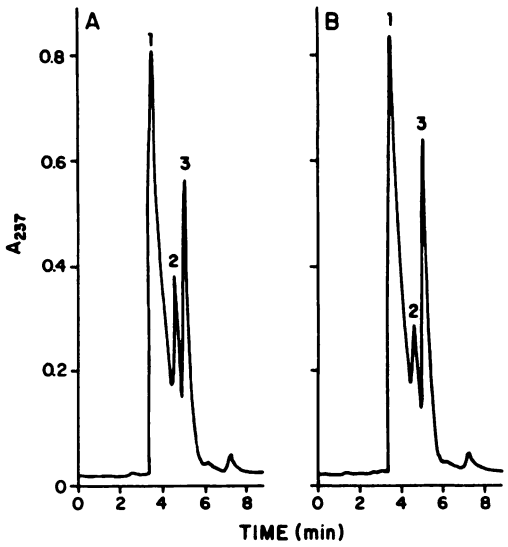
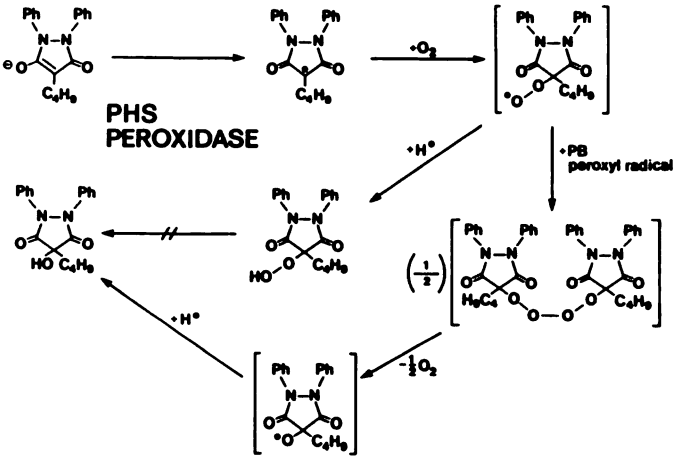


Fig. 9. HPLC chromatograms of the ethyl acetate extracts of incubations (37°, 2 min) consisting of 1 mM aminopyrine and 200 μM 4-hydroperoxyphenylbutazone in the presence (A) and absence (B) of 0.25 mg/ml solubilized RSV microsomes. The extract was analyzed by the isocratic HPLC system described in Experimental Procedures. The labeled peaks are: 1) aminopyrine; 2) 4-hydroxyphenylbutazone; and 3) 4-hydroperoxyphenylbutazone.



Scheme 1. PHS peroxidase-mediated oxidation of phenylbutazone and the proposed subsequent reactions resulting in formation of 4-hydroperoxyphenylbutazone and 4-hydroxyphenylbutazone.

We were able to isolate and identify 4-hydroperoxyphenylbutazone in incubations with RSV microsomes and HRP. Ichihara *et al.* (31) have isolated and identified 4-hydroperoxyphenylbutazone in incubations with HRP and leukocyte extracts. The isolated material was characterized as 4-hydroperoxyphenylbutazone by mass spectroscopy and nuclear magnetic resonance (31). The analysis by nuclear magnetic resonance indicated the presence of a hydroperoxy group at carbon 4 and eliminated diphenylbutazone peroxide as a possible structure. 4-Hydroxyphenylbutazone was observed in incubations with HRP and RSV microsomes. Marnett *et al.* (10, 13) have also identified 4-hydroxyphenylbutazone in RSV microsomal incubations. Ichihara *et al.* (31) have identified this metabolite in incubations with leukocyte extracts. The mechanism for the formation of 4-hydroxyphenylbutazone is not clear. Incubating 4-hydroperoxyphenylbutazone with RSV microsomes resulted in negligible enzymatic formation of 4-hydroxyphenylbutazone.

Thus this hydroperoxide was a poor substrate for PHS hydroperoxidase. Ichihara *et al.* (31) have reported similar observations during incubations of 4-hydroperoxyphenylbutazone with leukocyte extracts and proposed that 4-hydroxyphenylbutazone was formed by a mechanism distinct from that forming 4-hydroperoxyphenylbutazone. Interestingly, cumene hydroperoxide and *t*-butyl hydroperoxide, which like 4-hydroperoxyphenylbutazone are tertiary hydroperoxides, are also poor substrates for PHS hydroperoxidase (6, 28) and HRP[1]. A potential mechanism for the formation of 4-hydroxyphenylbutazone could be via the "self-reaction," a chain termination reaction between two phenylbutazone peroxyl radicals, forming a tetroxide (41). The tetroxide rearranges to two alkoxy radicals and molecular oxygen in the triplet state (42). 4-Hydroxyphenylbutazone is formed after the reactive alkoxy radicals abstract hydrogen atoms. 4-Hydroxyphenylbutazone was detected during the chemical oxidation of phenylbutazone to 4-hydroperoxyphenylbutazone (43), indicating 4-hydroxyphenylbutazone can arise in the absence of peroxidase activity. Thus the phenylbutazone peroxyl radical may either abstract a hydrogen atom, forming 4-hydroperoxyphenylbutazone, or react with a second peroxyl radical and eventually rearrange to 4-hydroxyphenylbutazone.

Phenylbutazone and other nonsteroidal anti-inflammatory drugs inhibit formation of PGs by inhibiting PHS. Although the actual mechanism of the inhibition of PHS by phenylbutazone is unknown, Reed *et al.* (20) have reported that this inhibition is dependent upon metabolism of phenylbutazone by PHS hydroperoxidase. Thus the inhibitor of PHS is not phenylbutazone but an oxidized metabolite or intermediate. Our observations showed that phenylbutazone essentially had little inhibitory effect on the cyclooxygenase activity in preparations of purified PHS devoid of peroxidase activity. Phenylbutazone significantly inhibited cyclooxygenase activity in preparations of purified PHS that possessed peroxidase activity, thus showing the requirement for peroxidative metabolism of phenylbutazone in order for inhibition to occur.

The question arose as to what oxidized metabolite of phenylbutazone was the inhibitor. The stable products of the hydroperoxidase-mediated oxidation of phenylbutazone were considered. 4-Hydroxyphenylbutazone was ineffective as a PHS cyclooxygenase inhibitor. Portoghese *et al.* (14) have reported similar results with 4-hydroxyoxyphenbutazone. Studies have shown the sensitivity of PHS to peroxides (21, 22). However, 4-hydroperoxyphenylbutazone was a weak inhibitor of PHS cyclooxygenase activity present in RSV microsomes. Other peroxides examined were more potent inhibitors of cyclooxygenase activity than 4-hydroperoxyphenylbutazone. The inhibitory activity in decreasing order of the peroxides tested was 12-HPETE = 15-HPETE > H_2O_2 ≫ *t*-butyl hydroperoxide ≫ 4-hydroperoxyphenylbutazone. The results with the hydroperoxides other than 4-hydroperoxyphenylbutazone were similar to those reported by Markey *et al.* (12), who used purified PHS in their incubations. The potency of the lipid hydroperoxides 12- and 15-HPETE was most likely due to their ease in rearranging to peroxyl radicals catalyzed by hematin (36). In contrast, *t*-butyl hydroperoxide, cumene hydroperoxide, and, most likely, 4-hydroperoxyphenylbutazone undergo negligible hematin-catalyzed hydrogen abstraction to a peroxyl radical. This is based on observations that *t*-butyl hydroperoxide and cumene hydroperoxide do not support hematin-catalyzed epoxidation of BP-7,8-diol (36). In view of the potency of phenylbutazone to inhibit PHS and prostacyclin synthase (20), these data suggest that the inhibitor of PHS may be the phenylbutazone peroxyl radical. Also, if the peroxyl radicals self-react to form the tetroxide, the decomposition products of this intermediate, the alkoxy radicals may be the inhibitor.

The potent cyclooxygenase inhibitors 15-HPETE and hydrogen peroxide are efficient substrates for PHS hydroperoxidase (28), whereas the weaker inhibitors *t*-butyl hydroperoxide and 4-hydroperoxyphenylbutazone are inefficient substrates for this enzyme. Thus there appears to be a relationship between substrate efficiency for PHS hydroperoxidase and inactivation of PHS cyclooxygenase by hydroperoxides. The hydroperoxidase can protect the enzyme from inhibition by peroxides in the presence of a reducing cofactor yet is required for peroxide inhibitory action. The dual action of the hydroperoxidase has been noted previously by Markey *et al.* (12). They observed that in incubation mixtures containing PHS holo- and apoenzyme, only the holoenzyme, which possesses hydroperoxidase activity, was inactivated by peroxides. Also, increasing PHS holoenzyme concentrations increased the I_{50} concentration of 15-HPETE.

Thus phenylbutazone is oxidized by PHS hydroperoxidase to a carbon-centered radical, which in turns reacts with molecular oxygen to form a peroxyl radical. The peroxyl radical abstracts a hydrogen atom forming 4-hydroperoxyphenylbutazone. It appears that the peroxyl radical and not 4-hydroperoxyphenylbutazone may be the inhibitor of PHS cyclooxygenase. The peroxyl radical may also react with a second peroxyl radical forming a tetroxide, which rearranges to form 4-hydroxyphenylbutazone. Clearly, further experiments are necessary to fully understand the mechanism responsible for the hydroperoxidase-dependent inactivation of PHS cyclooxygenase.

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