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Mechanism of Inhibition of Prostaglandin H Synthase by Eugenol and Other Phenolic Peroxidase Substrates

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SUMMARY

The mechanism of inhibition of prostaglandin H synthase (PHS) by eugenol was investigated using purified apoenzyme reconstituted with either manganese protoporphyrin IX (Mn-PHS) or hematin (Fe-PHS). Eugenol stimulated Fe-PHS activity at low concentrations and inhibited at higher concentrations, an activity typical of many phenolic compounds. Eugenol was also an excellent reducing cosubstrate for the peroxidase, being cooxidized to a reactive quinone methide in the process. Higher concentrations of eugenol were required to inhibit Fe-PHS than Mn-PHS (which retains cyclooxygenase activity but not peroxidase activity). Inhibition by eugenol was highly dependent on arachidonic acid concentration. In experiments using Mn-PHS, eugenol increased the time required for the initiation of O_2 consumption after addition of arachidonic acid and also inhibited the rate of O_2 uptake. Eugenol did not, however, affect the total amount of O_2 consumed. The addition of 10 μM hydroperoxide (prostaglandin G_2) to these incubations did not prevent the inhibitory effects of eugenol. Other phenolic compounds, including guaiacol, butylated hydroxyanisole, and acetaminophen inhibited Mn-PHS in a manner similar to eugenol. These results demonstrate that eugenol and other phenolic compounds specifically inhibit the cyclooxygenase component of PHS and that this inhibition occurs in addition to, or independent of, the effect of these compounds on peroxide tone or their peroxidative metabolism. We suggest that this inhibition is due to competition with arachidonic acid for the active site of PHS.

Phenol is a well known example of compounds that have dual effects on PHS activity. At low concentrations (200 μM) phenol stimulates PHS activity, whereas at much higher concentrations (4500 μM) it inhibits (1). The great majority of phenolic compounds tested for their effects on PHS activity show a similar phenomenon, although the concentrations at which stimulation and inhibition occur vary greatly. This dual effect is not limited to phenolic compounds, however, but also includes aromatic amines, antioxidants, and other compounds as well (1–5). The stimulation of PHS activity by these compounds has been attributed to their ability to provide reducing equivalents to the peroxidase and to their ability to scavenge radicals and thus protect the enzyme from self-inactivation (4, 6). On the other hand, inhibition at high concentrations has been explained by the requirement for hydroperoxide to initiate the catalytic cycle of PHS (7, 8). These compounds may prevent the level of hydroperoxide present (the "peroxide tone") from increasing to a point where PHS activity can be initiated. Phenolic antioxidants are capable of quenching radical intermediates in the cyclooxygenase reaction and high concentrations of these phenolic compounds would necessitate continuous reactivation of the enzyme (6, 9). This would require an increased level of hydroperoxide to initiate the accelerative phase of the cyclooxygenase reaction. Conversely, other investigators have argued, through structure-activity studies, that certain phenolic compounds are competitive inhibitors of PHS (5). Supporting evidence comes from studies that show increased inhibition of these compounds at low substrate (arachidonic acid) concentrations versus high substrate concentrations (10).

Studies on the inhibition of PHS by various compounds have been complicated by at least three problems, 1) use of microsomal preparations of PHS rather than purified enzyme, 2) inclusion of various activator compounds, such as phenol, glutathione, or tryptophan into inhibition assays, and 3) the fact that PHS holoenzyme contains two distinct catalytic components, cyclooxygenase and peroxidase, which makes proper kinetic studies difficult to perform and interpret. The availability of PHS apoenzyme circumvents these problems. The apoenzyme can be reconstituted with either manganese protoporphyrin IX, which restores only cyclooxygenase activity, or with hematin, which restores both functions (11). Thus, the effects of a compound on the cyclooxygenase alone, peroxidase alone (by using a peroxide as substrate rather than arachidonic acid), or the holoenzyme can be studied.

In the present study we have used the PHS apoenzyme, reconstituted with either manganese protoporphyrin IX or hematin, to study the mechanism of inhibition of PHS by

ABBREVIATIONS: PHS, prostaglandin H synthase; PPHP, 5-phenyl-4-pentenyl hydroperoxide; BHA, butylated hydroxyanisole; PGG_2, prostaglandin G_2; DETAPAC, diethylenetriamine pentaacetic acid; HPLC, high pressure liquid chromatography.

eugenol and several other phenolic compounds. Eugenol (4-allyl-2-methoxyphenol) is a phenolic compound used as a flavoring agent in foods and medicines and also as an analgesic in dentistry (12). The analgesic properties of eugenol have been attributed to its ability to inhibit PHS (13). *In vitro* assays have demonstrated that eugenol inhibits PHS at concentrations as low as 5×10^{-7} M and is slightly more potent than indomethacin (5, 14, 15). Eugenol is also oxidized by peroxidases to a reactive intermediate that is thought to be a quinone methide (16). Our results suggest that eugenol and other phenolic compounds are direct inhibitors of the cyclooxygenase component of PHS and that this inhibition occurs in addition to, or independent of, the effects on peroxide tone or peroxidase-dependent metabolism.

Experimental Procedures

Materials. PHS holoenzyme (purity > 90% as determined by sodium dodecyl sulfate gel electrophoresis) and PPHP were obtained from Oxford Biomedical Research, Inc. (Oxford, MI) whereas PHS apoenzyme (purity > 95% as determined by sodium dodecyl sulfate gel electrophoresis) was obtained from Biomol Research Laboratories (Philadelphia, PA). Arachidonic acid was purchased from Nu Chek Prep (Elysian, MN), whereas PGG_2 was from Calbiochem (La Jolla, CA) and [^{3}H]arachidonic acid (100 Ci/mmol) was from New England Nuclear (Boston, MA). Eugenol, guaiacol, flufenamic acid, hydroquinone, acetaminophen, aminopyrine, *n*-propyl gallate, DETAPAC, and hematin were obtained from Sigma Chemical Co. (St. Louis, MO). Phenol was purchased from Baker Chemical Co. (Phillipsburg, NJ) and BHA was ordered from Fluka Chemical Co. (Ronkonkoma, NY). Manganese protoporphyrin IX was obtained from Porphyrin Products, Inc. (Logan, UT) and hydrogen peroxide (30% solution) was from Fisher Scientific (Fair Lawn, NJ). Ram seminal vesicle microsomes were prepared as described earlier (17).

PPHP assay. The reduction of PPHP to its corresponding alcohol in the presence of PHS (holoenzyme) and various reducing cosubstrates was carried out as described by Markey *et al.* (4). Reactions contained 154 nM PHS and 200 μM reducing cofactor in 1 ml of 0.1 M phosphate buffer, pH 7.8, containing 200 μM Tween 20 and 1 mM DETAPAC. Reactions were preincubated for 3 min, initiated by the addition of 100 μM PPHP, and incubated at 37°C for 3 min. Reactions were terminated and analyzed for PPHP reduction by HPLC as described.

Peroxidase assays. Mn-PHS is deficient in peroxidase activity (7, 11). We tested our Mn-PHS for residual peroxidase activity by three different criteria. First, the PPHP assay described above was carried out using PHS apoenzyme and 1 μM manganese protoporphyrin IX in the absence of reducing cosubstrates. Controls consisted of manganese protoporphyrin IX alone and PHS alone. Reaction conditions were the same as described above. Second, the metabolism of [^{3}H]arachidonic acid by Mn-PHS was analyzed. Reactions contained 10 μg of PHS apoenzyme, 5 μM manganese protoporphyrin IX, and 100 μM arachidonic acid (0.5 μCi) in a total volume of 1 ml of 0.1 M Tris buffer, pH 7.8, with 1 mM DETAPAC. A separate reaction also contained 200 μM eugenol. Reactions were incubated at 37° for 3 min and stopped by acidification with 20 μl of glacial acetic acid. Samples were diluted 1:1 with methanol and analyzed for prostaglandin metabolites by HPLC (18). Mn-PHS, without peroxidase activity, should theoretically yield 15-hydroperoxy-prostaglandin E_2 (the hydrolysis product of PGG_2) rather than prostaglandin E_2 (the hydrolysis product of prostaglandin H_2). The ratio of 15-hydroperoxy-prostaglandin E_2/prostaglandin E_2 was determined. Third, the oxidation of two peroxidase cosubstrates, eugenol and aminopyrine, was followed using spectrophotometric methods. Reactions contained 1 mM aminopyrine (or various concentrations of eugenol), 10 μg/ml PHS apoenzyme, and 1 μM hematin or manganese protoporphyrin IX in a total volume of 2 ml of 0.1 M Tris buffer, pH 7.8, with 1 mM DETAPAC. Reactions were incubated at 37° with stirring and were started by the addition of 200 μM hydrogen peroxide. The absorbance change at 350 nm (eugenol) or 566 nm (aminopyrine) was followed for 2 min on a Hewlett Packard 8450A diode array spectrophotometer.

PHS inhibition assays. The effect of eugenol and other phenolic compounds on PHS activity was determined using three types of enzyme, ram seminal vesicle microsomes, Fe-PHS, and Mn-PHS. Incubations with microsomal PHS contained 0.25 mg of ram seminal vesicles, 500 μM phenol (when present), 10 or 100 μM arachidonic acid, and various concentrations of eugenol in a total volume of 1.5 ml of 0.1 M Tris buffer, pH 7.8, with 1 mM DETAPAC. The effect of eugenol on enzyme activity was measured by following oxygen uptake using a Yellow Springs, Inc. Model 53 oxygen-monitoring system. The oxygen uptake experiments were conducted with air-saturated buffer (100% air saturation equals 240 μM oxygen). Reactants were preincubated for 1 min before the addition of arachidonic acid.

The effect of eugenol and several other phenolic compounds on Mn-PHS and/or Fe-PHS was determined using the same system, with the exception of substituting 10 μg/ml PHS apoenzyme and 1–5 μM hematin or manganese protoporphyrin IX for the microsomal enzyme. Phenol was omitted from these incubations and arachidonic acid concentrations were varied as indicated. In some incubations with Mn-PHS, PGG_2 (1–10 μM) or ascorbic acid (0–50 mM) was preincubated for 1 min with the enzyme and eugenol (where indicated) before the addition of arachidonic acid.

Results

Effect of eugenol on microsomal PHS. The effects of eugenol on PHS using ram seminal vesicle microsomes as the source of enzyme were investigated. Because many previous investigations have used phenol as an activator of PHS, these assays were carried out in both the presence and absence of 500 μM phenol. Using 100 μM arachidonic acid as the substrate, eugenol stimulated oxygen consumption at concentrations up to 100 μM (Fig. 1). Oxygen uptake was due only to incorporation of oxygen into arachidonic acid because hydrogen peroxide-dependent metabolism of eugenol did not elicit oxygen uptake (not shown). At higher concentrations, eugenol began to inhibit oxygen consumption. The bell-shaped curve resulting from both the stimulation and inhibition of PHS is similar to that reported for several other phenolic compounds. The stimulation of oxygen consumption was not seen in the presence of 500 μM phenol, as would be expected. The I_{50} values for eugenol inhibition were 4.6 and 2.8×10^{-4} M for unstimulated versus phenol-stimulated, respectively. These inhibitory concentrations for eugenol were much higher than those previously reported for eugenol by Dewhirst (1.2×10^{-5} M) (5). Therefore, we tried a lower concentration of arachidonic acid (10 μM) in order to approximate his conditions more closely. Using this lower concentration of substrate, eugenol was a more potent inhibitor in both the absence (8.6×10^{-5} M) and presence (6.9×10^{-5} M) of phenol. In each instance, the inhibition by eugenol began at a eugenol:arachidonic acid ratio of >1:1. Thus, the I_{50} for eugenol inhibition appeared to be highly dependent on substrate concentration.

Metabolism of eugenol by PHS peroxidase. Eugenol is oxidized by horseradish peroxidase to a reactive intermediate that is thought to be a quinone methide (16). This reactive metabolite has the capacity to covalently bind to thiols and proteins, and, therefore, represents a potential mechanism for the inactivation or inhibition of PHS. We tested the ability of PHS peroxidase to oxidize eugenol to a quinone methide by monitoring the absorbance change at 350 nm. In the presence

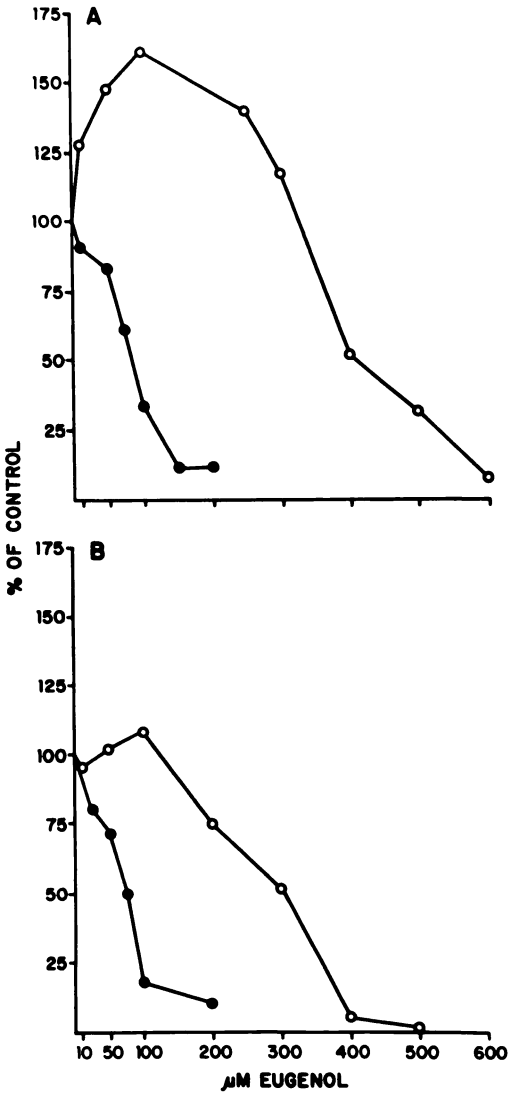

Fig. 1. Effect of eugenol on prostaglandin formation in phenol-stimulated or unstimulated ram seminal vesicle microsomes. Prostaglandin formation was monitored by measuring oxygen incorporation into arachidonic acid. Reactions contained 0.25 mg/ml ram seminal vesicle microsomes, 500 μM phenol (when present), 10 or 100 μM arachidonic acid, and various concentrations of eugenol in 1.5 ml of 0.1 mM Tris buffer (pH 7.8) containing 1 mM DETAPAC. Reactants were preincubated for 1 min at 37°C before the addition of arachidonic acid. A, Unstimulated; B, phenol-stimulated, ○, 100 μM arachidonic acid; ●, 10 μM arachidonic acid.

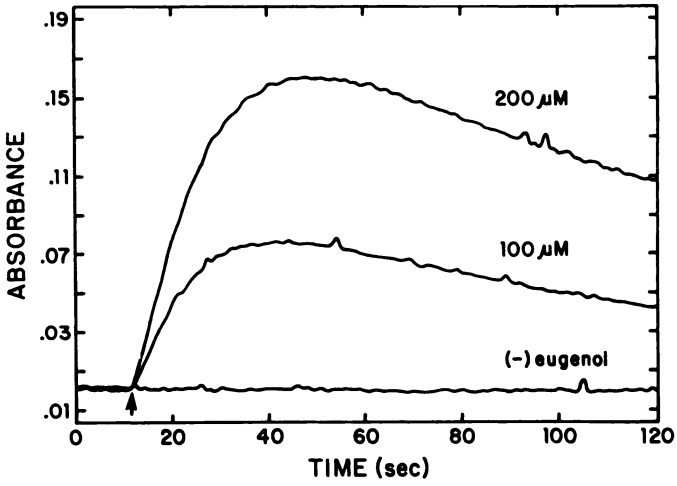

Fig. 2. Eugenol oxidation by the peroxidase component of Fe-PHS. Reactions contained 10 μg/ml PHS apoenzyme, 1 μM hematin, and various concentrations of eugenol in 2 ml of 0.1 M Tris buffer (pH 7.8) with 1 mM DETAPAC. Reactions were incubated at 37° with stirring and were started by the addition of 200 μM hydrogen peroxide (*arrow*). Absorbance changes at 350 nm were measured over time.

TABLE 1

Ability of eugenol to provide reducing equivalents to PHS peroxidase

Reactions contained 154 nM PHS and 200 μM test compound in 1 ml of 0.1 M phosphate buffer, pH 7.8, containing 200 μM Tween 20 and 1 mM DETAPAC. Reactions were preincubated for 3 min, initiated by the addition of 100 μM PPHP, and incubated at 37° for 3 min. Reactions were terminated and analyzed for PPHP reduction by HPLC as described by Markey *et al.* (4). Index refers to the percentage of PPHP that was reduced in the reaction (index = 1.00 means 100% of PPHP was reduced; index = 0.50 means 50%, etc.). Values are mean ± standard deviation.

Compound	Index
Control (no reducing substrate)	0.09 ± 0.01
Eugenol	0.67 ± 0.04
BHA	0.50 ± 0.01
Phenol	0.23 ± 0.01

of 200 μM hydrogen peroxide, we observed that Fe-PHS oxidized eugenol in a concentration-dependent manner (Fig. 2). The rate of formation of the quinone methide with 200 μM eugenol was 54 absorbance units/min/mg of PHS.

Eugenol as a reducing cosubstrate for PHS peroxidase. With few exceptions, compounds that are cooxidized by PHS are efficient electron donors to the peroxidase. The ability of a compound to serve as a reducing cosubstrate can be conveniently measured by following the reduction of the hydroperoxide PPHP to its corresponding alcohol in the presence and absence of the test substance (4). The results are usually expressed as an index (PPHP reduced/PPHP reduced + PPHP), where a value of 1.00 indicates complete reduction of PPHP to the alcohol and hence an excellent reducing cosubstrate. The ability of eugenol to donate electrons to PHS peroxidase (holoenzyme) was assayed using this method along with phenol and BHA to serve as comparison compounds. As seen in Table 1, eugenol was the most effective electron donor of the three compounds. Markey *et al.* (4) have reported values of 0.50 and 1.00 for phenol and BHA, respectively. In their experiments, the enzyme concentration was adjusted to give phenol an index of 0.50. Our results are similar to theirs in that BHA was approximately twice as effective as phenol but, due to lower enzyme activity, our results also show that eugenol is more effective than BHA, which would not be apparent at higher PHS concentrations. Thus, eugenol would have an index of 1.00 if compared with their results. In any event, eugenol is a very effective electron donor to PHS peroxidase.

Peroxidase activity of Mn-PHS. Mn-PHS has only cyclooxygenase activity, with little or no peroxidase activity (11). In order to test our enzyme preparations for residual peroxidase activity from possible contaminating holoenzyme, we employed three different test criteria. First, we compared the ability of Mn-PHS and Fe-PHS to oxidize eugenol to the quinone methide metabolite. As Fig. 3 demonstrates, there was no detectable quinone methide formed in the presence of Mn-PHS. We also used aminopyrine as a peroxidase substrate and were only able to detect product formation in the presence of Fe-PHS (not shown). Second, we measured the formation of prostaglandins

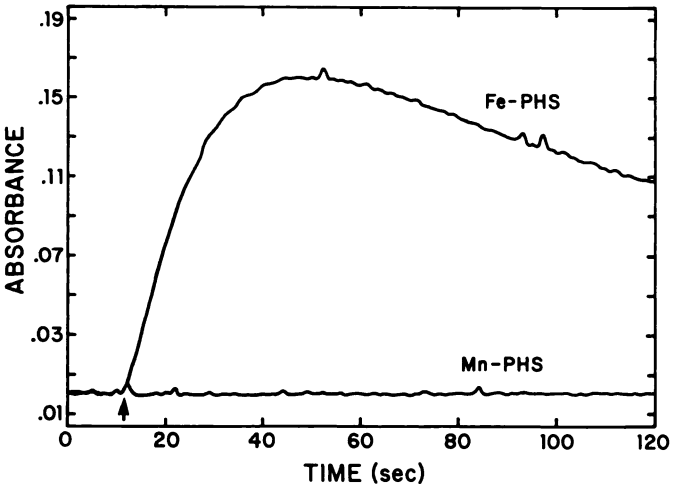

Fig. 3. Dependence of eugenol oxidation by PHS apoenzyme on hematin. Reactions contained 200 μM eugenol, 10 μg/ml PHS apoenzyme, and 1 μM hematin or manganese protoporphyrin IX. Other conditions were the same as in Fig. 2.

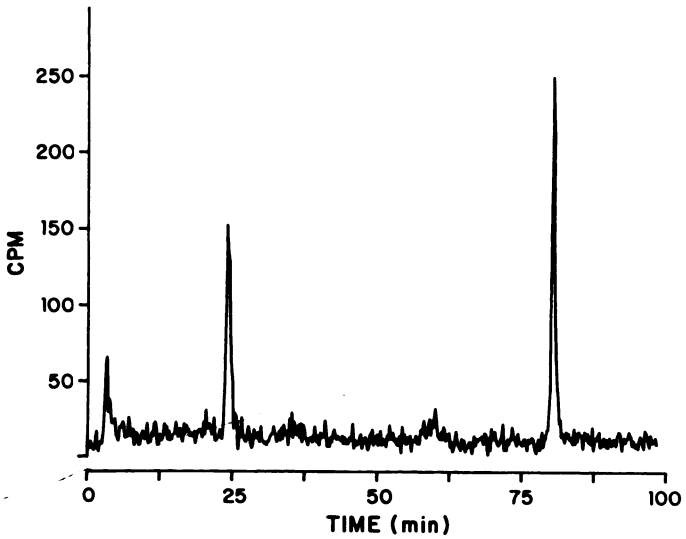

Fig. 4. Arachidonic acid-derived metabolites from incubations with Mn-PHS. Reactions contained 10 μg/ml PHS apoenzyme, 5 μM manganese protoporphyrin IX, and 100 μM arachidonic acid (0.05 μCi of [^{3}H]arachidonic acid) in 1 ml of 0.1 Tris buffer (pH 7.8) with 1 mM DETAPAC. Reactions were incubated at 37° for 3 min and stopped by acidification with 20 μl of glacial acetic acid. Samples were diluted 1:1 with methanol and analyzed by HPLC. Retention times: PGE_2, 21 min; 15-hydroperoxy-prostaglandin E_2 25 min; arachidonic acid, 82 min.

15-hydroperoxy-E_2 and E_2 in incubations containing Mn-PHS and [^{3}H]arachidonic acid. Fig. 4 illustrates the HPLC profile of radioactive metabolites obtained. Product from Mn-PHS was over 98% 15-hydroperoxy-prostaglandin E_2 (retention time, 25 min), indicating lack of peroxidase activity. In the presence of 200 μM eugenol, all product formation was inhibited. Third, using the aforementioned PPHP assay, negligible peroxidase activity was detected with manganese protoporphyrin IX alone, PHS apoenzyme alone, or Mn-PHS (not shown). Together, these results provide strong evidence that our preparation of Mn-PHS was virtually 100% free of residual peroxidase activity.

Effects of eugenol on Mn-PHS versus Fe-PHS. The effects of eugenol on either Mn-PHS or Fe-PHS were measured (Fig. 5). In Fig. 5A, eugenol caused a concentration-dependent

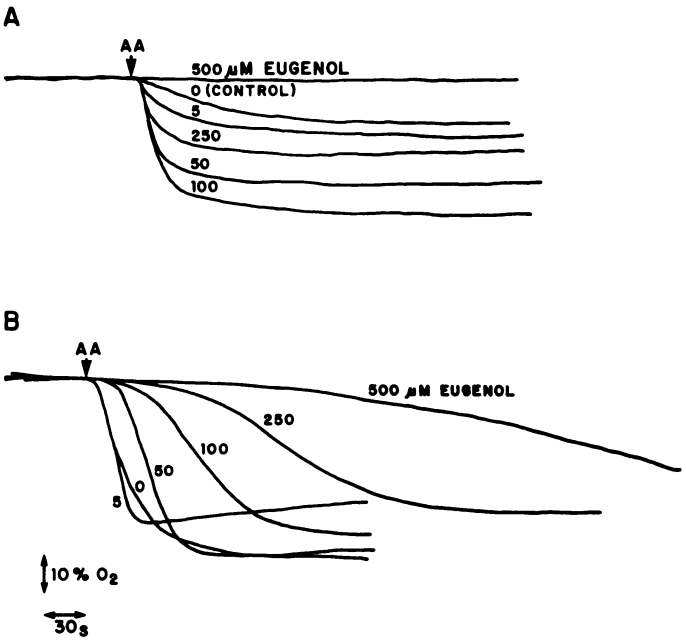

Fig. 5. Effect of eugenol on arachidonic acid-dependent O_2 consumption by PHS apoenzyme reconstituted with either hematin (A) or manganese protoporphyrin IX (B). Reactions contained 10 μg/ml PHS apoenzyme, 1 μM hematin or manganese protoporphyrin IX, and 5 to 500 μM eugenol in 1.5 ml of 0.1 M Tris buffer (pH 7.8) with 1 mM DETAPAC. Reactions were carried out at 37° and started by the addition of 50 μM arachidonic acid (*arrow*).

enhancement of oxygen uptake using Fe-PHS. At concentrations above 100 μM, eugenol began to inhibit the enzyme activity. At 500 μM eugenol, there was no detectable enzyme activity. These results parallel those seen earlier with microsomal PHS (Fig. 1). In Fig. 5B, results using Mn-PHS are presented. At concentrations of 50 μM and higher, eugenol inhibited both the initiation of oxygen uptake and the rate of oxygen uptake (I_{50} = 85 μM) that occurred after the addition of arachidonic acid. The inhibition appeared to be reversible, because the total amount of oxygen consumed in each incubation was about the same. Even in incubations containing 500 μM eugenol, oxygen consumption eventually reached the control level if followed for a long enough time. A concentration of 100 μM eugenol had opposite effects on Fe-PHS and Mn-PHS. At this concentration, eugenol stimulated Fe-PHS activity while it inhibited Mn-PHS. Thus, the cooxidation of eugenol by the peroxidase activity of Fe-PHS interfered with (or masked) the ability of eugenol to inhibit the cyclooxygenase. These results indicated that eugenol is capable of inhibiting the cyclooxygenase component of PHS and that this inhibition is independent of its ability to serve as a reducing cosubstrate for the peroxidase component of PHS.

The effect of eugenol on Mn-PHS activity in the presence of various concentrations of arachidonic acid was also determined. As can be seen in Fig. 6, eugenol was a progressively more potent inhibitor at lower concentrations of arachidonic acid. This suggested that eugenol might be acting as a competitive inhibitor. A double-reciprocal plot of data using three different eugenol concentrations (Fig. 7) demonstrated that this was indeed the case. These data were calculated from the rates of oxygen uptake and did not take into account the lag period before oxygen uptake began, which also progressively increased.

Effect of eugenol on peroxide tone. Both Fe-PHS and

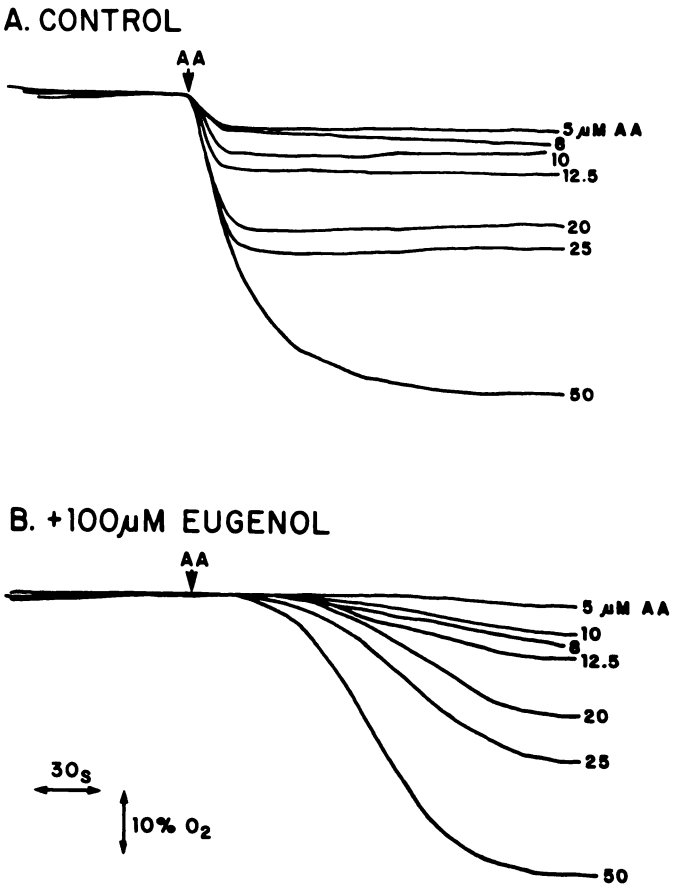

Fig. 6. Arachidonic acid-dependent O_2 consumption by Mn-PHS in the presence or absence of 100 μM eugenol. Reactions contained 10 μg/ml PHS apoenzyme, 1 μM manganese protoporphyrin IX, 100 μM eugenol (when present), and 5 to 50 μM arachidonic acid (*AA*) in 1.5 ml of 0.1 M Tris buffer (pH 7.8) with 1 mM DETAPAC. Reactions were carried out at 37° and started by the addition of arachidonic acid (*arrow*).

Mn-PHS require low levels of hydroperoxide (approximately 10^{-8} M) to initiate and sustain the enzyme reaction (9, 19). We tested the possibility that eugenol inhibits PHS by increasing the requirement for hydroperoxide activator by testing the effect of exogenous PGG_2 on Mn-PHS activity in the presence of eugenol (Fig. 8). Concentrations of up to 10 μM PGG_2 had no effect on the eugenol-dependent inhibition of the rate of oxygen uptake. The concentrations of hydroperoxide used in these experiments (10^{-6} to 10^{-5} M) far exceed the levels needed for enzyme activation (10^{-8} M) and, therefore, it appeared that the inhibitory effect of eugenol on Mn-PHS was independent of any effect on peroxide tone.

We also observed that ascorbic acid had a stimulatory effect on Mn-PHS activity. This was unexpected, because stimulation of the holoenzyme is thought to occur through peroxidase activity. Nevertheless, ascorbic acid exhibited a marked ability to increase oxygen consumption (Fig. 9). This stimulation continued even at high ascorbate concentrations (50 mM). No inhibition of Mn-PHS was seen with ascorbate. The increase in oxygen consumption was correlated with an increase in arachidonic acid-derived metabolites (not shown). Thus, ascorbic acid apparently protected the Mn-PHS from self-inactivation. This may occur via the reduction of oxidized enzyme intermediates back to a nondestructive state. Because this has been proposed as a possible site of action for the inhibition by phenolic compounds of PHS (9), we tested the effect of ascorbate on eugenol-dependent inhibition of Mn-PHS. As Fig. 9 illustrates, ascorbate had no effect on inhibition of PHS by eugenol, either on the lag phase or the rate of oxygen uptake. These results suggest that eugenol and ascorbate are acting at independent sites.

Effects of other phenolic compounds on Mn-PHS. The effects of several other phenolic compounds and flufenamic acid on Mn-PHS activity were also tested. The phenolic compounds tested included phenol, guaiacol, acetaminophen, hydroquinone, BHA, and *n*-propyl gallate. Each of these compounds was observed to inhibit Mn-PHS in a manner similar to eugenol. Each compound caused an increased lag time before oxygen incorporation began and also inhibited the rate of oxygen uptake but had little effect on total oxygen consumed during the experiment, although in some cases a little stimulation was observed. Flufenamic acid, a member of an entirely different class of PHS inhibitors than the phenols, was also observed to inhibit Mn-PHS in the same manner. The oxygraph traces for flufenamic acid, guaiacol, and BHA are shown in Fig. 10. The rest are omitted for the sake of brevity. Flufenamic acid has previously been reported to be a competitive inhibitor of PHS with respect to arachidonic acid (10). These results demonstrate that the various phenolic compounds tested appeared to inhibit Mn-PHS by a common mechanism and that this is similar to the mechanism of inhibition of PHS by fenamic acid derivatives.

Discussion

In this study we examined the various effects of eugenol on PHS in order to determine its mechanism of inhibition. *A priori*, we considered three possible mechanisms of inhibition, 1) peroxidase oxidation of eugenol to a reactive intermediate that inhibits the enzyme, 2) inhibition by lowering of peroxide tone, or 3) interference with the interaction of arachidonic acid with enzyme.

We observed that eugenol had both stimulatory and inhibitory effects on PHS. This occurred with both the microsomal PHS from ram seminal vesicles and purified Fe-PHS. The stimulation of PHS by eugenol was due to its ability to serve as a peroxidase cosubstrate. Eugenol was an excellent source of reducing equivalents to PHS peroxidase but the resulting formation of the quinone methide was not involved in the inhibition of PHS. In fact, oxidation of eugenol masked the ability of eugenol to inhibit PHS, which was apparent in incubations comparing Fe-PHS with Mn-PHS. This confirms an earlier suggestion that inhibition of PHS by phenolic compounds is independent of their ability to serve as cosubstrates of PHS peroxidase (9) and rules out the first possible mechanism of PHS inhibition by eugenol. PHS appears to be quite resistant to inactivation (or inhibition) by reactive metabolites produced during the cooxidation of xenobiotics, being regulated instead by a self-inactivation mechanism (4). For example, the reactive metabolite of acetaminophen, *N*-acetyl-*p*-benzoquinoneimine, does not inhibit PHS (2).

A second possible mechanism of PHS inhibition by eugenol involves lowering of peroxide tone. The concept of peroxide tone arose from observations that glutathione peroxidase, in the presence of glutathione, inhibited PHS activity and from observations that low concentrations of lipid hydroperoxide are required to initiate PHS activity (9, 20, 21). Glutathione per-

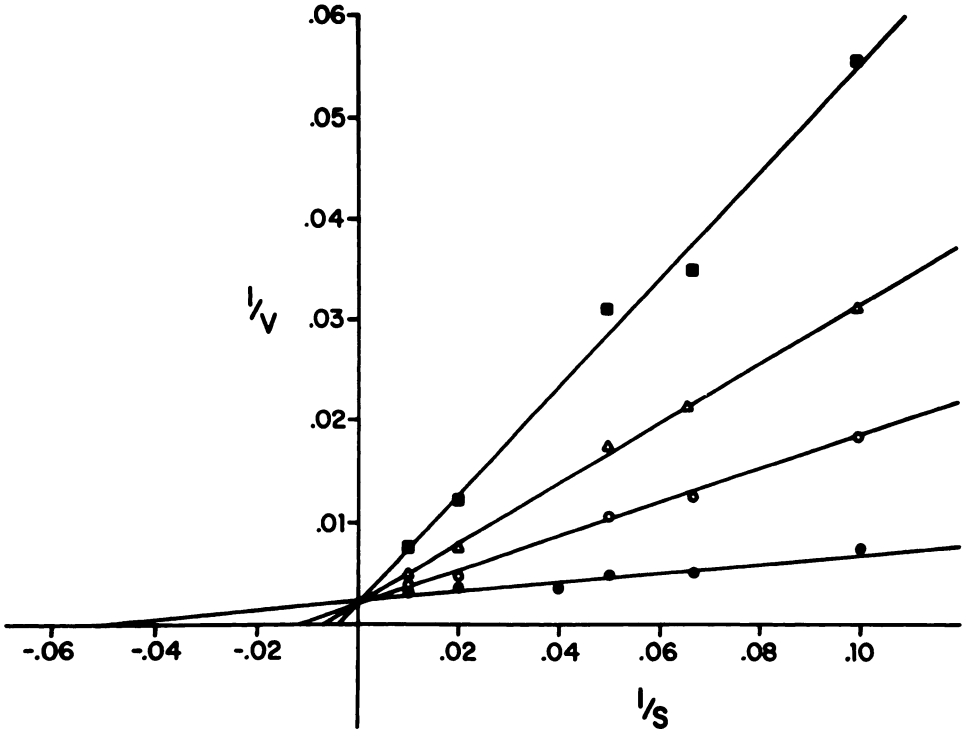

Fig. 7. Lineweaver-Burke plot of eugenol inhibition of Mn-PHS. Reactions were carried out as described in the legend to Fig. 6, except that arachidonic acid and eugenol concentrations were varied as indicated. ●, Control (no eugenol); ○, 25 μM; △, 50 μM; ■, 100 μM eugenol.

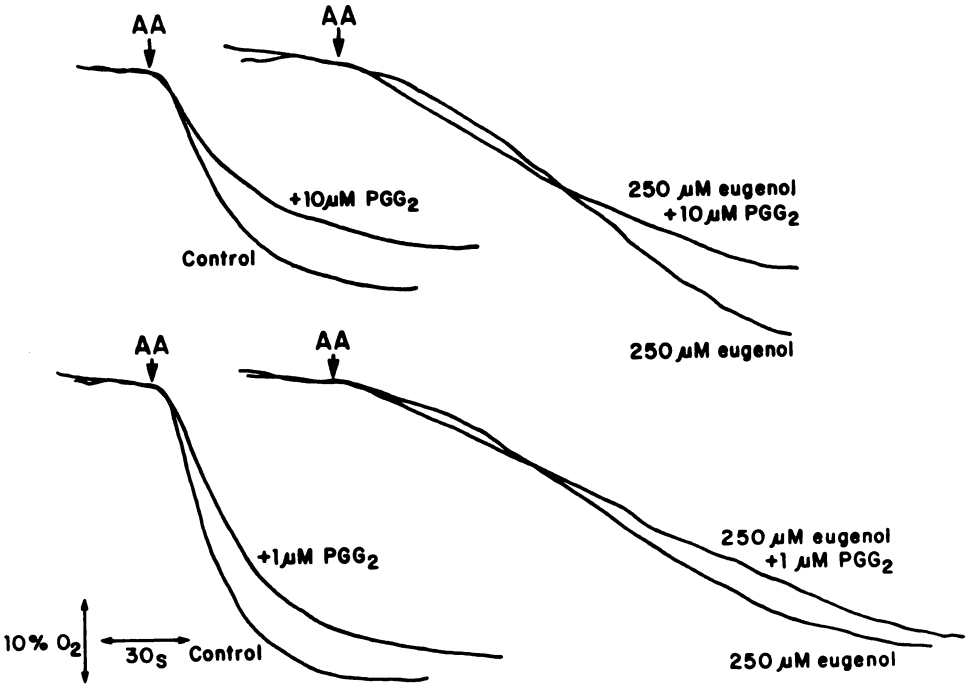

Fig. 8. Effect of PGG_2 on eugenol-dependent inhibition of oxygen consumption by Mn-PHS. Reactions contained 10 μg/ml PHS apoenzyme, 5 μM manganese protoporphyrin IX, 0 to 10 μM PGG_2, 0 or 250 μM eugenol, and 100 μM arachidonic acid (*AA*) in 1.5 ml of 0.1 M Tris buffer (pH 7.8) with 1 mM DETAPAC. Reactions were preincubated for 1 min before the addition of arachidonic acid (*arrow*).

oxidase/glutathione increases the inhibitory potency of several phenolic compounds, suggesting that phenolic compounds are better inhibitors at low levels of lipid hydroperoxide than at high levels (22). Arachidonic acid contains small amounts of hydroperoxide contaminants that nevertheless, may be present in high enough amounts to activate PHS. Thus, eugenol might be expected to have a greater inhibitory effect at low arachidonic acid concentrations than at high arachidonic acid concentrations simply because the amount of contaminating hydroperoxide would also be lower and more easily overcome by eugenol. Yet, we could not prevent the inhibitory effects of eugenol on the rate of arachidonic acid oxidation by Mn-PHS by adding back lipid hydroperoxide (PGG_2) in concentrations much higher than necessary to stimulate PHS activity.

In addition to inhibiting the rate of arachidonic acid oxidation, eugenol also increased the lag time that occurs between the addition of arachidonic acid and the initiation of oxygen uptake (Fig. 5B). The addition of PGG_2 to incubations containing eugenol and Mn-PHS had a slight stimulatory effect on the lag time (Fig. 8). Marshall and Kulmacz (23) recently suggested that the binding site for lipid hydroperoxide activators of PHS is distinct from the binding site for arachidonic acid. It is possible then that eugenol may be affecting both the binding of lipid hydroperoxide activator, which is overcome by adding

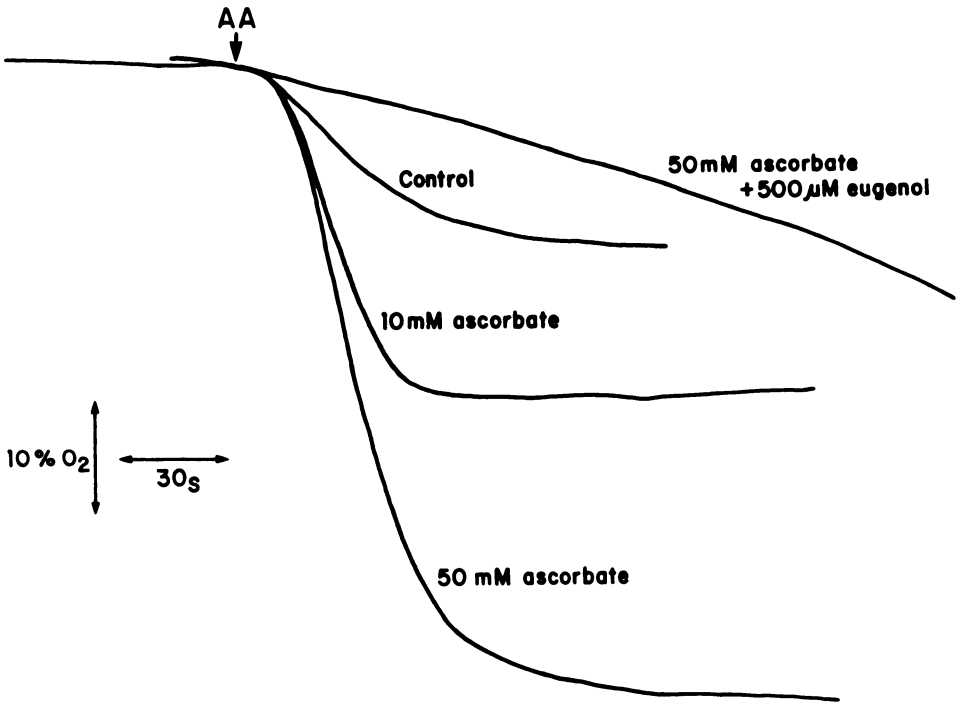

Fig. 9. Effect of ascorbic acid on Mn-PHS activity and eugenol inhibition of Mn-PHS. Reactions contained 10 μg/ml PHS apoenzyme, 1 μM manganese protoporphyrin IX, 0 to 50 mM ascorbic acid, and 50 μM arachidonic acid in 1.5 ml of 0.1 M Tris buffer (pH 7.8) with 1 mM DETAPAC. In one incubation, 500 μM eugenol was also present. Reactions were preincubated for 1 min before the addition of arachidonic acid (*arrow*).

extra hydroperoxide, and the binding of arachidonic acid, which is not influenced by hydroperoxide.

The peroxide tone hypothesis also suggests that eugenol inhibits PHS by quenching radical intermediates in the cyclooxygenase reaction thus requiring additional lipid hydroperoxide activation. Although the exact reaction mechanism of PHS is not known in detail, several radical intermediates have been identified. These include two arachidonic acid-derived radicals (an alkyl and a peroxyl radical), a protein-bound radical that may be involved in self-inactivation, and oxidized heme intermediates that resemble compounds I and II of horseradish peroxidase and may be associated with a tyrosyl radical (21, 24–27). It is unlikely that eugenol reacts with either the alkyl or peroxyl radical of arachidonic acid because alkyl radicals react extremely rapidly with oxygen (28) and the reaction of eugenol with the peroxyl radical would result in reduction of the radical to produce PGG_2 or an adduct, neither of which would inhibit the reaction rate. Eugenol does react with compounds I and II but this would not explain the greater inhibition seen with Mn-PHS compared with Fe-PHS, because manganese protoporphyrin IX-substituted peroxidases are reduced at a much slower rate by cosubstrates than are the heme-containing enzymes (29). Furthermore, reduction of the tyrosyl radical by eugenol would form compound II, which would also not explain the differences seen between Fe-PHS and Mn-PHS, or the lack of effect of added peroxide (PGG_2). Taken together, these observations suggest that eugenol inhibition of Mn-PHS is unrelated to hydroperoxide levels.

With regard to the possibility that eugenol quenches radical intermediates that lead to self-inactivation, we found that ascorbic acid stimulated Mn-PHS activity but did not inhibit, even at concentrations as high as 50 mM. A possible explanation for the effect of ascorbic acid is that it is quenching a specific protein-bound radical intermediate that leads to self-inactivation of Mn-PHS. In the presence of 50 mM ascorbic acid, eugenol-dependent inhibition of Mn-PHS was unaffected. These observations suggest that the effects of eugenol and ascorbic acid occur at different sites. In addition, our results with ascorbic acid demonstrate that one can dissociate stimulation of PHS activity by radical quenching from stimulation by electron donation to PHS peroxidase.

The third possible mechanism for inhibition of PHS by eugenol involves competition between eugenol and arachidonic acid for the active site of PHS. Inhibition of PHS by eugenol was highly dependent on arachidonic acid concentration. Using 100 μM arachidonic acid as substrate with unstimulated microsomal PHS, we observed an I_{50} of 4.6×10^{-4} M for eugenol, whereas with 10 μM substrate the I_{50} decreased to 8.6×10^{-5} M. Dewhirst (5) reported an I_{50} of 1.2×10^{-5} M in a similar system using 12 μM arachidonic acid. With substrate concentrations of around 1 μM, two reports of I_{50} values of around 1×10^{-6} M are published (14, 15). In our experiments, eugenol began inhibiting PHS whenever the ratio of eugenol:substrate became approximately >1:1. This likewise suggests an I_{50} of around 10^{-6} M when using 1 μM substrate. Kinetic plots of the inhibition of Mn-PHS by eugenol revealed that eugenol increased the apparent K_m of the Mn-PHS enzyme for arachidonic acid but had no effect on the V_{max} of the reaction.

These observations are consistent with the hypothesis that eugenol is acting as a competitive inhibitor of arachidonic acid binding in the active site of PHS. Although eugenol is structurally unrelated to arachidonic acid, little information is presently available about the nature and number of binding sites on PHS for substrates, hydroperoxides, and cosubstrates. How eugenol might fit into the active site in a way that would reversibly inhibit arachidonic acid binding must therefore remain speculative. Phenolic compounds that are the best inhibitors of PHS contain ring substituents that are electron donating and are hydrophobic (5). Eugenol fits both of these criteria. Our data suggest that eugenol interacts with PHS at more than one site, because eugenol stimulates Fe-PHS activity probably by binding at a site for cosubstrates yet inhibits Mn-PHS

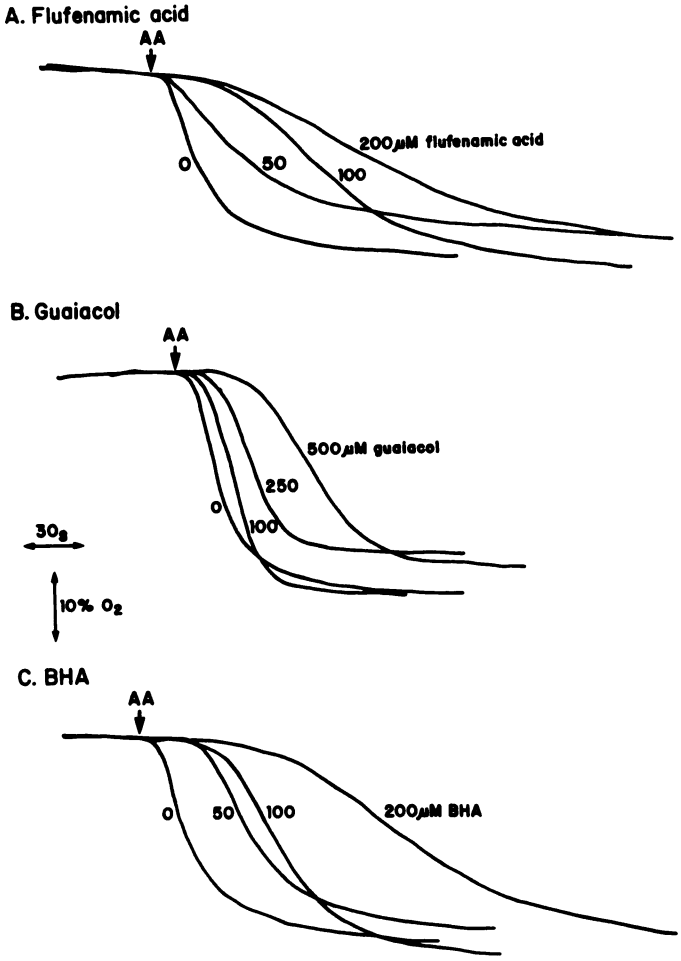

Fig. 10. Inhibition of Mn-PHS by flufenamic acid, guaiacol, and BHA. Reactions contained 10 μg/ml PHS apoenzyme, 1 μM manganese protoporphyrin IX, 50 μM arachidonic acid, and various concentrations of inhibitors in 1.5 ml of 0.1 M Tris buffer (pH 7.8) with 1 mM DETAPAC. Reactions were preincubated for 1 min before the addition of arachidonic acid (*arrow*).

activity probably by interfering with the arachidonic acid binding site. Our results also provide an alternative explanation for the observation that glutathione peroxidase/glutathione increases the inhibitory potency of phenolic compounds (22). In the presence of glutathione peroxidase, the PHS-dependent metabolism of phenolic compounds would be decreased, thus increasing the concentration of parent compound available to compete with arachidonic acid.

Our observations with other phenolic compounds demonstrate that the mechanism of PHS inhibition seen with eugenol is probably a common one with other phenolic chemicals. Of particular interest is the fact that flufenamic acid, a nonphenolic compound that has long been known to inhibit PHS (10), showed the same type of inhibition of Mn-PHS as eugenol and the other phenols. Interestingly, Egan *et al.* (30) have reported synergistic inhibition between fenamic acid derivatives and phenol, which is not seen with other types of inhibitors. Also, glutathione/glutathione peroxidase increased the inhibitory potency of fenamates as well as of phenol and acetaminophen, further implying that these agents all act to inhibit PHS in a similar manner (22). Markey *et al.* (4) demonstrated that flufenamic acid is a good electron donor to PHS peroxidase (index = 0.62).

In summary, we have presented evidence that eugenol and several other phenolic compounds are specific inhibitors of PHS cyclooxygenase. Their inhibitory activities appear to be unrelated to either peroxide tone or their ability to serve as reducing cosubstrates for PHS peroxidase. Rather, these compounds appear to inhibit by competing with arachidonic acid for the active site of the enzyme. These results help explain why certain phenols (e.g., eugenol and BHA) may be potent inhibitors of PHS activity *in vivo*, where arachidonic acid concentrations are low.

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