Inhibition of Prostaglandin H₂ Synthases by Salicylate Is Dependent on the Oxidative State of the Enzymes

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ABSTRACT

At antipyretic and analgesic doses, salicylate has no antiplatelet or anti-inflammatory effects, unlike typical inhibitors of the prostaglandin H synthases (PGHSs). We demonstrated that salicylate inhibits PGHS-1 and -2 with a potency inversely related to ambient hydroperoxide concentrations. Salicylate inhibition of PGHS-1 was prevented by 12-hydroperoxyeicosatetraenoic acid (12-HPETE). Increasing the production of prostaglandin G₂ (the peroxide product of PGHS-cyclooxygenase activity) by elevating the concentration of either enzyme or substrate reversed inhibition. Using analogs of benzoic acid differing only at the hydroxyl position, we revealed the importance of this moiety to salicylate’s inhibitory mechanism. Unlike typical phenolic inhibitors, e.g., acetaminophen, salicylate was ineffective as a reducing cosubstrate for PGHS-peroxidase activity, implicating the cyclooxygenase site as its putative target. PGHS-cyclooxygenase activity depends upon the oxidation of an active site tyrosine by electron transfer to the oxidized ferriprotoporphyrin of the peroxidase. The PGHS-1 apoenzyme reconstituted with manganese protoporphyrin instead of iron protoporphyrin has very little peroxidase activity. 12-HPETE does not prevent the inhibition of Mn-PGHS-1 by salicylate, indicating that reversal of salicylate inhibition by hydroperoxides depends upon electron transfer between the cyclooxygenase and peroxidase active sites. These results are consistent with an inhibitory action of salicylate at the PGHS-cyclooxygenase site that is dependent on the PGHS-peroxidase activity.
inhibited PGE₂ production by A459 cells with an IC₅₀ value of approximately 30 μM when exogenous arachidonic acid was absent or present in low concentrations; this occurred without changes in PGHS-2 expression. However, increasing the concentration of exogenous arachidonic acid abrogated the inhibition by salicylate, an effect considered to be consistent with salicylate acting as a competitive inhibitor of PGHS-2 in these cells. This inverse relationship between substrate concentration and the inhibitory potency of salicylate was confirmed in vivo (Giuliano et al., 2001).

An explanation of the unique human pharmacology of salicylate, however, cannot be readily derived from the above-mentioned findings. Of interest is the analogy between the pharmacological action of salicylate and that of acetaminophen, which also exerts antipyretic and analgesic effects at doses/concentrations having little or no antplatelet or anti-inflammatory action. We recently demonstrated that the cellular selectivity of acetaminophen as an inhibitor of PGHS-synthases is dependent on hydroperoxide concentrations within the cell (Boutaud et al., 2002).

The effect of peroxide concentration on the action of acetaminophen is consistent with the concept of PGHS isoforms as bifunctional enzymes operating in a branched chain mechanism in which a tyrosyl radical in the cyclooxygenase site is required for the dioxygenation of arachidonic acid to yield PGG₂, which is subsequently reduced to PGH₃ by the heme-containing peroxidase site (Dietz et al., 1988; Karthein et al., 1988) (see Discussion). Acetaminophen, acting as a cosubstrate of PGHS-peroxidase, reduces the higher oxidative state of the heme prosthetic group, inhibiting the regeneration of the catalytic tyrosyl radical within the cyclooxygenase site, thereby blocking arachidonic acid metabolism (Boutaud et al., 2002). Elevated levels of peroxide antagonize the reductant function of acetaminophen and prevent it from inhibiting the enzyme. Thus, cells with high levels of peroxide, such as the activated platelet, are resistant to the action of acetaminophen, whereas the drug blocks PG formation in endothelial cells at low micromolar concentrations (Boutaud et al., 2002). Because PGG₂ is a substrate for the PGHS-peroxidase, elevating the concentration of PGG₂ by increasing the concentration of arachidonic acid will antagonize the inhibitory action of acetaminophen (Boutaud et al., 2002).

Given the evidence that increasing the concentration of substrate similarly abrogates the inhibitory effect of salicylate, we considered whether salicylate inhibition could be suppressed by peroxides. These investigations address the hypothesis that inhibition of PGH-synthases by salicylate is contingent on a low concentration of the lipid peroxides that drive the enzyme to its higher oxidative state.

Materials and Methods

Materials. Diethyl ether was purchased from Aldrich Chemical Co. (Milwaukee, WI). Methanol was ordered from Burdick & Jackson (Muskegan, MI). 12-HPETE, 12-HETE, and 5-phenyl-4-pentenyl hydroperoxide (PPh) were supplied by Cayman Chemicals (Ann Arbor, MI). Acetaminophen, o-anisic acid, butylated hydroxyanisole, hematin, phenol, sodium salicylate, and Tris were purchased from Sigma-Aldrich (St. Louis, MO). Benzoic acid was supplied by Fisher Scientific (Pittsburgh, PA). Mn protoporphyrin IX was obtained from Porphyrin Products (Logan, UT). [¹⁴C]Arachidonic acid was from PerkinElmer Life Sciences (Boston, MA). Silica gel 60A thin layer chromatography plates were purchased from Whatman (Clifton, NJ).

Incubation of PGHS-1 and PGHS-2 with [¹⁴C]Arachidonic Acid and o-Anisic Acid, Benzoic Acid, or Salicylate. PGHS-1 was purified from ram seminal vesicles as described previously (Mar- nett et al., 1984). Wild-type murine PGHS-2 was expressed in 389 cells (Novagen, Madison, WI) and purified as described previously (Rowlinson et al., 1999).

Ovine PGHS-1 (specific activity 202 mol of arachidonic acid/min/mol of enzyme) or wild-type murine PGHS-2 (specific activity 109 mol of arachidonic acid/min/mol of enzyme) was preincubated on ice for 20 min with 2 M equivalents of hematin in Tris-HCl buffer, pH 8.0, 500 μM phenol. This solution was then warmed for 5 min at 37°C in the presence or absence of sodium salicylate (final concentrations 0.3, 0.5, 1, and 2 mM), o-anisic acid, or benzoic acid (final concentrations 1 and 2 mM). [¹⁴C]Arachidonic acid (20 μl) (4.8 nCi, 0.5 μM final concentration) in Tris-HCl buffer, pH 8.0, was preincubated at 37°C for 2 min. The reaction was initiated by adding equivalent activities of PGHS-1 (5.4 nM final concentration) or PGHS-2 (10 nM final concentration) to a total volume of 200 μl. The reaction was terminated after 8 s by the addition of 200 μl of ice-cold diethyl ether/methanol/4 M citric acid (30:4:1) containing 8 μg of butyryl hydroxynaphtoate as antioxidant and 8 μg of unlauned arachidonic acid as a carrier.

The organic layer was loaded on a silica plate and eluted with the organic phase of ethyl acetate/isooctane/water/glacial acetic acid (45:25:50:1). Thin layer chromatography plates were analyzed for radioactivity by a Bioscan AR-2000 imaging scanner (Bioscan, Washington, DC). Graphical analysis was performed using Win-Scan software (Bioscan). PGHS-1 and PGHS-2 activity was expressed as activity relative to control (no sodium salicylate added). Activity was determined as pimcomoles of arachidonic acid converted into product per minute per picomole of enzyme. Salicylate experiments were performed in duplicate on three separate occasions. o-Anisic acid and benzoic acid experiments were performed in duplicate on two separate occasions.

Incubation of PGHS-2 with Salicylate and Variable Substrate Concentrations. Murine PGHS-2 was prepared and preincubated as described above and then warmed for 5 min at 37°C in the presence or absence of sodium salicylate (final concentrations 0.5, 1, and 2 mM). [¹⁴C]Arachidonic acid (20 μl) (0.5, 1, 2, and 10 μM final concentration) in Tris-HCl buffer, pH 8.0, was preincubated at 37°C for 2 min. The reaction was initiated by adding PGHS-2 (10 nM final concentration) to a total volume of 200 μl and was terminated and products analyzed by thin layer chromatography as described above. Experiments were performed in duplicate on three separate occasions.

Incubation of Variable Concentrations of PGHS-2 with Salicylate. Experimental protocol was similar to that described above with the exception that the final [¹⁴C]arachidonic acid concentration was 10 μM and the reaction was initiated by adding PGHS-2 to achieve final concentrations of 10, 20, 30, 50, and 100 nM. Reactions were terminated after 8 s and products analyzed as noted above. The 10 nM PGHS-2 reaction was terminated after 15 s to allow for sufficient substrate utilization. Experiments were performed in duplicate on three separate occasions.

Incubation of Mn Protoporphyrin IX-Reconstituted PGHS-1 with Salicylate. Before reconstitution with hematin, ovine PGHS-1 purified as described above demonstrated 36% holoenzyme activity compared with the activity in the presence of hematin (data not shown). Further removal of heme was accomplished as published previously (Odenwaller et al., 1990) and resulted in a preparation of PGHS-1 with no detectable holoenzyme activity (data not shown). We used this apoenzyme preparation for reconstitution with Mn protoporphyrin IX.

Apo-PGHS-1 was preincubated on ice for 20 min with 2 M equivalents of Mn protoporphyrin IX in Tris-HCl buffer, pH 8.0, 500 μM phenol. This solution was then warmed for 5 min at 37°C in the presence or absence of sodium salicylate (final concentrations 0.3, 0.5, 1, and 2 mM). [¹⁴C]Arachidonic acid (20 μl) (4.8 nCi, 0.5 μM final concentration) in Tris-HCl buffer, pH 8.0, was preincubated at 37°C for 2 min. The reaction was initiated by adding equivalent activities of PGHS-1 (5.4 nM final concentration) or PGHS-2 (10 nM final concentration) to a total volume of 200 μl and was terminated and products analyzed by thin layer chromatography as described above. Experiments were performed in duplicate on three separate occasions.

Incubation of PGHS-2 with Salicylate and Variable Substrate Concentrations. Murine PGHS-2 was prepared and preincubated as described above and then warmed for 5 min at 37°C in the presence or absence of sodium salicylate (final concentrations 0.5, 1, and 2 mM). [¹⁴C]Arachidonic acid (20 μl) (0.5, 1, 2, and 10 μM final concentration) in Tris-HCl buffer, pH 8.0, was preincubated at 37°C for 2 min. The reaction was initiated by adding PGHS-2 (10 nM final concentration) to a total volume of 200 μl and was terminated and products analyzed by thin layer chromatography as described above. Experiments were performed in duplicate on three separate occasions.
containing [14C]arachidonic acid immediately before the initiation of the reaction. Experiments were performed in duplicate on three separate occasions. Histamine reconstituted PGHS-1 (Fe-PGHS-1; data not shown). Specimens described above, permitting a comparable amount of substrate utilization (as the absence of inhibitor) as seen within 8 s using hematin-reconstituted PGHS-1 (Fe-PGHS-1; data not shown). Specimens were then processed as described for Fe-PGHS-1. Experiments were performed in duplicate on three separate occasions.

### Incubation of PGHS-1 with 12-HPETE or 12-HETE

When needed, 10 μl of 12-HPETE or 12-HETE in Tris-HCl buffer, pH 8.0 (final concentration 0.3 μM for both), was added to the 20-μl solution containing [14C]arachidonic acid immediately before the initiation of the reaction with Fe-PGHS-1 or Mn-PGHS-1. Tris-HCl buffer, pH 8.0, was added to control samples. All experiments were performed in duplicate on three separate occasions.

### Incubation of PGHS-2 with 12-HPETE

Where indicated, 10 μl of 12-HPETE in Tris-HCl buffer, pH 8.0 (final concentration 0.2 μM), was added to the 20-μl solution containing [14C]arachidonic acid immediately before the initiation of the reaction with PGHS-2. Tris-HCl buffer, pH 8.0, was added to control samples. All experiments were performed in duplicate on three separate occasions.

### Assay of PGHS Peroxidase Activity with PPHP and Inhibitors

Peroxidase activity was determined by the reduction of PPHP to 5-phenyl-4-pentenyl alcohol (PPA) with a modification of a previously described method (Markey et al., 1987). PGHS-1 was preincubated on ice for 20 min with 2 M equivalents of hematin in Tris-HCl buffer, pH 8.0, 500 μM phenol. This solution was then warmed for 5 min at 37°C in the presence or absence of sodium salicylate (final concentrations 0.3, 0.5, 1, and 2 mM) or acetaminophen (final concentration 0.2 mM) and the peroxidase activity was determined.

### Isoelectric Focusing

Ultraviolet peaks for PPHP and PPA were detected at 250 nm and 280 nm by the reduction of PPHP by PGHS-1 peroxidase activity was expressed as activity relative to control (no inhibitor added). Activity was determined as picomoles of PPHP converted to PPA per minute per picomole of enzyme.

### Results

**Inhibition of Both PGHS-1 and -2 by Salicylate; the ortho-Hydroxyl Group Is a Determinant.** Salicylate demonstrated a similar inhibitory potency against PGHS-1 and -2 in experiments utilizing equivalent activities of both isoforms in cell-free preparations (IC50 value of 648 ± 130 μM for PGHS-1 and 763 ± 257 μM for PGHS-2; mean ± S.D.; Fig. 1A). To assess the importance of the hydroxyl group in PGHS inhibition by salicylate, o-anisic acid and benzoic acid were each incubated with PGHS-1 and -2. o-Anisic acid is structurally similar to salicylate with the exception of a substitution of the o-hydroxyl group with an o-methoxyl group, whereas benzoic acid differs from salicylate by the absence of the o-hydroxyl moiety (Fig. 1B). Both compounds were much less inhibitory than salicylate (IC50 value > 2 mM for both compounds against either PGHS isozyme; Fig. 1A).

**12-HPETE Opposes Salicylate Inhibition of PGHS-1 and PGHS-2.** Studies have reported that the inhibitory potency of many phenolic PGHS inhibitors is inversely related to ambient peroxide concentrations (Hemler and Lands, 1980; Hanel and Lands, 1982; Ouellet and Percival, 2001; Boutaud et al., 2002). We therefore added the platelet-derived lipid hydroperoxide (and PGHS-peroxide substrate) 12-HPETE to the PGHS assay. As illustrated in Fig. 2A, a concentration of 12-HPETE (0.3 μM) below that of substrate fully reversed PGHS-1 inhibition by salicylate. Only a slight decrease in inhibitory potency for salicylate was seen with

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**Fig. 1.** Salicylate inhibits PGHS-1 and -2 in vitro. A, equimolar amounts of hematin-reconstituted 5.4 nM ovine PGHS-1 (open squares) and 10 nM murine PGHS-2 (closed squares) were preincubated with salicylate ( ), o-anisic acid ( ), or benzoic acid ( ) at the concentrations indicated, as described under Materials and Methods, and then [14C]arachidonic acid (0.5 μM final concentration) was added. The reaction was stopped after 8 s. The PGHS activity is expressed as a percentage of the control to which no inhibitor was added. Each data point represents the mean ± S.E.M. of six values for salicylate and four values for o-anisic acid and benzoic acid. B, chemical structures of inhibitors tested against PGHS-1 and -2.
the reduced form of 12-HPETE, 12-HETE. A similar effect was observed when 12-HPETE was added to PGHS-2 (Fig. 2B). We limited the concentration of 12-HPETE to 0.2 mM because we have shown that it inactivates PGHS-2 at higher concentrations (Boutaud et al., 2002). At this concentration of hydroperoxide, the potency of salicylate as an inhibitor of PGHS-2 was substantially and significantly reduced, even though there was not the complete reversal of salicylate effect that was produced by addition of 0.3 mM 12-HPETE to PGHS-1.

Increasing PGG2 Production Reverses Salicylate Inhibition of PGHS-2. The primary substrate of PGHS-peroxidase in investigations of the purified enzyme in vitro is PGG2, the hydroperoxide product of arachidonic acid oxygenation by PGHS-cyclooxygenase. Because increased peroxidase activity is capable of reversing salicylate inhibition, it follows that enhancing PGG2 production should diminish the inhibitory effect of salicylate. We therefore modified our in vitro assay to assess the effect of increasing either arachidonic acid or PGHS-2 concentration on salicylate inhibition. For these studies the salicylate level was kept constant at 2 mM, because this amount showed greater than 75% inhibition of PGHS-2 under usual conditions (Fig. 1A). Increasing either substrate (Fig. 3A) or enzyme concentrations (Fig. 3B) largely reversed the inhibition of 2 mM salicylate.

Salicylate Is Not a PGHS-Peroxidase Cosubstrate and Does Not Inhibit Peroxidase Activity. Although many phenolic PGHS inhibitors serve as reducing cosubstrates for the PGHS-peroxidase active site, it has been reported that salicylate does not (Markey et al., 1987). We...
sought to determine whether salicylate had important redox interactions with PGHS-peroxidase cycling under our in vitro conditions. Reduction of the PGHS-peroxidase substrate PPHP to the alcohol PPA can be followed chromatographically (Markey et al., 1987). Under the same reaction conditions used for testing salicylate against ovine PGHS-1, we substituted PPHP as the substrate and confirmed that salicylate fails to serve as a reducing cosubstrate for the peroxidase activity (Fig. 4). As illustrated, this contrasts with acetaminophen, an effective reducing cosubstrate for the peroxidase (Markey et al., 1987). Importantly, salicylate does not inhibit PPHP reduction, implicating the PGHS-cyclooxygenase site as the primary target for the drug.

Salicylate Inhibition of Mn-PGHS-1 Is Not Reversed by 12-HPETE. To assess whether the effect of 12-HPETE on salicylate inhibition observed with Fe-PGHS-1 is mediated through an electron transfer between the peroxidase ferrirhodoporphyrin radical and the catalytic tyrosine of the cyclooxygenase site (Tyr385 in PGHS-1), we repeated the 12-HPETE experiments using Mn-PGHS-1, which has little peroxidase activity. Reconstituting apo-PGHS-1 with Mn protoporphyrin instead of iron protoporphyrin (hematin) generates an enzyme retaining full cyclooxygenase function but \(<1\%\) of normal peroxidase activity (Odenwaller et al., 1990, 1992), permitting a study of cyclooxygenase inhibition with minimal contribution from the peroxidase site. We found that inhibition of Mn-PGHS-1 by salicylate was slightly greater than that observed with Fe-PGHS-1 (IC\(_{50}\) value of 437 ± 34 μM for Mn-PGHS-1; mean ± S.D.; IC\(_{50}\) value 648 ± 130 μM for Fe-PGHS-1). Notably, in the presence of 0.3 μM 12-HPETE, salicylate does not inhibit the Fe-PGHS-1, whereas salicylate is able to inhibit Mn-PGHS-1 in the presence of 12-HPETE (Fig. 5).

**Discussion**

These results support a conclusion that the magnitude of inhibition of PGH synthases by salicylate is determined by the extent to which hydroperoxides are reduced by the PGHS-peroxidase. This is indicated by the finding that inhibition of PGHS-cyclooxygenase activity by salicylate is abrogated by 12-HPETE, a hydroperoxide substrate of the PGHS-peroxidase. Moreover, increasing the concentration of PGG\(_{2\alpha}\), the hydroperoxide product of the cyclooxygenase, by elevating the concentration of either arachidonic acid or enzyme, reverses the inhibition of the purified enzyme by salicylate. The effect of substrate concentration is similar to the observations of Mitchell et al. (1997) that inhibition of PGE\(_2\) synthesis by salicylate in A549 cells is reversed by elevating the concentration of exogenous substrate.

The influence of PGHS-peroxidase activity on the action of salicylate is further substantiated by the observation that 12-HPETE does not prevent salicylate from inhibiting the Mn-PGHS-1, which cannot reduce the hydroperoxide effectively, whereas this hydroperoxide completely abrogates the effect of salicylate on the Fe-PGHS-1. Taken together, these results indicate that the molecular activity of the PGHS-peroxidase determines the inhibition of PGHS-cyclooxygenase by salicylate. Thus, it can be predicted that salicylate will act as an inhibitor of PGH synthases only in cellular environments in which the levels of lipid hydroperoxide are low.

Inhibition of the PGHS-cyclooxygenase by both salicylate and acetaminophen is reversed by high concentrations of hydroperoxide. This similarity of salicylate and acetaminophen, as well as fundamental differences between their mechanism of action, are best considered in the context of the current understanding of the molecular linkage between PGHS-peroxidase activity and catalysis by the PGHS-cyclooxygenase site.
Oxygenation of arachidonic acid by the PGHS-cyclooxygenase requires the generation of a tyrosyl radical within this active site. Activation of this cyclooxygenase tyrosine to the radical state is initiated within the PGHS-peroxidase site, as a hydroperoxide oxidizes the ferriprotohemin (heme) prosthetic group by two electrons to a ferrylprotohemin cation radical (known as compound I) (1). Intramolecular electron transfer yields the tyrosyl radical (Y), reducing the heme to its fully covalent state (compound II) (2). A one-electron reduction of compound II regenerates the resting Fe(III) state of the enzyme (eq. 3) (Landino and Marnett, 1996; Marnett, 2000).

\[
\text{Fe}^3\text{PPIX} + \text{ROOH (PGG}_2) \rightarrow \text{O} = \text{Fe}^4\text{PPIX}^+ + \text{ROH (PGH}_2)
\]

(1)

\[
\text{O} = \text{Fe}^4\text{PPIX}^+ + \text{Y} \rightarrow \text{O} = \text{Fe}^4\text{PPIX} + \text{Y}
\]

(2)

\[
\text{O} = \text{Fe}^4\text{PPIX} + \text{e} \rightarrow \text{Fe}^3\text{PPIX} + \text{H}_2\text{O}
\]

(3)

The tyrosyl radical abstracts the pro-S-hydrogen from carbon-13 of arachidonic acid to initiate its oxygenation, and thereby is reduced to tyrosine (eq. 4). Regeneration of the tyrosyl radical occurs, however, in the final step of the cyclooxygenase catalytic cycle when tyrosine is oxidized by the peroxyl radical precursor to PGG2 (eqs. 5 and 6) (Marnett, 2000). PGG2 itself then serves as a substrate for the peroxidase site (Hemler et al., 1978) where it is reduced to the alcohol product of the PGHS, PGH2 (eq. 1).

\[
\text{Y} + \text{AA} \rightarrow \text{AA}^+ + \text{Y}
\]

(4)

\[
\text{AA}^+ + 2\text{O}_2 \rightarrow \text{PGG}_2^-
\]

(5)

\[
\text{PGG}_2^+ + \text{Y} \rightarrow \text{PGG}_2 + \text{Y}^-
\]

(6)

Some “leakage” of the PGG2 peroxyl radical from the cyclooxygenase active site can occur, thereby interrupting the cyclooxygenase cycle that regenerates the necessary tyrosyl radical; this necessitates reactivation of the cyclooxygenase activity by heme-dependent hydroperoxide reduction (eq. 2) (Marnett, 2000).

Our findings have confirmed that acetaminophen serves as a PGHS-peroxidase cosubstrate, supplying the electrons that return the ferrylprotohemin to the ferric or resting state necessary for another cycle of peroxide reduction (Markey et al., 1987). Thus, acetaminophen effectively competes with the cyclooxygenase active site tyrosine for reduction of the heme radical and thereby inhibits the catalytic function of the PGHS-cyclooxygenase; this inhibition is evident at low concentrations of peroxide, but increasing peroxide concentration drives the enzyme to the protoporphyrin radical state and antagonizes the inhibitory action of acetaminophen (Boutaud et al., 2002).

Salicylate, however, is not a cosubstrate for the peroxidase, as demonstrated by Markey et al. (1987) and confirmed under the conditions used in the present study. Rather, the evidence indicates that the action of salicylate is linked to occupancy of the PGHS-cyclooxygenase active site. Examination of the crystal structure of the PGH synthases has localized salicylic acid within the cyclooxygenase pocket just above the narrowest part of the channel (Loll et al., 1995). Localization of salicylate in the active site is consistent with the evidence that it antagonizes the acetylation of PGHS by aspirin (Vargafig, 1978; Merino et al., 1980; Dahl et al., 1983; Rizk and Abdel-Rahman, 1994) and blocks the inhibition of the enzyme by indomethacin (Humes et al., 1981; Dahl et al., 1983).

The mechanism whereby the oxidative state of the PGHS synthases determines the action of salicylate has not been elucidated. The hypotheses include reduction of the cyclooxygenase tyrosyl radical by salicylate, an effect that would be overcome by high concentrations of peroxides that drive the regeneration of the tyrosyl radical. Although salicylate is not a cosubstrate for the PGHS-peroxidase, the ability for the drug to act as a reductant has been observed with other hemoproteins with peroxidase activity. Formation of a phenoxy radical of salicylate by methemoglobin and horseradish peroxidase has been demonstrated by electron paramagnetic resonance under peroxidative conditions (Shiga and Imaizumi, 1973, 1975), and salicylate has been shown to serve as a reducing cosubstrate for myeloperoxidase (Hermann et al., 1999).

Alternatively, the binding affinity of salicylate in the PGHS-cyclooxygenase site could be determined by hydrogen bonding with the reduced catalytic tyrosine, in which case peroxide-driven tyrosyl radical formation would prohibit hydrogen bonding. By analogy, hydrogen bonding of aspirin with Tyr-385 of PGHS-1 has been proposed as a requisite step in the acetylation of the active site serine by aspirin (Hochgesang et al., 2000). Our finding that the function of salicylate as a PGHS inhibitor is dependent on its hydroxyl group would be consistent with either an action as a phenolic reductant of the tyrosyl radical or with a binding affinity that is determined by hydrogen bonding of the hydroxyl to tyrosine.

In conclusion, the finding that inhibition of the catalytic activity of the PGHS synthases by salicylate is antagonized by lipid hydroperoxides provides a basis for considering that cellular levels of hydroperoxides determine the selective action of the drug in vivo.

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