Resveratrol is a Peroxidase-mediated Inactivator of COX-1 but Not COX-2

A MECHANISTIC APPROACH TO THE DESIGN OF COX-1 SELECTIVE AGENTS*

Lawrence M. Szewczuk‡‡, Luca Forti¶, Lucia A. Stivala, and Trevor M. Penning**‡‡†

Received for publication, December 30, 2003, and in revised form, March 12, 2004
Published, JBC Papers in Press, March 12, 2004, DOI 10.1074/jbc.M314302200

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) is a phytoalexin found in grapes that has anti-inflammatory, cardiovascular protective, and cancer chemopreventive properties. It has been shown to target prostaglandin H₂ synthase (COX-1) and COX-2, which catalyze the first committed step in the synthesis of prostaglandins via sequential cyclooxygenase and peroxidase reactions. Resveratrol discriminates between both COX isoforms. It is a potent inhibitor of both catalytic activities of COX-1, the desired drug target for the prevention of cardiovascular disease, but only a weak inhibitor of the peroxidase activity of COX-2, the isoform target for non-steroidal anti-inflammatory drugs. We have investigated the unique inhibitory properties of resveratrol. We find that it is a potent peroxidase-mediated mechanism-based inactivator of COX-1 only \( K_{i \text{inact}} = 0.069 \pm 0.004 \text{ s}^{-1} \), with a calculated partition ratio of 22. Inactivation of COX-1 was time- and concentration-dependent, it had an absolute requirement for a peroxide substrate, and it was accompanied by a concomitant oxidation of resveratrol. Resveratrol-inactivated COX-1 was devoid of both the cyclooxygenase and peroxidase activities, neither of which could be restored upon gel-filtration chromatography. Inactivation of COX-1 by \( ^{3} \text{H}\text{resveratrol} \) was not accompanied by stable covalent modification as evident by both SDS-PAGE and reverse phase-high performance liquid chromatography analysis. Structure activity relationships on methoxy-resveratrol analogs showed that the m-hydroquinone moiety was essential for irreversible inactivation of COX-1. We propose that resveratrol inactivates COX-1 by a “hit-and-run” mechanism, and offers a basis for the design of selective COX-1 inactivators that work through a mechanism-based event at the peroxidae active site.

3,4′,5-Trihydroxy-trans-stilbene (resveratrol); (Scheme 1) is a natural product found in grapes, which is present at concentrations up to 100 \( \mu \text{M} \) in red wines and to a much lesser extent in white wines (1). It is reported to have anti-inflammatory, cardiovascular protective, and cancer chemopreventive properties and was shown to target prostaglandin H₂ synthases (COX-1 and COX-2) (2, 3). Resveratrol is unique in that it is a potent inhibitor of both the cyclooxygenase and peroxidase reactions of COX-1 (Table I) (2, 3). By contrast, classical non-steroidal anti-inflammatory drugs (NSAIDs) target the cyclooxygenase reaction only (4, 5). Resveratrol was also noncompetitive with arachidonic acid (AA), indicating that drug binding occurred at a site other than the cyclooxygenase active site of COX-1 (3). Finally, resveratrol was able to discriminate between the two COX isoforms because it only weakly inhibited the peroxidase activity of COX-2 (Table I) (2, 3). The mechanism by which resveratrol selectively inhibits the cyclooxygenase and peroxidase reactions of COX-1 is unknown.

COX-1 and COX-2 catalyze the first committed steps in the synthesis of all prostaglandins (PGs). They convert AA to PGH₂ (cyclooxygenase reaction), and the second reaction involves peroxidative cleavage of PGH₂ to yield PGH₃ (peroxidase reaction) (6–8). The catalytic mechanism of these heme-dependent enzymes is novel and requires the peroxidase activity to initiate the cyclooxygenase reaction by generating a tyrosyl radical (Scheme 2A). After initiation, the cyclooxygenase activity becomes autocatalytic. In contrast, the peroxidase activity requires a co-reductant to return the heme iron from the higher oxidation states generated during peroxidase catalysis (compound I (Fe⁴⁺) and compound II (Fe³⁺)) to its resting state (Fe²⁺) before peroxide bond cleavage can occur again. COX enzymes catalyze a branched-chain mechanism whereby peroxide (PG₉) generated at one active site can activate latent enzyme molecules to produce the tyrosyl radical (9–11). Both enzyme isoforms self-inactivate over time because of protein radical intermediates generated when there is insufficient co-reductant to reduce the heme iron back to its resting state (Ref. 12; for a recent review see Ref. 13). For resveratrol to be a selective inhibitor, it is likely to block this sequence in one isoform and not the other.

PGs are local mediators of vascular homeostasis; for example...
thromboxane A2 (TxA2) is a potent vasoconstrictor and platelet aggregator synthesized in activated platelets (8, 14), whereas prostacyclin (PGI2) is an anti-platelet aggregator and potent vasodilator synthesized in the vascular endothelial cells (15, 16). Vascular homeostasis is believed to result from a dynamic balance between TxA2 and PGI2 because they have opposing actions (17, 18). Imbalances in this ratio can explain some of the changes that occur in various pathological conditions including thrombosis (17, 18). Both TxA2 and PGI2 are synthesized from the precursor PGG2; however, different COX isoforms contribute to their formation. Platelets contain only COX-1, which is an obligate enzyme for TxA2 formation. In contrast, COX-2 in the vascular endothelial cells is the primary source of systemic PGI2 biosynthesis because selective COX-2 inhibitors reduce PGI2 levels but have little effect on COX-1-dependent platelet aggregation (19). Therefore, selective inhibition of COX-1 offers a viable mechanism for cardioprotective agents, which can act by tilting the TxA2-PGI2 balance in favor of PGI2. It is through this mechanism that low dose aspirin exerts its cardioprotective effects (4, 20, 21). By contrast, COX-2 selective inhibitors (i.e. Celebrex and Vioxx) are used for the treatment of inflammation.

We set out to dissect the basis of the unique inhibitory properties of resveratrol on COX-1. We hypothesized that resveratrol might exert its inhibitory actions by binding at the peroxidase active site. In this manner it would be possible for resveratrol to interact with the heme co-factor, which is required for both catalytic activities of both isoforms. Our results show that resveratrol is a mechanism-based inactivator of the peroxidase activity of COX-1 but not COX-2. Irreversible inactivation of COX-1 is achieved concomitantly with the oxidation of resveratrol at the peroxidase active site. Using a series of structural analogs (Scheme 1), we determined that the minimum requirement for mechanism-based inactivation was the m-hydroquinone moiety. Inactivation is not accompanied by covalent modification of COX-1, suggesting that resveratrol inactivates via a “hit-and-run” mechanism. Based on our results with resveratrol and its analogs, we predict that m-hydroquinones offer a route to COX-1-specific inactivators that target the peroxidase active site. Their potential cardioprotective role is discussed.

EXPERIMENTAL PROCEDURES

Materials—Fe-protoporphyrin-IX (FePPIX), Mn-protoporphyrin-IX (MnPPIX), AA, H2O2 (30% v/v), 1,1’-CIAA (51 mCi/mM), Sephadex G-25, and Tween 20 were purchased from Sigma. PGF2α, PGE2, and PGD2 were purchased from Biomol Research Laboratories. solvable and Ultima Gold were purchased from Packard Biosciences. 4

COX-1 was purified to homogeneity from ram seminal vesicles as described previously (23). The purified enzymes were obtained predominantly in their apo forms (˃85%) and were reconstituted with at least 1 equivalent of co-factor (FePPIX or MnPPIX) in the assay system prior to reaction initiation.

Cyclooxygenase Assay—The bis-dioxogeneration of AA to yield PGG2 was followed by measuring oxygen consumption using a Clark-style oxygen microelectrode (Instech). The standard assay chamber (600 μl) contained 100 mM Tris-HCl (pH 8.0), 1 mM phenol, 2 μM FePPIX (or MnPPIX), and 150 μM AA. The assays were initiated by the addition of AA. By using this procedure, our FePPIX-reconstituted COX-1 and COX-2 had specific activities of 27 and 20 μmol of O2 consumed/min/mg, respectively, whereas the MnPPIX-reconstituted COX-1 had a specific activity of 16 μmol of O2 consumed/min/mg.

Peroxidase Assay—The two-electron reduction of peroxide using TMPD as the reducing co-substrate was measured spectrophotometrically. The cuvette (1.0 ml) contained 100 mM Tris-HCl (pH 8.0), 2 μM FePPIX, 80 μM TMPD, and 300 μM H2O2 (EtOOH can be substituted). The assays were initiated by the addition of peroxide. The formation of N,N,N,N-tetramethyl-1,4-phenylene-diamine (E406 = 12,000 M⁻¹ cm⁻¹) was complete within 60 s. By using this procedure, our COX-1 and COX-2 enzymes had specific activities of 34 and 23 μmol of TMPD oxidized/min/mg, respectively, whereas the MnPPIX-reconstituted COX-1 had a specific activity of 0.25 μmol of TMPD oxidized/min/mg.

Reversible Inhibition of COX-1 by Resveratrol—The reversible inhibition of either the cyclooxygenase or peroxidase activity was determined in assays containing either AA or ETOOH as substrates, respectively, whereas the resveratrol concentration was varied. Six different substrate concentrations (AA = 5–83.33 μM, ETOOH = 20–300 μM) and five inhibitor concentrations (for cyclooxygenase 0–250 μM, for peroxidase 0–1.0 μM) were employed for the analysis. Resveratrol was dissolved in Me2SO, and the final concentration of organic solvent, which was 2%, had no effect on the initial velocities. Initial velocity data were fit to competitive, noncompetitive, and uncompetitive models using the program GraFit 4.0 (Erithacus Software).

Scheme 1. Structure of resveratrol and its methoxy analogs.

† Selective Inactivation of COX-1 by Resveratrol

Expression of COX-1 and COX-2 in bacteria, using the lacZ promoter, were calculated by linear regression to single wavelength data.

Difference Spectroscopy—Difference spectroscopy was used to characterize the formation of an enzyme-resveratrol complex. The cuvettes (1 ml) contained 100 mM Tris-HCl (pH 8.0), 5 μM FePPIX, 2.5 μM enzyme. Difference spectra were generated by subtracting the absorbance spectrum of holoenzyme from an identical sample that was treated with inhibitor (100 μM). In this portion of the spectrum, resveratrol is UV-visible transparent. Kd values were determined in assays containing either AA or EtOOH as substrates, respectively. Full scan spectra were recorded every 15 s for 3 min, and single wavelength data were recorded at 306 nm (λmax for trans-stilbene, Emax = 29,900 M⁻¹ cm⁻¹) every 0.1 s for 1 min. Initial velocities were calculated by linear regression to single wavelength data.

Oxidation of Resveratrol by the Peroxidase Activity of COX—The oxidation of resveratrol and its analogs by COX-1 and COX-2 was monitored spectrophotometrically by recording either full scan spectra or the absorbance change at a single wavelength over time. The cuvettes (1.0 ml) contained 100 mM Tris-HCl (pH 8.0), 2 μM FePPIX, 0–7 μg of enzyme, 25 μM analog, and the reactions were initiated with either H2O2 or 300 μM H2O2 to obtain the background and enzymatic rates, respectively. Full scan spectra were recorded every 15 s for 3 min, and single wavelength data were recorded at 306 nm (λmax for trans-stilbene, E406 = 29,900 M⁻¹ cm⁻¹) every 0.1 s for 1 min. Initial velocities were calculated by linear regression to single wavelength data.

To further characterize the enzymatic oxidation of resveratrol and its analogs, an HPLC method was employed. Half-COX-1 or half-COX-2 (0.2 units) was mixed with 50 μM resveratrol or analog in 100 mM Tris-HCl (pH 8.0). The 1-nl reactions were initiated with the addition of 300 μM H2O2 and quenched after 2 min by the addition of 250 μl of 1 M sodium citrate (pH 4.0). Samples (100 μl) were injected onto a Waters Symmetry C18 column (3.5 μM, 4.6 X 75 mm) equilibrated with solvent A (20% methanol in water) at a flow rate of 1.0 ml/min. Beginning at 2 min, a linear gradient was run to solvent B (80% methanol in water) over 10 min to separate compounds I–IV. The column was returned to its initial conditions and equilibrated for 5 min prior to the next injection. The percentage of enzymatic oxidation of resveratrol and its ana-
logs was quantified by monitoring the disappearance of compound in the presence of H2O2 with respect to a sample which contained no H2O2. The RP-HPLC analysis was performed using a Waters model 2695 pump equipped with a model 996 photodiode array detector.

** Peroxide-dependent Inactivation of COX by Resveratrol—** COX-1 or COX-2 (10 μM) was preincubated with mixtures of 100 μM H2O2, 100 μM resveratrol (or analog), and 1 μM phenol in 100 μM Tris-HCl (pH 8.0) supplemented with 10 μM FePPIX for 5 min at 25 °C. The complete system contained all ingredients, whereas other systems lacked one or more ingredients. Preincubations were initiated with H2O2 (or H2O when peroxide was not a reagent). Immediately following preincubation, the samples were diluted 40-fold into the cyclooxygenase assay or 200-fold into the peroxidase assay. Activity measurements were corrected for resveratrol carryover according to IC50 curves, and the percentage of activity remaining was computed with respect to an enzyme.

** Time-dependent Inactivation of COX-1 by Resveratrol—** The steady-state TMPD peroxidase assay was suitable for the accurate estimation of time-dependent inactivation of the COX-1 peroxidase because the rate of resveratrol oxidation was much lower than that observed with TMPD (126 versus 34 μmol/min/mg) when TMPD was saturating. Progress curves were corrected for the nonenzymatic rate of TMPD oxidation. kobs values for the time-dependent inactivation of the enzyme were obtained by fitting progress curves to a single exponential.

\[
\text{Absorbance}_{\text{obs}_{\text{start}}} = A_0 (1 - e^{-k_{\text{obs}}/t}) + \text{offset} \tag{Eq. 2}
\]

The kobs values were subsequently corrected for the rate of self-inactivation (0 μM resveratrol, 0.0142 ± 0.0002 s−1) and analyzed by the method of Kitz and Wilson to yield kobs (ΔKobs (Kitz and Wilson) = 256). The following equation was used to extract kinetic constants from the Kitz-Wilson analysis.

\[
k_{\text{obs}} = \left(\frac{k_{\text{inact}}}{v(\text{inact})}\right) + \left(\frac{1}{v(\text{inact})}\right) \tag{Eq. 3}
\]

The t1/2 for inactivation at saturation was obtained from Equation 4.

\[
t_{1/2} = \left(\ln 2\right)/k_{\text{inact}} \tag{Eq. 4}
\]

** Tryptic Resveratrol Incorporation into COX—** COX-1 was extensively dialyzed into 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% glycerol (v/v), and 0.2% Tween 20 (v/v) to remove contaminating reducing co-substrates. [1H]Resveratrol was prepared as a 10 mM solution in [3H]resveratrol and the total amount of protein applied to the gel was determined whether radioactivity was associated with either analyte. RP-HPLC method separated COX-1 from its FePPIX co-factor and [3H]resveratrol-inactivated COX-1. The resultant TMS ether PFB ester had a retention time of 17.33 min on the GC column, consistent with a dependence on peroxide substrate. This TMS ether PFB ester was dried under N2, and dissolved in pyridine (10 μl) and bis-(trimethylsilyl)trifluoroacetamide (10 μl), and allowed to stand at room temperature for 5 min. The resultant PFB ester was dried under N2, and dissolved in pyridine (10 μl) and bis-(trimethylsilyl)trifluoroacetamide (10 μl), and allowed to stand at room temperature for 5 min. The resultant PFB ester was dried under N2, and dissolved in pyridine (10 μl) and bis-(trimethylsilyl)trifluoroacetamide (10 μl), and allowed to stand at room temperature for 5 min. The resultant PFB ester was dried under N2, and dissolved in pyridine (10 μl) and bis-(trimethylsilyl)trifluoroacetamide (10 μl), and allowed to stand at room temperature for 5 min.

** RESULTS**

** Reversible Inhibition of COX-1 by Resveratrol—** The mechanism of reversible inhibition of COX-1 by resveratrol was reexamined because of its ability to inhibit the cyclooxygenase and peroxidase activities of this isoform. Attention to the mechanism of inhibition of COX-2 by resveratrol was of limited interest because the compound did not inhibit the cyclooxygenase activity of this isoform and had only a minimal effect on its peroxidase activity (IC50 = 280 μM, see Table 1). To determine the mechanism of reversible inhibition for the cyclooxygenase and peroxidase activities of COX-1, initial velocity studies were performed with AA and EtOOH, respectively. It was found that resveratrol was a noncompetitive inhibitor versus AA (Ki = 100.1 ± 10.0 μM, Fig. 1A), confirming the previous finding of Johnson and Maddipati (3). In contrast, resveratrol was an uncompetitive inhibitor versus EtOOH (Ki = 0.6 ± 0.1 μM, Fig. 1B), providing evidence for a COX-1-resveratrol-peroxide complex, consistent with a dependence on peroxide substrate. This phenomenon was not previously observed.

** Binding of Resveratrol to COX-1 and COX-2—** Holo-COX-1 and COX-2 were incubated with resveratrol, and changes in the absorbance of the Soret band were examined by difference spectroscopy (Fig. 2A). The difference spectrum revealed an increase in intensity of the Soret band plus the appearance of a new chromophore at 530 nm (Fig. 2A, inset). In contrast, no such spectral changes were observed upon incubating COX-2 with resveratrol, indicating a different mode of ligand binding (Fig. 2B). The increase in absorbance of the Soret band with COX-1 was concentration-dependent and saturable, allowing a Kd determination (Fig. 2C). Binding affinity for resveratrol (Kd = 11.7 ±
Selective Inactivation of COX-1 by Resveratrol

TABLE I
Summary of kinetic parameters for the inhibition of COX by resveratrol

| Kinetic parameter                      | COX-1                   | COX-2                   |
|----------------------------------------|-------------------------|-------------------------|
| Cyclooxygenase inhibition              | IC50 = 3.6 μM (2)       | No effect (2)           |
|                                        | Kci = 100 μM (noncompetitive) | Enhanced activity (3) |
| Peroxidase inhibition                  | ED50 = 15 μM (2)        | No inactivation (2)     |
|                                        | Kci = 100 μM            | ED50 ≥ 200 μM (3)      |
|                                        | (noncompetitive)        | Kci = 280 μM           |
| Chromophore                            | Ye; Kci = 12 μM         | No                     |
| Specific activity (resveratrol oxidation) | 1.26 μmol/min/mg         | 8.41 μmol/min/mg       |
| Effect on self-inactivation rate       | Increased 5-fold        | Protection             |
| Kinet(2)                               | 0.096 s−1              | No inactivation        |
| Kinit(2)                               | 1.52 μM                 | No inactivation        |
| Effect on PG synthesis                 | Peroxidase-mediated mechanism-based inactivator | Reducing co-substrate |
| Mechanism of action                    |                         |                         |

Time-resolved absorbance spectra of resveratrol upon incubation with holo-COX-1 in the absence and presence of H2O2 are shown in Fig. 3 (A and B). Upon addition of H2O2, resveratrol was rapidly oxidized (1.26 μmol/min/mg), as evident by the disappearance of its absorbance spectrum (time-resolved spectra generated with COX-2 were identical). Oxidation occurred at the peroxidase active site of COX-1 because pretreating the enzyme with either indomethacin or aspirin had little effect on the specific activity for resveratrol turnover (1.56 and 1.12 μmol/min/mg, respectively; data not shown). Although both COX isoforms were capable of catalyzing the oxidation of resveratrol, COX-2 was the more robust catalyst with a specific activity for resveratrol turnover of 8.41 μmol/min/mg (Fig. 3C).

Further evidence that resveratrol was oxidized by the peroxidase activity of COX-1 and COX-2 came from an end point analysis using RP-HPLC. The enzymatic depletion of resveratrol was only observed in the presence of peroxide substrate. COX-1 was able to oxidize 47% of the resveratrol in the assay system (50 nmol) before it was inactivated, whereas COX-2 was able to oxidize all 50 nmol during the 2-min assay (Table II). The RP-HPLC analysis confirmed the findings that COX-2 is a more robust catalyst for resveratrol turnover; however, with both COX-1 and COX-2, the UV-visible detector failed to identify new peaks that corresponded to the products of enzymatic oxidation.

Peroxidase-dependent Inactivation of COX by Resveratrol—A series of preincubation/dilution studies were performed to determine whether the oxidation of resveratrol was coincident with irreversible inactivation of either COX-1 or COX-2 (see Fig. 4). Several key findings were observed for COX-1 (Fig. 4A). First, resveratrol alone had no effect on the enzyme during a 5-min preincubation period (control). Second, in the presence of H2O2 alone, a small amount of enzyme self-inactivation was observed. However, under conditions in which resveratrol is rapidly oxidized (e.g., in the presence of H2O2), there was a significant increase in the amount of enzyme inactivation observed. Phenol, a prototypical reducing co-substrate, was able to protect against both self-inactivation and resveratrol-mediated enzyme inactivation. This same pattern was not observed for COX-2 (Fig. 4B), and instead resveratrol behaved identically to phenol. In an extension of the studies with COX-1, we showed that irreversible inactivation of both the cyclooxygenase and peroxidase activities occurred simultaneously and in a concentration-dependent fashion (Fig. 4C). Further evidence that inactivation by resveratrol was a peroxidase-mediated event was provided by using the MnPPIX-reconstituted form of COX-1. In this analysis, resveratrol was found to be a much less potent inactivator of the enzyme. At a single drug concentration (100 μM), the percentage of inhibition decreased from 1.8 μM was virtually unchanged when the measurements were repeated on indomethacin-treated (Kci = 17.7 ± 3.5 μM) and aspirin-treated (Kci = 16.3 ± 3.9 μM) forms of COX-1 in which the cyclooxygenase active site is rendered unavailable (data not shown).

Oxidation of Resveratrol by the Peroxidase Activity of COX—Resveratrol is a polyphenolic compound and could be easily oxidized by compounds I and II during peroxidase catalysis.

![Fig. 1: Reversible inhibition profiles of resveratrol with COX-1](image)

A, resveratrol is a noncompetitive inhibitor of the COX-1 cyclooxygenase activity. Resveratrol (○, 0 μM; □, 25 μM; △, 50 μM; ■, 100 μM; ▲, 250 μM), FePPIX, and COX-1 were mixed in 100 mM Tris-HCl (pH 8.0) supplemented with 1 mM phenol. Reactions were initiated with AA (6.33–83.33 μM), and oxygen consumption during the cyclooxygenase reaction was followed using an oxygen microelectrode. B, resveratrol is a noncompetitive inhibitor of the COX-1 peroxidase activity. Resveratrol (○, 0 μM; □, 0.1 μM; △, 0.25 μM; ■, 0.5 μM; ▲, 1 μM), FePPIX, and COX-1 were mixed in 100 mM Tris-HCl (pH 8.0) supplemented with 80 μM TMPD. Reactions were initiated with EtOOH (20–300 μM), and TMPD oxidation was monitored at 610 nm spectrophotometrically.
81.7 ± 5.1 for FePPIX-reconstituted COX-1 to 15.1 ± 3.3 for MnPPIX-reconstituted COX-1 in a standard cyclooxygenase activity assay (data not shown). This was anticipated because the MnPPIX-reconstituted form of COX-1 has near native cyclooxygenase activity, but lacks most of its peroxidase activity (26).

**Time-dependent Inactivation of COX-1 by Resveratrol**—To better characterize the mechanism-based inactivation of COX-1, we used a series of steady-state peroxidase assays. Progress curves obtained in the presence of increasing amounts of resveratrol clearly show a time- and concentration-dependent inactivation event (Fig. 5A). These curves were fit to a single exponential equation to yield $k_{\text{obs for inactivation}}$ at each resveratrol concentration. The $k_{\text{obs}}$ values for inactivation were replotted in a Kitz-Wilson analysis, which showed saturation kinetics (25). This analysis gave a $k_{\text{inact}}$ of 0.069 ± 0.004 s⁻¹, $K_{\text{inact}}$ of 1.52 ± 0.15 μM, and a calculated $t_{1/2}$ for inactivation of 10.04 s at saturation when the H₂O₂ concentration was held constant at 300 μM ($K_m = 287 μM$) (Fig. 5B) (13). We estimated a partition ratio of 22 by using $k_{\text{cat}}/k_{\text{inact}}$, where $k_{\text{cat}}$ for res-
veratrol oxidation was estimated to be 1.52 s⁻¹ from specific activity measurements (Fig. 3C). Furthermore, using the same steady-state assay, we showed that the ratio between enzyme inactivated by resveratrol and enzyme inactivated by peroxide remained unchanged over a wide range of H₂O₂ concentrations (Fig. 5C) and was greater than 3-fold. This finding is important because it implies that resveratrol can act as a mechanism-based inactivator of the COX-1 peroxidase over a dynamic range of peroxide concentrations expected in vivo.

Tritiated Resveratrol Incorporation into COX-1—[³H]Resveratrol was used to determine whether mechanism-based inactivation of COX-1 resulted in covalent modification of the enzyme. In the first set of experiments, Sephadex G-25 gel-filtration chromatography was used to separate bound and free [³H]resveratrol. Under these facile conditions, elution of tritium with the enzyme was observed as evident by a significant increase in the amount of radioactivity associated with the protein fractions when H₂O₂ and [³H]resveratrol were present in the reaction (Fig. 6). Under these conditions, COX-1 was inactivated by 60% and an estimate of the stoichiometry indicated that 6.0 mol of [³H]-labeled compound were bound/mol of synthase monomer (i.e. 10.0 mol of [³H]-labeled compound were bound/mol of inactivated synthase monomer) (Fig. 6B). The presence of H₂O₂ alone, COX-1 inactivation was <10%, indicating that the role of self-inactivation in the analysis was minimal. Nonspecific binding of [³H]resveratrol was observed in the absence of H₂O₂, yielding a stoichiometry of 1.4 mol of [³H]resveratrol bound/mol of synthase monomer (Fig. 6A); however, this nonspecific binding did not result in a loss of enzyme activity. These data indicated that inactivation of COX-1 resulted in co-elution of the enzyme with radioactivity derived from [³H]resveratrol on a Sephadex G-25 gel-filtration column.

To further assess covalent modification of COX-1 by resveratrol, SDS-PAGE and RP-HPLC methods were employed. In the SDS-PAGE experiment, resveratrol-inactivated COX-1 was boiled in SDS for 10 min and then subjected to PAGE. Following solubilization of the gel fragments containing COX-1, the amount of [³H]-labeled compound bound to inactivated COX-1 was estimated. A significant decrease in the stoichiometry was observed, and it was found that only 0.25 mol of [³H]-labeled compound were bound/mol of inactivated synthase monomer. In addition, the amount of [³H]resveratrol bound to COX-1 incubated with resveratrol alone decreased to 0.008 mol bound/mol of synthase monomer (Fig. 7).

RP-HPLC analysis was performed on resveratrol-inactivated COX-1 isolated by Sephadex G-25 gel-filtration chromatography. Two peaks of radioactivity were detected (Fig. 8A). One peak eluted in the void volume, and the other peak eluted with a retention time of 21.75 min and corresponded to a peak seen at 20.57 min when the absorbance was monitored at 306 nm (Fig. 8C). No radioactivity co-eluted with either the FePPIX co-factor or COX-1 (Fig. 8). The SDS-PAGE and RP-HPLC analyses show that the mechanism-based inactivation of COX-1 by resveratrol is not accompanied by stable covalent modification of the enzyme.

**COX-1-specific Loss of Prostanoid Synthesis—[¹⁴C]AA was used to show that saturating concentrations of resveratrol were able to eliminate PG synthesis by COX-1, but had no effect on PG synthesis by COX-2 (Fig. 9A). Product identity was established independently by GC/MS of the PGF₂α, TMS ether from the reaction with unlabeled AA. In this analysis, COX-2-generated PGF₂α had the same retention time (17.33 min) on the GC column and the same molecular ion (m/z = 569) as the authentic standard (data not shown). By contrast, resveratrol-inactivated COX-1 failed to produce any PG products. Evidence that resveratrol acted only as a co-reductant for the COX-2 reaction was further supported by AA-dependent oxygen uptake measurements. Here saturating concentrations of resveratrol caused an apparent increase in cyclooxygenase activity in a manner similar to phenol (Fig. 9B).

**Structure Activity Relationships (SAR) with Resveratrol Analogs**—Resveratrol and three of its methoxy analogs (I-IV) were used to delineate the SAR required for COX-1 inactivation (see Table II). Several key findings were observed. First, inactivation of the peroxidase and cyclooxygenase activities of COX-1 required the presence of the m-hydroquinone moiety (3,5-di-OH group). With respect to COX-2, analogs containing the m-hydroquinone moiety were not inactivators of either the peroxidase or cyclooxygenase activities. Second, the Kᵣ for changes in Soret band absorbance with COX-1 increased with the number of methoxy groups present on resveratrol (I-IV). Although binding affinity is significantly decreased with IV, the fact that binding is observed indicates that the free hydroxyl groups on the trans-stilbene scaffold are not the sole determinants of heme interaction. By contrast, no changes in Soret band absorbance were observed with COX-2 using any of the resveratrol analogs, indicating a different mode of binding. Third, all of the methoxy analogs were oxidized by both COX-1 and COX-2 with the exception of the tri-methoxy analog (IV). This indicates that any one of the three hydroxy groups on resveratrol (I) can be enzymatically oxidized by COX-1 and COX-2, but the trans double bond is not oxidized. These results were confirmed by RP-HPLC analysis, which showed that COX-1 and COX-2 catalyzed the disappearance of all the analogs except IV (Table II). COX-2 was found to be the more robust catalyst of each analog except for the di-methoxy analog (III). This analog had the unique property of acting as a reducing co-substrate for both COX-1 and COX-2 and protected both enzymes against self-inactivation. This finding indicates that the phenol moiety (4'-OH group) of resveratrol acts as a reducing co-substrate for COX-1 and is not the moiety responsible for

| Analog | Enzyme | IC₅₀ Peroxidase | IC₅₀ Cyclooxygenase | Kᵣ Oxidation of compound | Compound oxidized (HPLC) | Mode of action | t₀ | µM | s⁻¹ |
|--------|--------|----------------|--------------------|-------------------------|-------------------------|----------------|----|----|----|
| I      | COX-1  | 2.8 ± 0.6      | 67 ± 19            | 11.7 ± 1.8              | 1.26                    | 46.89          | Inactivator | 0.069 ± 0.004 | 1.52 ± 0.15 | 10.04 |
| II     | COX-2  | ND             | ND                 | ND                       | 8.41                    | 100            | Co-substrate | ND            | ND            | ND     |
| III    | COX-1  | 5.1 ± 0.9      | 30 ± 5             | 17.7 ± 3.4              | 0.06                    | 9.38           | Inactivator | 0.046 ± 0.002 | 1.26 ± 0.16 | 15.07 |
| IV     | COX-2  | ND             | ND                 | ND                       | 2.98                    | 17.93          | Co-substrate | ND            | ND            | ND     |

* Values elevated because 1 mM phenol is present in the assay and no peroxide co-substrate is present.
* Plots of velocity vs. enzyme amount (µM) gave linear lines with a correlation coefficient (r²) of >0.975.
* ND, not detectable.
inactivation. With the exception of the tri-methoxy analog (IV), which cannot be oxidized, all the analogs acted as reducing co-substrates for COX-2 and protected this enzyme from self-inactivation.

DISCUSSION

Resveratrol was found to be a potent inhibitor of both the cyclooxygenase and peroxidase activities of COX-1, but the drug acted only as a reducing co-substrate for COX-2 (Table I). The observation that resveratrol acted as a noncompetitive inhibitor of the cyclooxygenase activity of COX-1 suggests that AA and resveratrol bind at different sites that correspond to the cyclooxygenase and peroxidase active sites, respectively. This confirms a novel mode of inhibition for resveratrol because all known NSAIDs are competitive with AA (4, 5). The observation that resveratrol acted as an uncompetitive inhibitor of the peroxidase activity of COX-1 suggests that resveratrol requires a peroxide substrate to exert its inhibitory effects via the formation of an E-SI complex.

Resveratrol interacts with the FePPIX co-factor of COX-1 but not COX-2 as measured by difference absorbance spectroscopy. These changes in the Soret band absorbance spectrum are not related to changes in the oxidation state of iron (reduction to Fe²⁺ is associated with a large bathochromic shift in Soret band absorbance to ~430–440 nm), but instead may be related
Resveratrol was rapidly oxidized by the peroxidase activity of both COX-1 and COX-2. The oxidation of resveratrol occurred at both the peroxidase active site and had an obligatory requirement for peroxide substrate. Although characterization of a mechanism-based inactivator for COX is complicated by the fact that the enzymes undergo self-inactivation (12, 13), it was found that in the presence of peroxide and resveratrol there was significantly more COX-1 inactivation than could be accounted for by self-inactivation alone (Fig. 4A). By contrast, COX-2 was a superior catalyst of resveratrol oxidation, but it was not inactivated by resveratrol.

The kinetic characterization of a mechanism-based inactivator requires a series of preincubation/dilution experiments in which inactivator concentration is varied and loss of enzymatic activity is monitored over time. With resveratrol this was not possible because the $t_{1/2}$ for inactivation was 10 s. To obtain reasonable estimates of $k_{inact}$, a steady-state approach was employed in which enzyme inactivation was monitored by measuring the decrease in the rate for the enzymatic oxidation of TMPD. These assays showed a time- and concentration-dependent inactivation, which was observed over a dynamic range of peroxide concentrations as would be expected in vivo. Steady-state $k_{inact}$ (0.069 ± 0.004 s$^{-1}$) and $K_{inact}$ (1.52 ± 0.15 μM) were obtained, and the partition ratio was calculated to be 22. The mechanism-based inactivation of COX-1 by resveratrol was irreversible because activity was not restored by rapid dilution into an activity assay or by Sephadex G-25 gel-filtration chromatography, which separates enzyme from free resveratrol. With COX-2 resveratrol behaved like a reducing co-substrate only. This result was confirmed by AA-dependent O$_2$ uptake measurements, which showed that resveratrol stimulated O$_2$ uptake for COX-2, like phenol (Fig. 9B).

Although a significant amount of tritium was associated with $[^3H]$resveratrol-inactivated COX-1 by Sephadex G-25 gel-filtration chromatography (Fig. 6), this radioactivity was lost under the denaturing conditions of SDS-PAGE and also by RP-HPLC (Figs. 7 and 8). We conclude that the mechanism-based inactivation of COX-1 is not accompanied by stable covalent modification of the enzyme. During the mechanism-based inactivation of lactoperoxidase (LPO) by resorcinol (m-hydroquinone), a stoichiometry of 10.0 mol of resorcinol incorporated/monomer was reported (27). However, SDS-PAGE and RP-HPLC were not used to determine whether stable covalent modification had occurred.

**SAR with Resveratrol Analogs**—SAR analysis using methoxy-resveratrol analogs was revealing (Table II). First, the m-hydroquinone moiety (3,5-di-OH group) of resveratrol is required for mechanism-based inactivation of COX-1, a finding that was confirmed by studies with resorcinol (m-hydroquinone), which identified it as the minimal structure for inactivation of COX-1 (data not shown). The m-hydroquinone is unique because oxidation of one hydroxy group results in a semiquinone radical that cannot be stabilized through the ring structure to the remaining hydroxy group, as is the case for o- and p-hydroquinones. Second, any of the three hydroxy groups on resveratrol can be oxidized by both COX-1 and COX-2; however, the outcome of these events differs with both the position of the hydroxy group and by enzyme isoform. With COX-2, all of the hydroxy groups on resveratrol can serve as reducing co-substrates (Scheme 2A). However, with COX-1, oxidation of the m-hydroquinone moiety leads to inactivation (Scheme 2B), whereas oxidation of the phenol moiety leads to reducing co-substrate activity (Scheme 2A). Therefore, with respect to COX-1, resveratrol contains moieties that make it both a mechanism-based inactivator and a reducing co-substrate, namely a m-hydroquinone and a phenol moiety on op-
posite rings. These findings give insight into the design of more efficient mechanism-based inactivators of COX-1; these could contain a di-m-hydroquinone moiety on the trans-stilbene scaffold rather than contain functionally opposed moieties as is the case for resveratrol.

**Mechanism of COX-1 Inactivation by Resveratrol**—Mechanism-based inactivation of the peroxidase activity of COX-1 by resveratrol leads to the elimination of PG synthesis, whereas PG synthesis by COX-2 is unaltered (Fig. 9). Both enzymes are likely to oxidize the m-hydroquinone to an unstabilized radical species, yet only in COX-1 does enzyme inactivation occur. Such an unstabilized radical was proposed for the inactivation of LPO and thyroid peroxidase (TPO) by resorcinol (27). Because stable covalent modification of COX-1 was not observed, inactivation must result from a "hit-and-run" mechanism in which the unstabilized m-hydroquinone radical generates a protein radical that goes on to inactivate the enzyme. Such protein radicals are believed to be responsible for the normal phenomenon of peroxidase self-inactivation (12, 13). In this mechanism, the oxidized m-hydroquinone product leaves the enzyme (Scheme 2B).

Support for a radical mechanism comes from two observations. First, in the presence of saturating amounts of phenol, the specific activity of resveratrol oxidation by COX-1 increased 10-fold to 12.4 μmol/min/mg (data not shown), whereas inactivation was almost entirely prevented (Fig. 4A). This finding suggests that phenol protects against resveratrol-mediated inactivation of COX-1 by quenching either the m-hydroquinone radical species or the protein radical species necessary for inactivation to occur. Second, preliminary liquid chromatography/mass spectrometry (LC/MS) data on the retained radioactive peak from the RP-HPLC analysis (Fig. 8A) showed a resveratrol oxidation product with a mass of 454 ([M+H]+ = 453). This mass is consistent with the formation of a resveratrol dihydrodimer (28, 29), which can only occur through a radical mechanism (the complete characterization of this product will be the subject of another article).

It is interesting to ponder why some peroxidases are sensitive to inactivation by the m-hydroquinone moiety (COX-1, LPO, and TPO) and others immune (COX-2, chloroperoxidase, myeloperoxidase, and horseradish peroxidase) (Ref. 27 and this work). The answer may lie in the residues surrounding the peroxidase active sites. For example, several His residues at the bottom of the peroxidase active site of COX-1 are changed to aliphatic residues in the peroxidase active site of COX-2 (His-442 → Ile, His-443 → Ala, and His-445 → Ala) (30, 31). The prevalence of His residues in the peroxidase active site of COX-1 provides a source of oxidizable residues to generate damaging protein radicals for inactivation. Histidinyl radicals were observed during the reaction between bovine superoxide dismutase and H₂O₂. In this system, the histidinyl radical reacts with molecular oxygen to form an intermediate peroxyl radical that rapidly decays to form 2-oxohistidine. This oxidized amino acid is implicated in the inactivation of Cu,Zn-

---

**Fig. 8. Determination of covalent modification of COX-1 by resveratrol via RP-HPLC.** Protein fractions corresponding to resveratrol-inactivated COX-1 were obtained by Sephadex G-25 gel-filtration chromatography. Aliquots of peak protein fractions (100 μl containing 10–20 μg of COX-1) were injected onto a Vydac C₄ column (5 μm; 2.1 × 150 mm) and analyzed by RP-HPLC with in-line diode array and radiometric detection. A, radiochromatogram (delay from diode array to radiometric detector was 1 min). B, A²₂₂ nm chromatogram (used to detect COX-1). C, A₃₀₆ nm chromatogram (used to detect trans-stilbenes, which have λₘₐₓ of 306 nm). D, A₄₀₀ nm chromatogram (used to detect FePPIX co-factor). No radioactivity is associated with the COX-1 or FePPIX peaks.
superoxide dismutase by its own reaction product, H$_2$O$_2$ (32, 33).

Pharmacology of m-Hydroquinones—There has been little focus on the development of selective COX-1 inactivators because it is accepted that COX-2 is the desired target for NSAIDs (34). However, aspirin is an effective cardioprotective agent that targets platelet-specific COX-1. Although it is not selective for this isoform, extremely high efficacy as an anti-platelet agent results from its ability to irreversibly inactivate platelet COX-1 (for a recent review, see Ref. 35). Irreversible inhibition can only be surmounted by new protein synthesis. Because platelets are unable to synthesize new protein the effect of aspirin is governed by the $t_{1/2}$ of the platelet, which is 7 days. Thus, a single low dose of aspirin can eliminate platelet TxA$_2$ synthesis for an extended period, whereas PGI$_2$ synthesis in the vascular endothelial cells can recover quickly (20, 35). In this manner, aspirin shifts the TxA$_2$-PGI$_2$ balance to favor cardioprotection over thrombosis.

We have shown that resveratrol, and other m-hydroquinones, are selective mechanism-based inactivators of COX-1. Although the in vitro kinetic parameters for the inactivation of COX-1 by m-hydroquinones are favorable, their efficacy as cardioprotective agents relies on several factors other than $k_{\text{inact}}/K_{\text{co-substrate}}$. First, m-hydroquinones require a peroxide substrate to exert their effects. In the resting platelet, the peroxide tone is likely to exist at a basal level until the platelets become activated at which point the production of peroxides, namely PGG$_2$ by COX-1 and 12-hydroperoxyeicosatetraenoic acid by 12-lipoxygenase, could drive the mechanism-based inactivation of COX-1 with the observed rate constants. Prior to platelet activation, the mechanism-based inactivation of COX-1 by m-hydroquinones will be less. Second, we have demonstrated that the ability of m-hydroquinones to inactivate COX-1 is significantly impeded by the presence of saturating amounts of the reducing co-substrate phenol. Naturally occurring reducing co-substrates in vivo may act as antagonists to the inactivation event. Such antagonism may explain the loss in potency of resveratrol as an anti-platelet agent in whole blood versus washed platelets (36). These findings would suggest that low peroxide tone and high co-reductant tone might limit the efficacy of m-hydroquinones to be cardioprotective. Despite these arguments, there are examples in which m-hydroquinones (namely resveratrol) show significant anti-platelet activity in vivo (37, 38).

Conclusions—The data presented herein offer a basis for the design of a new class of selective COX-1 inactivators, namely...
m-hydroquinones, that are mechanism-based inactivators of the COX-1 peroxidase. These compounds are unique because they prevent the formation of prostaglandins by acting at a site different from where classical NSAIDs exert their effects.

Acknowledgments—We thank John Lawson (Center for Experimental Therapeutics, University of Pennsylvania School of Medicine, Philadelphia, PA) for help with the GC/MS analysis of PGE2. We thank Dr. Sridhar Gopishetty for insightful conversations on this project. We thank Dr. Ian Blair and Dr. Seon Hwa Lee (Center for Cancer Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA) for the preliminary LC/MS analysis of the resveratrol dihydrodimer. We thank Dr. Robert Copeland (Glaxo-SmithKline) for recombinant COX-2 expressed in S2/1 insect cells.

REFERENCES

1. Pace-Asciak, C. R., Hahn, S., Diamandis, E. P., Soleas, G., and Goldberg, D. M. (1995) Clin. Chem. Acta 235, 297–219
2. Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W., Fong, H. H., Farnsworth, N. R., Kinghorn, A. D., Mehta, R. G., Moon, R. C., and Pezzuto, J. M. (1997) J. Agric. Food Chem. 45, 5488–5492
3. Selinsky, B. S., Gupta, K., Sharkey, C. T., and Loll, P. J. (2001) Biochemistry 40, 5172–5180
4. Urbaniai, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildhaus, D., Miyashita, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1996) Nature 384, 644–648
5. Cichewicz, R. H., Rouzi, S. A., and Hamann, M. T. (2000) J. Nat. Prod. 63, 29–33
6. Patrono, C. (1994) J. Biol. Chem. 269, 2890–2896
7. Sirtori, C., Taddei, S., Polentarutti, N., Tadini, G., and Polentarutti, F. (1995) J. Biol. Chem. 269, 2755–2760
8. Grady, J., Salmon, J. A., and Moncada, S. (1979) Blood Cells Mol. Dis. 12, 987–913
9. Moncada, S., Gryglewski, R., Bunting, S., and Vane, J. R. (1976) Nature 263, 663–665
10. Korbut, R., and Moncada, S. (1976) Thrombosis Res. 13, 489–500
11. Bunting, S., Moncada, S., and Vane, J. R. (1983) Br. Med. Bull. 39, 271–276
12. McAdam, B. F., Catella-Lawson, F., Mardini, I. A., Kapoor, S., Lawson, J. A., and FitzGerald, G. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 272–277
13. Amezquita, J. L., O'Grady, J., Salmon, J. A., and Moncada, S. (1979) Thrombosis Res. 16, 69–79
14. Patruno, C. (1994) N. Engl. J. Med. 330, 1287–1294
15. Strieder, S., Schraiber, K., Scherrer, H. J., Dietz, R., and Ruf, H. H. (1992) J. Biol. Chem. 267, 13870–13878
16. Divi, R. L., and Doerge, D. R. (1994) Biochemistry 33, 9668–9674
17. Cechewicz, R. H., Rouzi, S. A., and Hamann, M. T. (2000) J. Nat. Prod. 63, 29–33
18. Gunther, M. R., Gupta, K., Sharkey, C. T., and Loll, P. J. (2001) Biochemistry 40, 5172–5180
19. Uchida, K., and Kawakishi, S. (1994) J. Biol. Chem. 269, 2755–2760
20. Otsuka, K., and Kawakishi, S. (1994) J. Biol. Chem. 269, 2890–2896
21. Bunting, S., Gryglewski, R., Moncada, S., and Vane, J. R. (1976) Prostaglan-