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

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

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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 H_2 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 nonsteroidal 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_{inact} = 0.069 ± 0.004 s^{-1}, $K_{i(inact)}$ = 1.52 ± 0.15 μM), 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}H]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 peroxidase active site.

3,4′,5-Trihydroxy-*trans*-stilbene (resveratrol[1]; Scheme 1) is a natural product found in grapes, which is present at concentrations up to 100 μM 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 H_2 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 nonsteroidal 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 PGH_2 by two sequential reactions that occur at spatially distinct active sites on the enzymes. The first reaction involves the *bis*-dioxygenation of AA to yield PGG_2 (cyclooxygenase reaction), and the second reaction involves peroxidative cleavage of PGG_2 to yield PGH_2 (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 2*A*). 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 (Fe^{5+}) and compound II (Fe^{4+})) to its resting state (Fe^{3+}) before peroxide bond cleavage can occur again. COX enzymes catalyze a branched-chain mechanism whereby peroxide (PGG_2) 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

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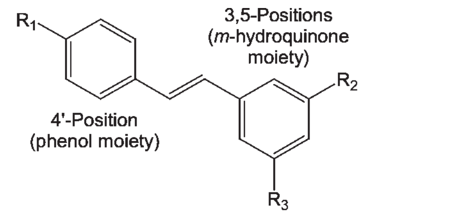
[1] The abbreviations used are: resveratrol, 3,4′,5-trihydroxy-*trans*-stilbene; COX, prostaglandin H_2 synthase (EC 1.14.99.1); LPO, lactoperoxidase; TPO, thyroid peroxidase; FePPIX, Fe-protoporphyrin-IX; MnPPIX, Mn-protoporphyrin-IX; NSAID, nonsteroidal anti-inflammatory drug; AA, arachidonic acid; PG, prostaglandin; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine; EtOOH, ethyl hydroperoxide; resorcinol, *m*-dihydroxybenzene; RP-HPLC, reverse phase high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; TMS, trimethylsilyl; PFB, pentafluorobenzyl; LC/MS, liquid chromatography/mass spectrometry; SAR, structure activity relationship; TxA_2, thromboxane A_2.

thromboxane A_2 (TxA_2) is a potent vasoconstrictor and platelet aggregator synthesized in activated platelets (8, 14), whereas prostacyclin (PGI_2) 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 TxA_2 and PGI_2 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 TxA_2 and PGI_2 are synthesized from the precursor PGH_2; however, different COX isoforms contribute to their formation. Platelets contain only COX-1, which is an obligate enzyme for TxA_2 formation. In contrast, COX-2 in the vascular endothelial cells is the primary source of systemic PGI_2 biosynthesis because selective COX-2 inhibitors reduce PGI_2 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 TxA_2-PGI_2 balance in favor of PGI_2. 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.



$R_1=R_2=R_3=OH$ = resveratrol (**I**)
$R_1=OCH_3$, $R_2=R_3=OH$ = 4'-OMe-resveratrol (**II**)
$R_1=OH$, $R_2=R_3=OCH_3$ = 3,5-di-OMe-resveratrol (**III**)
$R_1=R_2=R_3=OCH_3$ = 3,4',5-tri-OMe-resveratrol (**IV**)

SCHEME 1. **Structure of resveratrol and its methoxy analogs.**

EXPERIMENTAL PROCEDURES

Materials—Fe-protoporphyrin-IX (FePPIX), Mn-protoporphyrin-IX (MnPPIX), AA, H_2O_2 (30% v/v), [1-^{14}C]AA (51 mCi/mmol), Sephadex G-25, and Tween 20 were purchased from Sigma. $PGF_{2\alpha}$, PGE_2, and PGD_2 were purchased from Biomol Research Laboratories. *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine (TMPD) was purchased from Arcos Organics. Resveratrol (**I**) was purchased from Cayman Chemical, and [U-^{3}H]resveratrol (3.6 Ci/mmol) was purchased from Moravek Biochemicals and Radiochemicals. Solvable and Ultima Gold were purchased from Packard Biosciences. 4′-Methoxy-3,5-dihydroxy-*trans*-stilbene (**II**), 4′-hydroxy-3,5-dimethoxy-*trans*-stilbene (**III**), and 3,4′,5-trimethoxy-*trans*-stilbene (**IV**) were synthesized according to published methods (Scheme 1) (22). *m*-Dihydroxybenzene (resorcinol) was purchased from Aldrich. Ethyl hydroperoxide (EtOOH) was purchased as a 5% solution in H_2O from Polysciences. Phenol was purchased from Fisher Scientific.

Enzymes—COX-1 was purified to homogeneity from ram seminal vesicles as described previously (23), and human COX-2 was purified from baculovirus infected *Sf*-21 cells as described (24). 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*-dioxygenation of AA to yield PGG_2 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 O_2 consumed/min/mg, respectively, whereas the MnPPIX-reconstituted COX-1 had a specific activity of 16 μmol of O_2 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 H_2O_2 (EtOOH can be substituted). The assays were initiated by the addition of peroxide. The formation of *N,N,N′,N′*-tetramethyl-1,4-phenylene-diimine (E_{610} = 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 0.22 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 = 8.33–83.33 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 Me_2SO, 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).

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. K_d values were determined by adding resveratrol or its analogs (10–250 μM) incrementally while monitoring complex formation at 404 nm with respect to an untreated sample. Hyperbolic plots of Δabsorbance at 404 nm *versus* resveratrol (or analog) concentration were obtained. Best estimates of $\Delta absorbance_{max}$ and K_d were obtained by iterative fits to the following equation for a hyperbola (the fits gave a mean ± standard deviation).

$$\Delta absorbance = (\Delta absorbance_{max} * [I])/(K_d + [I]) \quad \text{(Eq. 1)}$$

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 H_2O or 300 μM H_2O_2 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 (λ_{max} for *trans*-stilbene, E_{306} = 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, a RP-HPLC method was employed. Holo-COX-1 or holo-COX-2 (0.2 units) was mixed with 50 μM resveratrol or analog in 100 mM Tris-HCl (pH 8.0). The 1-ml reactions were initiated with the addition of 300 μM H_2O_2 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 C_{18} column (3.5 μm; 4.6 × 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 analogs was quantified by monitoring the disappearance of compound in the presence of H_2O_2 with respect to a sample, which contained no H_2O_2. The RP-HPLC analysis was performed using a Waters model 2695 pump equipped with a model 996 photodiode array detector.

Peroxidase-dependent Inactivation of COX by Resveratrol—COX-1 or COX-2 (10 μM) was preincubated with mixtures of 100 μM H_2O_2, 100 μM resveratrol (or analog), and 1 mM phenol in 100 mM 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 H_2O_2 (or H_2O 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 IC_{50} curves, and the percentage of activity remaining was computed with respect to an enzyme control.

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 (1.26 *versus* 34 μmol/min/mg) when TMPD was saturating. Progress curves were corrected for the nonenzymatic rate of TMPD oxidation. k_{obs} values for the time-dependent inactivation of the enzyme were obtained by fitting progress curves to a single exponential.

$$\Delta\text{absorbance}_{610\text{ nm}} = A_0(1 - e^{-kt})/k + \text{offset} \qquad \text{(Eq. 2)}$$

The k_{obs} 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 k_{inact} and $K_{i(inact)}$ (25). The following equation was used to extract kinetic constants from the Kitz-Wilson analysis.

$$k_{obs} = (k_{inact}{*}[I])/(K_{i(inact)} + [I]) \qquad \text{(Eq. 3)}$$

The $t_{1/2}$ for inactivation at saturation was obtained from Equation 4.

$$t_{1/2} = (\ln 2)/k_{inact} \qquad \text{(Eq. 4)}$$

Tritiated Resveratrol Incorporation into COX-1—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. [^{3}H]Resveratrol was prepared as a 10 mM solution in Me_2SO with a specific radioactivity of 25,000 cpm/nmol and used to determine whether inactivation of COX-1 by resveratrol was accompanied by covalent modification. The reactions contained 100 mM Tris-HCl (pH 8.0), 20 μM FePPIX, 10 μM COX-1, 500 μM [^{3}H]resveratrol (5 × 10^6 cpm/assay) and were initiated with either water or 125 μM H_2O_2. The reactions were quenched on ice after 5 min and immediately loaded onto a Sephadex G-25 gel-filtration column (1 × 45 cm) equilibrated in 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2% Tween 20, and 1 mM phenol. The eluant was monitored for absorbance at 280 nm, whereas each fraction was monitored for protein concentration (MicroBCA assay, Pierce), peroxidase activity, and radioactivity. Additional controls were performed in the absence of [^{3}H]resveratrol to determine the contribution of self-inactivation in this analysis, which was minimal. Stoichiometry of incorporation was determined by using the specific radioactivity of [^{3}H]resveratrol and the protein concentration as conversion factors.

To further assess covalent modification of COX-1 by resveratrol, SDS-PAGE and RP-HPLC methods were employed. Radiolabeled samples, identical to those used for Sephadex G-25 gel-filtration chromatography, were prepared for SDS-PAGE analysis. The control (enzyme containing no peroxide) and experimental (enzyme inactivated in the presence of peroxide substrate) samples were mixed with SDS loading buffer and boiled for 10 min. The denatured samples were separated by SDS-PAGE (12% polyacrylamide separating gel) and visualized by staining with Coomassie Blue. The gel was subsequently cut into 32 pieces, and the radioactivity of each piece was eluted by treatment with 0.5 ml of Solvable at 55 °C for 3 h. Radioactivity was quantified by scintillation counting using Ultima Gold scintillant. Stoichiometry of incorporation was determined by using the specific radioactivity of [^{3}H]resveratrol and the total amount of protein applied to the gel (assuming 100% recovery) as conversion factors.

RP-HPLC was performed on [^{3}H]resveratrol-inactivated COX-1. Prior to the analysis, resveratrol-inactivated COX-1 was purified by Sephadex G-25 gel-filtration chromatography, as previously described, to remove unbound ^{3}H-labeled ligands. Aliquots of peak protein fractions (100 μl containing 10–20 μg of COX-1) were injected onto a Vydac C_4 column (5 μm; 2.1 × 150 mm) equilibrated with solvent A (0.1% trifluoroacetic acid in water) at a flow rate of 0.3 ml/min. Beginning at 2 min, a linear gradient was run to solvent B (0.1% trifluoroacetic acid in acetonitrile) over 30 min to separate COX-1 (27 min) from FePPIX (24 min) and resveratrol (16 min). Eluant was monitored at 220 nm (COX-1), 400 nm (FePPIX), 306 nm (resveratrol) and for tritium. The RP-HPLC method separated COX-1 from its FePPIX co-factor and determined whether radioactivity was associated with either analyte. RP-HPLC analysis was performed using a Waters model 2695 pump equipped with a model 996 photodiode array detector and an in-line β-RAM model 3 radio flow-through detector (IN/US Systems).

Formation of Prostanoids by COX-1 and COX-2—COX-1 or COX-2 (2 μM) was incubated with either 250 μM resveratrol or 1 mM phenol in 100 mM Tris-HCl (pH 8.0) containing 5 μM FePPIX. The 100-μl reactions were initiated by the addition of 150 μM [^{14}C]AA (25 nCi/reaction) and quenched after 1 min with stannous chloride in HCl (50 mg in 5 ml of 0.04 N HCl) to reduce the PG products to $PGF_{2\alpha}$. PG products from each reaction were extracted twice with 400 μl of ethyl acetate, dried *in vacuo*, and separated by thin layer chromatography (ethyl acetate:2,2,4-trimethylpentane:acetic acid, 110:50:20, v/v/v). The resulting plate was visualized by autoradiography after an overnight exposure at −80 °C.

Product confirmation was achieved by gas chromatography/mass spectrometry (GC/MS) of the trimethylsilyl (TMS) ether pentafluorobenzyl (PFB) ester derivatives. Briefly, enzymatically generated product or authentic $PGF_{2\alpha}$ standard were dried under N_2, dissolved in diisopropylethylamine (10 μl) and 10% PFB bromide in acetonitrile (20 μl), and allowed to stand for 15 min at room temperature. The resultant PFB ester was dried under N_2, and dissolved in pyridine (10 μl) and *bis*-(trimethylsilyl)trifluoroacetamide (10 μl), and allowed to stand at room temperature for 5 min. The resultant TMS ether PFB ester was dried under N_2, resuspended in dodecane at a concentration of ~5 μg/ml, and used for GC/MS analysis. A Fisons MD-800 mass spectrometer, equipped with a Fisons 8000 gas chromatograph and a Fisons AS-800 autosampler, was used for all analyses. The MS was operated in the negative ion electron capture mode, using ammonia as the moderating gas. The ion monitored was *m/z* 569 for $PGF_{2\alpha}$ TMS ether PFB ester. A DB5-MS column (0.25 mm × 0.25 μm × 30 m) was used with a temperature program of 1 min isothermal at 190 °C followed by heating at 20 °C/min to 320 °C. The carrier gas was helium. The $PGF_{2\alpha}$ TMS ether PFB ester had a retention time of 17.33 min on the GC column. The enzymatic product displayed GC and MS characteristics identical to authentic $PGF_{2\alpha}$.

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 (IC_{50} = 280 μM, see Table I). 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 (K_i = 100.1 ± 10.0 μM, Fig. 1*A*), confirming the previous finding of Johnson and Maddipati (3). In contrast, resveratrol was an uncompetitive inhibitor *versus* EtOOH (K_i = 0.6 ± 0.1 μM, Fig. 1*B*), 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 was incubated with resveratrol, and changes in the absorbance of the Soret band were examined by difference spectroscopy (Fig. 2*A*). The difference spectrum revealed an increase in intensity of the Soret band plus the appearance of a new chromophore at 530 nm (Fig. 2*A*, inset). In contrast, no such spectral changes were observed upon incubating COX-2 with resveratrol, indicating a different mode of ligand binding (Fig. 2*B*). The increase in absorbance of the Soret band with COX-1 was concentration-dependent and saturable, allowing a K_d determination (Fig. 2*C*). Binding affinity for resveratrol (K_d = 11.7 ±

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

Kinetic parameter	COX-1	COX-2
Cyclooxygenase inhibition	ED_{50} = 15 μM (2) K_i = 26 μM (3) K_i = 100 μM (noncompetitive)	No effect (2) Enhanced activity (3)
Peroxidase inhibition	ED_{50} = 3.6 μM (2) IC_{50} = 15 μM (3) K_i = 0.6 μM (uncompetitive)	ED_{50} = 85 μM (2) IC_{50} ≥ 200 μM (3) IC_{50} = 280 μM
Chromophore	Yes; K_d = 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
K_{inact}	0.069 s^{-1}	No inactivation
$K_{i(inact)}$	1.52 μM	No inactivation
Effect on PG synthesis	Eliminates	No effect
Mechanism of action	Peroxidase-mediated mechanism-based inactivator	Reducing co-substrate

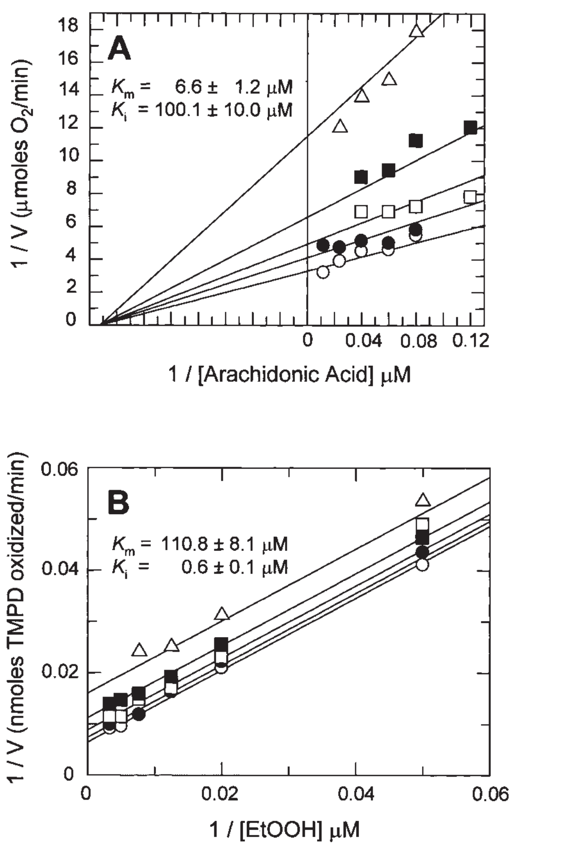


FIG. 1. **Reversible inhibition profiles of resveratrol with COX-1.** *A*, resveratrol is a noncompetitive inhibitor of the COX-1 cyclooxygenase activity. Resveratrol (○, 0 μM; ●, 25 μM; □, 50 μM; ■, 100 μM; △, 250 μM), FePPIX, and COX-1 were mixed in 100 mM Tris-HCl (pH 8.0) supplemented with 1 mM phenol. Reactions were initiated with AA (8.33–83.33 μM), and oxygen consumption during the cyclooxygenase reaction was followed using an oxygen microelectrode. *B*, resveratrol is an uncompetitive 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.

1.8 μM) was virtually unchanged when the measurements were repeated on indomethacin-treated (K_d = 17.7 ± 3.5 μM) and aspirin-treated (K_d = 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. Time-resolved absorbance spectra of resveratrol upon incubation with holo-COX-1 in the absence and presence of H_2O_2 are shown in Fig. 3 (*A* and *B*). Upon addition of H_2O_2 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. 3*C*).

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. 4*A*). First, resveratrol alone had no effect on the enzyme during a 5-min preincubation period (control). Second, in the presence of H_2O_2 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 H_2O_2), 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. 4*B*), 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. 4*C*). 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

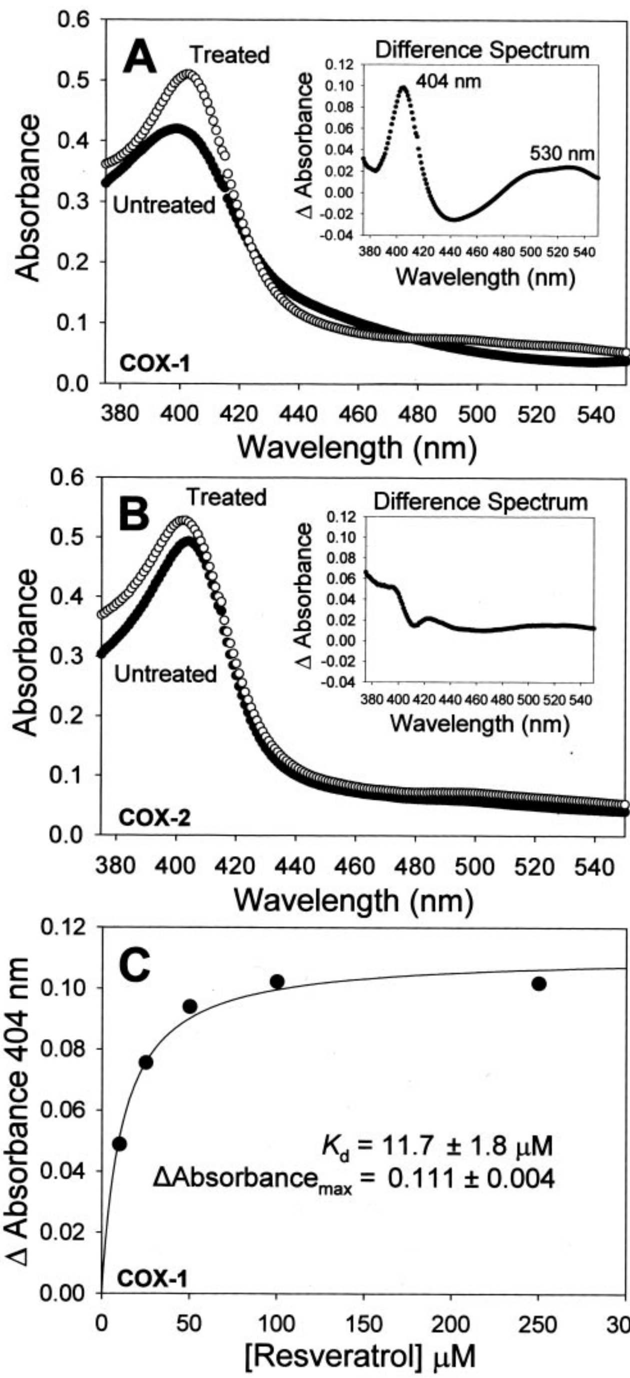


FIG. 2. **Resveratrol binding to COX-1 generates a unique chromophore that is not present in COX-2.** COX-1 (2.5 μM) (*A*) or COX-2 (2.5 μM) (*B*) plus FePPIX (5 μM), and either Me_2SO or resveratrol (100 μM) were mixed in 100 mM Tris-HCl (pH 8.0), and absorbance spectra were collected from 375 to 550 nm on a diode array spectrophotometer. *Insets*, difference spectra were generated by subtracting the absorbance spectrum of solvent-treated enzyme from that of resveratrol-treated enzyme. *C*, K_d for resveratrol binding to COX-1 was determined by monitoring Δabsorbance at 404 nm, while the concentration of resveratrol was incrementally increased (10–250 μM).

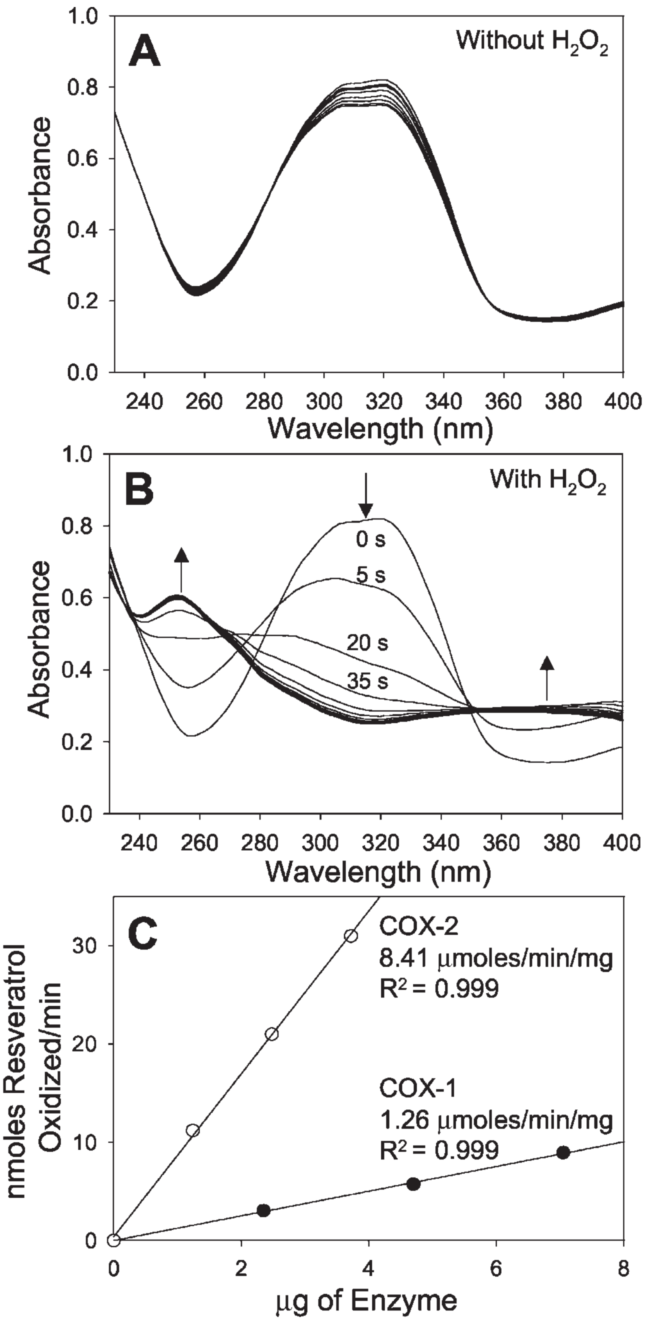


FIG. 3. **Spectrophotometric analysis of resveratrol oxidation catalyzed by the COX peroxidase.** Holo-COX-1 (0.3 μM) was incubated with 30 μM resveratrol in 100 mM Tris-HCl (pH 8.0) in the absence (*A*) or presence (*B*) of H_2O_2 (300 μM), and time-resolved spectra were collected. *C*, specific activity for resveratrol oxidation catalyzed by COX-1 or COX-2 was determined by monitoring the disappearance of the resveratrol (30 μM) spectrum at 306 nm over time while enzyme concentration was varied. Reactions were initiated with 300 μM H_2O_2.

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. 5*A*). These curves were fit to a single exponential equation to yield k_{obs} for inactivation at each resveratrol concentration. The k_{obs} values for inactivation were replotted in a Kitz-Wilson analysis, which showed saturation kinetics (25). This analysis gave a k_{inact} of 0.069 ± 0.004 s^{-1}, $K_{i(inact)}$ of 1.52 ± 0.15 μM, and a calculated $t_{1/2}$ for inactivation of 10.04 s at saturation when the H_2O_2 concentration was held constant at 300 μM (K_m = 287 μM) (Fig. 5*B*) (13). We estimated a partition ratio of 22 by using k_{cat}/k_{inact}, where k_{cat} for res-

TABLE II
SAR analysis of the inhibition of COX by resveratrol and its methoxy analogs

Analog	Enzyme	IC_{50}		K_d	Oxidation of compound[b]	Compound oxidized (HPLC)	Mode of action	k_{inact}	$K_{i(inact)}$	$t_{½}$
		Peroxidase	Cyclooxygenase[a]							
		μM	μM	μM	$\mu mol/min/mg$	%		s^{-1}	μM	s
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
	COX-2	ND[c]	ND	ND	8.41	100	Co-substrate	ND	ND	ND
II	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
	COX-2	ND	ND	ND	2.98	17.93	Co-substrate	ND	ND	ND
III	COX-1	ND	ND	57.0 ± 4.3	9.75	71.66	Co-substrate	ND	ND	ND
	COX-2	ND	ND	ND	5.23	19.36	Co-substrate	ND	ND	ND
IV	COX-1	ND	ND	82.6 ± 7.7	0.00	0.00	No effect	ND	ND	ND
	COX-2	ND	ND	ND	0.00	0.00	No effect	ND	ND	ND

[a] Values elevated because 1 mM phenol is present in the assay and no peroxide co-substrate is present.
[b] Plots of velocity *vs.* enzyme amount (μg) gave linear lines with a correlation coefficient (r^2) of >0.975.
[c] ND, not detectable.

veratrol oxidation was estimated to be 1.52 s^{-1} from specific activity measurements (Fig. 3*C*). 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 H_2O_2 concentrations (Fig. 5*C*) 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—[^{3}H]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 [^{3}H]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 H_2O_2 and [^{3}H]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 ^{3}H-labeled compound were bound/mol of synthase monomer (*i.e.* 10.0 mol of ^{3}H-labeled compound were bound/mol of inactivated synthase monomer) (Fig. 6*B*). In the presence of H_2O_2 alone, COX-1 inactivation was <10%, indicating that the role of self-inactivation in the analysis was minimal. Nonspecific binding of [^{3}H]resveratrol was observed in the absence of H_2O_2, yielding a stoichiometry of 1.4 mol of [^{3}H]resveratrol bound/mol of synthase monomer (Fig. 6*A*); 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 [^{3}H]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 ^{3}H-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 [^{3}H]-labeled compound were bound/mol of inactivated synthase monomer. In addition, the amount of [^{3}H]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. 8*A*). 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. 8*C*). 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—[^{14}C]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. 9*A*). Product identity was established independently by GC/MS of the $PGF_{2\alpha}$ TMS ether PFB ester formed from the reaction with unlabeled AA. In this analysis, COX-2 generated $PGF_{2\alpha}$ 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. 9*B*).

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 K_d for changes in Soret band absorbance with COX-1 increased with the number of methoxy groups present on resveratrol, the highest K_d being 83 μM for the tri-methoxy analog (**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

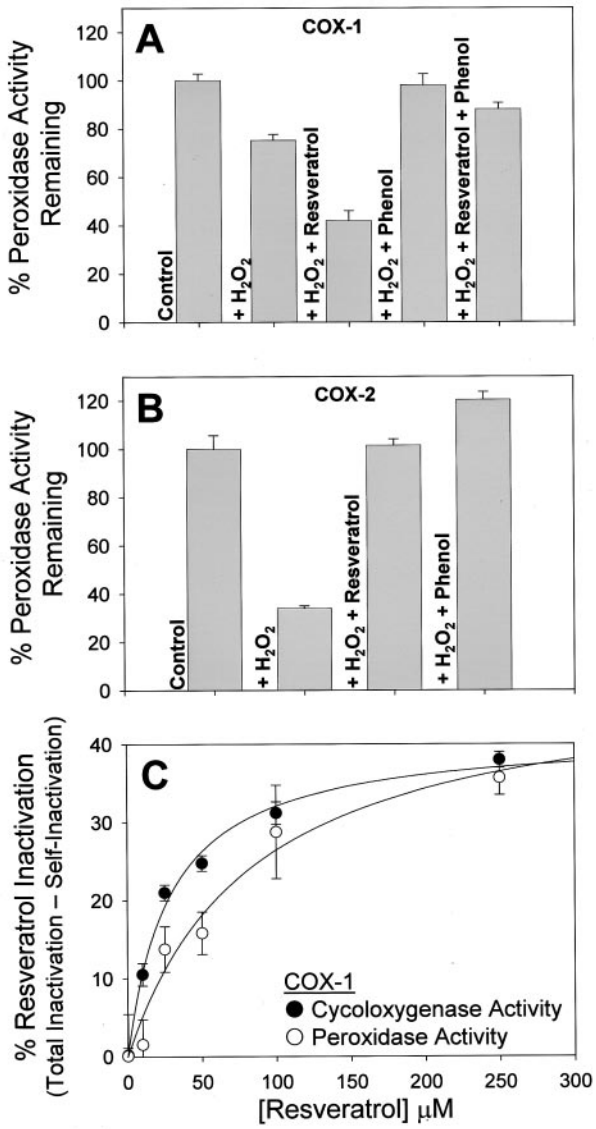


FIG. 4. **Mechanism-based inactivation of COX by resveratrol.** *A* and *B*, holo-COX-1 or holo-COX-2 (10 μM) was preincubated with mixtures of 100 μM resveratrol, 100 μM H_2O_2, and 1 mM phenol in 100 mM Tris-HCl (pH 8.0) for 5 min at 25 °C. The samples were then diluted into the peroxidase assay (200-fold), and the percentage of activity remaining was determined. Values were corrected for resveratrol carryover based on IC_{50} curves. *C*, holo-Cox-1 (10 μM) was preincubated with 100 μM H_2O_2 and resveratrol (0–250 μM) in 100 mM Tris-HCl (pH 8.0) for 5 min at 25 °C. The samples were then diluted 40-fold into the cyclooxygenase assay (●) and 200-fold into peroxidase assay (○). Percentage of resveratrol mediated inactivation was determined by subtracting the amount of self-inactivation (0 μM resveratrol) from the amount of total inactivation observed. Values were corrected for resveratrol carryover based on IC_{50} curves (n = 2).

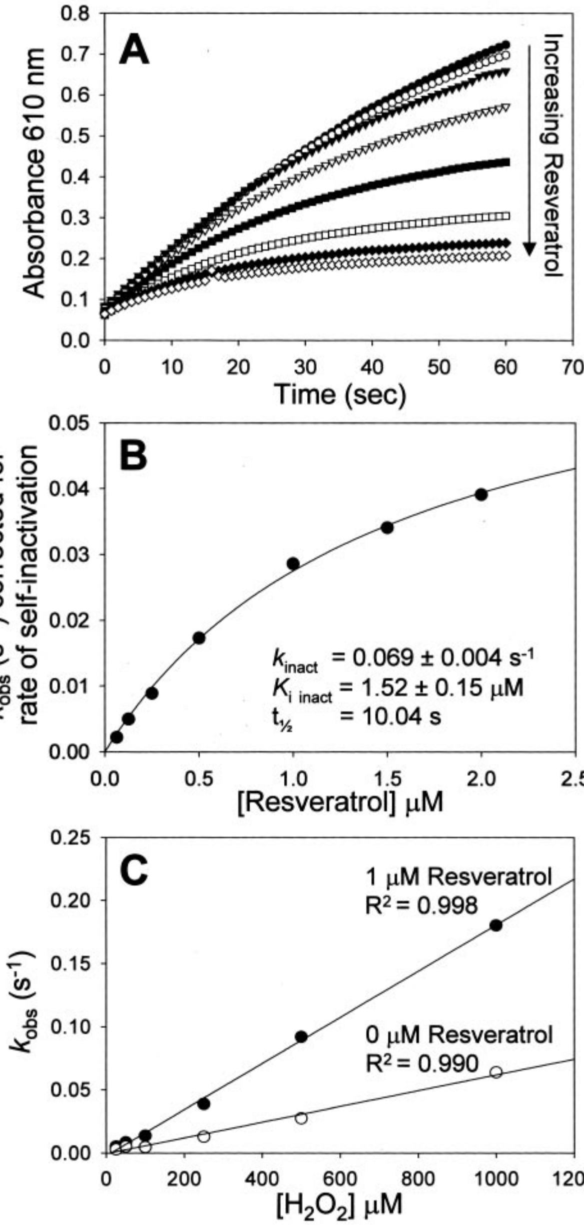


FIG. 5. **Time- and concentration-dependent inactivation of COX-1 by resveratrol.** *A*, steady-state progress curves obtained for the peroxidase-dependent oxidation of TMPD (80 μM) by COX-1 (30 nM) in the presence of 300 μM H_2O_2 while resveratrol concentration was increased (●, 0.0 μM; ○, 0.0625 μM; ▼, 0.125 μM; ▽, 0.25 μM; ■, 0.5 μM; □, 1.0 μM; ♦, 1.5 μM; ◇, 2.0 μM). *B*, rate constants (k_{obs}) for the time-dependent inactivation of the COX-1 peroxidase by resveratrol were extracted from steady-state data by single exponential fits and analyzed by the method of Kitz and Wilson. *C*, rate constants (k_{obs}) for the time-dependent inactivation of the COX-1 peroxidase by either 0 or 1 μM resveratrol were determined over a wide range of H_2O_2 concentrations (25–1000 μM).

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·S·I 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 Fe^{2+} is associated with a large bathochromic shift in Soret band absorbance to ~430–440 nm), but instead may be related

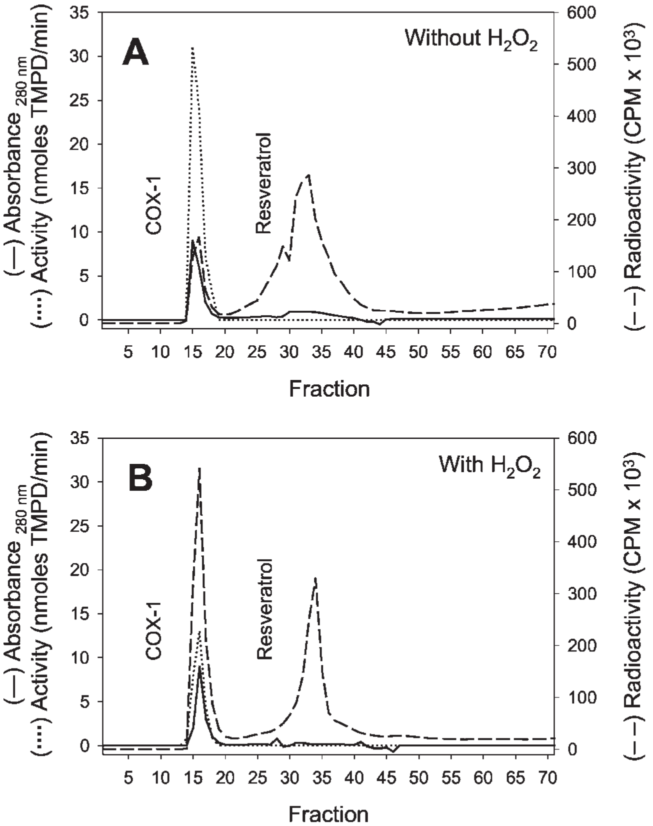


FIG. 6. **Determination of covalent modification of COX-1 by resveratrol via gel-filtration chromatography.** COX-1 (10 μM, extensively dialyzed to remove reducing co-substrates) was mixed with 20 μM FePPIX and 500 μM [^{3}H]resveratrol (25,000 cpm/nmol) in 100 mM Tris-HCl (pH 8.0). Reactions were initiated with either water (*A*) or 125 μM H_2O_2 (*B*). After 5 min, the reactions were quenched on ice and immediately loaded onto a Sephadex G-25 gel-filtration column to separate COX-1 from free [^{3}H]resveratrol. The column eluant was monitored for absorbance at 280 nm (—), enzyme activity (···), radioactivity (- - -), and protein concentration.

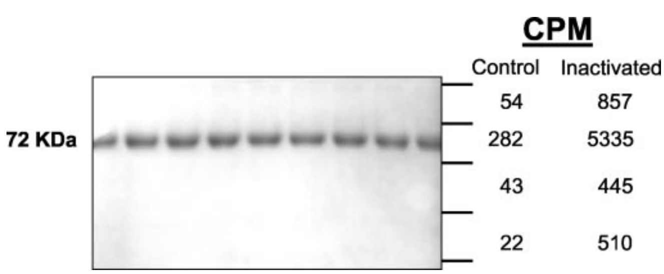


FIG. 7. **Determination of covalent modification of COX-1 by resveratrol via SDS-PAGE.** COX-1 (10 μM, extensively dialyzed to remove reducing co-substrates) was mixed with 20 μM FePPIX and 500 μM [^{3}H]resveratrol (25,000 cpm/nmol) in 100 mM Tris-HCl (pH 8.0). Reactions were initiated with either water (control) or 125 μM H_2O_2 (inactivated). After 5 min, the reactions were quenched on ice and immediately mixed with SDS-PAGE loading buffer. The samples (103.2 μg of COX-1) were boiled for 10 min and separated by SDS-PAGE on a 12% separating gel. Following electrophoresis, the gels were stained with Coomassie Blue (shown), cut into ~1-cm slices, and the radioactivity eluted with Solvable. Radioactivity was quantified by scintillation counting. Radioactivity corresponding to the analyzed slices is shown.

to a change in the axial ligation of the heme iron. The spectroscopically derived K_d value for resveratrol remained unchanged when the cyclooxygenase active site of COX-1 was rendered unavailable by treatment with indomethacin or aspirin. These findings showed that chromophore formation occurred at the peroxidase active site of COX-1.

Mechanism-based Inactivation of COX-1 by Resveratrol—Resveratrol was rapidly oxidized by the peroxidase activity of both COX-1 and COX-2. The oxidation of resveratrol occurred at 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. 4*A*). 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 $K_{i(\text{inact})}$ (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. 9*B*).

Although a significant amount of tritium was associated with [^{3}H]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 2*A*). However, with COX-1, oxidation of the *m*-hydroquinone moiety leads to inactivation (Scheme 2*B*), whereas oxidation of the phenol moiety leads to reducing co-substrate activity (Scheme 2*A*). 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-

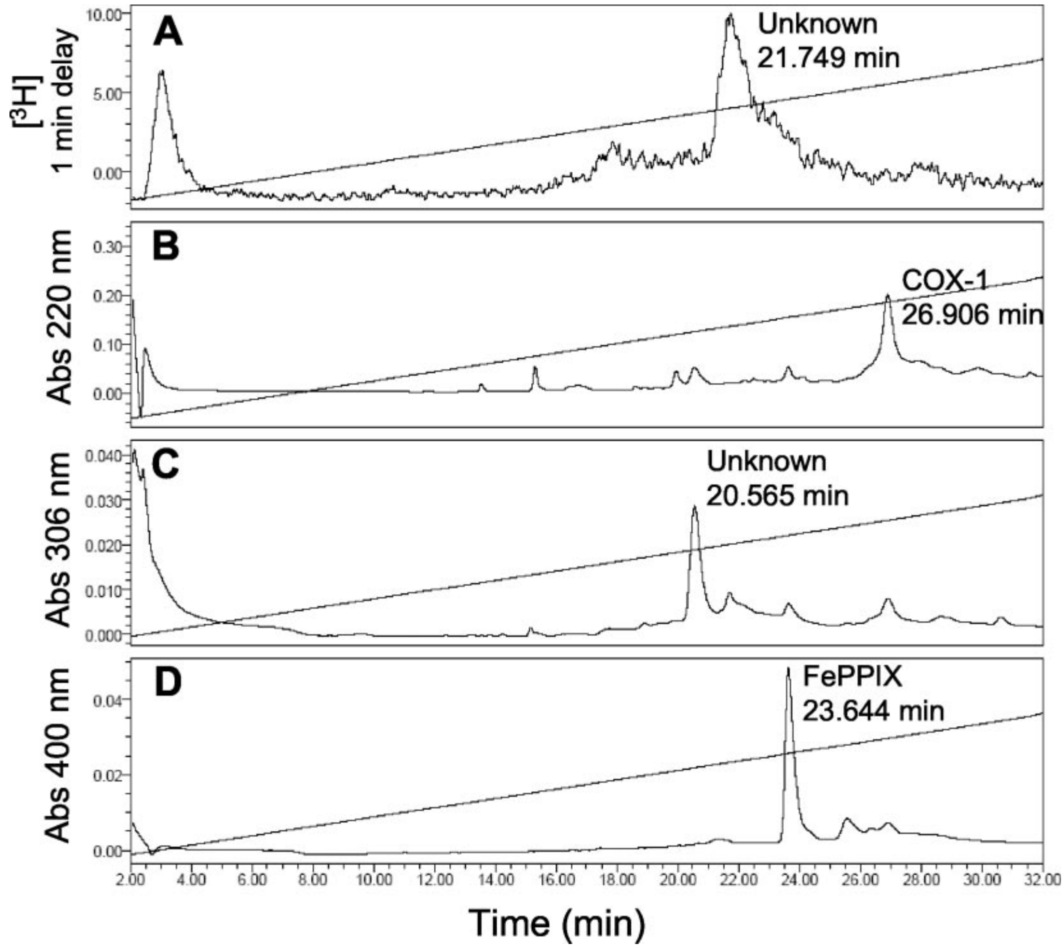


FIG. 8. **Determination of covalent modification of COX-1 by resveratrol via RP-HPLC.** Protein fractions corresponding to resveratrol-inactivated COX-1 were obtained by Sephadex G-25 gel-filtration chromatography. Aliquots of peak protein fractions (100 μl containing 10–20 μg of COX-1) were injected onto a Vydac C_4 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*, $A_{220\ nm}$ chromatogram (used to detect COX-1). *C*, $A_{306\ nm}$ chromatogram (used to detect *trans*-stilbenes, which have λ_{max} of 306 nm). *D*, $A_{400\ nm}$ chromatogram (used to detect FePPIX co-factor). No radioactivity is associated with the COX-1 or FePPIX peaks.

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*A*). 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 2*B*).

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. 4*A*). 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. 8*A*) 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 H_2O_2. 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-

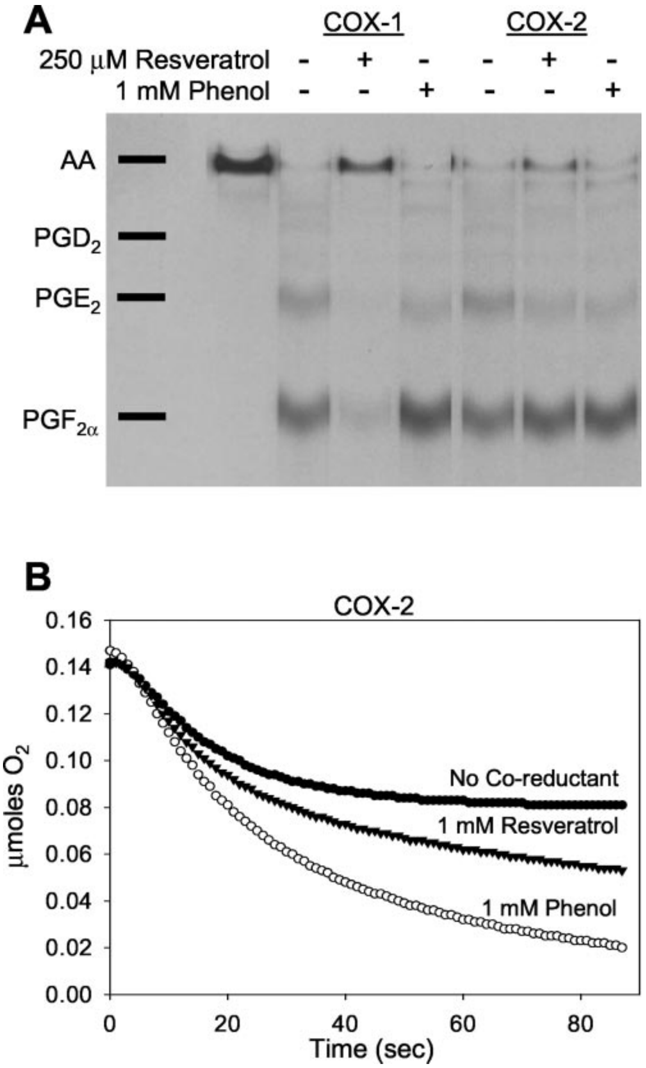
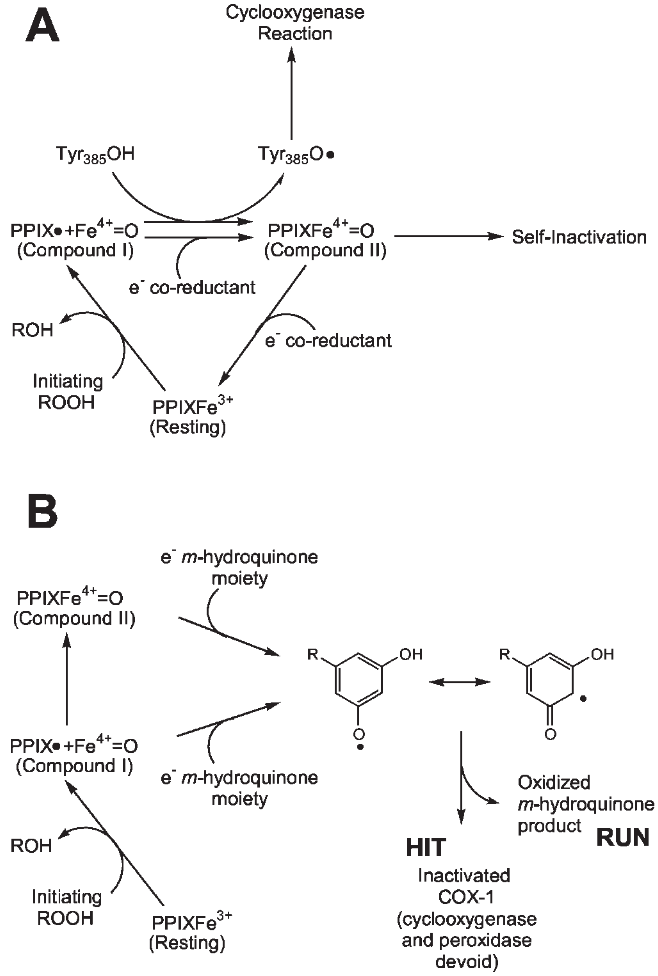


FIG. 9. **Effect of resveratrol on COX-dependent prostanoid synthesis.** *A*, holo-COX-1 or holo-COX-2 (2 μM) was incubated with either 250 μM resveratrol or 1 mM phenol in 100 mM Tris-HCl (pH 8.0). Reactions were initiated by the addition of 150 μM [^{14}C]AA (25 nCi/reaction) and quenched after 1 min with stannous chloride in HCl. PG products were separated by TLC (ethyl acetate:2,2,4-trimethylpentane: acetic acid, 110:50:20, v/v/v), visualized by autoradiography, and quantitated by scintillation counting. *B*, holo-COX-2 (0.6 μM) was incubated with either 1 mM resveratrol or 1 mM phenol in 100 mM Tris-HCl (pH 8.0). Cyclooxygenase assays were initiated by the addition of 150 μM AA, and oxygen uptake during the *bis*-dioxygenation reaction was monitored by oxygen microelectrode.



SCHEME 2. **Accepted mechanism of COX catalysis (*A*) and proposed scheme for *m*-hydroquinone inactivation of COX-1 (*B*).**

superoxide dismutase by its own reaction product, H_2O_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 $k_{\text{inact}}/K_{i(\text{inact})}$. 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.

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